An-Najah National University

Faculty of Graduate Studies

CD44 EXPRESSION LEVELS AS A BIOMARKER FOR THE EARLY DETECTION OF VARIOUS CANCERS IN BIOLOGICAL SAMPLES: A CASE-CONTROL STUDY

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This thesis was Defended Successfully on 06/02/2024 and approved by

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Dedication

I dedicate my dissertation to the land that has nurtured my roots and the people who have shaped my journey, with the deepest gratitude and respect to Palestine, a land rich in history, resilience, and unwavering spirit. This work serves as a respectful tribute to the enduring spirit of a nation that flourishes through the resilience of its citizens. To the educators, healers, farmers, and visionaries who, despite challenges, work diligently to create a brighter future each day, and to every individual who embodies the spirit of Palestine—this is dedicated to you.

To my beloved family, whose endless love and support have been my guiding light in times of challenge and triumph, you are the foundation upon which my dreams are built.

This master's dissertation is dedicated to my beloved parents. Thank you to the unwavering support and encouragement of those who have been by my side; I have found my guiding light throughout this journey. Their dedication, expertise, and unwavering support helped develop me into who I am today.

I would like to express my sincere gratitude to my brother and sisters, whose consistent support, immense love, and unwavering confidence in me have been fundamental to my journey. Your companionship has provided significant comfort and joy, your wit has illuminated challenging times, and your robust work ethic serves as a lasting model of perseverance and commitment. Your contributions have motivated me to pursue excellence, maintain humility, and face challenges with both courage and determination. Your belief in my capabilities has strengthened my determination, and your support in my life serves as a continual reminder of the importance of family solidarity. This dissertation serves as a testament to my efforts and reflects the significant impact you have had on my personal and career development. I sincerely appreciate your sacrifices, encouragement, and steadfast belief in my potential. This project is equally your responsibility as it is mine.

To my family, for your love, guidance, and sacrifices, I am eternally grateful. This achievement is as much yours as it is mine.

To the resilient people of Palestine, who courageously face adversity and maintain hope amidst hardship. Your strength and perseverance inspire me to contribute meaningfully to our shared heritage and the global understanding of it.

This journey is not just my own but a reflection of our collective aspirations and enduring spirit. May this work contribute to the rich tapestry of Palestinian history and serve as a testament to the power of perseverance, education, and unwavering determination.

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Last, I would like to express my gratitude to the people and the land of Palestine for their unwavering inspiration and motivation throughout my academic journey. This journey has been a profound experience, encompassing both personal and collective growth.

Declaration

I, the undersigned, declare that I submitted the thesis entitled**:**

CD44 EXPRESSION LEVELS AS A BIOMARKER FOR THE EARLY DETECTION OF VARIOUS CANCERS IN BIOLOGICAL SAMPLES: A CASE-CONTROL STUDY

I declare that the work provided in this thesis, unless otherwise referenced, is the researcher's work, and has not been submitted elsewhere for any other degree or qualification.

Student's Name: **Samah Mohammad Ahmad Bsharat**

Signature: *Samah Bsharat*

Data: 6/2/2025

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CD44 EXPRESSION LEVELS AS A BIOMARKER FOR THE EARLY DETECTION OF VARIOUS CANCERS IN BIOLOGICAL SAMPLES: A CASE-CONTROL STUDY

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Abstract

Background: Cancer stem cells (CSCs) contribute to tumor progression, metastasis, and therapy resistance, with CD44 playing a key role in CSC function. As a transmembrane glycoprotein, CD44 interacts with ligands like hyaluronic acid, regulating adhesion, migration, and signaling pathways (Ras-MAPK/ERK). Its alternative splicing and modifications enhance functional diversity, promoting chemoresistance and metastasis. Elevated CD44 expression in aggressive cancers, including breast and colorectal malignancies, highlights its potential as a diagnostic biomarker and therapeutic target.

Given its role in tumor progression, this study aims to evaluate soluble CD44 as a biomarker for the early detection and differentiation of breast and colorectal cancer. By analyzing its expression levels in blood and urine across different cancer stages and types, this research seeks to establish CD44's diagnostic value and potential contribution to precision medicine in oncology.

Methods: This case-control study (2023–2024) examined CD44 expression as a biomarker for breast cancer (BC) and colorectal cancer (CRC) at three hospitals in Nablus, including 40 BC patients, 23 CRC patients, and 70 healthy controls. Blood and urine samples were analyzed for CD44 gene and soluble forms, with exosomal RNA quantified via real-time PCR (normalized to GAPDH) and soluble CD44 levels measured using ELISA. Statistical analyses (two-way ANOVA, t-tests, $p \leq 0.05$) confirmed significant differences, ensuring accuracy through standardized protocols.

Results: The research indicates a very strong statistically significant elevation in CD44 expression in breast cancer patients when compared to healthy controls ($p < 0.0001$) in both fold change (gene expression) and soluble CD44 (sCD44) serum and urine concentrations. Similarly, a moderate statistically significant elevation ($p < 0.01$) in

CD44 gene expression form and soluble form for CRC patients compared to healthy controls. Furthermore, there is a strong statistically significant difference in CD44 expression between patients with breast cancer and those with colorectal cancer ($p < 0.001$).

Conclusions: The study identified significantly elevated sCD44 levels in serum and urine of breast and colorectal cancer patients, supporting its potential as a cancer biomarker. In contrast, αFP showed minimal diagnostic value, with negligible serum changes and absence in urine, reinforcing its association with liver-related cancers.

Keywords: sCD44-Soluble CD44, breast cancer, colorectal cancer, case-control study, alpha-fetoprotein (αFP), Palestine.

Chapter one
Introduction and Background

1.1 Introduction and Background

1.1.1 Overview of Cancer Stem Cells and its role in tumor progression

Cancer is a complicated set of diseases characterized by the uncontrolled division and proliferation of aberrant cells within the body. It is one of the most formidable foes humanity has ever faced. Millions of people are terrified by this word, which stands for a wide variety of ailments that can afflict almost every tissue or organ. The body's natural regulatory systems are upset by cancer, which can result in the growth of cancerous tumors or the invasion of neighboring tissues and organs.

Cancer, in all its manifestations, is a major global health concern that affects individuals, families, and communities everywhere. The illness has no borders; it affects people of all ages, genders, races, and socioeconomic backgrounds. Its effects are felt in research facilities, healthcare systems, and the very fabric of societies; scientists, healthcare workers, and carers must remain committed to this field and innovate continuously (1).

One prevalent trait of tumors is their heterogeneity, wherein individual cancer cells within a tumor mass display different properties. This poses difficulties for disease pathogenesis and therapeutic approaches. Tumor metastasis, recurrence, progression, and initiation are all caused by cancer stem cells (CSCs) (2). CSCs, or cancer stem cells, are a subset of cells in a tumor that have characteristics of stem cells; this includes the capacity to self-renew, differentiate into many cell types, and initiate tumors. They also have the ability to adapt and survive in negative circumstances like hypoxia, starvation of nutrients, and radiation or chemotherapeutic exposure (3).

It is believed that CSCs developed as a result of either reprogramming differentiated cancer cells into a stem cell-like state or mutations in stem or progenitor cells (4).

The growth of CSCs has been linked to fibroblasts and extracellular matrix elements found in tumor microenvironments (TME), such as fibronectin, laminin, collagen, osteopontin, and hyaluronan. These elements create a supportive environment for CSCs to thrive and promote tumor progression (5). Finding the surface marker, the associated

intracellular signaling, and the interactions between the tumor microenvironment and CSCs may be crucial to developing novel treatment approaches for afflicted individuals. These indicators fall into two categories: surface markers and intracellular signaling pathways. Surface markers are known to be proteins or molecules expressed on the outside membrane of CSCs, acting as markers that set CSCs apart from other cancerous cells. They are also involved in cell signaling, supporting CSC survival and function. The most significant is CD166, alternatively referred to as Activated Leukocyte Cell Adhesion Molecule (ALCAM) (6), CD24, which is involved in immune evasion and metastatic dissemination, CD44, CD133 (Prominin-1), which is commonly linked to CSC in glioblastoma and colorectal cancer; it promotes the survival of cancer stem cells and is associated with resistance to apoptosis (7).

CD29 and EpCAM (Epithelial Cell Adhesion Molecule) contribute to the preservation of stem-like characteristics in epithelial cancers while promoting proliferation and migration (8). Of these markers, CD44 is the most widely used (9).

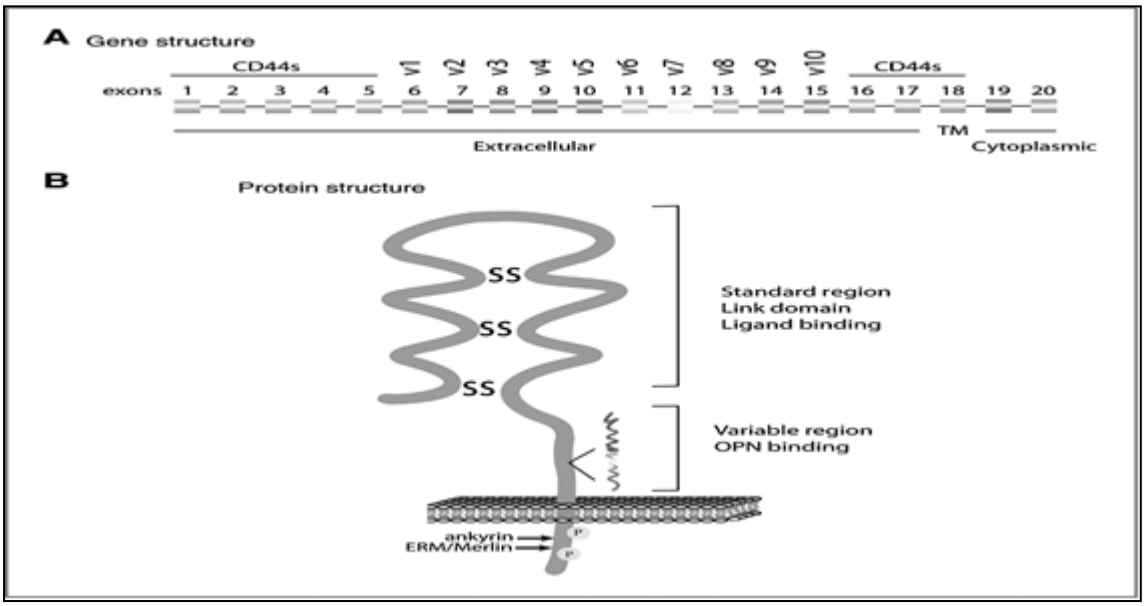
1.1.2 CD44: Structure, Ligands, and Functional Importance

CD44 (Cluster of Differentiation 44) was first discovered to be a glycoprotein expressed on human and mouse mesenchymal cells in the early 1980s. It was cloned later and identified as a member of the cartilage link protein family. Non-kinase cell surface transmembrane glycoprotein is encoded by a large, intricate gene that is highly conserved and found on either the human chromosome 11 or the mouse chromosome 2 (10).

There are 20 exons in total. Ten of them, called "constant" exons (exons 1–5 and 16–18), are expressed in all isoforms. These exons encode the nonvariant standard isoform or CD44s. The other 10 are central and are called "variable" exons. They go through a lot of different types of splicing of the CD44 pre-mRNA, where they are added or taken out in different ways in the membrane-proximal stem region to make splicing variants (CD44v isoforms), which demonstrate tissue-specific expression and specialized functions (2). Tissue- and environment-specific variables control variant expression.

Figure 1

Structure of the CD44 gene and protein



Source: Louderbough JM V, Schroeder JA. Understanding the Dual Nature of CD44 in Breast Cancer Progression. Mol Cancer Res [Internet]. 2011 Dec 1;9(12):1573–86. Available from: https://doi.org/10.1158/1541-7786.MCR-11-0156

The extracellular (consisting of the first 17 exons), stem, transmembrane, and short C-terminal intracellular/cytoplasmic sections make up the four primary domains of the CD44 protein. Its stem region has variable exons, and its extracellular domain comprises seven domains. Exons 10 or 9 encode the cytoplasmic area, whereas a single exon (exon 18) encodes the transmembrane domain (10).

CD44's extracellular domain is a highly adaptable and glycosylated area that facilitates essential interactions with extracellular matrix components, including hyaluronic acid (HA), osteopontin, and fibronectin, along with growth factors such as Vascular Endothelial Growth Factor (VEGF) and Hepatocyte Growth Factor (HGF). By these engagements, CD44 governs cellular processes such as adhesion, migration, and signal transduction, playing a crucial role in tissue homeostasis and in pathological conditions such as cancer and inflammation. Alternative splicing and post-translational modifications, including glycosylation, improve the functional variety of CD44, enabling it to adjust its ligand-binding characteristics to particular cellular environments. This extracellular domain is crucial for tumor progression, metastasis, and immune regulation (12).

The second domain of CD44 is the transmembrane domain (TMD), which consists of a single α-helical segment that includes roughly 23 hydrophobic amino acids. It is

responsible for anchoring the glycoprotein within the plasma membrane and facilitating its function as a receptor for hyaluronic acid and other ligands. The TMD is significantly preserved across various species and plays a crucial role in maintaining the structural integrity of CD44, facilitating its involvement in cell-extracellular matrix interactions and signal transduction processes. It is also integral to the oligomerization of CD44, which is vital for its role in cell adhesion and migration. Moreover, when TDM interacts with lipid rafts (which are specialized membrane microdomains), it affects the localization and activity of CD44, thereby influencing various cellular responses, including those related to tumor progression and metastasis (13).

The cytoplasmic domain of CD44, measuring around 70–75 amino acids, serves as an essential intracellular segment that connects extracellular signals to intracellular responses through its interaction with cytoskeletal proteins, like the ezrin-radixin-moesin (ERM) family, along with signaling molecules that participate in pathways such as Ras-MAPK/ERK (Ras-Mitogen-Activated Protein Kinase/Extracellular Signal-Regulated Kinase) and PI3K-Akt (Phosphoinositide 3-Kinase-Akt). This domain governs processes including cell adhesion, migration, and proliferation by anchoring CD44 to the actin cytoskeleton and facilitating dynamic cytoskeletal rearrangements. Phosphorylation and palmitoylation are post-translational modifications that modulate the function of CD44 by influencing signal transduction and lipid raft localization. The dysregulation of the cytoplasmic domain plays a significant role in various pathological conditions, such as cancer metastasis and inflammatory disorders (14).

