ADDIS ABABA UNIVERSITY

COLLEGE OF HEALTH SCIENCES

SCHOOL OF MEDICINE

DEPARTMENT OF MEDICAL BIOCHEMISTRY

ASSESSMENT OF SERUM FERRITIN AND hs-CRP AS DIAGNOSTIC MARKER AMONG BREAST, CERVICAL, AND COLORECTAL CANCER PATIENTS ATTENDED AT TIKUR ANBESA SPECIALIZED HOSPITAL, ADDIS ABABA, ETHIOPIA.

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A THESIS SUBMITTED TO THE DEPARTMENT OF MEDICAL BIOCHEMISTRY, SCHOOL OF MEDICINE, COLLEGE OF HEALTH SCIENCES, ADDIS ABABA UNIVERSITY, IN PARTIAL FULFILLMENT OF MASTER OF SCIENCE DEGREE IN MEDICAL BIOCHEMISTRY.

SEPTEMBER, 2021

ADDIS ABABA, ETHIOPIA

ADDIS ABABA UNIVERSITY

COLLEGE OF HEALTH SCIENCES

SCHOOL OF GRADUATE STUDIES

DEPARTMENT OF MEDICAL BIOCHEMISTRY

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SEPTEMBER, 2021

ADDIS ABABA, ETHIOPIA

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Declaration Sheet

This is to certify that this Masters thesis entitled: ***Assessment of serum ferritin and hs CRP as diagnostic marker among breast, cervical and colorectal cancer patients attended Tikur Anbessa Specialized Hospital, Addis Ababa, Ethiopia***, a cross sectional study conducted by **Amanuel Wotera Wogga**, and Submitted to the Department of Biochemistry for partial fulfillment of the requirements for the degree of Master of Science in Medical Biochemistry complies with the regulation of the university and meets accepted standards with respect to originality and quality.

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Chair of Department or Graduate Program Coordinator

ACKNOWLEDGEMENTS

Above all, I would like to express my first and foremost thanks to the almighty God for his unconditional love, care, mercy and grace.

I am very grateful for study participants for their good collaboration during study period.

It is also my pleasure to express my heartfelt thanks to my family for their unceasing support throughout my life.

I am extremely grateful to my respected advisors Dr. Maria Degef and Dr. Solomon Tebeje Gizaw for their friendly approach, constructive comments, valuable scientific guidance and persistent support which can hardly be expressed in words.

It is also my pleasure to express my deepest gratitude to the Breast Cancer Thematic Research Group (BCTRG) at CHS for their partial financial support.

I am very thankful to the Department of Medical Biochemistry, School of Medicine, College of Health Sciences, Addis Ababa University for giving me this opportunity.

Finally, I extend my special thanks to Arbaminch University for sponsoring me through my graduate study.

ACRONYMS AND ABBREVIATIONS

CA	Cancer antigen
CA19-9	carbohydrate antigen 19-9
CCL2	C–C motif chemokine ligand 2
CEA	carcino-embryogenic antigen
COX2	cyclooxygenase 2
CpG	5'-C-Phosphate-G-3'
CRC	Colorectal Cancer
CSCs	cancer stem cells
CXCL3	Chemokine C-X-C motif ligand 3
CXCL8	chemokine C-X-C motif ligand 8
CXCR4	C-X-C chemokine receptor type 4
CYFRA	cytokeratin fragment 21-1
DAMPs	damage-associated molecular patterns
DNMT	DNA methyltransferase
EMT	epithelial to mesenchymal transition
FTH	heavy chain ferritin
FTL	light chained ferritin
GLOBOCAN	Global Cancer Incidence, Mortality and Prevalence
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIF1α	Hypoxia-inducible factor 1-alpha
HPV	human papilloma virus
hs-CRP	high sensitive C-reactive protein
ICAM-1	Intercellular adhesion molecule
IKK	IKKβ kinase
IL	Interleukin
IRE	iron responsive element
IRP	iron regulatory protein
JAK-STAT	Janus kinase/signal transducers or activators of transcription
JNK1	c-Jun N-terminal kinase pathway

MAPK	mitogen-activated protein kinase
MET	mesenchymal to epithelium transition
MMP	matrix metalloproteinases
MVB	multi-vesicular body
NCOA4	nuclear receptor coactivator 4
NF-κB	Necrosis factor kappa B
NOS	nitrogen oxide synthase
PAMPs	pathogen-associated molecular patterns
RAS	rat sarcoma
RNI	Reactive nitrogen species
ROS	Reactive oxygen species
SCC	serum squamous cell carcinoma
STAT	signal transducers or activators of transcription
TAM	tumor-associated macrophages
TfR1	transferrin receptor1
TGFβ	Transforming Growth Factor-Beta
TIM2	T cell immunoglobulin and the mucin-domainreceptor-2
TLR	Toll like receptor
TME	tumor microenvironment
TNF	Tumor necrosis factor
UTR	untranslated region of the mRNA
VCAM-1	vascular cell adhesion molecule

TABLE OF CONTENTS

Contents

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Background: Cancer is defined as group of diseases with uncontrolled cell growth. Inflammation is linked with cancer and stated as one of the hallmarks of cancer. hs-CRP, an acute-phase plasma protein that increases during systemic inflammation, is one of the most frequently used inflammatory biomarker. Similarly, serum ferritin, which is commonly used and convenient biomarker to assess iron level in the body is recently used as an important marker of inflammation. However, both hsCRP and serum ferritin level was not commonly used for early screening, prognosis or diagnosis of cancer.

Objective: The aim of this study was to assess serum ferritin and hs-CRP level in breast, cervical and colorectal cancer before any treatment.

Methods: Hospital based cross-sectional study was used as an appropriate study design. A total of 120 breast, cervical and colorectal cancer patients (40 in each cancer type), who did not take any type of treatment were included in this study. Biochemical tests of serum ferritin and hsCRP level were determined using COBAS c 501 and COBAS e 411 rack systems, respectively. Comparison of medians of serum ferritin and hsCRP among cancer types and among cancer stage was assessed using Kruskal- Wallis H tests. P-values less than 0.05 were considered to be statistically significant.

Results: Increased level of hsCRP was observed on breast cancer (22.5 %), cervical cancer (50%), and CRC (37.3%) patients. Similarly, elevated serum ferritin level was observed on 12.5% breast, 30% cervical and 42.5% of colorectal cancer patients. Based on the stage of cancer, higher median (interquartile) value of hsCRP and serum ferritin was noticed on stage IV and II cancer patients respectively. Significantly higher median value of hsCRP was observed on cervical cancer patients ($p < 0.005$). But, insignificantly higher level of serum ferritin and higher level of serum hsCRP was seen on stage II ($p=0.259$) and stage IV ($p=0.702$) cancer patients, respectively.

Conclusion: The current study proved that there was increment of serum ferritin and hsCRP in the three cancer cases. As a result, serum ferritin and hsCRP can be used as potential diagnostic biomarkers in breast, cervical and colorectal cancer.

Keywords: Breast cancer, Cervical cancer, CRC, Diagnostic markers, serum ferritin, hsCRP.

1. INTRODUCTION

1.1. Background of the study

Cancer is defined as group of diseases with uncontrolled cell growth and fundamentally characterized by resistance to apoptosis, invasion of neighboring tissues and spread to other parts of the body (via direct cell migration or through the blood and lymph systems), and eventually cause death (Hanahan and Weinberg, 2011). It begins due to abnormal changes in a single cell's genetic material that affect the mechanisms of normal cell growth, regulation, and cell death, which finally leads to uninhibited cell growth. In most cases abnormal changes are caused by combination of both hereditary factors (inheritance of one or more genetically inactive proteins) and environmental factors such as physical carcinogens (e.g. ionizing radiation), biological carcinogens (e.g., parasites, certain viruses, and bacteria) and chemical carcinogens (e.g. components of tobacco smoke, asbestos, and radon) (Nelson and Cox, 2013; Federal Ministry of Health Ethiopia, 2015).

Cancer has a capability to affect almost any part of the body and has its own name according to tissue type where it begins. For example, cancer that begins in the skin or tissues that cover organs is known as Carcinoma (WebMed, 2020) and those group of cancers that begin in the connective tissues and in the bones is called soft tissue sarcoma and sarcoma respectively. Soft tissue sarcoma forms in the tissues that support connect and surround body structures. This includes muscle, tendons, blood vessels, fat, cartilage, nerves and the lining of joints (Mayo clinic, 2021). Similarly, cancer that usually involves in the white blood cells is leukemia and those that begin in cells of the immune system are known as Lymphoma (cancer that begins in lymphocytes) and multiple myeloma (malignancy which start in clonal plasma cells of the bone marrow) (WebMed, 2021).

Most and perhaps all forms of human cancer cells have developed characteristics that differentiate them from normal cells. The gradual transformation of normal human cells into highly malignant derivatives is distinguished by a limited number of biochemical, genetic and cellular traits-acquired capabilities called hallmarks of cancer, which form a basis for understanding the dynamics of neoplastic disease. One of such hallmarks of cancer is inflammation, the body's biological response to fight against things that damage it (Hanahan and Weinberg, 2011). In 1863, Rudolff Virchow proposed the association of inflammation with cancer for the first time and he stated that the site of chronic inflammation is the origin of cancer (Balkwill and Mantovani, 2001). Since then, a number of researchers have been studying the role of inflammation in the different types of cancer and proposed causative and diagnostic hypotheses to explain the relationship between inflammation and cancer (Heikkilä *et al*., 2007).

According to the causative hypothesis of inflammation and cancer, tumor cells draw immune cells and stimulate the production of cytokines and chemokine creating tumor microenvironment, thus oxidative damage related with inflammation cause mutation on tumor suppressor genes and proteins involved in apoptotic control and DNA repair. In addition, signaling of inflammatory cytokines through transcription factors and intracellular enzymes restrain apoptosis and promote growth and proliferation of cancer cells, which results in acceleration of tumor progression (Coussens and Werb, 2002; Heikkilä *et al*., 2009). But the diagnostic hypothesis states that tumor-stimulated inflammation infilters cells (mainly tumor associated macrophages), express genes and immunological marks that are helpful in informing the prognosis and diagnosis of cancer and sensitivity of therapies (Riaz *et al*., 2017; Jerby-Arnon *et al*., 2018).

1.2. Statement of the problem

Cancer is a rapidly growing disease and the second leading cause of death globally, which was responsible for almost 10 million deaths and 19.3 million new cases in 2020 (Sung *et al*., 2021). One out of six deaths in the world is because of cancer (WHO, 2018). Cancer is also an increasing public health burden for Ethiopia and Sub-Saharan Africa at large as a result of population aging and growth, and, increasing adoption of cancer-associated lifestyle choices such as smoking, ‘‘westernized’’ diets and physical inactivity (Stewart and Wild, 2014). According to the GLOBOCAN report of 2020, Ethiopian annual incidence and mortality of cancer were 77,352 and 51,865, respectively. From these, the first dominant malignancies are breast, cervical and colorectal, each cover a number of new cases 16,133; 7,445 and 6,048, respectively. Breast cancer is the leading cause of death accounting for 17.1% of total cancer mortality followed by cervical (10.2%) and colorectal cancer (7.15%). Combination of these three cancers cover 29,626 of newly diagnosed cases (38.9% of total incidence) and 18,965 number of deaths (36.5% of total mortality) in Ethiopia (Sung *et al*., 2021). Thus, discovery of biomarkers can help in reducing mortality number, because of its prognostic or diagnostic use.

1.3. Significances of the study

Since the linkage of inflammation with malignancy is postulated in around the mid-19thcentury, several studies have been done and some shown that inflammatory markers like hsCRP and ferritin are elevated though few shown reduced ferritin and raised hsCRP in different types of cancer. But to our knowledge, there is no study conducted in our country regarding the association of multiple malignancies with ferritin and hs-CRP. Therefore, this study is aimed to assess the level of hsCRP and ferritin in top three malignancies of Ethiopia (breast, cervical and colorectal cancer) to generate information that can serve as an input for the scientific world and physicians in revealing the level of ferritin and hsCRP in Ethiopian people. Thus, the output of this study might have a role in early detection, screening, prognosis or diagnosis of patients. The result of this study can also show the potential use of serum ferritin as inflammatory biomarker in three cancer cases. Moreover, considering the limitation, this study could serve as a baseline information for further investigation on the same area.

2. LITERATURE REVIEW

2.1. Cancer and Inflammation

Inflammation is a biological protective response of the body to inflammatory stimuli. Though the perception of connecting inflammation with cancer started in 19th century, it was declined for a prolonged time. But from different lines of work the birth of inflammation-malignancy association was revealed in the last few decades (Medzhitov, 2008). It is believed that immune system and inflammation contribute to malignancy initiation, progression and metastasis. Thus, cancer biology is recently understood from an inclusive concept that puts cancer cells in a network of stromal cells, inflammatory immune cells and vascular vessels which all together make tumor microenvironment (TME). Cancer associated inflammation is mainly characterized by: WBC infiltration (mainly tumor-associated macrophages), occurrence of polypeptide messengers of inflammation (cytokines like TNF, IL-1, IL-6, chemokines such as CCL2 and CXCL8) and presence of angiogenesis (Rosenthal *et al*., 2019).

Irrespective of its existence, tumor-stimulated inflammation or chronic inflammation has countless effects on tumor microenvironment composition and plasticity of stromal and tumor cells. Cell plasticity in malignancy describes a molecular and phenotypic change that contributes in enhancing tumor heterogeneity and therapy resistance. Immune system driven inflammatory process during tumorigenesis have either anti-tumorigenic function (exerts immunological sculpting and immunosurveillance of tumor heterogeneity) or pro-tumorigenic function, which supports cancer by influencing tumor microenvironment towards more tumor-progressive state, delaying anti-tumor immunity and applying direct tumor-promoting signal into cancer and epithelial cells (McGranahan and Swanton, 2017; Zilionis *et al*., 2019).

Infiltration of cell type, expression of gene and other immunological biomarkers are helpful in informing cancer prognosis and sensitivity to therapies (Riaz *et al*., 2017; Jerby-Arnon *et al*., 2018). Immunologically inflamed/hot tumor microenvironment illustrate tumors having an elevated level of infiltrating T cells and amplified presence of components important for antitumor immune function whereas "cold" or "infiltration-excluded" TME is coined to describe tumors that do not display cellular or gene expression features satisfactory for anti-tumor action mainly by T cells (Binnewies *et al*., 2018). But tumors with the later term may still exhibit upregulated inflammatory mediators and recruited tumor promoting immune cells like neutrophils, monocytes, macrophages and innate lymphoid cells (Rothwell *et al.*, 2012; Ridker *et al.*, 2017).

2.2. Stimuli of inflammation in tumorigenesis

Inducers which can trigger inflammatory response during carcinogenesis of different malignancies include; inactivation of tumor suppressors, loss of barrier function/commensal microorganisms, oncogene activation, carcinogenic microbes, environment, obesity, hypoxia, pollutants and cell death. Loss of tumor suppressor function/limitless replicative potential/ is mentioned as one of hallmarks of cancer. P53 gene which encodes p53protein is the most commonly mutated tumor suppressor gene. One multifaceted role of p53 protein in cellular homeostasis is its antagonism with NF-kB, an important orchestrator of inflammation in both tumor and inflammatory cells, (Karin, 2006). An activated NF-κB induce the expression of genes encoding adhesion molecules, inflammatory cytokines, key enzymes in prostaglandin synthesis pathway(COX-2), NO synthase, angiogenic factors; and promote cell survival by promoting anti-apoptotic gene(Bcl2) (Pikarsky *et al.*, 2004; Karin, 2006). Thus, stimulated NF-κB signaling due to loss of p53 protein function involved in tumor initiation and progression (Komarova *et al.*, 2005; Schwitalla *et al.*, 2013b). These inflammatory entities and signaling support tumor development and metastasis in colorectal cancer (Pribluda *et al*., 2013; Schwitalla *et al*., 2013b). Damage of tumor suppressors also hinder an accurate DNA repair and increase DNA injury which results in DNA-damage driven inflammatory pathways (Andriani *et al*., 2016).

Another stimulant of inflammatory response during tumorigenesis is activation of oncogenes, which is systematically associated with the mass production of chemokines, cytokines and recruitment of myeloid cells that results either tumor promotion or immunosuppression. For instance, K-Ras oncogenic signaling regulates CXCL3 expression, basic chemokine for myeloid cell recruitment (Liao et al., 2019). K-Ras stimulation also increases production of cytokines and chemokines such as IL-1α, IL-1β, CXCL1 and CCL2 that are involved in “senescence associated secretory phenotype” (Davalos *et al.*, 2010). c-Myc and K-Ras activation cooperatively also induce IL-23, CCL9 and other inflammatory bodies in pancreatic cancer. Oncogene activation mechanism, which produces massive inflammatory chemokines and cytokines is postulated as a unifying mechanism how inflammation is activated in many malignancies (Kortlever *et al.*, 2017)

Tumor initiation and progression driven by persistent recognition of carcinogenic pathogens like HPV, HBV, HCV, H. pylori and H. hepaticus promote inflammatory response mechanisms distinct from other inducers. Sensors like STING, cGAS, TLR2, TLR4 and multiple sensors linked to inflammasomes involve in recognition of conserved molecular patterns in pathogens and elicit innate inflammatory response (Woo *et al.*, 2015). Conversely, an alternative developing paradigm claims that commensal microbiota promote cancer, either by releasing distance discharge of inflammation inducing microbial metabolites or translocation and adherence of microbes to malignant cells. These microorganisms and microbial yields can move to the site of metastasis with tumors and function as a source of irritation in metastasis (Bullman *et al*., 2017). For example, commensal microbiota affects tumor development and progression in colon cancer. This is because bacteria directly interact with tumor surface and make hyper propagating cells unable to correctly differentiate, form adherence junction, make well-built mucus and separate compartment of immune from bacteria; Thus, it results intestinal transformation followed by bacterial deterioration in the intestine (Grivennikov *et al*., 2012: Dejea *et al*., 2018).

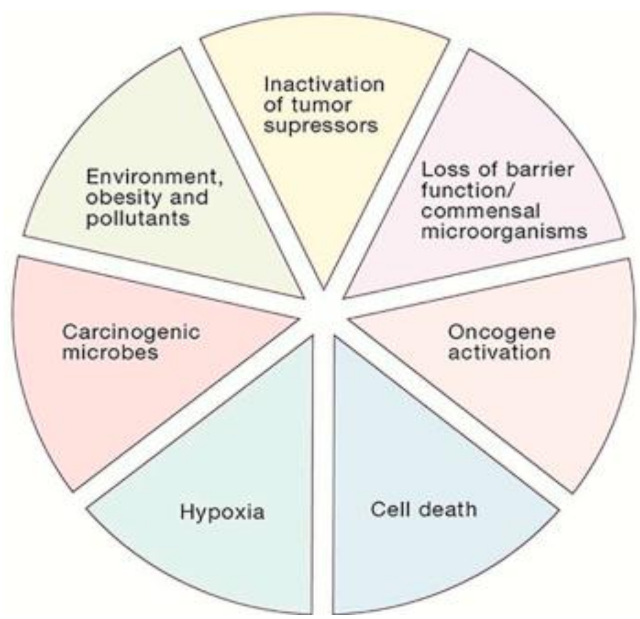

Figure 1: Sources/stimulus of tumor-associated inflammation in different tumor types. (Tomkovich et al., 2019).

2.3. Role of inflammation in tumor initiation and promotion

Two key codependent events are important in effective tumor initiation. The first event involves the accumulation of mutations and epigenetic changes in genes and signaling pathways that results in inactivation of tumor suppressor and activation of oncogenes. Though conventionally these have been mostly associated with environmental factors and genetic errors in DNA replication and repair, inflammatory reactions use influential mechanisms that results in collection of different mutations and epigenetic alteration in epithelial cells. Reactive oxygen species (ROS) and reactive nitrogen species (RNI) made from macrophages and neutrophils cause production of mutagenic DNA lesions such as 8-nitroguanine and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG). Thus, stimulation of inflammation leads to tumorigenesis, predisposing of the collection of mutations in healthy tissues. In fact, Chronic intestinal inflammation mutates Tp53 and other cancer associated genes and initiates tumor growth without presence of extra mutagen (Meira *et al*., 2008; Robles *et al*., 2016; Canli *et al*., 2017).

Interestingly, cytokines like IL-6, IL-1β and TNFα participate in inflammatory cell signaling that triggers epigenetic machinery of epithelial cells, including miRNA, lncRNA, components of DNA and histone modifications (OTL1, Dnmt1, Dnmt3) that alters manifestation of tumor suppressor genes and oncogenes (Grivennikov, 2013). In a normal microenvironment, the antioxidant system balances pro-oxidants and antioxidant. Promoter CpG sites of tumor suppressor genes and microRNA are hypomethylated and normally expressed. But in inflammatory microenvironment, exposure to pro-inflammatory cytokines like IL-6 or ROS/RNS transcriptionally influences DNA methyltransferase 1(DNMT1) protein and results in epigenetic silencing via hypermethylation of DNA of microRNAs and tumor suppressor genes (Wilson,2008). Generally, DNA methylation pattern of human malignancy is characterized by hypomethylated tumor cell genome and hypermethylated CpG promoter site, which leads to silencing of genes that favors tumor-relevant pathways (Rokavec *et al.*, 2016).

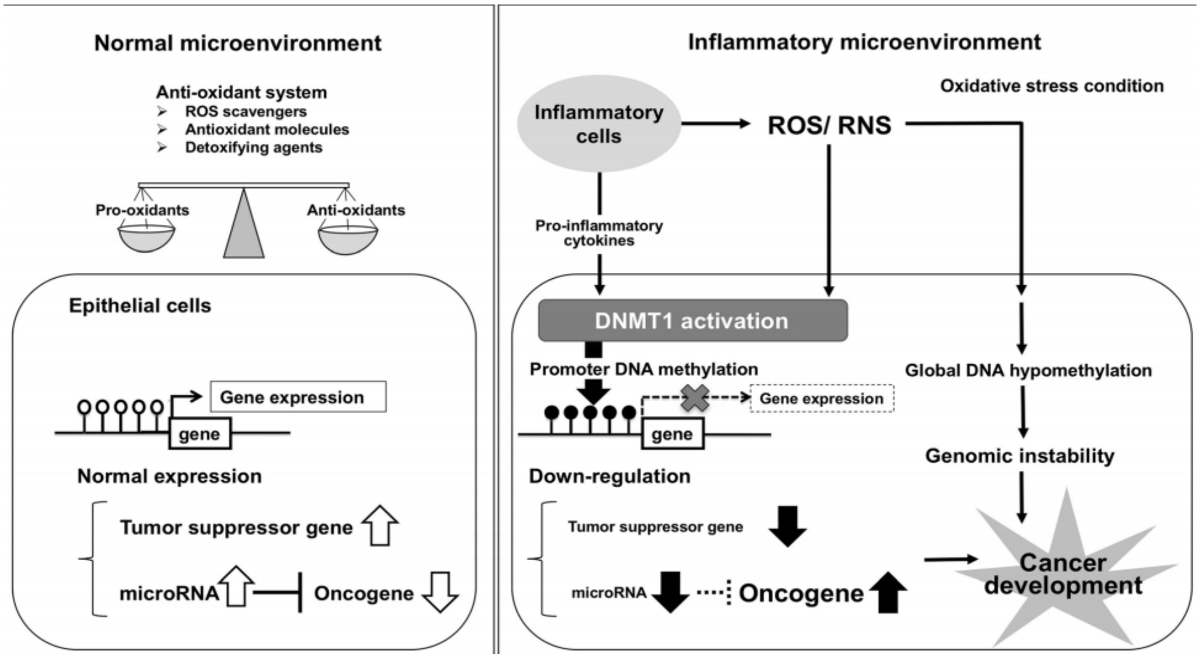

Fig. 2. Epigenetic alterations under an inflammatory microenvironment. (*White circles indicate unmethylated CpG sites, and black circles show methylated CpG sites in the promoter region*). (*Mariko, 2018*).