Hyaluronan (HA) is the most prevalent and direct ligand for CD44, the membrane receptor CD44 variations' common ligand-binding domain for HA is shared by all isoforms (15). The negatively charged polysaccharide hyaluronan (also hyaluronate or hyaluronic acid), which is made up of 2,000–25,000 repeating glucuronic acid and N-acetylglucosamine disaccharide subunits, plays a critical role in the microenvironment of cancer cells, impacting both the prognosis and the course of the tumor (5). Through its interaction with CD44, the HA mediates nonreceptor kinases and Ras family GTPases, as well as activating and regulating many signaling domains inside the plasma membrane. Through this connection, adapter proteins like Vav2, Grb2, and Gab-1 are made easier to produce. These adapter proteins then go on to activate oncogenic pathways, including mitogen-activated protein kinases and PI3 kinases/Akt, which in

turn stimulate the growth, survival, migration, invasion, and chemoresistance of tumor cells. Chains of heparan sulfate may bind to regulatory growth factors and stimulate carcinogenic pathways. Moreover, the HA-CD44 connection activates metabolic and multidrug transporters, which enhance tumor cell invasion and motility (15).

Many malignancies, including those of the breast, lung, colon, prostate, and ovary, have been shown to express CD44 aberrantly.

As mentioned previously, CD44 is a cell surface molecule, so proteolytic enzymatic processes have the ability to release or shed CD44 into the bloodstream (16). As a result, both plasma and cell culture supernatants contain soluble CD44. Because of this bloodstream-based CD44 shedding, soluble CD44 levels in a variety of biological materials can be found and measured. Given that increased levels of soluble CD44 have been linked to a number of illnesses and ailments, including cancer and inflammation, this is very helpful in clinical settings. Additionally, researching soluble CD44 can shed important new light on how CD44 functions in various physiological and pathological processes. This can lead to a deeper understanding of the role of CD44 in health and disease (16).

1.1.3 Introduction to Breast and Colorectal Cancer

Breast cancer is a serious malignant disease that arises from the cells within breast tissue. This cancer is among the most common globally and stands as the primary cause of cancer-related fatalities in women. Breast cancer primarily impacts women, though it can also manifest in men, though infrequently. About 1% of all breast cancer cases in the United States are identified in men, according to the American Cancer Society, 2024. The median age at diagnosis for men is 69 years, with risk escalating as men grow older. This risk is raised by several factors like family history of breast cancer and radiation exposure (17).

Breast cancer is a complex, multifactorial disease. Its development is affected by several factors, like genetic predispositions, including mutations in the BRCA1 and BRCA2 genes; unbalanced hormones, such as extended exposure to estrogen; AND environmental and lifestyle factors like obesity, smoking, and drinking alcohol (18).

Mutations in BRCA1 and BRCA2 are significant factors in hereditary breast cancer, accounting for 2–4% of all breast cancer cases worldwide. They interfere with essential DNA repair mechanisms, resulting in genomic instability and the development of aggressive tumor traits. Breast cancers associated with BRCA1 are generally characterized by high-grade pathology, absence of hormone receptors such as ER and PR, and frequently display aggressive traits, including the overexpression of p53, cyclin E, and basal epithelial markers like cytokeratin 5/6. In contrast, cancers associated with BRCA2 frequently exhibit similarities to non-hereditary breast cancers, including elevated hormone receptor expression and unique morphological traits, such as increased tubule formation and reduced mitotic activity (19).

Cancer detection, especially in its early stages, remains one of the most critical steps in the fight against cancer; when cancer is detected early, this improves survival rates and enhances the quality of patients' lives. For many years, breast cancer detection relied on several methods: mammography, which is considered the standard screening instrument for detecting breast cancer, works by utilizing a low dose of X-rays to generate images of the breast, facilitating the identification of tumors and abnormalities. It is advisable for women over 40 to undergo regular mammograms, as these screenings can identify cancer at an early stage when treatment options are most effective (20).

Ultrasound (US) technique is based on employing sound waves to generate images of the internal structures of the breast. It is especially effective in differentiating solid masses from fluid-filled cysts and acts as an additional resource to mammography, particularly for women with dense breast tissue (21). Digital breast tomosynthesis (DBT), or 3D mammography, is a technique that works by acquiring various images of the breast from multiple perspectives and then synthesizing them into a three-dimensional representation (22). Magnetic resonance imaging (MRI) technique works by using nonionizing electromagnetic radiation and seems to be free from exposure-related hazards. It utilizes radio frequency (RF) radiation alongside meticulously regulated magnetic fields to generate high-quality cross-sectional images of the body in any orientation. This method is frequently employed for patients at high risk, delivering detailed images capable of identifying cancers that may not be detectable via mammography or ultrasound (23). Molecular Breast Imaging (MBI) relies on administering a minimal quantity of radioactive tracer that is preferentially absorbed by

cancerous cells compared to healthy cells, then a specialized camera identifies these regions, emphasizing potential tumors (24).

Histopathological imaging is the standard method for diagnosing breast cancer, where hematoxylin and eosin (H&E) are used to stain the extracted tissue to maximize visibility prior to imaging analysis (25). However, each of these methods has benefits and drawbacks of its own.

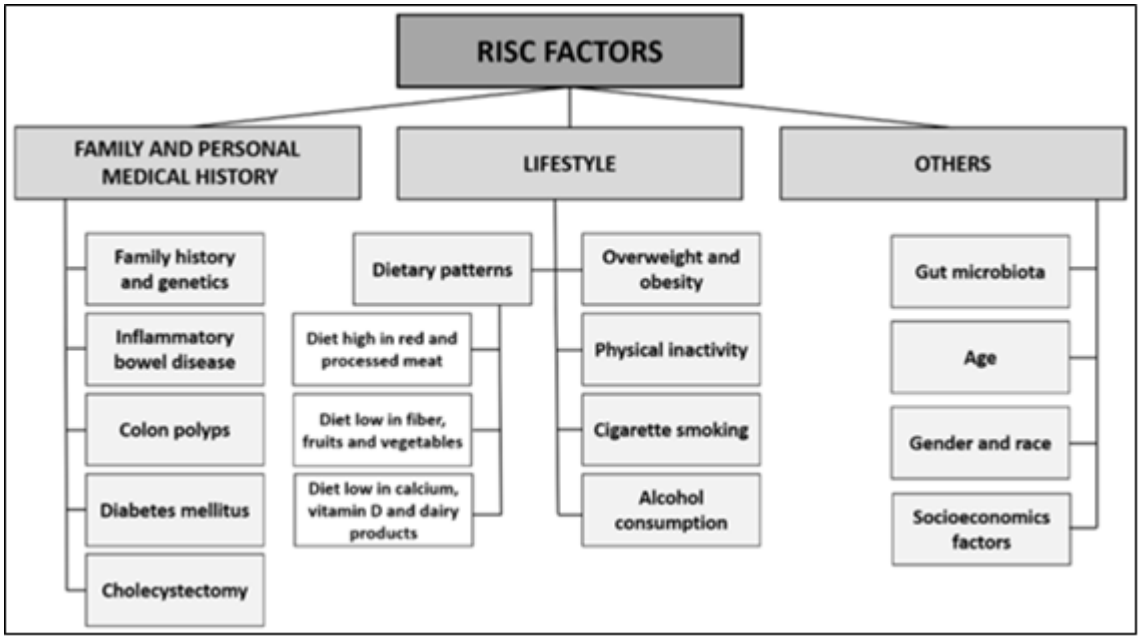
Biomarkers can also offer insights into tumor characteristics and inform treatment decisions. These biomarkers include estrogen receptor (ER) and progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), Ki-67, circulating tumor DNA (ctDNA), CA 15-3, and CA 27-29 (26), (27).

Colorectal cancer can result from a mix of environmental and genetic causes. In Palestine, CRC represents a serious health issue, being the second most prevalent cancer and a primary contributor to cancer-related deaths. It represented 13.9% of all cancer-related fatalities in the region in the year 2020 (28). However, its incidence rate is not the same in all areas of Palestine, with an incidence rate of 15.3 per 100,000 individuals in the West Bank and Jerusalem and a 10.2 per 100,000 incidence rate for people in the Gaza Strip (29).

Researchers have discovered that the incidence of colorectal cancer (CRC) is much higher in nations that are embracing the "Western" way of life. This is because variables such as obesity, a sedentary lifestyle, and the intake of red meat, alcohol, and tobacco are thought to be the main causes of CRC. Figure 2 lists additional factors that have been linked to the development of colorectal cancer, such as colon polyps, inflammatory bowel illnesses, diabetes, or cholecystectomy (30).

Figure 2

Factors that have been linked to the emergence of colorectal cancer



Source: Sawicki T, Ruszkowska M, Danielewicz A, Niedźwiedzka E, Arłukowicz T, Przybyłowicz KE. A Review of Colorectal Cancer in Terms of Epidemiology, Risk Factors, Development, Symptoms and Diagnosis. Vol. 13, Cancers. 2021

Colorectal cancer (CRC) diagnosis requires a multimodal approach. Its identification and assessment depend on a blend of screening tests, sophisticated imaging methods, and histological evaluation.

Primary screening tests are used to catch the disease in its early stages, even before the onset of symptoms. These methods include the Fecal Occult Blood Test (FOBT), which is an easy non-invasive method based on detecting the hidden blood in stool samples. This test is essential in the early detection of CRC. Its advantages rely on its non-invasiveness, cost-effectiveness, availability, and reduction of CRC mortality because of regular screening and detection the disease before the emergence of symptoms (31).

Another non-invasive method for screening and detecting CRC in the early stages is the stool DNA test (sDNA), this test is based on examining the genetic material that is released from the surface of tumors or pre-cancerous polyps into the stool. It is a highly sensitive and convenient method; however, it is considered expensive and costs much when compared with other screening methods like FOB, and it requires confirmatory colonoscopy when positive (32).

Colonoscopy is an essential imaging method for the diagnosis of colorectal cancer (CRC) because it enables tumor identification, biopsies, and inspection of the entire large intestine. Modern technology improves the efficacy of colonoscopies, such as computer-aided diagnostic systems. In some circumstances, non-invasive techniques like computed tomographic colonography (CTC) and colon capsule endoscopy (CCE) are options. Further diagnostic procedures include sigmoidoscopy, CT colonography (virtual colonoscopy), genetic biomarker analysis, and individualized chemotherapy based on genetic variables (30).

Early and accurate diagnosis is essential for improving cancer survival rates by choosing appropriate treatments and lowering the risk of cancer metastasis. And so, finding more clinical biomarkers is essential to further categorize patients, provide more details about the initial diagnosis, and track the course, metastasis, and recurrence of the disease.

The continuous fight against cancer is a fundamental human struggle as much as a scientific one. It requires the fortitude of those receiving the diagnosis, the empathy of those offering support, and the tenacity of scientists and medical professionals working toward a day when cancer is no longer a fatal illness.

1.2 Problem statement

Cancer is still a major worldwide health concern. According to WHO in 2022, around 20 million new cancer cases were identified, resulting in 9.7 million deaths worldwide. The incidence is anticipated to increase in the forthcoming decades. It is expected that by 2050 the annual number of new cancer cases will reach 35 million, representing a 77% increase from the figures recorded in 2022s (33).

Regarding statistics in Palestine, cancer represented around 14% of all fatalities in 2020, positioning it as the second leading cause of death, following cardiovascular diseases, which comprised 30.6% of deaths. From 2000 to 2016, there was a 136% increase in cancer mortality, followed by an additional 14% rise from 2016 to 2020 (34). The most common form of cancer among Palestinian women is breast cancer, constituting a notable share of cancer diagnoses and fatalities, accounting for 35.6% of all documented cases of cancer among females (35). Palestine also demonstrates elevated incidence and mortality rates in comparison to the regional averages for Western Asia, with 46.6 per

100,000 females as the incident rate and 16.0 per 100,000 females as the mortality rate (28). These elevated rates in Palestine indicate significant demographic and health system challenges that necessitate prompt attention.

In 2018, breast cancer emerged as the third leading cause of cancer-related fatalities in Palestine, representing 12% of total cancer deaths. Lung cancer accounted for 20%, followed by colon cancer at 13% (36).

In the context of CRC, its incidence rate in the West Bank and Jerusalem is approximately 15.2 per 100,000 individuals, whereas in the Gaza Strip, it is recorded at 11.5 per 100,000. Colorectal cancer represents approximately 13.9% of all cancer-related fatalities in the region (37).

Researchers must take these statistics on breast cancer and CRC morbidity and mortality into consideration for formulating strategies grounded in evidence.

The benchmark for breast cancer screening continues to be mammography. However, its availability in Palestine is limited. There are many obstacles related to the use of mammograms as a screening tool for breast cancer: these machines have limited availability, there are insufficient numbers of trained radiologists, and the irregular upkeep of current equipment obstructs extensive implementation.

Regarding colorectal cancer diagnosis, colonoscopy is the standard diagnostic tool. Its use also has many limitations. First, the procedure is costly and invasive. It is also not readily accessible in numerous regions of Palestine. This procedure is invasive and needs to be performed by qualified gastroenterologists, and the number of experts in this domain is restricted.

Histopathological examination also has some limitations; the shortage of sophisticated pathology laboratories hinders the diagnosis.

When talking about Palestine, is considered a developing country and one of the third-world countries with high poverty, high unemployment, and a lack of natural resources. Palestine is under Israeli occupation that enforces considerable limitations on economic growth and development, including constraints on movement, settlement activities, and

resource management. So, these procedures are considered highly expensive for most Palestinians and difficult to access sometimes.

Alternative, cost-effective, less invasive screening methods are the goal that should be the attention of researchers to bridge the gap in the diagnosis and treatment of cancer.

CD44 which is a cell surface glycoprotein, is engaged in many cellular processes like cell adhesion, migration, and signaling pathways. It is involved in cancer growth and metastasis. Our study focuses on measuring its soluble levels in biological samples such as blood and urine as an early diagnostic marker for BC and CRC. This could be a suitable non-invasive, easily accessible screening method, especially in limited resource areas such as Palestine.

1.3 Purpose of the study

This study aims to examine the potential of soluble CD44 expression levels as a biomarker for the early detection of breast and colorectal cancer in different biological samples like blood and urine.