The second event involves the formation of transformed or malignant clones followed by development of frank tumor, where inflammatory mechanisms contribute significantly. There are two pathways (extrinsic and intrinsic) that connect cancer and inflammation. The extrinsic pathway, driven by inflammatory or infectious conditions and the intrinsic pathway, activated by genetic alteration are capable of causing malignancy. Genetic events like activation of oncogenes, chromosomal amplification or rearrangement and inactivation of tumor suppressor gene, transform a cell and make it to produce inflammatory mediators through stimulation of transcription factors, NF-κB, STAT3 and HIF1α in tumor cells. These transcription factors direct the production of cytokines, chemokines and cyclooxygenase 2 (COX2). These factors in turn, recruit and trigger different leukocytes, prominently cells of myelomonocytic family. Cytokines again elicit key transcription factors from tumor cells, inflammatory cells and stromal cells, which results in production of more inflammatory mediators and generation of tumor-related inflammatory microenvironment (Alberto *et al.*, 2008). Additionally, cytokines increase survival probability of transformed cells through stimulation of pro-survival pathways which are mainly mediated by NF-κB, STAT3, and other types of signaling (Grivennikov *et al*., 2009; Dmitrieva-Posocco *et al*., 2019).

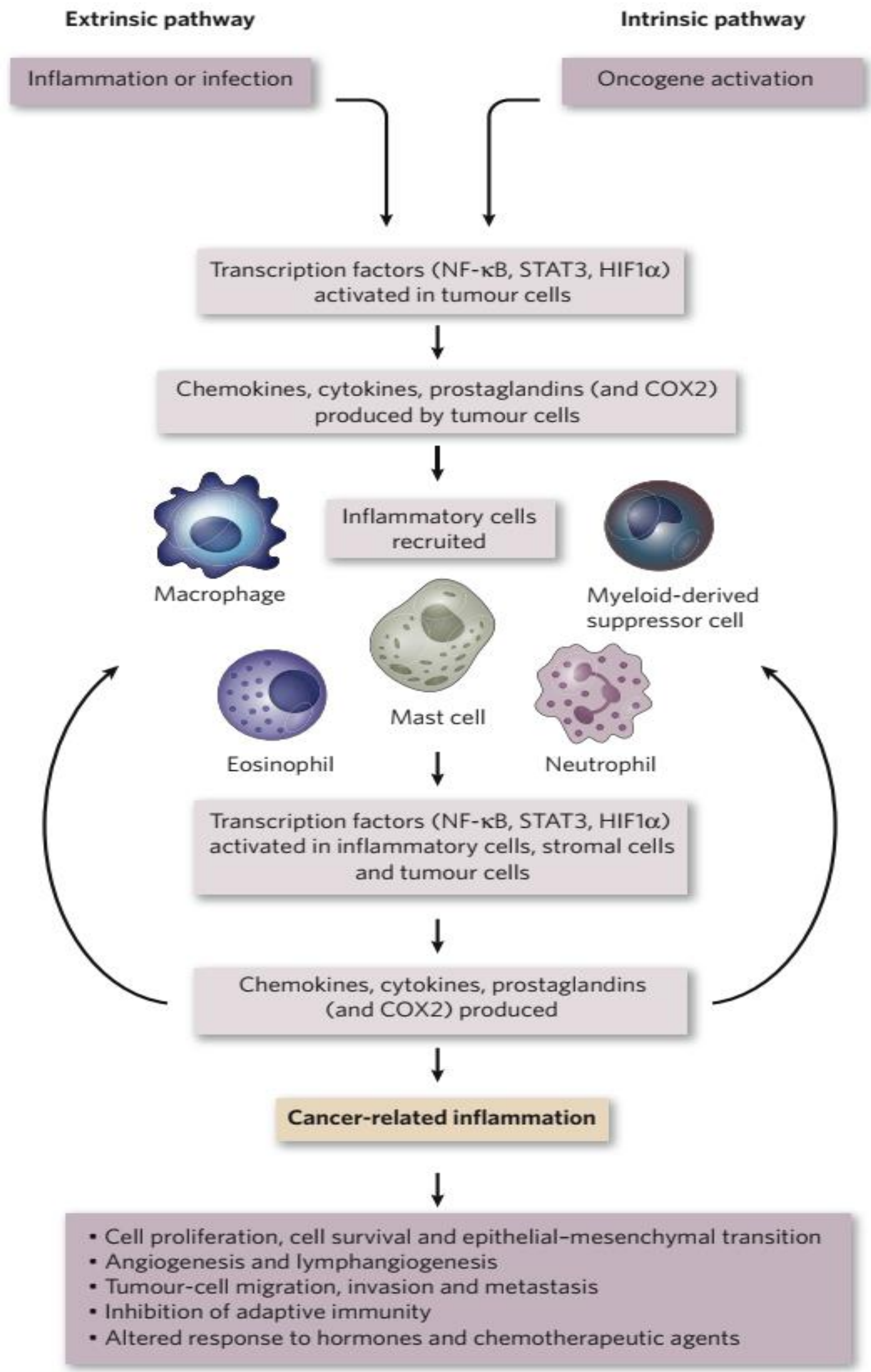

Figure 3. Pathways that connect inflammation and its role in initiation of cancer. (*Alberto et al.,2008*).

Following tumor initiation, tumor promotion, another key step in carcinogenesis is preceded by interdependent cellular and molecular mechanisms. Like tumor initiation, inflammatory factors function as direct growth factors for developing tumors and shape cell plasticity in the tumor microenvironment. One of the first pieces of evidence that indicates function of inflammation in tumor promotion came from studies that showed inactivation of NF-κB in myeloid cells, where removal of IKKβ kinase(IKK) results in decrease of tumor growth in colitis-linked cancer. IKK is a complex enzyme that phosphorylates inhibitors of NF-κB (IκB) causing it to detach from NF-κB and allowing NF-κB to function well (Greten et al., 2004). Signaling induced by IL-6, IL-11and IL-17 also rises proliferation of cancer cells under suboptimal conditions like: hypoxia, lack of nutrients, anti-tumor immunity and growth factors scarce environment (Huber *et al.*, 2012; Putoczki *et al.*, 2013).

2.4. Inflammation function in tumor progression and metastasis

The progression of metastasis begins with acquisition of cell plasticity, particularly epithelial to mesenchymal transition (EMT) and invasion of malignant cells in neighboring tissues. Though epithelium to 'fibroblast' cell transition may be partial and incomplete, it reduces mobility of cancerous cells and permits them to penetrate the basal membrane, invade the tissues and spread to the body through blood vessels and lymphatics (Varga and Greten, 2017). Altered epithelial and transient amplifying cells undergo dedifferentiation and cell plasticity to attain the phenotype of cancer stem cells in response to inflammatory signaling (Schwitalla *et al*., 2013a). Thus, cancer stem cells(CSCs) that are functionally and transcriptionally closer to mesenchymal cells, are efficient and capable of being used as metastatic seeds (de Sousa *et al*., 2017).

Growth factors and inflammatory cytokines influence and regulate cancer cell plasticity (states of epithelium to mesenchymal transition(EMT) and mesenchymal to epithelial transition(MET)), cancer invasion and re-establishment of CSC pool during metastasis. For example, cytokines like TNF and IL-1β activate transcription factors such as Slug and Twist and directly influence EMT expression (Suarez-Carmona *et al*., 2017; Francart *et al*., 2018). Additionally, IL-11 in colon cancer is involved in recruitment of fibroblast and TGFβ; and assist in immune escape and tumor invasion (Calon *et al*., 2012; Calon *et al*., 2015). Similarly, IL-11 within breast cancer tumor microenvironment acts on malignant cells and drives clonal section of most malignant and invasive clones. (Marusyk *et al*., 2014). Myeloid cells recruitment in the invasive section of the tumor produces matrix metalloproteinases (MMP) and other enzymes which degrade extracellular matrix and help cell migration (Akkari *et al.*, 2014; Sevenich *et al.*, 2014). Similar myeloid cells, myeloid derived suppressor cells (pathologically activated neutrophils and monocytes) have a contribution on suppression of anti-tumor response (Yang *et al*., 2008; Veglia *et al*., 2018). Macrophages also play an important various role in chemoattraction, immunosuppression and degradation of extracellular matrix while invasion proceeds (Gatenbee *et al.*, 2019).

As metastasis spread through blood stream or lymphatics, intravasation and extravasation is mediated by expressing adequate set of integrin and adhesion molecules (such as VCAM-1 and ICAM-1), to permit heterotypic cell-cell contact, adhesion and passage. Inflammatory cytokines trigger selectins, integrin and adhesion molecules. The progression of extravasation and adhesion are additionally supported by neutrophils and monocytes (Kersten *et al*., 2017), that trigger adherence of metastatic seeds, form complexes with cancer cells and mediate their adhesion and translocation in the vessel wall as well as establishment and maintenance of the metastatic niche. Thus, oligocellular complexes between monocyte-cancer cells, cancer cells themselves and neutrophil-cancer cell protect metastatic seeds from immunosurveillance (Wolf *et al*., 2012; Aceto *et al*., 2014; Szczerba *et al*., 2019). Besides this, studies claim that inflammatory signals either from obesity, microbes or tumor-specific inflammatory stimuli can enhance cell-cell contact and raise the rate of metastasis. For example, breast cancer metastasis is activated by IL-17 driven neutrophils activation which enhances cellular interaction in circulation and establishment of pre-metastatic niche (Coffelt *et al.*, 2015) while metastatic load of lung cancer is increased by inflammations elicited by tobacco smoke, obesity and microbial compounds through activation of neutrophils and raised contact of neutrophil-cancer cells (Albrengues *et al.*, 2018).

2.5. The biochemistry of ferritin

Ferritin, the known iron-storage protein, was initially expressed as iron-rich constituent of horse liver in the late 19th century and later it purified from horse spleen (Arosio *et al*, 2017). Ferritin is synthesized by many body cells and found mainly in the spleen, liver, bone marrow and muscle with only a small amount exist in blood. The amount of serum ferritin is used as an indicator of iron reserve, showing iron deficiency anemia if too little iron or hemochromatosis if too much iron is available (Thomas, 1998; Wick *et al*., 2011). Structurally, apoferritin (iron free ferritin) is highly conserved spherical shell glycoprotein that is assemble of 24 monomer subunits that consist of either heavy chain (FTH) or light chain (FTL) polypeptides. Each subunit in ferritin contains 5 α-helix secondary structures, with an extended loop among second and third helices (Harrison and Arosio, 1996). In addition to providing intracellular storage of a huge amount of iron in a soluble, safe and bioavailable form, ferritin prevents cells from oxidative damage of free iron and toxicity (Recalcati *et al*., 2008). Though commonly ferritin is cytosolic protein, it has also been found in mitochondria, serum and nucleus (Thompson *et al*., 2002). The cytosolic ferritin, an approximately 450kDa hollow-cage protein, can store up to 4500 Fe^{3+} ions and is made of two types of subunits: FTH (21kDa) and FTL (19kDa). In humans, the genes that code for H-sub unit is found in eleventh chromosome and L-sub unit is found in nineteenth chromosome (Worwood *et al*.,1985).

Both subunits (H and L-sub units) have a particular function and are found in a various H/L ratio and expression level in different tissues (White and Munro, 1988). The FTH (heavy chain sub unit) protein contain a ferroxidase moiety that is responsible to convert ferrous Fe to inert ferric Fe form, permitting ferritin to sequester iron inside the shell (Chasteen and Harrison,1999). The Fe2+ion enters into ferritin through the support of electrostatic gradient, and once internalized, the Fe2+ iron moves to ferroxidase center on H-sub unit of ferritin where Fe2+ reacts hydrogen peroxide and oxidized to Fe3+ atom (Arosio *et al*., 2015). Since FTL does not have ferroxidase moiety, ferroxidase activity is exclusively executing by FTH (Ferreira *et al*., 2000). Due to higher requirement of ferroxidase activity in heart and brain tissues, FTH is the major sub unit expressed in these tissues. Although light chain subunit (FTL) lacks iron-oxidizing ability, it improves protein stability and helps iron integration. FTL is highly expressed in liver and spleen (Luscieti *et al*., 2010).

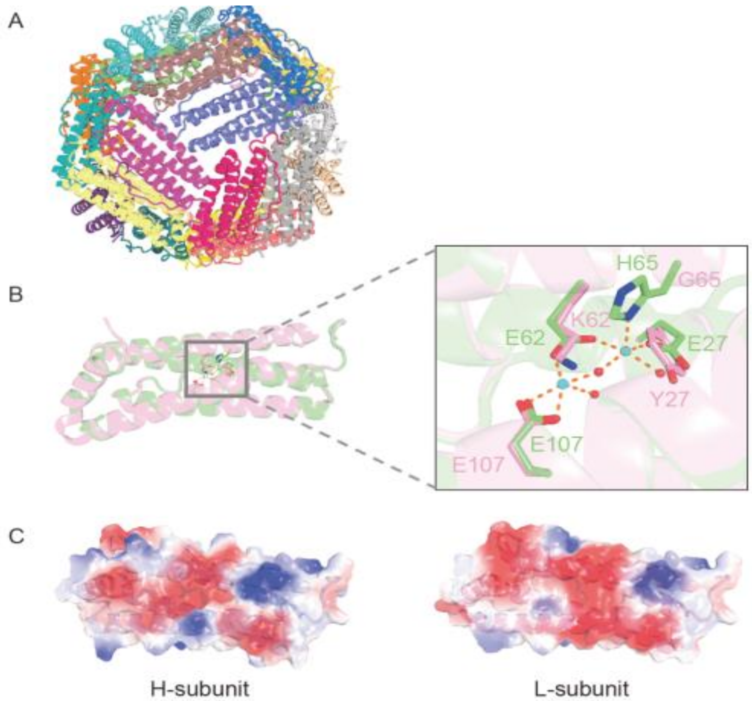

Figure 4. Typical structure of ferritin. A, Typical ferritin is a spherical shell composed of 24 subunits. B, H-subunit (lime) and L-subunit (pink) of human ferritin. C, Internal surface electrostatic potential properties (blue: positive; red: negative) in H-subunit and L-subunit of human ferritin. (*Source: Zhang et al.,2020*).

Regulation of ferritin at transcriptional and translational levels results in different H/L ratios in different tissues (White *et al.*, 1988). One of the well-known regulations of ferritin at post transcriptional level is iron availability based interaction of iron regulatory protein (IRP) with iron responsive element (IRE) in ferritin encoding transcript. There are two types of IRPs, namely IRP1 and IRP2, which bind iron responsive element, located at the 5′ UTR (untranslated region) of the mRNA, and restrain ferritin translation under low-iron condition but translation is facilitated under iron abundant condition through dramatical change of IRP1 conformation and proteasomal degradation of IRP2 from IRE which finally results adjustment of ferritin amount according to intracellular iron level (Torti, 2002).

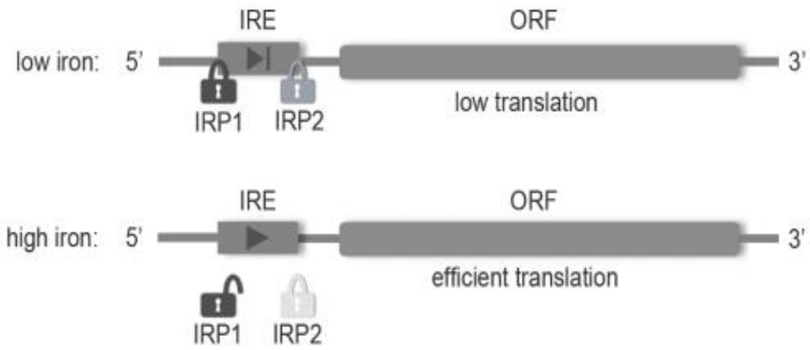

Figure 5. Schematic representation of the regulation of ferritin expression at the post-transcriptional level. (*Zhang et al.,2020*).

Besides iron availability, pro-inflammatory cytokines like IL-1β, IL-6 and TNF-α trigger transcription of ferritin through NF-κB pathway (Miller *et al*, 2001; Ruddell *et al.*, 2009). IFN-γ and lipopolysaccharide (LPS) stimulates IRP2 degradation in NO-dependent manner and leads to an enhanced ferritin production in macrophages (Kim and Ponka,2000); and IL-6 increases FTH & FTL production in liver cells (Miller *et al*.,2001). Furthermore, the intracellular amount of ferritin is regulated by a level of degradation through ferritinophagy. Lysosomal degradation of ferritin through cargo nuclear receptor coactivator 4 (NCOA4) increases the release and availability of iron inside the cell (Mancias *et al.*, 2014; Hou *et al.*, 2016). TNF-α involves in cytosolic ferritin degradation via JNK1 signaling, with a subsequent increase in oxidative stress, which further induces ferroptosis (a novel form of regulated cell death, characterized by increased intracellular iron availability, oxidative stress and lipid peroxidation) (Gao *et al.*, 2016). Ferroptosis has been indicated in pathological cell death linked with degenerative diseases and carcinogenesis (Stockwell *et al.*, 2017).

2.5.1. Serum ferritin

Though ferritin is mainly recognized as a cytosolic iron storage protein, it is present in extracellular fluids like serum, cerebrospinal fluid and urine in various amounts. Serum ferritin, an acute phase protein, is composed of few H sub units and large L-chain sub units. 50-80% of serum ferritin is glycosylated and its half-life is 30 hours. Since glycosylation occurs in the intracellular compartment of a cell it implies that serum ferritin is a secretory protein (Cullis *et al.*, 2018). Despite the cellular source and pathway of ferritin is mostly unknown, some studies indicated that ferritin core is accumulated in lysosomes and autophagosomal compartment of macrophages, which ferritin secrete through non-classic secretion such as autophagy-related ferritin secretion with involvement of galectin-8, TRIM16 and Sec22b proteins and multi-vesicular body (MVB)–exosome pathway (Truman-Rosentsvit *et al.,* 2018). It was also reported that hepatocytes are able to secrete ferritin but the mechanism and pathway remain far from clarified (Wang *et al*., 2010).

Serum ferritin is traditionally used as a commonly deployed and convenient method to assess tissue iron level in the body (Daru *et al.,* 2017b). Besides indicating body iron status, serum ferritin secreted from hepatocytes and macrophages act as another iron donor protein next to ferroportin, the only protein known in facilitating cellular iron export (Leimberg *et al.,* 2005). Though it thought that serum ferritin is an iron-poor protein, it carries more iron atoms than transferrin (Cohen *et al*.,2010). Since last decades' serum ferritin is clinically used as serum markers for many inflammatory pathologies and in that regard it help in diagnosis of various condition including, Still's disease, autoimmune diseases, neurologic disorders and cancers (Vanarsa *et al*., 2012; Seyhan *et al.,* 2014).

Various ferritin receptors help secreted serum ferritin to have a possible effect on target cells. For example, in mouse T Cells, TIM2 (T cell immunoglobulin and the mucin-domain receptor-2) bind FTH peptide, which causes endocytosis of ferritin and uptake of iron into cell cytosol (Chen *et al.,* 2005). Another receptor in mice called Scara5 also binds L-ferritin polypeptides in kidney capsular compartment (Li *et al.,* 2009). In humans, receptors such as TIM-1, C-X-C chemokine receptor type 4 (CXCR4) and transferrin receptor1 (TfR1) were able to bind and internalize FTH and causes uptake of ferritin in neuron cells, embryonic kidney cell and erythroblasts respectively. FTH binding to transferrin receptor1 occurs through protein domains which are distinct from those involved in transferrin; and internalization of FTH through this process is important for the production of hemoglobin through the use of iron (Sakamoto, 2015).

2.5.2. Ferritin in inflammation and malignancy

Inflammation is the biological response of an organism to infection or damaged cell/tissue, which is characterized by pain, swelling, redness, heat and function loss. Depending on the type and duration of stimuli it can be harmful or advantageous (Chen *et al.,* 2018). The characteristics and origin of physiologic and pathologic inflammation stimuli is very diverse but the most common and important ones are damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs), which are sensed by various molecules and results in stimulation of inflammatory mediators in a given tissue through mitogen-activated protein kinase (MAPK), NF-κB or Janus kinase/signal transducers or activators of transcription (JAK-STAT) pathways (Medzhitov, 2008; Chen *et al.*, 2018). Besides being regulated by inflammatory stimuli, ferritin also enhances inflammatory response in the body. For example, Ruddell and his colleagues shown that FTH stimulate a signaling cascade that leads to NF-κB activation, which results in enhanced expression of various pro-inflammatory mediators in hepatocytes and increase cytokines and other mediators' production in immune cells (Zarjou *et al*., 2019).

Serum ferritin is mostly detected at a higher level in cancer patients than healthy individuals. Studies indicated that ferritin has a possible significant role in malignancy development through modulation of immunosuppression, angiogenesis and cell proliferation (Li, 2010; Alkhateeb and Connor, 2013). Tumor-associated macrophages actively secrete pro-inflammatory mediators like TNF-α and degrade binding of IRP that causes increased ferritin expression and lowered labile iron pool, which suggested that it has a role in the progression of tumor (Alkhateeb, and Connor, 2013). Overexpressed ferritin level is observed in tissues of various type of malignancy. For example, ferritin level in breast cancer is 6-fold greater than normal breast tissue, which is associated with histopathological dedifferentiation, epithelial proliferation and shorter survival (Alkhateeb, and Connor, 2013). Similarly, higher expression of ferritin in hypoxic conditions (hallmark of solid tumors) independent of iron level, was demonstrated from studies done in alveolar cell of colon tumors (Parks, 2017; Smith *et al*., 2003). Recently, studies describe that higher level of ferritin, especially H subunit in ovarian cancer contribute to chemoresistance of drugs used in the treatment of cancer. This is because FTH reduce the effect of drug through decrease of reactive oxygen species levels, which helps anti-tumor drugs to mediate their activity (Salatino *et al*., 2019).