1.4 Specific Objectives

1. To examine CD44 expression levels in blood and urine from patients diagnosed with colorectal and breast cancer and healthy individuals.
2. To analyze the variations in CD44 expression levels among different stages of breast cancer.
3. To compare the expression level of soluble CD44 across BC and CRC. And differentiate its role in these cancers.
4. To quantify alpha-fetoprotein (AFP) levels in blood and urine samples from cases and control groups.
5. To compare the expression level of soluble CD44 in the blood and urine of BC patients.

1.5 Study hypothesis

H11. There is a significant difference in soluble CD44 expression levels in the blood of breast cancer patients and healthy controls.

H12. There is a significant difference in soluble CD44 expression levels between CRC patients' serum and healthy controls.

H13. There is a significant difference in soluble CD44 levels in breast cancer patients' urine and the urine of healthy controls.

H14. There is a significant difference in alpha-fetoprotein levels between cancer groups (BC and CRC) and the healthy group.

1.6 Research Questions

1. Is there a difference in sCD44 expression level between cancer patients (BC and CRC) and healthy participants?
2. How do CD44 expression levels in biological samples serve as a reliable tool for the early detection of different types of cancers?
3. What relationship exists between the stage of cancer and CD44 expression patterns in breast cancer?
4. Can we consider alpha-fetoprotein an essential biomarker in BC and CRC?

1.7 Importance of the study

To transform cancer diagnosis and treatment, research on CD44 expression levels as a tool for early colorectal and breast cancer detection is essential. In the context of cancer prevention and healthcare developments, this research challenge is extremely important because of its potential to improve patient outcomes, lower healthcare costs, and impact global health.

One of the most important aspects of successful cancer treatment is early detection. When CD44 expression is identified as a valid biomarker, patients with breast and colorectal malignancies may benefit from early detection, prompt therapies, and higher survival rates. Investigators seek to detect the existence of cancerous tissues or cells prior to the development of tumors that may be seen or felt.

Early identification also makes less-invasive treatments and higher treatment success rates possible. Patients may need fewer, more targeted therapies if they use CD44 expression as an early marker, which will improve their overall health and quality of life while also avoiding the need for protracted treatments.

By understanding the levels of CD44 expression, we can enhance the personalized treatment paradigm. Treatments can be more effectively administered and possibly have fewer adverse effects if they are customized based on particular biomarkers, which guarantees that patients receive therapies most appropriate for their cancer profile.

Unfortunately, the presence of visible tumors or the need for intrusive procedures are common requirements of conventional diagnostic approaches, which can lead to delayed diagnosis and worse prognoses. And if CD44 works as intended, it could make non-invasive diagnostic techniques like blood tests possible. Regular screenings for early cancer detection are now more accessible and less intrusive because of non-invasive diagnostics, which also increase patient comfort, compliance, and the overall effectiveness of healthcare services.

When CD44-based early detection techniques are successfully applied, the financial and medical costs related to cancer can be greatly decreased. Early case detection allows for more effective use of healthcare resources, which reduces costs significantly and improves public health outcomes overall.

The disparity in cancer outcomes across different locations can be closed by implementing an early detection method that is accessible, inexpensive, and effective worldwide. This research could significantly reduce global inequities in cancer-related morbidity and mortality rates by providing precise and accessible diagnostic techniques.

1.8 Literature review

With more than 200 different forms and 1500 fatalities each day worldwide, cancer has a poor prognosis and late diagnosis rates, which contribute to low survival rates. Since traditional techniques like ultrasound and biopsy rely on the phenotypic characteristics of the tumor, they are ineffective for early detection. Therefore, there is a need for more accurate and non-invasive methods for the early detection of cancer (38).

Nucleic acids, proteins, metabolites, isoenzymes, and hormones are examples of biomarkers—molecules that experience substantial alterations over the course of cancer. They are categorized as prognostic (used to inform about disease recurrence), predictive (used to estimate treatment response), and diagnostic (used for the detection of the disease). Alterations in particular biomarkers frequently signal the onset of cancer, so they might be useful in the early monitoring and diagnosis of the disease's course. These biomarkers are found in tissues, blood, or other body fluids (39). Serum protein biomarkers like AFP and CD44 are extensively utilized for cancer staging and assessing treatment response (40) (41).

Alpha-fetoprotein (AFP) is mainly linked to liver cancer and specific germ cell tumors. The elevation in breast cancer and CRC patients is rare and is not generally utilized as a biomarker for this condition. However, Villacampa et al. did research to examine the existence of alpha-fetoprotein (AFP) receptors in a human breast cancer cell line, indicating a possible involvement of AFP in the biology of breast cancer. His research establishes a direct correlation between AFP and breast cancer through the detection of specific AFP receptors on MCF-7 breast cancer cells. The receptors facilitate the binding and uptake of AFP, potentially impacting cellular processes like lipid transport. He also demonstrated that the presence of these receptors could act as a potential diagnostic marker for detecting malignant transformation in breast cancer (42).

Bennett et al. said that AFP has demonstrated the ability to inhibit the growth of estrogen receptor-positive tumors by disrupting estrogen-stimulated signaling, despite not directly binding to estrogen receptors (43). A reaction product of AFP and estradiol has been reported by Jacobson HI (44) to suppress the growth of estrogen-dependent breast cancer xenografts. Bennett et al. also observed that the administration of AFP at a dosage of 100 µg/mouse/day significantly suppressed the growth of estrogen-dependent human breast cancer xenografts.

Alpha-fetoprotein (AFP) is a recognized serum biomarker; however, its effectiveness as a urinary biomarker is still being explored. A 2023 review in Cancer Cell International analyzes various urinary biomarkers, including AFP, linked to hepatocellular carcinoma (HCC). This article emphasizes that effective urinary biomarkers must exhibit specific characteristics: low molecular weight (≤ 20 kDa), suitable electric charge, cancer specificity, and adequate concentration for reliable detection. Considering that AFP is a

sizable glycoprotein (approximately 70 kDa), its filtration into urine may be restricted, which could diminish its utility as a urinary biomarker (45).

There is a distinct category of malignancies, known as AFP-producing tumors, has the capability to secrete AFP even when they arise from non-hepatic tissues. The clinical significance of these tumors arises from their aggressive nature and the challenges they present in diagnosis. These tumors can be distinguished by the production of ectopic AFP, frequently associated with hepatoid differentiation, wherein tumor cells resemble liver tissue. Histopathological examination and AFP immunohistochemical staining are essential for diagnosis, with cytoplasmic AFP positivity indicating the source. These tumors encompass gastrointestinal and urological cancers (46).

Colorectal cancer (CRC) that produces AFP is extremely uncommon, and research on this topic is limited, but its propensity for liver metastases and quick progression emphasizes the importance of early identification and careful pathological assessment.

An article entitled "Alpha-fetoprotein-producing early rectal carcinoma: a rare case report and review." described a 41-year-old woman diagnosed with AFP-producing early rectal carcinoma, representing the first documented case of early-stage detection of rectal cancer that produces AFP. Histopathological analysis indicated that the carcinoma demonstrated hepatoid differentiation. This case showed that full resection and early diagnosis can have a beneficial effect since the tumor was restricted locally in contrast to most AFP-producing tumors, and this enabled total resection and a positive prognosis (47).

CD44 is a well-known CSC (cancer stem cell) marker that takes part in the control of numerous cellular processes, such as adhesion, migration, and cell division. It is also crucial for the development of tumors, invasion, and metastasis, as well as the reaction to therapy. CD44 expression may be relevant as a biomarker for breast cancer, as its expression level was found to be elevated at both the protein and mRNA levels in breast cancer compared to normal breast tissues (48). It has also been found that the quantity of CD44 protein in high-grade breast cancer tissues was noticeably greater than that in low-grade tissue, in line with a study conducted by Hanxiao Xu (49). CD44 is also correlated with decreased disease-free survival and distant metastases (10). This

demonstrates that patients with high CD44 protein may not have a good prognosis, and high-grade tumors are more likely to return quickly and with more aggression.

Among the groundbreaking research on the correlation between the prognosis of cancer and CD44 expression is the G0nthert et al. study. First, this research revealed that CD44 variants with an extracellular domain were uniquely present in metastatic tumor cells, while they were not found in non-metastatic cells or normal tissues. And, over-expression of the CD44 variant in non-metastatic tumor cells resulted in their transformation into highly metastatic cells. The study further revealed that the extracellular domain in the CD44 variant facilitates interactions with elements of the extracellular matrix and various cellular structures. The interactions contribute to cell adhesion, invasion, and migration, all of which are essential for metastasis. This domain probably interacts with particular ligands, facilitating the shift of tumor cells toward a more aggressive phenotype (50).

CD44 may have a role in the development of breast cancer tumors, as its level is elevated in the serum of breast cancer patients. It is advised that sCD44 detection in peripheral blood is considered a promising technique that may have predictive value in the treatment of breast cancer patients, particularly following tumor removal, as soluble CD44 found inside the cancer sufferers' bloodstreams most likely originated from tumor cells rather than typical cells. So increased levels of serum CD44 in cancer sufferers could be a result of ongoing tumor cells secreting CD44 molecules. This is supported by Sanaa A. El-Benhawy, who proved that there is a substantial positive correlation between tissue and serum CD44 (48).

Sebastian Mayer found in his study that breast cancer patients had notably elevated levels of sCD44v6 in their serum when compared to healthy controls. Furthermore, increased sCD44v6 levels correlated with larger tumor sizes and a greater occurrence of lymph node metastasis. The findings indicate that sCD44v6 is indicative of aggressive tumor behavior and may signify potential disruptions in the tumor's nuclear splicing machinery (51).

Jong-Min Baek conducted a study that was the first to demonstrate a correlation between serum sCD44 levels and survival outcomes in HER2-positive breast cancer. His study showed that elevated serum levels of sCD44 were associated with advanced

tumor stages, as well as liver metastases, in patients with breast cancer. Although sCD44 levels did not serve as predictors for treatment response or recurrence-free survival, they were recognized as a significant prognostic marker for overall survival in patients with HER2-positive breast cancer, with elevated levels correlating with worse outcomes. No correlation was observed between sCD44 levels and overall survival in patients who were HER2-negative. sCD44 may serve as a significant biomarker for the identification of high-risk HER2-positive breast cancer patients, highlighting its correlation with aggressive tumor characteristics and metastatic capabilities. (52).

Carolin Lackner said that individuals diagnosed with breast cancer exhibited elevated serum levels of soluble CD44v5 and CD44v6 in comparison to their healthy counterparts. Increased levels of sCD44v6 were notably linked to liver and bone metastases, whereas sCD44v5 levels exhibited a more varied distribution among metastatic sites. This study also found that elevated expression of CD44v5 and CD44v6 in primary tumors was associated with increased serum levels of these variants in the majority of cases, indicating that their release into circulation may be enhanced by tumor-associated proteases or factors specific to metastatic sites (53).

Colorectal cancer survival varies according to stage. It was found that there was a 92% survival rate for stage I and 10% for stage IV. The 60–69-year age range has shown an improvement in survival, which has been linked to screening (54).

Twenty exons and nineteen introns make up the CD44 gene; stable exons (CD44s) encode the standard isoform, whereas intermediate exons (CD44v) encode variable isoforms. There are three isoforms of CD44 found in the human gut epithelium: CD44s, CD44v6, and CD44v4-10. The prognosis of patients with colorectal cancer (CRC) is adversely affected by the presence of CD44v6. Research indicates that CD44v6-expressing CRC cells cause metastatic lesions in recipient mice, emphasizing the function of this protein in invasion, metastasis, and colonization (55).

Asieh Sadeghi's investigation into CD44 expression revealed a surprising negative correlation between lymphatic invasion, CD44 expression, and the stage of colorectal cancer (CRC). In contrast to predictions, patients with positive lymphatic invasion had reduced local CD44 expression, and higher expression of CD44 was seen in the earlier stages of CRC, which could be caused by distinct CD44 isoforms expressed in cancer

stem-like cells (56). In the mentioned study, they focused on CD44 isoforms. Another research for Rina Fujiwara-Tani about the expression of CD47 and CD44 in colorectal cancer showed highly elevated CD44 levels in CRC when compared with normal tissues, as well as high CD44 expression is related to poor prognosis and greater rates of recurring (57) .

Qian Zhang performed an article titled “Pan-cancer and single-cell analyses identify CD44 as an immunotherapy response predictor and regulating macrophage polarization and tumor progression in colorectal cancer” that emphasizes the role of CD44 as a crucial regulator in colorectal cancer (CRC), affecting tumor progression and the tumor immune microenvironment. Elevated CD44 expression in colorectal cancer is linked to macrophage polarization, fostering a pro-tumor M2 phenotype, while the knockdown of CD44 leads to a shift in macrophages towards the anti-tumor M1 phenotype, resulting in a reduction of tumor cell proliferation, migration, and invasion. This highlights its essential role in tumor growth and metastasis. The study also showed that single-cell RNA sequencing indicates that CD44 is primarily expressed in malignant cells and tumor-associated macrophages, highlighting its dual function in promoting immune evasion and advancing tumor progression. These findings indicate that CD44 may serve as a promising target for therapeutic strategies designed to enhance immunotherapy responses and influence the tumor microenvironment in colorectal cancer (58).

Ziranu P highlights the complex function of CD44 in colorectal cancer (CRC), he also mentioned in his study the role of CD44 in tumor progression, metastasis, chemoresistance, and prognosis.

This study emphasizes that elevated CD44 expression is linked to advanced disease stages, unfavorable prognosis, and reduced relapse-free survival, whereas its absence in certain instances unexpectedly correlates with poorer outcomes. It also turned out that CD44 promotes chemoresistance through its interaction with signaling pathways such as PI3K/Akt and the upregulation of drug-resistance genes, establishing it as a significant factor in therapy failure. The findings of Ziranu P study highlight CD44's significant role as a regulator in CRC progression and its potential as a target for therapeutic interventions (59).

Urine can be used as a non-invasive diagnostic tool in conjunction with additional clinical assessments to enhance diagnostic precision. Tumor markers identified in urine originate from tumor cells or host responses and may include proteins, metabolites, or nucleic acids.