2.6. Cancer and hs-CRP

The acute-phase response is a leading systemic reaction of the host to local and systemic disorders caused by tissue injury, infection, trauma, neoplasmic growth or immunological disorders (Gruys *et al.*, 2005). The function of this response to remove the cause of the disturbance and to restore homeostasis (Ebersole and Cappelli, 2000). A macrophage within a tissue initiate acute phase response by inducing secretion of different cell communicating chemical such as pro-inflammatory cytokines, nitric oxide, and glucocorticoids (Ebersole and Cappelli, 2000). These chemicals stimulate and modulate hepatic acute phase response and systemic acute phase reaction. Acute phase response is stimulated by the release of IL-1, IL-6, and TNF-α from monocytes and macrophages at the place of tissue injury or infection (Gruys *et al.*, 2005).

Acute phase proteins(APPs) are a class of proteins which are synthesized from hepatocytes and increased during inflammation. APPs are categorized into two groups based on protein concentration. Proteins which reduce production during acute phase response are referred as "negative" APPs, which include transferrin, albumin, transthyretin and retinol-binding protein. Whereas proteins which increase synthesis in response to acute phase response are called "positive" APPs, which are CRP, mannose-binding protein, D-dimer protein, alpha 1 antitrypsin, fibrinogen, alpha 2 macroglobulin, prothrombin, plasminogen, complement factors, ferritin, serum amyloid P component complement and serum amyloid A (Grover, 2016). Positive acute phase proteins functions in activating the complement system, microorganism optimization and trapping, binding of cellular remnants, scavenging of radicals and free hemoglobin, neutralizing enzymes and modulating the host's immune response (Gruys *et al.*, 2005).

CRP is the first known acute phase protein which play an important role in innate immunity *(*Jain *et al.*, 2011). It is discovered by Tillett and Francis in 1930 as a serum substance in patients with acute inflammation (Roitt and Delves, 1998). Human CRP is made of five non-covalently arranged identical polypeptide which make cyclic pentamer around a Ca-binding cavity (Fritz and Giarardin, 2005). It directly binds different types of microorganism and activate the classical C1q pathway of complement system and involve in opsonization *(*Jain *et al.*, 2011*)*. During inflammatory processes CRP levels increase dramatically which, rises up to 50,000 fold in acute inflammation. The elevation of CRP above normal level initiates within 6 h, and climaxes at 48 h. It helps binding of damaged cells and foreign molecules, and increase phagocytosis by macrophages, through CRP receptor within macrophages (Jain *et al.*, 2011).

Elevated hs-CRP concentrations have been reported in many diseases such as cardiovascular diseases, infectious diseases, diabetes, inflammatory bowel diseases, autoimmune disorders, arthritis, and many cancers (Coventry *et al.*, 2009). Several studies have attempted to identify associations between baseline hs-CRP and incidence of human carcinomas, some have shown positive associations that may be site specific (Sasazuki et al., 2010). According to Heikkilä and his colleagues' meta-analysis of 12 prospective studies have shown that elevated hs-CRP was associated with an increased risk of lung, colorectal, breast, and ovarian cancers (Heikkilä *et al.*, 2009). Another studies also indicated that hs-CRP is much higher in cancer patients than in healthy subjects (Zhang *et al.*, 2010) i.e. elevated hs-CRP is related with progression of disease and decreased survival in patients having malignancies of esophageal, gastric, colorectal, liver, pancreatic, urinary bladder, kidney, ovarian, and cervical (Wang and Sun, 2009).

3. OBJECTIVES

3.1. General Objective

To assess serum ferritin and hs-CRP level in breast, cervical and colorectal cancer patients attending Tikur Anbessa Specialized Hospital, Addis Ababa, Ethiopia.

3.2. Specific Objectives

- ✓ To measure serum ferritin and hs-CRP level among breast, cervical and colorectal cancer patients.
- ✓ To evaluate the level of serum ferritin and hs-CRP level in different stages of cancer
- ✓ To compare the difference of serum ferritin and hs-CRP level between breast, cervical and colorectal neoplasm.
- ✓ To compare the difference of serum ferritin and hs-CRP level between cancer stages.

4. MATERIALS AND METHODS

4.1. Study area and period

This study was conducted from September 2020 to July 2020 in Medical Biochemistry Department, School of Medicine, College of Health Sciences, AAU. Tikur Anbessa Specialized Hospital, teaching hospital of the University, is main central hospital in Ethiopia and accepts referrals from all over the country. The oncology unit of TASH is the cancer referral hospital in the country and has OPD, which provides service to new and follow-up patients and an in-patients department. TASH offers comprehensive cancer treatment for patients who came from each corner of the country.

4.2. Study design

Hospital based cross-sectional study was used as an appropriate study design.

4.3. Source population

All cancer patients attending Tikur Anbessa Specialized Hospital, Addis Ababa, Ethiopia.

4.4. Study population

Voluntary breast, colorectal and cervical cancer patients attending TASH during the study period.

4.5. Sampling technique

Convenient sampling technique was used and study participants who met inclusion criteria were selected for this study.

4.6. Sample size determination

Sample size determination was made by using G* Power statistical software for Windows (version 3.1.9.4) analyses with selection of Test family: F tests; Statistical test: ANOVA, fixed effects, omnibus, one-way and; type of power analysis: a priori: compute required sample size – given α, power, and effect size. By considering the assumption of: effect size 40%, power (1-β error probability) 95%, α-error probability 0.05, and number of groups 3; the minimum sample size calculated was 102. But to increase the validity of data we deliberately added 18 number of participants and the total sample size became 120 (40 breast cancer patients, 40 cervical cancer patients and 40 colorectal cancer patients).

4.7. Eligibility

4.7.1. Inclusion criteria

Breast, cervical and colorectal cancer patients of both sexes (≥18 years of age) with no prior chemotherapy, surgery, radiation, or hormonal therapy were included in this study.

4.7.2. Exclusion criteria

- ✓ Breast, colorectal and cervical cancer patients under any type of treatment (surgery, radiotherapy, hormonal therapy or chemotherapy).
- ✓ Breast, colorectal and cervical cancer patients with the age below 18 years old.
- ✓ The above-mentioned cancer patients with a known history of anemia, infection, viral or alcoholic hepatitis, CVD, Pregnancy, renal disorders and hemochromatosis.

4.8. Study variables

4.8.1. Dependent variables

- Serum ferritin level

- Serum hs-CRP level

4.8.2. Independent variables

- ✓ Socio-demographic characteristics (Age, sex, marital status, educational level, religion, residence, BMI).
- ✓ Behavioral factors (Alcohol consumption).
- ✓ Clinical characteristics (type of cancer and stage of cancer).

4.9. Operational definition

- hs-CRP concentration equal or exceeding 10 mg/L is considered to be marker of elevated inflammation on malignancy (Hart *et al.*, 2020)
- Normal range of serum ferritin in adult human is considered as, 30-200 µg/L (ng/mL) in Men and 13-150 µg/L (ng/mL) in Women (WHO, 2017).

4.10. Data and blood sample collection procedure

Each visitor who came to the oncology clinic during the study period was evaluated for eligibility criteria. After we obtained informed consent from each participants, socio-demographic characteristics, behavioral factors and clinical data from each eligible individual was gained through an interview of Amharic version structured questionnaire and medical chart of the participant. In parallel, 5 mL of venous blood was aseptically drawn from each participant by experienced oncology nurses and added it to serum separator tube (SST), which was properly labeled with participant identification code. After waiting for 30 minutes the blood inside SST was centrifuged at 4000rpm for 10 minutes. The serum obtained was then poured into 1.5 mL Eppendorf tube and kept at optimum temperature until required. Chemistry analysis was done at Ethiopian Public Health Institute (EPHI), National References Laboratory for Clinical Chemistry.

4.11. Data quality control and management

To keep the quality and reproducibility of the data, collected samples were checked every day by principal investigator for its completeness and remaining sample was kept in the secure and biochemically convenient place. Biochemical test of hsCRP and ferritin was made after internal quality control was done (IQC) on calibrated machines, COBAS c 501 and COBAS e 411 rack system respectively. Each test strictly followed standard operation procedure of the laboratory and done by well experienced laboratory professionals. The laboratory received ISO 15189 standard accreditation certificate from Ethiopian National Accreditation Office (ENAO) in 2017. Coding, entering, verification and cleaning of data was performed with the utmost care by the investigator.

4.11.1. Test principles of the laboratory analyte

Measurement of ferritin

Current study used electrochemiluminescence immunoassay “ECLIA” method on cobas e immunoassay analyzer. This method uses different test principles such as sandwich, competitive and bridging for measurement, but the most commonly used one for assessing ferritin concentration is competitive principle.

Test principle

Sandwich principle: Total duration of assay: 18 minutes.

- First incubation: by incubating 10 µL of sample, a monoclonal ferritin-specific antibody labeled with a ruthenium and biotinylated monoclonal ferritin-specific antibody form a sandwich complex.
- Second incubation: by adding streptavidin-coated microparticles, sandwich complex bound to solid phase through biotin and streptavidin interaction.
- The mixture of reaction extracted into measuring cell, where microparticles magnetically trapped onto electrode surface.
- Substances which unbound to electrode surface are then eliminated by ProCell/ProCell M.
- Then, voltage application on electrode induce chemiluminescent emission and photomultiplier measure it at 410nm.
- Result are determined with calibration curve, which is generated by 2-point calibration and master curve provided through reagent barcode.

The analyzer automatically calculates the analyte concentration of each sample either in µg/L or ng/ml. (EPHI reference, 2015).

Measurement of hsCRP

This study used Roche CRP turbidity assay based on the principle of particle-enhanced immunological agglutination. In turbidimetry, cause of turbidity of sample is existence of antigen-antibody complexes. The method uses a beam of light that passes through turbid sample and measure transmitted light intensity through the sample (Unscattered light) to assess the concentration of antigen in the sample.

Test principle: Particle enhanced immunoturbidimetric assay.
Human CRP agglutinates with latex particles coated with monoclonal anti-CRP antibodies. The precipitate is determined turbidimetrically at 540 ± 20nm.

The analyzer automatically calculates the analyte concentration of each sample either in mg/Lor mg/dL (Price et al., 1987; Eda et al., 1998).

Conversion factors: mg/L* 9.52= nmol/L

mg/L*0.1= mg/dL

4.12. Data processing and analysis

Data obtained from study population was entered into Microsoft excel and then imported and analyzed by using SPSS version 25. Descriptive analysis such as minimum, maximum, range, mean, quartiles, median, frequency, standard deviation and percentage were used to display socio-demographic characteristics and magnitude of hsCRP and serum ferritin levels. Mann-Whitney U test was used to define median differences of variables. Data with normal distribution was shown by mean ± standard deviation while non-normal distribution data was presented with median (Interquartile). P-values less than 0.05 were considered to be statistically significant.

4.13. Ethical consideration

Participants were enrolled into this study after explaining the purpose and aims of the study, and written informed consent was obtained from each participant before starting the questionnaire and sample collection. Ethical approval was attained from Research and Ethics Committee of the Department of Medical Biochemistry, School of Medicine, College of Health Sciences, Addis Ababa with Ref No. SOM/BCHM/089/2012 and protocol No. M.Sc. 03/20. Formal letter calling for collaboration was written from department of Biochemistry to oncology unit of TASH. Confidentiality of information provided in questionnaire and laboratory result was strictly maintained.