One of the frequently identified tumor markers in urine is NMP22 (Nuclear Matrix Protein 22). In bladder cancer patients it was found to be increased attributed to the release of nuclear matrix proteins from cancer cells. Duquesne et al demonstrated that the sensitivity of NMP22 as a urinary biomarker for bladder cancer detection varies significantly, ranging from 47% to 100%, while its specificity ranges from 60% to 90%, contingent upon the clinical context and the population under investigation, it even exhibits greater sensitivity than urinary cytology, especially in identifying low-grade urothelial tumors (60).

TMPRSS2-ERG Fusion Gene is a genetic marker identified in urine that signifies the presence of prostate cancer (61).

Methylated SEPT9 (mSEPT9) DNA is a biomarker primarily identified in blood for the screening of colorectal cancer (CRC). The exploration of its presence in urine has been conducted; however, research remains limited. Husain et al. (2017) conducted one of the significant research studies in the detection of circulating tumor DNA, including mSEPT9, in urine samples (62). This study illustrates the practicality of monitoring circulating tumor DNA (ctDNA) in urine to assess early tumor responses to therapy. This emphasizes a non-invasive approach for the real-time evaluation of treatment effectiveness.

Elevated levels of soluble CD44 (sCD44) in serum have garnered significant attention from researchers and studies regarding their potential as biomarkers for various cancers, including breast and colorectal cancer. Conversely, urinary sCD44 has been investigated in greater depth concerning bladder cancer.

Chapter Two
Materials and Methods

2.1 Study design, setting, and time

A case-control study was conducted at AL-Najah National University Hospital, Rafidia Hospital, and Al Watani Hospital in Nablus between 2023 and 2024. The study included patients diagnosed with breast cancer (BC) and colorectal cancer (CRC) as the case group, alongside a control group of healthy individuals, who were recruited from patients' companions or individuals coming to these hospitals for routine health check-ups matched for age and gender. (n=70 as the control group, 40 as the breast cancer group, and 23 as the colorectal cancer group). Urine and serum samples were collected from both groups to assess the expression levels of CD44 in the gene expression levels and soluble form. Standardized procedures were followed for sample collection, processing, and storage to ensure consistency and reliability in detecting gene expression levels across all participants.

2.2 Data Collection

Variables collected from study participants' files included demographic and clinical information such as age, gender, prior cancer diagnoses, other chronic conditions, cancer type, and stage at diagnosis for BC. Additionally, quantitative levels of CD44 expression were measured in the collected clinical follow-up data.

2.3 Inclusion and Exclusion Criteria

2.3.1 Inclusion Criteria

- Female patients diagnosed with primary BC.
- Age over 30 years.
- Patients aged over 30 years with a confirmed diagnosis of CRC (colorectal cancer)
- Conscious, in good mental health, and able to provide written informed consent.
- Healthy controls were confirmed to be free of BC, CRC, and other types of cancer.
- A newly diagnosed patient who has not started chemotherapy to make sure that CD44 expression levels accurately represent the disease's untreated state.

2.3.2 Exclusion Criteria

- Patients with coexisting degenerative diseases.
- Patients with metastatic cancer.
- Patients with other malignancies which could make it harder to tell if CD44 is a good predictor for the type of cancer being studied.
- Patients with active inflammatory conditions.
- Patients with co-existing diabetes mellitus (DM) or hypertension (HTN).
- People who have already undergone therapy for the cancer they currently have, since certain medications can change the expression levels of CD44.

2.4 Sample size and sampling technique

Random sampling techniques were employed to select participants while considering the constraints of the hospital settings and population availability. Patients were chosen randomly to minimize selection bias and ensure representativeness. The study included patients diagnosed with breast cancer (BC) and colorectal cancer (CRC) as the case group, comprising 40 BC patients and 23 CRC patients. A control group of 70 healthy individuals was matched with the case group based on age and gender to enable meaningful comparisons.

2.5 study variables

2.5.1 Dependent Variable

The main dependent variable in this study is the level of CD44 expression, which is measured quantitatively in the blood and urine samples collected from the participants.

2.5.2 Independent Variables

Cancer Types: This variable represents which type of cancer is being investigated.

Biological Samples: This indicates the type of biological samples that are gathered for analysis, serum or urine.

Clinical Stage of Cancer: Stage 0, Stage I, Stage II, Stage III, and Stage IV This variable represents the stage of cancer at the time of diagnosis for cancer-diagnosed participants.

2.6 Procedures

2.6.1 Total RNA extraction from exosomes

An essential first step in comprehending exosomes' function in intercellular communication and biomarker identification is the isolation of total RNA from them. By separating high-quality RNA from the lipid bilayer vesicles, this procedure makes transcriptomic analysis and diagnostic studies possible. This makes it possible for scientists to look into the molecular signatures found in exosomes, which may reveal important information about disease conditions and possible treatment targets.

2.6.1.1 Exosomes isolation from Urine and serum sample

Urine and serum samples were collected from participants and processed for exosome isolation. Initially, samples were centrifuged at a low speed (300 to 2,000 × g) for 10 minutes to remove cells and debris, yielding a supernatant containing potential exosomes. The supernatant was then filtered using a sterile 0.22 μm filter to remove larger particles and cysts. Following filtration, ultracentrifugation was performed at a high speed (100,000 to 120,000 × g) for 1 to 2 hours to pellet the exosomes. After centrifugation, the supernatant was carefully discarded to minimize disruption of the exosome pellet. The exosome pellet was resuspended in phosphate buffer saline (PBS) to maintain integrity and viability. Additional ultracentrifugation steps were performed to purify the sample further, effectively isolating the exosomes while removing residual contaminants. The final exosome preparation was stored under appropriate conditions for downstream analyses.

2.6.1.2 RNA isolation from exosomes

Exosomal RNA was purified using the EXORNEASY MIDI KIT (cat# 20-77144T QIAGEN, Germany) to isolate total RNA from urine and serum samples from BC, CRC patients, and healthy individuals according to the manufacturer’s protocol. The isolated exosome pellet was resuspended in a lysis buffer, allowing the release of RNA from the exosomes. Proteinase K was added to the resuspended exosomes to digest any protein contaminants. The exosome lysate was mixed with a phenol-chloroform solution and subjected to centrifugation, causing phase separation. The aqueous phase containing the RNA was carefully collected. The RNA-containing aqueous phase was combined with an equal volume of isopropanol or ethanol to precipitate the RNA. The RNA precipitate

was pelleted by centrifugation and washed with ethanol to remove impurities. The RNA pellet was air-dried or dried briefly under vacuum to remove residual ethanol. The RNA pellet was then resuspended in an appropriate buffer or RNase-free water to obtain the purified RNA. The concentration and quality of the isolated RNA will be assessed using Nanodrop (Thermo Fisher Scientific, United States) (RNA concentrations: 100 ng/ml-1000 ng/ml; purity: 80%-95%). Total RNA samples will be stored at -80°C until use.

2.6.1.3 cDNA synthesis

Complementary DNA (cDNA) was synthesized from mRNA samples using the miScript II RT Kit cat. No. 218161 (Qiagen, Germany) following the manufacturer's guidelines. RNA samples were treated with a reverse transcriptase enzyme and appropriate primers to initiate cDNA synthesis. RNA and reverse transcriptase enzymes were incubated at an optimal temperature to allow the reverse transcription reaction to occur. Nucleotides (dNTPs) will be added to provide the building blocks for cDNA synthesis. Reverse transcriptase enzyme catalyzed cDNA synthesis, using the RNA template as a guide. The reaction mixture was heated to inactivate the reverse transcriptase enzyme and stop cDNA synthesis. RNA template was then removed through treatment with an enzyme such as RNase H. The concentration and quality of the isolated cDNA were assessed using fluorometry (Thermo Fisher Scientific (USA)). Samples were stored at -20°C.

2.6.1.4 Real-time PCR

Real-time PCR was performed with TaqMan Fast Advanced Master Mix (cat# 4371130, Applied Biosystems) to quantify CD44 gene expression, which was normalized to the expression of GAPDH as housekeeping genes. The cycling conditions for RT–PCR using the Thermo Fisher One-Step RT–PCR Kit included an RT step for 30 min at 50°C and denaturation for 15 min at 95°C. Then, the reaction mixture was incubated for 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, followed by 72°C for 10 min. Data was analyzed using a QuantStudio™ 5 Real-Time PCR System (cat# A34322, Applied Biosystems).

2.6.2 sCD44 protein levels assessment using ELISA

Urine and serum samples from BC, CRC patients, and healthy individuals were centrifuged at 5000 rpm for 30 minutes at 4°C. According to the manufacturer's

protocols, sCD44 protein levels were measured using ELISA kits (Thermofisher; Cat# BMS209-2 Waltham, Massachusetts, USA). All reagents and samples were brought to room temperature (18-25°C) before use. A volume of 100 µL of each standard and sample was added to the appropriate wells and incubated for 2.5 hours at room temperature with gentle shaking. The solution was discarded, and the wells were washed 4 times with 1X wash solution; washing was performed by filling each well with wash buffer (300 µL) using a multichannel pipette or auto-washer. After washing, the liquid was completely removed at each step. One hundred (100 µL) of 1× prepared detection antibody was added to each well and incubated for 1 hour at room temperature with gentle shaking. One hundred microliters of a prepared streptavidin solution were added to each well and incubated for 45 minutes at room temperature with gentle shaking. One hundred (100 µL) of TMB One-Step Substrate Reagent (Item H) was added to each well and incubated for 30 minutes at room temperature in the dark with gentle shaking. Finally, 50 µL of Stop Solution (Item I) was added to each well. The absorbance at 450 nm was immediately read using an ELISA reader (Tecan M100 plate reader) Männedorf, Switzerland.

2.6.3 AFP levels assessment using ELISA

Alpha-fetoprotein (AFP) levels were determined using a Human AFP ELISA Kit (Thermo Fisher Scientific, USA, Cat. No. EHALFP). After being collected, serum samples were centrifuged for 10 minutes at 3000 rpm and kept at -20°C until analysis. The experiment was conducted using a 96-well microplate that had been pre-coated with anti-AFP antibodies. After adding 100 µL of diluted serum samples, controls, and AFP standards to each well, the wells were incubated for 60 minutes at 37°C. After that, the wells were cleaned three times using a wash buffer to get rid of any loose materials. After 30 minutes of incubation at 37°C with 100 µL of horseradish peroxidase (HRP)-conjugated secondary antibody, there were five washes. For color development, 100 µL of TMB substrate was added, and the mixture was incubated for 15 minutes at 37°C in the dark. 50 µL of stop solution (H_2SO_4) was added to stop the reaction, and a microplate reader was used to measure absorbance at 450 nm. By creating a standard curve using the established AFP standards, AFP concentrations were ascertained. Every sample was examined twice, and the test was deemed legitimate if the standard curve's correlation coefficient (R^2) was ≥ 0.99.

2.7 Statistical analysis

Differences were analyzed with two-way ANOVA for comparisons among multiple groups using GraphPad Prism 9.0 (GraphPad Software, La Jolla, CA). A p-value ≤ 0.05 according to a t-test indicated the statistical significance and was calculated as the difference in means between two variables. The results are presented as the mean ± SD.

2.8 Ethical Consideration

The study and procedures conformed with the ethical standards of the Institutional Review Board (IRB) at An-Najah National University (Approval No. Mas. Dec.2023/5). The researchers ensured that informed consent was given willingly and free from compulsion to all participants—cancer patients as well as healthy controls. They were completely aware of the nature, goals, potential dangers, and advantages of the research, protecting the confidentiality and privacy of participant data. To avoid unwanted access, all obtained data, including genetic and medical records, must be anonymized and securely kept, giving vulnerable groups—such as minors and economically disadvantaged groups—special safeguards. Participation and informed consent were sought in a morally and culturally appropriate way. We ensured that subjects endured as little discomfort or injury as possible during study procedures and sample collection. Throughout the investigation, we put the comfort and well-being of participants first. We maintained a rigorous and impartial study design, methods, and analysis while carrying out the research with scientific integrity. We did not alter or misconstrue results to further a specific goal. Everyone, regardless of financial class, race, or nationality, will be able to receive any benefits that result from the study, such as better diagnostic techniques. This ensures equity and justice in the results of healthcare. We conducted the study in accordance with the Declaration of Helsinki's recommendations for clinical practice.

Chapter Three

Results

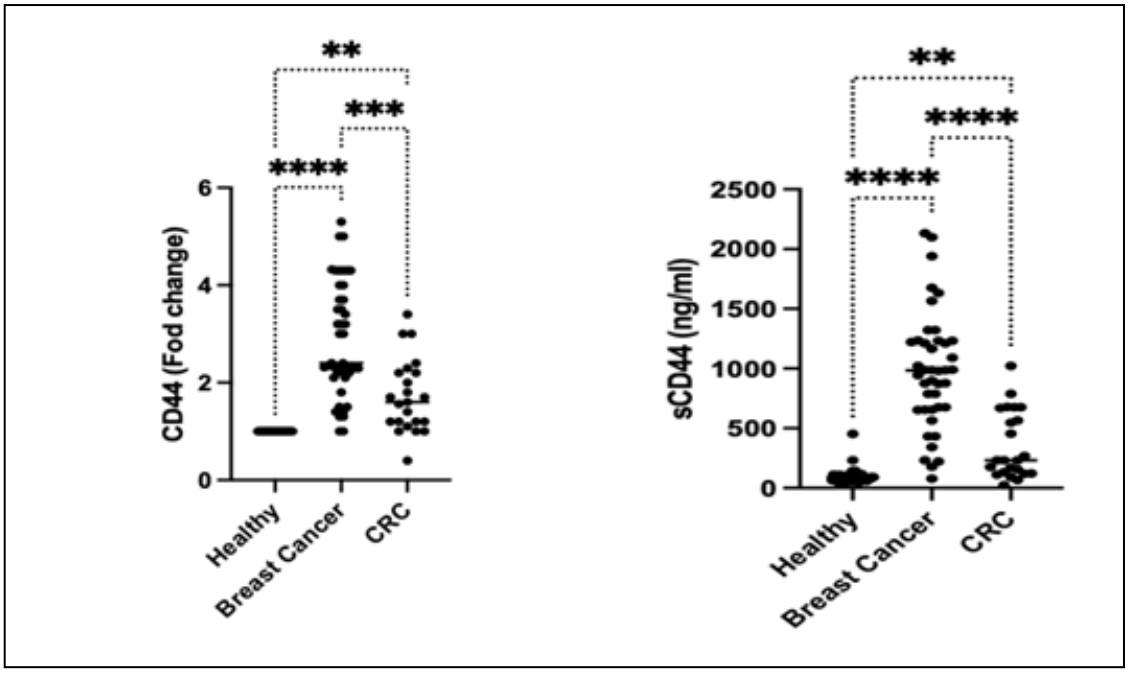
3.1 Introduction

The membrane glycoprotein CD44 is a potential biomarker for cancer diagnosis, prognosis, and disease monitoring since it is essential for cell adhesion, migration, and resistance to apoptosis. By contrasting these levels with those of healthy controls, the following findings offer a thorough examination of CD44 expression in blood and urine samples from patients with colorectal and breast cancer. Furthermore, the study assesses the significance of alpha-fetoprotein (αFP), which is frequently employed as a biomarker in liver and germ cell malignancies, across various cancer stages in serum and urine samples. The objective of this investigation is to improve our understanding of the diagnostic and prognostic importance of CD44 and αFP in patients with breast and colorectal cancer by looking at their expression patterns in these biological matrices.

3.2 Comparative Analysis of CD44 Expression in Healthy Individuals, Breast Cancer Patients, and Colorectal Cancer Patients

Figure 3

Expression Levels of CD44 in Healthy Controls, Breast Cancer Patients, and Colorectal Cancer (CRC) Patients



The figure illustrates a comparison of CD44 expression levels among three groups: healthy controls, patients diagnosed with breast cancer, and patients diagnosed with colorectal cancer (CRC). CD44 levels are assessed in two formats: as fold change (left panel) and as soluble CD44 (sCD44) concentrations in serum (ng/ml, right panel). The differences between groups are indicated by asterisks, with p-values represented as follows: **for $p < 0.01$, which refers to moderate statistical significance, *** for $p < 0.001$ refers to strong statistical significance, and **** for $p < 0.0001$, which means very strong statistical significance.

When compared to healthy controls, the figure shows that serum samples from people with colorectal cancer (CRC) and breast cancer have higher levels of CD44. The left panel indicates that CD44 in the gene expression level in healthy controls was remarkably low, nearly zero at the baseline. It turned out that there was a very strong statistically significant elevation in CD44 expression in breast cancer patients when compared to healthy controls ($p < 0.0001$) with a fourfold increase in CD44 expression in breast cancer patients relative to healthy controls. Furthermore, patients with colorectal cancer (CRC) exhibit a moderate statistically significant elevation in CD44 gene expression level when compared to healthy controls (**, $p < 0.01$) with numerous data points grouped above the 2-fold increase threshold. CD44 gene expression level is strongly statistically significantly elevated in breast cancer patients in comparison to those with CRC ($p < 0.001$), indicating a differential upregulation of CD44 in breast cancer that may be indicative of its unique tumor biology or microenvironment.

The right panel shows soluble CD44 (sCD44) levels in the serum, indicating that sCD44 levels in serum (ng/ml) are significantly increased in both breast cancer and CRC patients when compared to healthy controls. Patients with breast cancer demonstrate a very strong statistically significant elevation in sCD44 levels when compared to healthy controls ($p < 0.0001$). sCD44 is incredibly low in healthy people, with a median level of approximately 200 ng/ml, whereas in breast cancer patients, it is around 1500 ng/ml. This indicates a rise of 1300 ng/mL in sCD44 levels among breast cancer patients when compared to healthy controls. This represents a 650% increase in the sCD44 concentration in breast cancer patients relative to healthy individuals, indicating a significant rise in soluble CD44 (protein form of CD44) among those with breast cancer.

Similarly, patients with colorectal cancer also present moderate statistically significant higher sCD44 levels in comparison to healthy individuals ($p < 0.01$). With a median of approximately 1000 ng/ml, there is an increase of 800 ng/ml in sCD44 levels in CRC patients compared to healthy controls. This represents a 400% increase in sCD44 concentration in CRC patients relative to healthy individuals, highlighting a notable elevation in sCD44 associated with colorectal cancer.

Additionally, a very strong statistically significant difference has been noted between breast cancer and CRC patients, with breast cancer patients exhibiting elevated sCD44 levels (**** for $p < 0.0001$), suggesting that sCD44 may function as a more prominent biomarker in breast cancer relative to CRC.

3.3 CD44 Overexpression in Breast Cancer Serum and Cancer Score

Table 3.1

CD44 overexpression in Breast cancer serum associated with cancer score

No.	Sample Code	sCD44 (ng/ml)	a-FP (ng/ml)	Score
1	26 BC	222	12	2
2	ABC 6	988	3	2
3	ABC 5	789	3.2	2
4	ABC 4	1166	3	2
5	ABC 2	876	1.6	2
6	1BC W	78	12	2
7	6BC W	990	1.4	2
8	9BCW	980	3.3	2
9	27BC	1090	1	2
10	20BC	178	22	3
11	21 BC	899	23	3
12	22 BC	788	44	3
13	23 BC	677	12	3
14	24 BC	878	0.5	3
15	ABC 3	1234	2.3	3
16	ABC 1	1322	4.3	3
17	2BCW	655	44	3
18	3BC W	433	23	3
19	4BC W	1233	12	3
20	5BC W	987	1.2	3
21	7BC W	1233	7.2	3
22	8BCW	1211	2.4	3
23	11BC	987	1.2	3
Mean			864.9565217	10.4173913

Table 3.1 presents a review of serum levels of soluble CD44 (sCD44) and alpha-fetoprotein (αFP) in breast cancer patients, organized by stages of cancer progression (Stage II and Stage III). Soluble CD44 concentrations, ranging from 78 ng/mL to 1322

ng/mL, demonstrate a distinct rising pattern as cancer advances. Specifically, the mean sCD44 level in Stage II patients was 797.67 ng/mL, while Stage III patients had significantly higher levels, averaging 908.21 ng/mL. Samples 1BC W (78 ng/ml) and 26 BC (222 ng/ml), which have lower sCD44 values, are both given a score of 2. This implies that lower sCD44 levels could be a sign of an earlier stage of the disease's progression or a less severe disease state. Nevertheless, although this correlation is apparent in numerous samples, certain exceptions (such as sample number four ABC 4 with 1166 ng/ml and a score of 2) underscore the necessity for additional investigation to comprehensively understand the factors affecting the score and its association with sCD44 levels.

These findings demonstrate sCD44's potential value as a biomarker for evaluating illness. Higher sCD44 levels could be a sign of greater inflammation, tumor activity, or metastatic potential, which could account for some samples' higher clinical scores. sCD44 is a useful tool for clinical stratification since it can differentiate between various patient groups or disease severity. However, the observed exceptions imply that sCD44 might not be adequate as a single biomarker and should be taken into account in combination with additional elements, like αFP or other clinical characteristics, to offer a more thorough assessment.

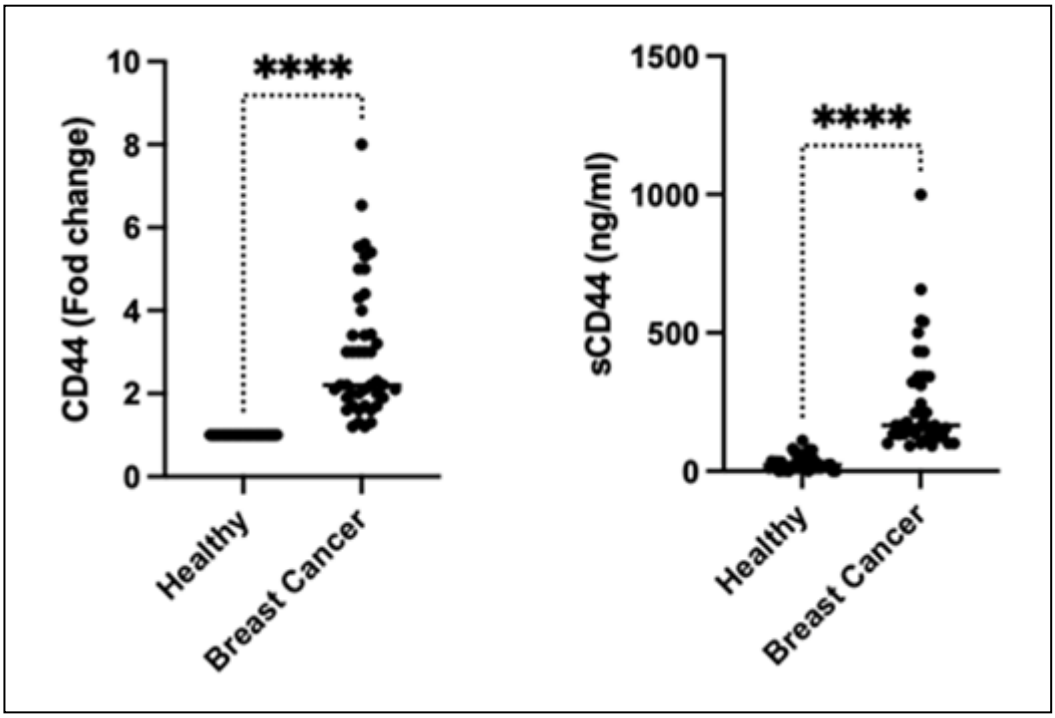
Additionally, αFP levels, ranging from 0.5 ng/mL to 44 ng/mL and with a mean of 10.4, were generally lower than sCD44 levels but still showed potential as a supplementary diagnostic marker.

The results also showed that patients with cancer score 3 had a greater mean a-FP concentration (13.43 ng/ml) than those with score 2 (4.06 ng/ml), with the lowest reported value (0.5 ng/ml) in a score 3 patient (Sample 24 BC) and the highest (44 ng/ml) in two score 3 patients (Samples 22 BC and 2BCW); the range of a-FP levels was quite wide. Although a-FP levels may be higher in more advanced disease states, this variability and the existence of outliers imply that a-FP may not be a reliable indicator of cancer severity in patients with breast cancer.

3.4 CD44 Overexpression in Breast Cancer Urine

Figure 4

CD44 overexpression in breast cancer urine, left panel (fold), right panel (sCD44)



for p < 0.01, which refers to moderate statistical significance, * for p < 0.001 refers to strong statistical significance, and **** for p < 0.0001, which means very strong statistical significance.

The accompanying figures present two different measures of CD44 overexpression in urine samples from patients with breast cancer relative to healthy controls. The left image displays the CD44 fold change, whereas the right figure displays the soluble CD44 (sCD44) concentration in ng/mL. The fold change in the breast cancer group is between about 2 and 10, which suggests that CD44 expression is significantly upregulated. With values around 1, the healthy control group, on the other hand, exhibits a negligible fold change. The hypothesis that CD44 is significantly overexpressed in breast cancer and may contribute to tumor growth, metastasis, and cancer progression is supported by a very strong statistical significance difference between the two groups (****, $p < 0.0001$).

The healthy group retains significantly lower values of DC44 in the protein form, usually below 200 ng/mL, but breast cancer patients show higher sCD44 concentrations, ranging from 200 ng/mL to over 1000 ng/mL, as seen in the right panel. The significance of sCD44 in differentiating breast cancer patients from healthy persons is further supported by the **** ($p < 0.0001$) statistical significance in this instance.

Both panels' data clearly imply that CD44 may be a trustworthy biomarker for breast cancer. The relationship between absolute concentration (ng/mL) and relative expression (fold change) suggests that CD44 is not only elevated but also much more prevalent in the urine of patients with breast cancer. According to these findings, CD44 may have a biologically significant impact on the pathophysiology of breast cancer by affecting cell adhesion, migration, and apoptosis resistance, all of which are critical for the growth of tumors.

3.5 CD44 Overexpression in breast cancer urine associated with cancer score

Table 3.2

CD44 overexpression in Breast cancer urine associated with cancer score

No.	Sample Code	sCD44 (ng/ml)	a-FP (ng/ml)	Score
1	CU9	100	N. D	2
2	R BCU 1	133	N. D	2
3	R BCU 2	117	N. D	2
4	W BCU 28	122	N. D	2
5	W BCU 29	123	N. D	2
6	W BCU 30	144	N. D	2
7	W BCU 31	210	N. D	3
8	W BCU 32	134	N. D	3
9	W BCU 33	155	N. D	3
10	R BCU 6	165	N. D	3
11	R BCU 8	100	N. D	3
12	R BCU 9	92	N. D	3
13	R BCU 11	177	N. D	3
14	BCU20	145	N. D	3
15	R BCU4	166	N. D	3
16	R BCU3	134	N. D	3
17	R BCU 5	342	N. D	3
18	BCU39	244	N. D	5

Table 3.2 The soluble CD44 (sCD44) concentrations in urine samples from patients with breast cancer are shown in table 2. along with the accompanying cancer progression scores. The range of sCD44 values is 92 ng/mL to 342 ng/mL, and there is a discernible trend of rising concentrations linked to higher cancer scores. sCD44 levels are typically lower in patients with Stage II breast cancer (Score 2), typically less than 150 ng/mL. On the other hand, sCD44 concentrations are higher in individuals with Stage III malignancy (Score 3), surpassing 150 ng/mL and even reaching 342 ng/mL. A higher sCD44 concentration of 244 ng/mL is also seen in the patient with the highest score (Score 5).

For every sample, the αFP levels are recorded as N.D. (Not Detected). This implies that, at least in measurable levels, αFP, a biomarker commonly linked to liver cancer or germ cell tumors, is either absent or irrelevant in the setting of these breast cancer patients.

The significant variations between serum and urine CD44 levels demonstrate how this biomarker behaves differently in various bodily fluids. Urine levels of CD44 are much lower, ranging from 92 ng/mL to 342 ng/mL, but serum levels are consistently higher, ranging from 78 ng/mL to 1332 ng/mL. Both matrices demonstrate a strong association between rising CD44 levels and progressing cancer stages, especially between Stage II and Stage III, despite the lower quantities in urine. As cancer progresses, serum CD44 levels show a more noticeable rise, indicating a heightened sensitivity for identifying minute variations in disease severity. Urine samples, however, are useful and practical for monitoring CD44 due to the non-invasive nature, even though urine samples need more sensitive detection techniques.

There are significant changes in the detectability and significance of Alpha-Fetoprotein (αFP) as a biomarker between serum and urine when its levels are analyzed. A minority of patients with breast cancer had detectable amounts of αFP in their serum, ranging from 0.5 ng/mL to 44 ng/mL. These levels varied, but there was no discernible relationship between them and the advancement of the malignancy. However, no urine sample had αFP levels, indicating that either αFP does not filter into the urine in large quantities, or it is not a useful biomarker for breast cancer in this bodily fluid. According to these results, αFP does not seem to have the same diagnostic significance in urine as it does in serum for some cancer types.