5.RESULT

5.1. Socio-demographic characteristics of breast, cervical and colorectal cancer patients.

A total of 120 study participants (40 patients in each cancer type), aged between 27-80 years with no prior treatment were enrolled to the current study. Among 120 total sample population, 28 (23.33%) of them were from rural and 92 (76.67%) were from urban. Details of the sociodemographic data are shown in Table 1. The mean ±SD of participants' age was 50.83 ± 13.97, 50.83 ± 11.61 and 48.90 ± 13.31 for breast, cervical and colorectal cancer individuals respectively. Based on participants' marital status, married patients were greater in number in each type of cancer, which was 26(65%) on breast cancer, 23(57.5%) on cervical cancer and 28(70%) on CRC. From total study participants, cancer patients who do not use alcohol were 73(60.83%), have past alcohol history were 30(25%) and those who use alcohol currently were 17(14.16%). Among the cancers cases, 27.5% of breast, 7.5% of cervical, and 20% of CRC have a family history. Larger number of cancer patients were observed on stage II(n=56) and stage III(n=50) whereas, small number of cancer patients were seen on stage I(n=2) and stage IV (n=12).

Table 1. Socio-demographic characteristics of breast, cervical and colorectal cancer patients.

Variables	Breast cancer (n=40)	Cervical cancer (n=40)	Colorectal cancer (n=40)
Sex			
Female	39	40	31
Male	1	-	9
Age			
≤ 47	21	16	20
> 47	19	24	20
Marital status			
Single	0	3	3
Married	26	23	28
Divorced	1	1	3
Widowed	13	13	6
Residence			
Rural	9	8	11
Urban	31	32	29

Education status			
Illiterate	23	36	26
Literate	17	4	14
BMI			
< 25	36	36	39
25-30	4	4	1
> 30	0		0
Family History			
Yes	11	3	8
No	29	37	32
Alcohol use			
Never	23	25	25
Past	13	7	10
Current	4	8	5
Stage of cancer			
I	2	0	0
II	24	10	22
III	12	22	16
IV	2	8	2

5.2. hsCRP level on breast, cervical and colorectal cancer.

Among the study participants, elevated level of serum hsCRP was measured on 22.5% of breast cancer, 50% of cervical cancer and 37.2% of colorectal cancer. Based on the type of cancer, higher number of cancer patients with elevated level of hsCRP was observed on cervical cancer (n=20), which was followed by colorectal (n=15) and breast (n=9) cancer.

Table 2. hsCRP amount among breast, cervical and colorectal cancer.

Cancer type	**< 10 mg/L**		**≥ 10 mg/L**	
	Count	Percent	Count	Percent
Breast (n=40)	31	77.50%	9	22.5%
Cervical (n=40)	20	50%	20	50%
Colorectal (n=40)	25	25%	15	37.2%

The highest hsCRP value quantified on breast cancer was 186.6 mg/L, cervical cancer (185.41 mg/L) and CRC (320 mg/L). The median and IQR of breast cancer calculated was 2.78mg/L and 3.46 mg/L respectively. But on cervical cancer it was 9.785mg/L (median) and 26.47 mg/L (interquartile). Similarly, median and IQR of colorectal cancer was 4.89 mg/L and 32.54 mg/L respectively.

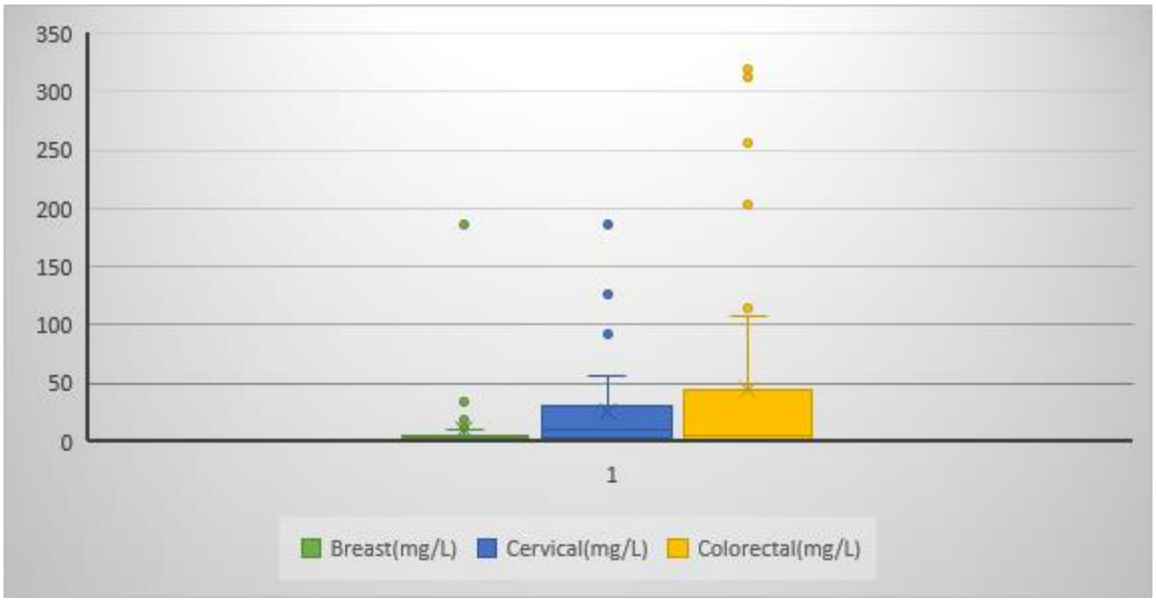

Figure 6. Comparison of hsCRP level based on the type of cancer, TASH, Addis Ababa, Ethiopia, 2020.

5.3. Serum ferritin level in breast, cervical and colorectal cancer.

Normal range of serum ferritin in adult human is considered as 30-200 µg/L (ng/mL) in Men and 13-150 µg/L (ng/mL) in Women. From a total of 40 breast cancer sample population, 5 female patients (12.5%) showed an elevated serum ferritin level. Similarly, in cervical cancer high level of serum ferritin was observed on 12 female patients (30%) out of 40 study participants since it is exclusively women disease. In colorectal cancer, high levels of ferritin were seen on 17 patients (42.5%) out of 40 patients, which were 13 from female (32.5%) and 4 from male (10%).

Table 3. Serum ferritin status based on the type of the cancer.

Sex	Ferritin level (ng/mL)	Breast (n=40)	Cervical (n=40)	Colorectal (n=40)
Female	< 13	0	3(7.5%)	0
	13-150	34(85%)	25(62.5%)	18(45%)
	>150	5(12.5%)	12(30%)	13(32.5%)
Male	< 30	0	-	3(7.5%)
	30-200	1(2.5%)	-	2(5%)
	> 200	0	-	4(10%)
Elevated serum ferritin in both sex		5(12.5%)	12(30%)	17(42.5%)

The minimum level of serum ferritin quantified on breast, cervical and colorectal cancer was 21.24ng/mL,10.6 ng/mL and 14.37 ng/mL respectively. But the maximum serum ferritin level observed on breast, cervical and colorectal cancer was 1261 ng/mL, 1304 ng/mL and 1698 ng/mL respectively. The median of serum ferritin measured on breast cancer was 142.5 ng/mL, cervical cancer (98.18 ng/mL) and CRC (125.85 ng/mL). From Quartile values measured, Q3 observed on breast, cervical and colorectal cancer was 216 ng/mL, 203.43 ng/mL and 273.75 ng/mL respectively.

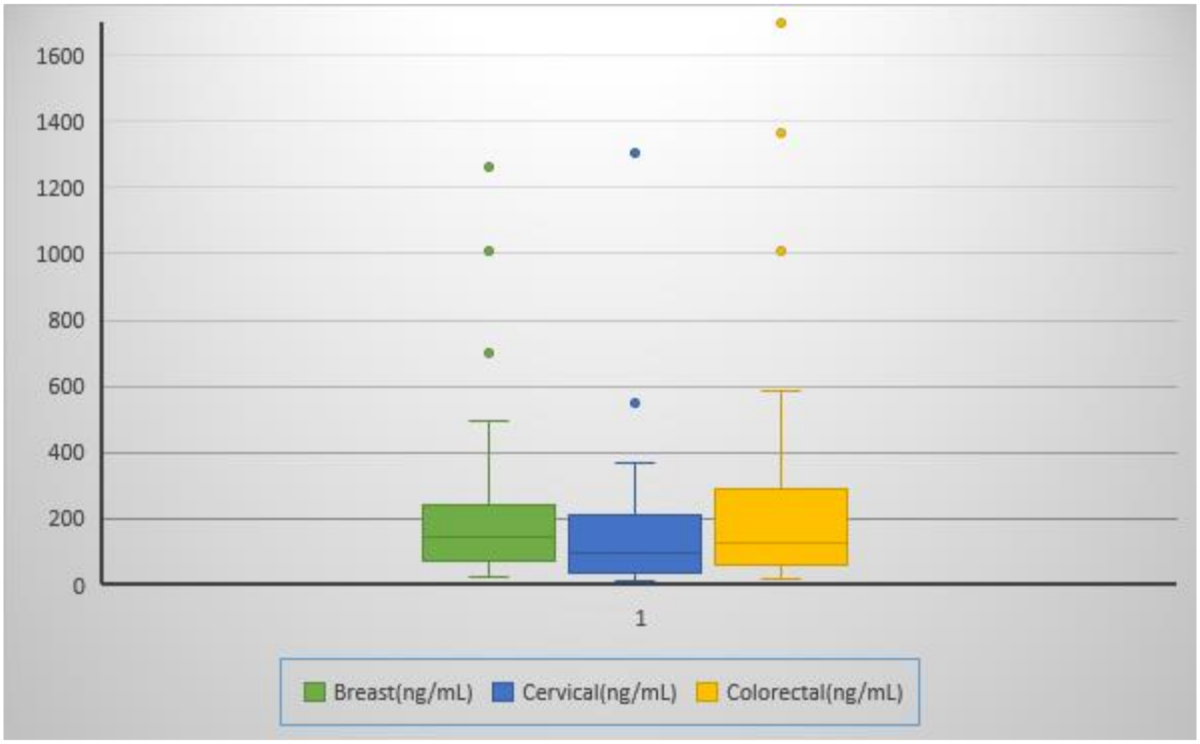

Figure 7. Comparison of serum ferritin level based on the type of malignancy, TASH, Addis Ababa, Ethiopia, 2020.

5.4. hsCRP and ferritin level based on stages of cancer.

The highest hs-CRP level observed on stage I, II, III and IV was 5.68mg/L, 257.05mg/L, 312.92mg/L and 320.19mg/L respectively. In other word, the highest amount of hsCRP was recorded on stage IV cancer patients that is followed by stage III, I and II. The highest median (interquartile) value of hs-CRP was noticed on stage IV cancer (15.9 (4.83-40.93) mg/L) and smallest amount of hs-CRP median (interquartile) was recorded on stage II cancer (3.39(1.48-10.83) mg/L). But the median (interquartile) value measured on stage III was 5.2(1.88-20.81) mg/L respectively.

Relative to other cancer stages, the highest value of serum ferritin (1698ng/ml) was observed on stage II. The highest computation of serum ferritin median (interquartile) value was recorded on stage II cancer (127.7(57.35-220.98) ng/ml), which is followed by stage III (121.25(57.68-230.2) ng/ml) and stage IV (103.77(59.01-166.63) ng/ml).

Table 4. Serum hsCRP and ferritin level in relation to cancer stage.