While both serum and urine can be used to assess CD44, serum levels are significantly greater, according to a comparison of serum and urine sCD44 levels. Urinary CD44 levels range from 92 ng/ml to 342 ng/ml, whereas serum CD44 levels are typically substantially higher, ranging from 78 ng/ml to 1322 ng/ml. The lower levels in urine may result from various mechanisms of excretion and clearance, along with the dilution effect in urine relative to the more concentrated environment found in blood. Although urine levels are lower; both serum and urinary CD44 levels show a positive correlation with clinical scores, suggesting that higher CD44 levels in both fluids may indicate greater disease severity in breast cancer.

Concerning alpha-fetoprotein (αFP), Table 3.1 indicates its presence in serum, with levels varying from 0.5 ng/ml to 44 ng/ml; however, it is not detectable (N.D.) in urine samples as presented in Table 3.2 The absence of αFP in urine indicates that αFP is a protein predominantly found in the bloodstream, possibly attributed to its larger molecular size or restricted filtration through the kidneys. The absence of αFP in urine restricts its effectiveness as a urinary biomarker for breast cancer, and this emphasizes the significance of choosing the correct biological fluid for the measurement of specific biomarkers in diagnostic or prognostic applications.

3.6 Alpha-fetoprotein (αFP) and Soluble CD44 (sCD44) Levels in CRC patients

Table 3.3

CD44 and aFP overexpression in Colorectal cancer serum

No.	Sample Code	sCD44 (ng/ml)	αFP
1	R CRC 1	670	5
2	R CRC 2	233	2.3
3	R CRC B 3	120	1
4	R CRC B 4	170	1
5	R CRC B 5	133	1
6	R CRC B 6	122.5	1.4
7	R CRC B 7	156	2.3
8	R CRC B 8	177	0.4
9	R CRC B 9	233	0.6
10	R CRC B 10	267	2.2
11	R CRC B 11	677	1.4
12	R CRC B 12	233	4
13	R CRC B 13	566	0.3
14	R CRC B 15	455	0.2
15	R CRC B 16	677	0.23
16	R CRCB 17	1022	0.6
17	R CRC B 18	788	0.34
18	R CRC B 19	678	0.33
19	R CRC B 20	111	0.23
20	1 CRC	90	0.12
21	B CRC 1 N	546	0.11
22	20 CRC	23	0.23
23	21 CRC	67	0.3
Mean		357.1521739	1.112608696

Data presented in Table 3.3 shows the levels of sCD44 range from 23 ng/mL to 1022 ng/mL, whereas αFP levels fluctuate between 0.1 and 5 ng/mL. The study includes a total of 23 CRC patients. The sCD44 levels were found to be significantly higher in CRC patients compared to healthy controls, with a mean value of 357 ng/mL. Additionally, there was no significant difference in αFP levels between the two groups.

The patient with the highest sCD44 level (1022 ng/mL) also has a low αFP concentration (0.34 ng/mL), suggesting that these biomarkers may not be in agreement. Likewise, an αFP level of just 0.4 ng/mL is associated with the second-highest sCD44 concentration, which is 788 ng/mL. This discrepancy suggests that in this study, a high sCD44 level does not always correspond to a high αFP level. The patient with the highest αFP level, 5 ng/mL, had a sCD44 value of 670 ng/which is comparatively high. This patient is one of the rare instances where both biomarkers exhibit increased levels. Interestingly, even in cases when sCD44 concentrations surpass 500 ng/mL, most patients had αFP levels below 1 ng/mL, indicating that sCD44 and αFP express themselves independently in the CRC population. This variation suggests that sCD44 and αFP might have different functions in the pathophysiology of colorectal cancer (CRC), with sCD44 possibly acting as a more accurate gauge for the burden of the disease or progression than αFP.

3.7 Alpha-Fetoprotein (αFP) and Soluble CD44 (sCD44) Levels in Healthy Individuals' Serum

Table 3.4

Alpha-fetoprotein (αFP) and Soluble CD44 (sCD44) Levels in healthy individuals

No.	Sample Code	sCD44 (ng/ml)	α-FP (ng/ml)
1	C.B.1	70	0.5
2	C.B.2	88	0.54
3	C.B.3	60	0.4
4	C.B.4	45	0.9
5	C.B.5	45	1
6	C.B.6	110	1.2
7	C.B.7	55	1.7
8	C.B.8	80	1.44
9	C.B.9	90	3
10	C.B.10	56	9
11	C.B.11	78	6
12	C.B.12	77	1
13	C.B.13	90	1
14	C.B.14	90	1.4
15	C.B.15	65	8.6
16	C.B.16	110	9
17	C.B.17	115	7
18	C.B.18	135	4.5
19	C.B.19	88	3
20	C.B.20	233	1.5
21	C.B.21	453	1.2
22	C.B.22	90	0.6
23	C.B.23	45	1.2
24	C.B.24	66	1
25	C.B.25	112	1.2
26	C.B.26	146	3.3
27	C.B.27	77	1
28	C.B.28	44	1.4
29	C.B.29	90	0
30	C.B.30	112	0
31	C.B.31	145	0
32	C.B.32	122	0
Mean		102.5625	2.299375

The findings in Table 3.4 show the amounts of soluble CD44 (sCD44) and alpha-fetoprotein (αFP) in 32 healthy individuals. The samples' αFP levels range from 0 to 9 ng/mL, with the majority of results falling within the lower end of this range, which denotes typical physiological levels. Interestingly, samples like C.B.28, C.B.30, and C.B.31 show an αFP level of 0 ng/mL, which is in accordance with what is likely to be the baseline for healthy patients. A number of samples have values below 1 ng/mL, which is regarded as being well within the usual reference range for healthy people. These samples include C.B.1 (0.5 ng/mL), C.B.2 (0.54 ng/mL), C.B.3 (0.4 ng/mL), and

others. The dataset's highest αFP value, 9 ng/mL (sample C.B.16), is nevertheless within permissible bounds for a healthy population, no notable outliers have been identified.

The levels of soluble CD44 exhibit a broad distribution, varying from 44 ng/mL to 453 ng/mL. The notable variability among individuals indicates a wider range for sCD44 within the healthy population, likely highlighting the protein's involvement in various physiological processes, including immune modulation, cell adhesion, and inflammation. The lowest sCD44 concentration is observed in sample C.B.28 (44 ng/mL), whereas the highest concentration is found in sample C.B.21 (453 ng/mL). This value is significantly greater than those of the other samples, highlighting individual variability in sCD44 levels among healthy individuals. The majority of samples, however, cluster between 70 and 150 ng/mL, establishing a general range of 102.5625 for sCD44 in this study.

3.8 Comparison of sCD44 and AFP levels between healthy individuals and breast cancer patients

When comparing the results between the αFP and sCD44 levels in healthy individuals' serum (Table 3.4) and breast cancer patients (Table 3.1), the analysis revealed notable variations in biomarker concentrations that could indicate the pathophysiological effects of breast cancer. As mentioned previously in Table 1, αFP levels vary from 0 to 9 ng/mL, with most samples concentrated at lower values, indicating stable, low concentrations commonly observed in non-diseased conditions, whereas breast cancer patients exhibit notably increased αFP levels, with values reaching up to 44 ng/mL. These findings are in accordance with suggestions that say elevated αFP is linked to malignancies, serving as a potential diagnostic marker for differentiating between healthy individuals and those impacted by cancer. This disparity is also indicated by sCD44 levels with a mean of 864.9565217 for breast cancer compared to 102.5625 for healthy individuals. Among healthy individuals, the majority of samples are grouped around lower values, with standard readings below 150 ng/mL. However, significantly increased sCD44 levels were observed in samples from patients with breast cancer, reaching up to 1322.

These elevated αFP levels are suggestive of the biomarker's potential role in cancer detection, as breast cancer, like other malignancies, may stimulate αFP production. This marked difference underscores αFP's potential utility in differentiating between healthy individuals and those with breast cancer. Conversely, the significant rise in sCD44 levels in breast cancer patients underscores its potential as a more reliable marker for the identification of breast cancer, especially in advanced cases where levels are notably elevated. The broad variation in sCD44 levels noted among cancer patients indicates a potential relationship with tumor heterogeneity or stage, with elevated levels possibly reflecting more advanced disease.

3.9 Alpha-fetoprotein (αFP) and Soluble CD44 (sCD44) Levels in healthy individuals' urine

Table 3.5

Alpha-fetoprotein (αFP) and Soluble CD44 (sCD44) Levels in Healthy Individuals' Urine

No.	Sample Code	sCD44 (ng/ml)	αFP (ng/ml)
1	C.U.1	25	N. D
2	C.U.2	19	N. D
3	C.U.3	12	N. D
4	C.U.4	45	N. D
5	C.U.5	66	N. D
6	C.U.6	34	N. D
7	C.U.7	56	N. D
8	C.U.8	22	N. D
9	C.U.9	78	N. D
10	C.U.10	45	N. D
11	C.U.11	77	N. D
12	C.U.12	43	N. D
13	C.U.13	67	N. D
14	C.U.14	70	N. D
15	C.U.15	80	N. D
16	C.U.16	110	N. D
17	C.U.17	12	N. D
18	C.U.18	15	N. D
19	C.U.19	16	N. D
20	C.U.20	12	N. D
21	C.U.21	12	N. D
22	C.U.22	12.3	N. D
23	C.U.23	15	N. D
24	C.U.24	16	N. D
25	C.U.25	23	N. D
26	C.U.26	33	N. D
27	C.U.27	35.3	N. D
28	C.U.28	34	N. D
29	C.U.29	33	N. D
30	C.U.30	12	N. D
31	C.U.31	24	N. D
32	C.U.32	23	N. D
33	C.U.33	0	N. D
34	C.U.34	0	N. D
35	C.U.35	1.2	N. D
36	C.U.36	2	N. D
37	C.U.37	1	N. D
38	C.U.38	1	N. D

Table 3.5 summarizes the levels of Alpha-Fetoprotein (αFP) and Soluble CD44 (sCD44) in urine samples collected from 38 healthy individuals.

In every sample analyzed, αFP levels were noted as "N.D.", which means the absence of αFP in these samples. The lack of αFP in these samples corresponds with its known

low baseline in healthy individuals, as αFP is generally not present in urine at detectable levels under non-pathological circumstances.

On the other hand, sCD44 levels were found in urine samples, ranging from 1.2 ng/mL to 110 ng/mL with a mean of 31.1

The differences in sCD44 levels among samples are significant, indicating a certain level of individual biological variation even within a healthy population.

3.10 Comparison of sCD44 and AFP levels between healthy individuals and CRC patients

As mentioned previously, sCD44 levels range from 44 ng/mL to 453 ng/mL among healthy individuals, sCD44 levels in CRC patients exhibit a significantly wider and notably increased range, from 23 ng/mL to 1022 ng/mL. The highest sCD44 level recorded among healthy individuals is 453 ng/mL (sample C.B.21), indicating an upper outlier within this group; however, it is still considerably lower than the levels seen in CRC patients. On the other hand, the majority of CRC samples demonstrate concentrations significantly exceeding the highest levels recorded in healthy individuals, with several samples attaining exceptionally high values.

When comparing alpha-fetoprotein (αFP) levels in the serum of healthy individuals versus colorectal cancer (CRC) patients, it was found that the average alpha-fetoprotein results among healthy individuals were 2.299375, while for CRC, patients the average of the results was 1.112608696, which is less than what is seen in healthy individuals. This unexpected finding, in which CRC patients exhibit slightly lower average αFP levels than healthy individuals, indicates that αFP may not be significantly elevated in CRC and that the condition may not lead to substantial alterations in αFP production. This further reinforces the notion that αFP is not directly linked to colorectal tumorigenesis or progression, and it is commonly utilized as a marker in liver cancers. The role of αFP in CRC diagnostics may be restricted, and it could prove more beneficial when used in conjunction with other biomarkers to improve diagnostic specificity and sensitivity.

3.11 Comparison of Alpha-Fetoprotein (αFP) and soluble CD44 (sCD44) levels in healthy individuals' urine (Table 3.5) versus breast cancer patients' urine (Table 3.2)

In the samples from healthy individuals (Table 3.5) and those from breast cancer patients (Table 2), αFP levels were consistently noted as "N.D." (non-detectable) throughout. This observation aligns with the anticipated behavior of αFP, which is generally not found in urine at detectable levels in conditions unrelated to liver tissues. On the contrary, the levels of sCD44 show a significant disparity between healthy individuals and those with breast cancer, suggesting its potential utility as a urinary biomarker for cancer detection. In BC samples presented in Table (3.2), sCD44 levels vary from 92 ng/mL to 244 ng/mL, with the majority of values concentrated below 200 ng/mL with a mean of 155.7222222. For the healthy individuals sCD44 levels vary between 1.2 ng/mL and 110 ng/mL with a mean of 31.1, the majority of samples demonstrate comparatively low levels of sCD44.

The lack of extremely low values in breast cancer patients is also noteworthy, with the minimum sCD44 value observed in breast cancer patients is 92 ng/mL, approaching the upper threshold found in healthy individuals, suggesting a baseline shift. This persistent increase in sCD44 levels observed in breast cancer patients, even at lower concentrations, indicates that malignancy may universally raise sCD44 levels.

Chapter Four

Discussion

This study emphasizes notable differences in serum CD44 expression between patients with breast cancer and colorectal cancer (CRC) when compared to healthy controls, indicating a possible role for CD44 as a biomarker in cancer diagnostics and differentiation between various cancer types. CD44 is a cell-surface glycoprotein initially identified on lymphocytes in 1982 (63) that plays a critical role in cell adhesion, migration, and interactions within the tumor microenvironment. It has been associated with several oncogenic processes, such as tumor growth, metastasis, and resistance to apoptosis (40).

The increase in CD44 expression observed in serum samples from colorectal cancer (CRC) and breast cancer patients when compared to healthy controls underscores the significant role of CD44 in cancer progression. Many studies provide evidence that CD44 and its soluble form (sCD44) are notably increased in several types of cancer, such as breast cancer and colorectal cancer (CRC) (64), (65).

However, the role of CD44 is much more complex. It can exhibit dual effects, acting as both a promoter and suppressor of tumor progression, contingent upon the cancer type, isoform, and microenvironmental factors. The hyaluronan/CD44 axis can modulate pathways in various ways, occasionally leading to a reduction in tumor progression when competing ligands are present (66).

The notable increase of CD44 in CRC patients, albeit to a lesser degree than in breast cancer, reinforces its correlation with cancer progression in gastrointestinal tumors. This rise suggests that CD44 may be a useful marker for early CRC identification even while it is not as noticeable as in breast cancer. Many studies also discovered a correlation between higher tumor aggressiveness and a worse prognosis for patients with colorectal cancer and elevated CD44 expression. A study performed by Zhang Q in 2024 found that most malignancies, including colorectal cancer, had increased CD44 expression where CD44 levels are associated with increased immune cell infiltration, such as that of neutrophils, dendritic cells, and macrophages, indicating that CD44 affects the tumor immune microenvironment (58). This study also demonstrates CD44's potential as a therapeutic target and a predictive marker for immunotherapy responses by showing

that it influences tumor cell behavior and the immune milieu, contributing to the advancement of colorectal cancer.

Ziranu P also found a relation between CRC and CD44 overexpression, which is associated with the presence of cancer stem cell-like characteristics, which play a role in tumor initiation and progression (59).

The recognized function of sCD44 in tumor cell adhesion, motility, and metastatic potential is consistent with the reported increase in sCD44 levels with cancer progression. According to this relationship, sCD44 may be a useful marker for detecting aggressive cancer types, which would help with patient stratification based on disease severity and the customization of treatment plans. A study conducted by Baek J confirmed that there is a correlation between higher sCD44 levels and liver metastases, as opposed to other metastatic sites, and advanced stages of breast cancer. This indicates a possible involvement of sCD44 in the advancement of aggressive disease and the occurrence of organ-specific metastasis (52).

Nonetheless, in numerous cancers, elevated CD44 expression has not consistently correlated with a negative prognosis. A variety of studies have produced differing results, with variations influenced by the type of cancer, the methodology employed, CD44 isoforms studied (e.g., CD44 standard vs. variants), or the sensitivity of molecular processing.

Horiguchi et al. revealed a positive correlation between CD44 expression and a better prognosis for breast cancer. He found a strong correlation between CD44 and improved relapse-free survival (RFS) and a higher frequency of expression in smaller tumors (67).

A meta-analysis study conducted by Qiao G identified a notable correlation between CD44v6 expression and poor overall survival (OS). The study indicated that patients exhibiting CD44v6 had 1.55 times increased risk of poor prognosis in comparison to those lacking CD44v6 expression. The findings also indicated that CD44v6 expression was associated with advanced histological grade and lymph node metastasis, highlighting its potential as a prognostic marker in breast cancer (68).

When we evaluated soluble CD44 (sCD44) in breast cancer patients' serum, the results showed a rise in concentrations as the disease stage progressed. The data show that

mean sCD44 levels increased significantly from Stage II to Stage III, moving from 797.67 ng/mL to 908.21 ng/mL. These results are consistent with previous research that points to increased sCD44 as a possible indicator of tumor aggressiveness and progression (52). This study supports the relation between advanced disease stage and elevated sCD44 levels. Baek J also showed that there are no significant differences in CD44 concentration between HER2-positive and HER2-negative breast cancer patients. However, his findings showed that sCD44 concentration was a significant predictor of survival only in HER2-positive cases, with greater sCD44 levels linked to a lower chance of survival.

Increased levels of sCD44 serve as indicators of tumor aggressiveness and metastasis. The interaction between CD44 and hyaluronic acid (HA), its main ligand, improves the self-renewal capabilities of cancer stem cells (CSCs). This interaction triggers multiple intracellular signaling pathways that enhance the proliferation, survival, and invasion of cancer cells. It also plays a crucial role into the invasion of cancer cells to adjacent tissues through its interaction with HA and various extracellular matrix components, which initiates downstream signaling pathways, including those related to matrix metalloproteinases (MMPs), which facilitate the degradation of the extracellular matrix, thereby enhancing the mobility of tumor cells (66). Consequently, the expression of CD44 is frequently heightened in invasive and metastatic tumor cells, which is associated with unfavorable outcomes in multiple cancer types.

There was no difference in soluble CD44 (sCD44std) between breast cancer patients and healthy controls, according to Sebastian Mayer's study (51). This study was conducted to examine the connection between blood levels of soluble CD44 (sCD44) isoforms and clinical characteristics in patients with breast cancer. It found increased levels of sCD44v6 and total protein TP in the sera of breast cancer patients as compared to healthy controls and greater sCD44v6 levels in breast cancer patients with lymph node metastases. Regarding CD44 (sCD44std), the results of this study conflict with ours.

Using urine as a non-invasive diagnostic tool for the evaluation of soluble CD44 (sCD44) level is not widely adopted. When compared to blood draws or biopsies, urine samples are non-invasive, easily obtained, and they also lessen patient discomfort. Additionally, this approach reduces the possibility of contamination or complications,

providing a workable substitute for early cancer detection and progression tracking. However, urinary CD44 levels are lower when compared to serum, and this is consistent with what was previously mentioned in our study. This lower level could be attributed to the distinct biological processes that regulate their release. Serum CD44 plays a significant role in systemic circulation and tumor biology, resulting in elevated concentrations, particularly in malignancies. It is involved in essential processes such as cell adhesion, migration, and invasion, which are fundamental to tumor progression and metastasis (66). In contrast, CD44 in urine is influenced by the filtration processes in the kidneys and may experience dilution or reduction during excretion, which limits its concentration in urine relative to serum. Furthermore, tumor-derived CD44 variants may exhibit reduced efficiency in urinary excretion.

Isamu Okamoto said that urine serves as an effective, non-invasive diagnostic method for identifying various cancers, particularly those of the bladder and kidneys. Urine-based biomarkers such as CD44, DNA fragments, and proteins present promising alternatives to invasive procedures like biopsies or cystoscopies (69).

Although αFP is primarily associated with liver cancer and germ cell tumors, recent studies suggest its relevance in other cancers, such as breast and colorectal cancers, albeit at lower and less consistent levels. The presence of αFP in these cancer types could indicate specific oncogenic pathway activation or reflect metastatic disease, particularly when elevated alongside sCD44. In this context, combining the monitoring of both sCD44 and αFP could offer a more comprehensive view of the tumor microenvironment. While sCD44 plays a more prominent role in cancer progression assessment, αFP may provide additional information, particularly in advanced cancer stages. Together, these biomarkers could enhance diagnostic accuracy, better differentiate cancer stages, and potentially guide therapeutic decisions, contributing to a more refined understanding of disease progression and metastatic potential in breast and colorectal cancers.

Certain studies indicate increased AFP levels in uncommon instances of AFP-producing colorectal adenocarcinoma. These tumors typically demonstrate aggressive characteristics, early metastasis, and a poor prognosis. This suggests a potential role for AFP as a prognostic marker in colorectal malignancies (70).

4.1 Conclusion and Recommendations

The findings indicate that sCD44 levels are markedly increased in breast cancer's serum and urine in comparison with healthy individuals; their levels are also elevated in colorectal cancer serum, which emphasizes its potential as a strong biomarker for cancer detection and progression monitoring.

However, their levels in breast cancer patients were significantly higher compared to CRC, with more pronounced increases noted in advanced stages.

On the contrary, alpha-fetoprotein (αFP) levels exhibited inconsistency, demonstrating limited use as a diagnostic tool in both BC and CRC with no detectable values in urine samples.

The consistent upregulation of soluble CD44 (sCD44) in various biological matrices demonstrates its significance in tumor biology, while αFP is more pertinent in other malignancies such as liver cancer.

The results of this study highlight sCD44's potential for non-invasive cancer diagnostics and its relationship with disease severity.

4.2 Recommendations

From what this study has revealed, numerous recommendations are presented. The study's insights and conclusions support these recommendations. To fill in the gaps and overcome the obstacles found, they offer insightful advice for upcoming actions and choices:

- First, the execution of extensive research to confirm the clinical relevance of sCD44 across various cancer demographics and enhance its function in differentiating between breast cancer and colorectal cancer.
- Investigate the biological role of the elevation of sCD44 in cancer patients to identify potential therapeutic targets.
- Utilize sCD44 as a biomarker for the early detection and diagnosis of breast and colorectal cancers (CRC), especially by taking advantage of its increased levels in serum and urine.

- We recommend the conduction of thorough investigation into the independent expression patterns of αFP and sCD44 to elucidate their complementary roles in disease characterization.
- Focus on advancing non-invasive urinary biomarkers such as sCD44 for regular cancer screening and monitoring, thereby minimizing the necessity for invasive blood sampling.
- The use of αFP as a standalone biomarker for CRC and breast cancer should be limited, given its restricted elevation and diagnostic effectiveness in these conditions, and focus on examining its function in liver and germ cell malignancies, where it demonstrates increased diagnostic potential.
- Examine the specificity and sensitivity of sCD44 in relation to established biomarkers across different cancer types.
- Broaden the research to investigate sCD44 expression in additional cancer types, including lung, ovarian, and pancreatic cancer, to discern similarities and differences in its diagnostic significance.

These recommendations are designed to enhance the comprehension and utilization of CD44 and sCD44 within the field of oncology, promoting advancements in cancer diagnostics, therapeutics, and patient care.

4.3 Strengths and Limitations of the Study

Strength:

Cancer continues to be a primary cause of death globally, highlighting the ongoing need for dependable biomarkers to enhance early detection, prognosis, and treatment monitoring. One of the prospective contenders is CD44, a membrane glycoprotein, and (sCD44), its soluble form; they have gained attention because of their vital functions in cell adhesion, migration, and apoptosis resistance. These processes are essential for the growth and spread of tumors. In addition, alpha-fetoprotein (αFP) has demonstrated promise in certain cancer situations, despite being mainly associated with liver and germ cell cancers. In this study, we intend to shed light on their clinical relevance and establish the foundation for upcoming advancements in cancer biomarker research by comparing levels between cancer patients and healthy controls.

A significant strength of this study lies in the inclusivity of various cancer types, including breast and colorectal cancer, thereby improving the applicability of findings across diverse malignancies. As well as it includes various biological samples, serum, and urine, which highlights the potential for non-invasive cancer diagnostics, enhancing the findings' relevance for patients and clinical application. It also reveals variations in biomarker behavior across biological matrices, providing valuable insights into their unique diagnostic applications.

This study focuses on investigating both sCD44 and αFP. This facilitates a multi-marker strategy, potentially enhancing diagnostic tools by integrating the advantages of each biomarker. On the other hand, it highlights the limited utility of αFP in breast and colorectal cancers, directing attention towards sCD44 as a more dependable biomarker, thereby facilitating more focused research efforts.

The strengths of the study collectively provide a significant foundation for future research regarding the role of CD44 in cancer biology. The findings present considerable potential for enhancing diagnostic and therapeutic strategies, especially via the advancement of non-invasive methods and more dependable biomarkers, contributing to enhanced cancer management and patient outcomes.

Limitations:

Every research study has intrinsic constraints that could affect how its findings are interpreted and applied more broadly. It is necessary to be aware of these limits in order to comprehend the scope of the study and identify topics for further research advancement. This study was conducted in Palestine on the diagnosis of breast and colorectal cancers, it presented particular difficulties for this investigation because of data accessibility, patient involvement, and geographic limitations. In this section we list the main restrictions that emerged throughout the research process, emphasizing how these constraints affected the study's findings and possible directions for improvement in subsequent studies.

The study's results are based on a comparatively small sample size, which limits the results' statistical power and generalizability, and this limits the capacity to make solid inferences that can be safely extrapolated to more diversified and sizable populations. Due to sample bias, the observed data may not accurately reflect the underlying

variability within the larger target population as a result of this limitation. Future studies should use bigger, multi-center cohorts that take into account a greater range of patient characteristics, such as age, sex, cancer stages, and underlying comorbidities, in order to validate these findings and guarantee their relevance to various demographic and clinical contexts.

Another limitation in this study is the lack of an extended design; this in turn, limits the ability to track variations in biomarker levels over time and their dynamic correlation with the course of the disease, the effectiveness of treatment, or recurrence. In order to better understand the temporal dynamics of biomarker expression, future studies should include repeated sampling at predetermined intervals throughout various treatment phases.

The study relies on measuring sCD44 levels and does not investigate the function of other CD44 isoforms, which may have diverse significance in cancer biology and diagnostics, instead concentrating on soluble CD44 (sCD44).

The study encountered difficulties because the data systems in Palestine lacked sufficient information. This made it more difficult to gather and examine bigger datasets, which might have had an impact on the results' statistical power and generalizability.

A significant number of potential participants chose not to engage in the study. There may be apprehensions related to privacy, cultural stigma, or a lack of trust in research methodologies. This resulted in a smaller sample size and could have led to selection bias.

It will be crucial to address these contextual and systemic limitations in future studies to enhance the reliability and applicability of research findings in the region.