Stage of cancer		hsCRP(mg/L)	Ferritin(ng/mL)
I(n=2)	Minimum	3.07	57.75
	Maximum	5.68	69.93
	Median(IQR)	-	-
II(n=56)	Minimum	0.28	10.6
	Maximum	257.05	1698
	Median(IQR)	3.39(1.48-10.83)	127.7(57.35-220.98)
III(n=50)	Minimum	0.13	11.94
	Maximum	312.92	1304
	Median(IQR)	5.2(1.88-20.81)	121.25(57.68-230.2)
IV(12)	Minimum	0.46	31.28
	Maximum	320.19	340.4
	Median(IQR)	15.9(4.83-40.93)	103.77(59.01-166.63)

5.5. Comparison of median serum level of hsCRP and ferritin in cancer types

Patients were divided into three groups based on the type of malignancy. The median difference of hsCRP level among three cancer cases was statistically significant (p=0.003). Between cancer types, the lower median level was observed on breast cancer patients whereas the highest median value of hsCRP was observed in cervical cancer. But, the median difference of serum ferritin observed among breast, cervical and colorectal cancer was statistically insignificant (p=0.172).

Table 5. Comparison of hsCRP and serum ferritin median in types of cancer.

Variables	Breast (n=40)	Cervical l(n=40)	Colorectal (n=40)	p-value
hsCRP(mg/L)	2.78	9.78	4.89	0.003
Ferritin(ng/ml)	142.5	98.175	125.85	0.172

*Kruskal- Wallis H test. The significance level is 0.05.

5.6. Comparison of median serum level of hsCRP and ferritin on stages of cancer

As shown on table 6, patients were classified according to the stages of cancer to compare the median value of serum ferritin and hs-CRP. Since Kruskal- Wallis H test is used to determine difference of median of three or more groups, it was applied on this study. The median difference observed on both serum hsCRP (p=0.259) and ferritin (p=0.702) was statistically insignificant.

Table 6. Comparison of hsCRP and serum ferritin median in cancer stages.

Variables	Stage I(n=2)	Stage II(n=56)	Stage III(n=50)	Stage IV(n=12)	P-value
hsCRP(mg/L)	-	3.39	5.2	15.89	0.259
Ferritin(ng/ml)	-	127.7	121.25	103.77	0.702

*Kruskal- Wallis H test. The significance level is 0.05.

6. DISCUSSION

Inflammation related to cancer has been proposed since the mid-19th century and it was stated that chronic inflammation is an indicator of cancer or its site is the origin of cancer (Heikkilä *et al.*, 2007). In addition to other biomarkers, serum ferritin and hsCRP were proposed as an inflammatory marker in different types of malignancy (Coventry *et al*.,2009; Alkhateeb, and Connor, 2013) though strong evidence and clear biochemical justification from various studies is yet to come. To fill the gap and to observe serum ferritin and hsCRP can be used as potential diagnostic biomarker of cancer, this study was conducted in Tikur Anbessa Specialized Hospital with the aim of assessing hsCRP and serum ferritin levels among breast, cervical and colorectal cancer patients before taking any type of treatment.

Coventry and his colleagues reported that increased hsCRP was observed in many types of cancers (Coventry *et al*.,2009). Shilpa and his friends showed, higher hsCRP level observed in breast cancer patients than control healthy individuals (Shilpa *et al*., 2014). In study from Wang and sun, hsCRP level was reported higher in malignant patients than healthy individuals (Wang and Sun, 2009). The result of this study indicated that 22.5% of breast cancer patients had elevated hsCRP level, which is in agreement with report of Allin and Nordestgaard, 2011; Han *et al*., 2011; Pierce et al., 2009; Ravishankaran and Karunanithi, 2011. This observation could be because of persistent inflammatory state of cancer due to vicious cycle and complex interplay between inflammation and cancer (Hanahan and Weinberg, 2011).

CRP, most commonly used inflammatory marker, has been examined in a number of human cancer as prognostic parameter (Brown *et al*., 2006; Helzlsouer *et al*., 2006; McSorley *et al*., 2007; Siemes *et al*., 2006; Tadahiro *et al*., 1998). A study conducted in Austria showed that patients with cervical cancer has high CRP, thus the study concluded that serum CRP could be used as independent prognostic parameter (Stephan *et al*., 2007). A study conducted in Japan by Tehadiro and his colleagues showed that the preoperative serum CRP was elevated on 41.7% of cervical cancer patients (Tehadiro *et al*., 1998). The result of current study showed that percentage of cervical cancer patients with elevated hsCRP level was 50%, which is in line with a report of Sproston *et al*., 1995; Fujiwaki *et al*., 2003; Srivani *et al*., 2003; McSorley *et al*., 2007. Elevation of hsCRP in cervical cancer might be due to genetic factors like inflammation-induced cytokines, which play significant role in cervical tumorigenesis, progression and prognosis (Kang *et al.,* 2007). Besides to this, inflammatory cells which mediates tumor microenvironment increases CRP level in cervical cancer (Coussens and Werb, 2002).

In contrast to other oncogenic parameters, which are expensive and require well-equipped laboratory, serum CRP can be analyzed more easily, cheaply and perform as routine parameter for inflammation diagnosis (Tehadiro *et al.,* 1998). Studies have been indicated that hs-CRP is much higher in cancer patients than in healthy subjects (Zhang *et al.,* 2010). Heikkilä and his colleagues done meta-analysis of 12 prospective studies, which showed that elevated hs-CRP was associated with various types of malignancies including colorectal cancer (Heikkilä *et al.,* 2009). The result of current study showed that 37.2% of colorectal cancer patients have elevated hsCRP level, which is in concordance with a study of Cooper *et al.,* 1979; Otsuji *et al.,* 1982; Gurleyik *et al.,* 1995; Yuceyar *et al.,* 1996. The higher synthesis of CRP in colorectal cancer was due to the high production of pro-inflammatory cytokines such as interleukin-6, interleukin-1 and tumor necrosis factor (Castell *et al.,* 1990).

Tissue (cytosolic) ferritin within breast cancer tissue showed six-fold higher than ferritin in normal/benign breast tissue (Weinstein *et al.,* 1982; Elliott *et al.,* 1993). Additionally, proteins of transferrin and transferrin receptor level were greater in breast cancer tissue in comparison to normal/benign breast tissue (Faulk *et al.,* 1980; Marcus and Zinberg, 1975; Rossiello *et al.,* 1984). Marcus and Zinberg informed that 41% of preoperative breast cancer women have elevated serum ferritin compared to healthy women, in whom serum ferritin level was normal (Marcus and Zinberg, 1975). The result of current study indicated that 12.5 % of breast cancer patients have elevated serum ferritin, which is in line with a report of Faulk *et al.,* 1980; Elliott *et al.,* 1993; Ionescu *et al.,* 2006; Marcus and Zinberg, 1975; Rossiello *et al.,* 1984; Reizenstein, 1991; Weinberg, 1996; Weinstein *et al.,* 1982. This is because macrophages and hepatocytes produce serum ferritin in response to inflammatory cytokines such as TNF-α and IL-1β, which activate NF-κB pathway (Alkhateeb *et al.,* 2013). Another reason of higher ferritin level in breast cancer is high expression of heme importing proteins like cluster of differentiation 163 (CD163) (Shabo et al., 2008).

Since iron has important role in DNA synthesis and proliferation, tumor cells need iron more than normal cells because of its high regenerative capacity (Alkhateeb *et al.,* 2013). Serum ferritin is synthesized from macrophages and hepatocytes and involve in tumor angiogenesis and proliferation, besides to storing iron and maintaining iron homeostasis (Tingting *et al.*, 2017; Koyama *et al.*, 2017; Facciorusso *et al.*, 2014; Le and Richardson, 2002). Serum ferritin is also capable to bind high molecular weight kininogen (HMWK), thus inhibit its antiangiogenic effect (Coffman *et al.*, 2009). The result of current study showed that 30 % of cervical cancer patients have raised serum ferritin. Elevation of serum ferritin in this finding could be because of its significant role in cancer development through modulation of immunosuppression, angiogenesis and cell proliferation and/or degradation of IRP due to production of pro-inflammatory mediators from tumor-associated macrophages (Li, 2010; Alkhateeb, and Connor, 2013). Besides to this, production of cytokines within tumor cell increases serum ferritin (Tingting *et al*., 2017).

Cancer cells exhibit alteration of iron metabolism and have high affinity for iron. Because of high iron uptake, cancer cells have aberrant iron storage and trafficking, including higher expression of serum ferritin (Torti, 2020). Current study showed that 42.5% of colorectal cancer patients have elevated serum ferritin, which is in agreement with report of Smith *et al*., 2003; Lee *et al.* 2004; Balder *et al.*, 2006; Kabat *et al.* 2007; Cross *et al.* 2010; Zhang *et al.* 2011; Hara *et al.* 2012; Parks, 2017, which showed that over expression of ferritin was observed in colorectal cancer. This is due to abnormally high expression of iron importing proteins like dimetal transporter 1 (DMT1) and heme importers such as; heme responsive gene 1 (HRG1), heme carrier protein 1 (HCP1), cluster of differentiation 91(CD91), and cluster of differentiation (CD163) (Xing *et al*, 2016; Ma *et al.*, 2018; Fiorito *et al.*, 2019). Iron imported through DMT1 activated cyclin-dependent kinase 1 (CDK1) and JAK1/STAT1 and involved in colorectal tumorigenesis whereas inhibition of DMT1 hinder tumor growth (Xue *et al.*, 2016). Another reason of higher ferritin level in colorectal cancer patients is because of overexpression of six-transmembrane epithelial antigen of prostate (STEAP) reductases 1 and 2, enzymes which reduce ferric iron to bioactive ferrous iron, which were involved in proliferation of cancer cell and resistance of apoptosis (Moreaux *et al*., 2012).

hsCRP level was found increased with the stage of cancer (Gockel *et al*., 2006; Guillem and Triboulet, 2005; Gunter *et al*.,2006; Hashimoto *et al*.,2005; Miyata *et al*., 2001; Shilpa *et al*., 2014; Stephan *et al*., 2007; Tadahiro *et al*.,1998). For example, the mean ± SD of stage I breast cancer patients was 1.34 ± 0.7mg/L, which is increased to 3.6 ± 1.4 mg/L in stage III breast cancer patients (Shilpa *et al.*, 2014). In our study, the median (interquartile) value of stage I and stage II cancer is recorded as 4.38(3.72-5.02) mg/L and 3.39(1.48-10.83) mg/L respectively. Reduction of hsCRP through stage I to stage II on this study might be because of wide variation of participants' number on these two stages, which was 2 and 56 respectively. Starting from stage II malignant patients, median (interquartile) value of hsCRP was consecutively raised in stage III and IV cancer patients in a dramatic pattern. This finding was in agreement with previous studies (Tadahiro *et al*.,1998; Stephan *et al*., 2007; Shilpa *et al*., 2014) that where higher hsCRP level was observed at higher stages of cancer. Serum ferritin among stage I, II, III and IV cancer patient was given as 63.84(60.79-66.88) ng/ml, 127.7(57.35-220.98) ng/ml, 121.25(57.68-230.2) and 103.77(59.01-166.63) ng/ml respectively. This finding is in concordance with study conducted by Guner and his colleagues in Turkey (Guner *et al*.,1992; Koyama et al., 2017; Facciorusso et al., 2014; Parks, 2017; Fiorito et al., 2019; Xing et al, 2016; Ma et al., 2018).

7. CONCLUSION

Based on the finding of this study, serum ferritin and hsCRP levels were increased in breast, cervical and colorectal cancer patients prior to treatment. Thus, we believe that physicians and clinicians can use serum ferritin and hsCRP as potential diagnostic markers on mentioned cancer cases. In addition, the result of current study showed that serum ferritin together with hsCRP can be used as inflammatory biomarkers for breast, cervical and colorectal cancer. Last but not least, we believe that this study will encourage researchers to engage in further research on this field.