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List of Abbreviations

Abbreviation	Definition
AFP	Alpha-fetoprotein
ALCAM	Activated Leukocyte Cell Adhesion Molecule
BC	Breast cancer
CCE	colon capsule endoscopy
CD44	Cluster of Differentiation 44
CRC	Colorectal cancer
CSCs	cancer stem cells
CTC	computed tomographic colonography
DBT	Digital Breast Tomosynthesis
DM	diabetes mellitus
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Estrogen Receptor
EpCAM	Epithelial Cell Adhesion Molecule
FOBT	Fecal Occult Blood Test
HA	Hyaluronan
HER2	Human Epidermal Growth Factor Receptor 2
HTN	hypertension
IRB	Institutional Review Board
MBI	Molecular Breast Imaging
MRI	Magnetic Resonance Imaging
PR	Progesterone Receptor
RT–PCR	Real-time PCR
TMD	Transmembrane domain
TME	tumor microenvironments
US	ultrasound
cDNA	Complementary DNA
ctDNA	Circulating Tumor DNA
sDNA	Stool DNA Test

Appendices

Appendix A

Institutional Review Board (IRB)

An-Najah National University
Faculty of Medicine & Health Sciences
Institutional Review Board

جامعة النجاح الوطنية
كلية الطب و علوم الصحة
لجنة اخلاقيات البحث العلمي

Ref: Mas. Dec. 2023/5

IRB Approval Letter

Title of Research:

CD44 Expression Levels as a Biomarker for the Early Detection of Various Cancers in Biological Samples: A Case-Control Study

Submitted by:
Samah Bsharat

Supervisor:
Nihad Al-Othman

Approved:
12th Dec. 2023

Your Study Title" **CD44 Expression Levels as a Biomarker for the Early Detection of Various Cancers in Biological Samples: A Case-Control Study**".reviewed by An-Najah National University IRB committee and was approved on 12th Dec. 2023

Hasan Fitian, MD

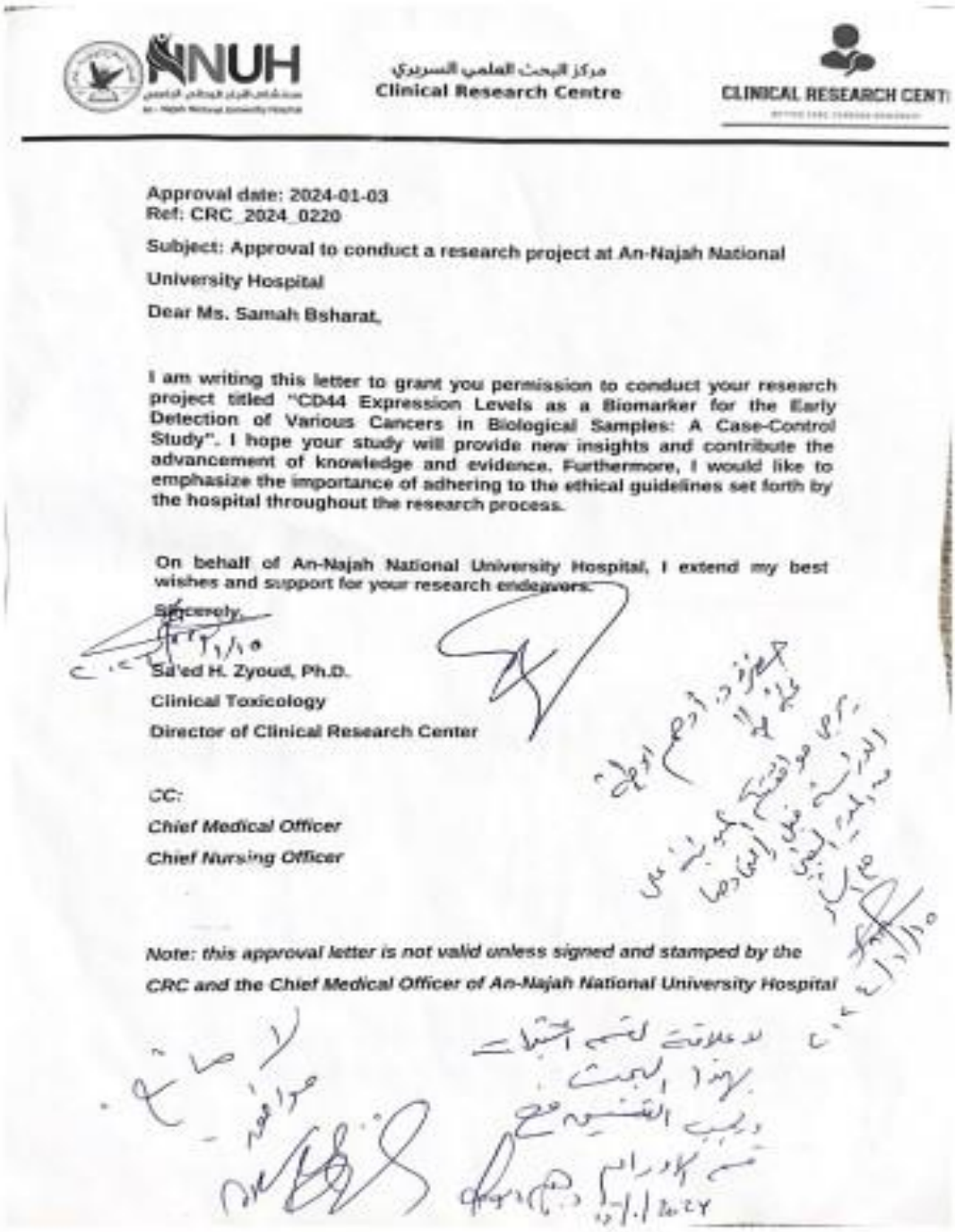
IRB Committee Chairman

IRB

Nablus - P.O Box 7 or 707 | Tel (970) (09) 2342902/4/7/8/14 | Fax/ele (970) (09) 2342910| E-mail : IRB@najah.edu

Appendix B

Approval to conduct the research at An-Najah National University Hospital



مركز البحث العلمي السريري
Clinical Research Centre

CLINICAL RESEARCH CENTI

Approval date: 2024-01-03
Ref: CRC_2024_0220

Subject: Approval to conduct a research project at An-Najah National University Hospital

Dear Ms. Samah Bsharat,

I am writing this letter to grant you permission to conduct your research project titled "CD44 Expression Levels as a Biomarker for the Early Detection of Various Cancers in Biological Samples: A Case-Control Study". I hope your study will provide new insights and contribute the advancement of knowledge and evidence. Furthermore, I would like to emphasize the importance of adhering to the ethical guidelines set forth by the hospital throughout the research process.

On behalf of An-Najah National University Hospital, I extend my best wishes and support for your research endeavors.

Sincerely,

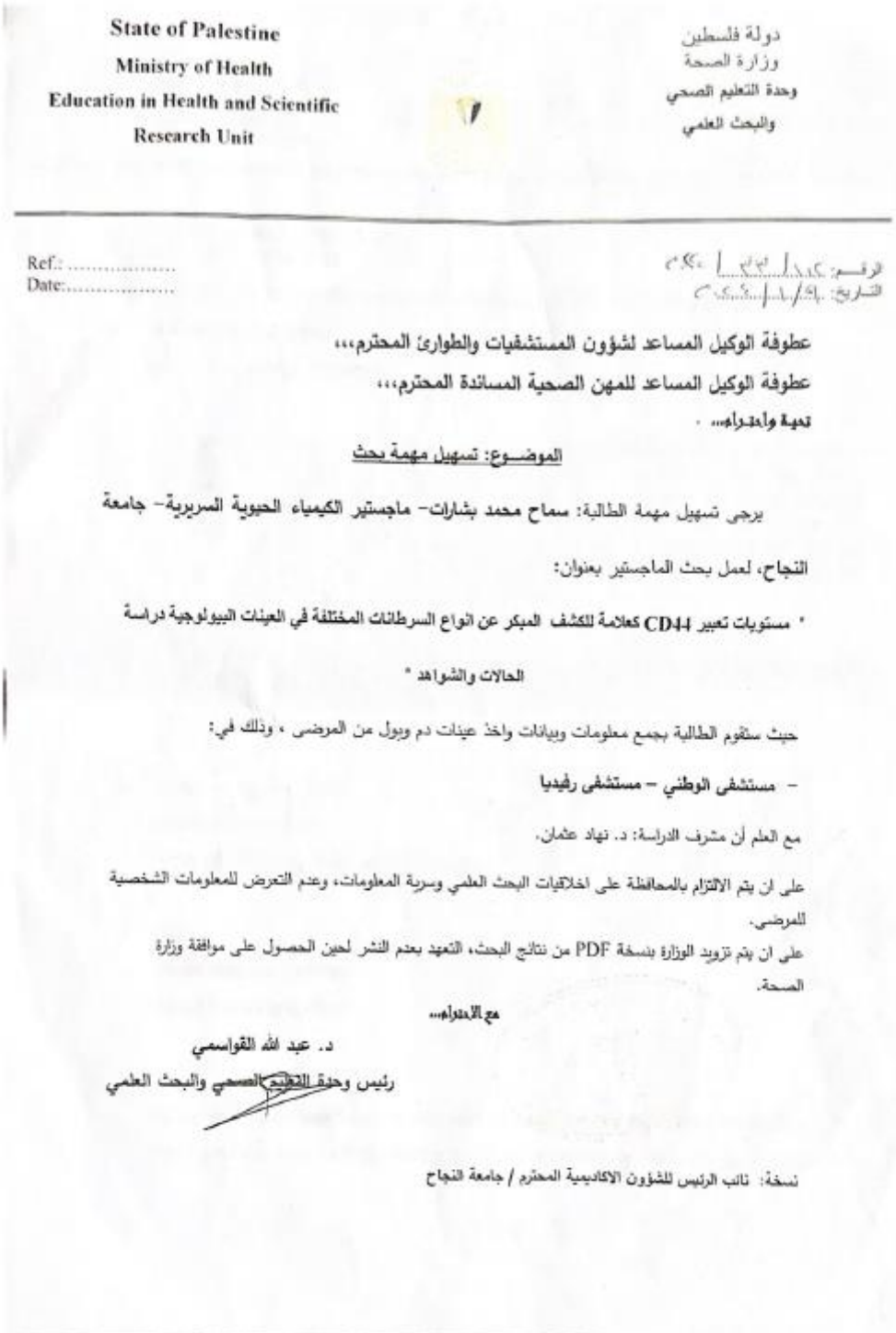
Sa'ed H. Zyoud, Ph.D.
Clinical Toxicology
Director of Clinical Research Center

CC:
Chief Medical Officer
Chief Nursing Officer

Note: this approval letter is not valid unless signed and stamped by the CRC and the Chief Medical Officer of An-Najah National University Hospital

Appendix C

Facilitating a research mission



State of Palestine
Ministry of Health
Education in Health and Scientific
Research Unit

دولة فلسطين
وزارة الصحة
وحدة التعليم الصحي
والبحث العلمي

Ref.: .....
Date:.....

الرقم: .....
التاريخ: .....

عطوفة الوكيل المساعد لشؤون المستشفيات والطوارئ المحترم،،،

عطوفة الوكيل المساعد للمهن الصحية المساندة المحترم،،،

تحية واحترام،،

الموضـــوع: تسهيل مهمة بحث

يرجى تسهيل مهمة الطالبة: سماح محمد بشارات- ماجستير الكيمياء الحيوية المريرية- جامعة النجاح، لعمل بحث الماجستير بعنوان:

" مستويات تعبير CD44 كعلامة للكشف المبكر عن انواع السرطانات المختلفة في العينات البيولوجية دراسة الحالات والشواهد "

حيث ستقوم الطالبة بجمع معلومات وبيانات واخذ عينات دم وبول من المرضى ، وذلك في:

- مستشفى الوطني – مستشفى رفيديا

مع العلم أن مشرف الدراسة: د. نهاد عثمان.

على ان يتم الالتزام بالمحافظة على اخلاقيات البحث العلمي وسرية المعلومات، وعدم التعرض للمعلومات الشخصية للمرضى.

على ان يتم تزويد الوزارة بنسخة PDF من نتائج البحث، التعهد بعدم النشر لحين الحصول على موافقة وزارة الصحة.

مع الاحترام،،

د. عبد الله القواسمي

رئيس وحدة التعليم الصحي والبحث العلمي

نسخة: نائب الرئيس للشؤون الاكاديمية المحترم / جامعة النجاح

جامعة النجاح الوطنية

كلية الدراسات العليا

مستويات تعبير CD44 كعلامة للكشف المبكر عن أنواع السرطان المختلفة في العينات البيولوجية: دراسة الحالات والشواهد

إعداد

سماح محمد بشارات

إشراف

د. نهاد العثمان

قدمت هذه الرسالة استكمالا لمتطلبات الحصول على درجة الماجستير في الكيمياء الحيوية السريرية، من كلية الدراسات العليا، في جامعة النجاح الوطنية، نابلس –فلسطين.

2024

مستويات تعبير CD44 كعلامة للكشف المبكر عن أنواع السرطان المختلفة في العينات البيولوجية: دراسة الحالات والشواهد

إعداد

سماح محمد بشارات

إشراف

د. نهاد العثمان

الملخص

الخلفية: تساهم الخلايا الجذعية السرطانية (CSCs) في تطور الأورام وانتشارها ومقاومتها للعلاج، حيث يلعب بروتين CD44 دورًا رئيسيًا في هذه العمليات من خلال تنظيم الالتصاق والهجرة ومسارات الإشارة الخلوية. تؤدي التعديلات الهيكلية لهذا البروتين إلى زيادة مقاومة العلاج وانتشار السرطان، خاصة في الأنواع العدوانية مثل سرطان الثدي والقولون والمستقيم، مما يجعله هدفًا واعدًا للتشخيص والعلاج.

يهدف هذا البحث إلى تقييم CD44 القابل للذوبان كعلامة حيوية للكشف المبكر عن سرطان الثدي وسرطان القولون والمستقيم، من خلال تحليل مستوياته في الدم والبول.

منهجية الدراسة: أُجريت الدراسة بين عامي 2023–2024 على 40 مريضًا بسرطان الثدي، 23 مريضًا بسرطان القولون والمستقيم، و70 شخصًا سليمًا كمجموعة ضابطة، وذلك في ثلاثة مستشفيات في نابلس. تم تحليل عينات الدم والبول باستخدام Real-time PCR لتحديد التعبير الجيني لـ CD44، و ELISA لقياس تركيزه القابل للذوبان.

النتائج: أظهرت الدراسة ارتفاعًا قويًا جدًا في تعبير CD44 لدى مرضى سرطان الثدي مقارنة بالأصحاء ($p < 0.0001$) في كلٍّ من التعبير الجيني وتركيز CD44 القابل للذوبان، و لوحظ ارتفاع معتدل في تعبير CD44 لدى مرضى سرطان القولون والمستقيم مقارنة بالأصحاء ($p < 0.01$) وُجد فرق إحصائي قوي بين تعبير CD44 في سرطان الثدي مقارنة بسرطان القولون والمستقيم ($p < 0.001$).

الاستنتاج: أكدت الدراسة ارتفاع مستويات sCD44 في الدم والبول لدى مرضى السرطان، مما يدعم استخدامه كعلامة حيوية واعدة للتشخيص المبكر. على العكس، لم يُظهر αFP قيمة تشخيصية كبيرة، مما يثبت ارتباطه بسرطانات الكبد أكثر من سرطان الثدي والقولون والمستقيم.

الكلمات المفتاحية: CD44 القابل للذوبان (sCD44)، سرطان الثدي، سرطان القولون والمستقيم، دراسة الحالات والشواهد، ألفا فيتوبروتين (αFP)، فلسطين.