8. STRENGTH AND LIMITATION OF THE STUDY

8.1. Strength of the study

This study has several limitations, thus it did not answer different questions, but we believe that it might attracts the attention of medical researchers and clinicians. Though the period of data collection was during the outbreak of COVID-19, we overcame the challenge by following recommended preventive measures in both sample collection and laboratory works. The present study on assessment of serum ferritin and hsCRP level was conducted in Ethiopia for the first time, thus it provides base-line information for further studies on prognosis or diagnosis of breast, cervical and colorectal malignancy.

8.2. Limitation of the study

The first limitation of this study was that it is conducted on small sample size with cross sectional study design. Second, age-matched individuals without breast, cervical and colorectal cancer as a control group were not included in this study. Third, association of ferritin structural subunit with breast, cervical and colorectal cancer was not observed due to unavailability of instrument and financial constraint.

9. RECOMMENDATIONS

Based on our finding, there is variation of serum ferritin and hs-CRP level in cancer cases and stages. Thus, we recommend researchers to observe the strength of association and details of biochemical mechanisms of two parameters with three cancer types and each cancer stage by using suitable research design and large sample size. We encourage researchers to look into whether serum ferritin and hsCRP can be used as prognostic marker in each cancer case. We would also recommend further studies should distinguish which structural ferritin was strongly link with each cancer cases and each cancer stages.

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11. ANNEXES

Annex I: Information sheet (English version)

You are kindly invited to take part in this study, which is supervised by the Department Medical Biochemistry, School of Medicine, College of Health Sciences, Addis Ababa University. Please take the time to read the following information and respond to each question. You have full right to not take part in the study if don't want to involve in it.

Research title: Assessment of serum ferritin and hs-CRP as diagnostic marker among breast, cervical and colorectal cancer patients attending TASH, AA, Ethiopia.

Sponsoring organization: Addis Ababa University, College of Health Science, Department of Medical Biochemistry.

Principal Investigator: Amanuel Wotera

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Advisors:

Dr. Maria Degef

Dr. Solomon Tebeje Gizaw

Dr. Wondimagegn Tigeneh

Procedure and things that will be expected from participants; If you agree to take part in this study, you will be given the consent form to sign, and interviewed by the principal investigator to assess whether you qualify to participate in the study or not. If you are fit for the study, the data collector will ask some questions which are important for the study and collect a blood sample for laboratory examination.

Potential Risks: You will be requested to give a 5 mL blood sample from your left upper arm by an experienced laboratory technician. You may have minor discomfort and pain during blood drawing and there may also be mild redness, or swelling on the site where blood is drawn. But this is minor and will resolve on the next couple of minutes. The whole procedure will be carried out by health professionals through standard clinical practice, so participating in this study has no major risks. Unfortunately, if anything happened, appropriate medical care will be provided for you.

Potential Benefits; you will not receive any payment during participation in this study as compensation. The outcome of the result also will help to generate the information necessary for clinicians in early screening or diagnostics of malignancies. Hence, you are indirectly benefiting other societies in this respect.

Confidentiality; the participation is completely based on your willingness and you have the right to not participate. If you are agreeing to involve in this study, we respect your privacy and confidentiality. Any information that identifies you will not be shared with anyone else outside the study team. Logbooks used in the laboratory will have no names but codes. Thus, your honest and genuine participation in this study is very important & highly appreciated.

Consent form (English version)

This is MSc student at Addis Ababa University, College of Health Sciences, Department of Medical Biochemistry and currently conducting research entitled "**assessment of the serum ferritin and hs-CRP as diagnostic marker among Breast, Cervical and Colorectal cancer patients attended Tikur Ambessa Referral Hospital**". This study has been approved by the Ethical and Review Committee of the Department of Medical Biochemistry in Addis Ababa University.

I understood the information of this study that has been explained to me by the data collector. I also understood the risk and benefit of participation in this study.

I am agree to participate in the study and here I approve my agreement with my signature.
Participant's signature______________ Date___________
Investigator's signature___________ Date_________ questionnaire code________

Thank you for your priceless support!

Questionnaires (English version)

Questionnaire identification code______, Cancer type_____________,

Date of interview_____________

S.No	Variables	Response	Remark
Part I: Sociodemographic characteristics			
101	Sex	1. Male 2. Female	
102	Age (yrs.)	1. < 50 2. 50-59 3.60-69 4. ≥ 70	
103	Marital status	1. Single 3. Divorced 2. Married 4. Widowed	
104	Residence	1. Rural 2. Urban	
105	Educational status	1. Illiterate 2. High school 3. Diploma & above	
106	BMI(Kg/m^2)	1.< 25 2. 25-30 3. ≥ 30	
107	Family history of malignancy?	1. Yes 2. No	
108	Alcohol consumption	1. Never 2. past 3. Current	
Part III: Clinical characteristics			
301	Type of cancer	1. Breast 2. Cervical 3. Colorectal	
302	Stage of cancer	1. Stage I 3. Stage III 2. Stage II 4. Stage IV	

Annexe II: **የታሳታፍ ፍቃድና መተማመኛ ቅፅ**

ሰላም ጤና ይስጥልኝ አማኑኤል ወተራ እባላለሁ። በአዲስ አበባ ዩኒቨርሲቲ ጤና ሳይንስ ኮሌጅ በባዮኬሚስትር ትምህርት ክፍል የሁለተኛ ዲግር ተመራቂ ተማር ስሆን የዚህ ጥናት ራዕስ "Assessment of serum ferritin and hs-CRP as diagnostic marker among breast, cervical and colorectal cancer patients attended at TASH, AA, Ethiopia" የሚል ነዉ።

እባክዎ በዚህ ጥናት ዉስጥ ለመሳተፍ ከመስማማትዎ በፍት ቀጥለዉ የምገኘዉን ሀሳብ በጥሞና ያንብቡና ግልፅ ያልሆነ ካለ ይጠይቁ።

የእርስዎ በዚህ ጥናት ላይ የሚኖርዎት ተሳትፎ ሙሉ በሙሉ በግል ፈቃደኝነት ላይ የተመሰረተ ሲሆን ለመሳተፍ አልያም ላለመሳተፍ ቢወስኑ እንኳ በዚህ ሆስፒታል ዉስጥ የሚያገኙት ማንኛዉም አገልግሎት አይቋረጥም።

የጥናቱ ተሳታፊ ከሆኑ የሚጠበቅበዎት

በዚህ ጥናት ለመሳተፍ ፈቃዶዎ ከሆኔ አንዳንድ ቃለ መጠይቆችን ለመመለስና በስተመጨረሻ ሁለት የሻይ ማንክያ ያክል የደም ናሙና ለመስጠት መስማማት ይጠበቅቦታል።

በዚህ ጥናት መሳተፍ የሚያስገኘዉ ጥቅም

ይህ ጥናት የሁለተኛ ዲግር መመረቂያ ፅሁፍ እንደመሆኑ መጠን የምከፈልዎት ገንዘብ ባይኖርም በዉጤቱ ተጠቃም ነዎት። ምክንያቱም የእርስዎ ተሳትፎ በእርስዎና በወገነዎ ደም ዉስጥ የሚገኝ Serum Ferritin እና hs-CRP የተሰኘ ፒሮትን መጠን ከጡት ካንሰር፣ ከማኽፀን ካንሰር፣ አሊያም ከትልቅ አንጀት ካንስር ጋር ያለዉን ተያያዥነት እና ስጋት ለጤና ባለሙያዎች ስለሚያሳይ እንደ እርሶ ያሉ ታካሚዎችን በአማራጭ መንገድ ይበልጥ እንዲረዱት ያግዛል።

በዚህ ጥናት መሳተፍ የሚያስክትለዉ ጉዳት

መጠነኛ የደም ናሙና ከእጆዎ በምወሰድበት ጊዜ አነስተኛ የህመም ስሜት ሊኖር ይችላል። ነገር ግን ናሙናዉ የሚወሰደዉ በከፍተኛ ጥንቃቄ ልምድ ባለዉ ባለሙያ ስለሆኔ ህመሙ በጥቂት ደቂቃዎች ዉስጥ ይተዋል።

የሚስጥር አጠባበቅ ሁኔታ

ከእርስዎ የሚገኘዉ መረጃና ናሙና ጥቅም ላይ የሚዉለዉ ለጥናቱ አላማ ብቻ ነዉ። ቀጥሎም የተሰበሰበዉ መረጃ ለስራዉ አግባቢነት ላላቸዉ ጥቂት ሰዎች ብቻ የሚደርስ ስሆን የራስዎን ማንነት የሚገልጡ መረጃዎች ማለትም ስም ፤ አድራሻ ፤ የስልክ ቁጥር እና የመሳሰሉትን ግን አያካትትም። ይልቁንም ለዚህ አገልግሎት ብቻ የሚዉል እርስዎን ለመለየት የምረዳ መለያ ቁጥር ጥቅም ላይ ይዉላል።

የፈቃደኝነት ቅፅ

ከላይ የተፃፈዉን ሀሳብ ፤ የጥናቱን ዓላማ፤ በጥናቱ ዉስጥ በመሳተፍ የሚገኘዉን ጥቅምና ጉዳት በግልጽ ተረድቻለሁ።

ስለዚህ በዚህ ጥናት ዉስጥ ለመሳተፍ ፈቃደኛ ነኝ።

አዎ   አይደለሁም______________.

ፊርማ.

የአጥኚዉ ፍርማ.

መለያ ቁጥር .

ስለፊቃደዎ አመሰግናለሁ!!!

ቃለ - መጠይቅ (Amharic Version)

መለያ ቁጥር .   ቀን ________________________________.

ክፍል አንድ ፡ የማህበረሰብና ስነህዝብ ባህርያት በተመለከተ

101. ፆታ   1. ወንድ   2. ሴት

102. ዕድሜ   1. ከ46 በታች   2. ከ47 በላይ

103. የጋብቻ ሁኔታ   1. ያላገባ   2. ያገባ   3. የተለያየ   4. ባለቤቱን በሞት ያጣ

104. የመኖርያ ቦታ   1. ገጠር   2. ከተማ

105. የትምህርት ሁኔታ   1. አልተማርኩም   2. ሁለተኛ ደረጃ   3. ድፒሎምና ከዛ በላይ

106. BMI(Kg/m^2).

107. ከዚህ በፍት ከቤተሰብ/ከቤተ ዘመድ መካከል ካንሰር የታመመ አለ?   1. አዎ   2. አይ

108. አልኮል እጠቀማለሁ   1. በፍፁም   2. በፊት   3. አሁን

ክፍል ሶስት፡ የህክምና ምርመራ ዉጤት በተመለከተ

201. የካንሰሩ ዓይነት   1. የጡት   2. የማህፀን   3. የትልቅ አንጀት

202. የካንሰሩ ደረጃ   1. ደረጃ አንድ   2. ደረጃ ሁለት   3. ደረጃ ሶስት   4. ደረጃ አራት

Declaration

I, Amanuel wotera Wogga, declare that this research paper entitled "**Assessment of the serum ferritin and hs-CRP as diagnostic marker among Breast, Cervical and Colorectal cancer patients attended Tikur Ambessa Referral Hospital"**, is my original work and has not previously been submitted for any degree in any other University and all sources of materials used for this research have been fully acknowledged.

Amanuel Wotera Wogga:

Signature: ________________________________

Date: __________________________________