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The correlation between Estrogen level and microbial interaction in Breast cancer

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The correlation between Estrogen level and microbial interaction in Breast cancer

Abstract

Background

Breast cancer incidence and mortality have increased globally over recent decades. Estrogen plays a pivotal role in breast cancer development, and growing evidence suggests that gut microbiota, particularly *Lactobacillus* species, may influence estrogen metabolism. However, the relationship between circulating estrogen levels, gut microbiota, and breast cancer remains unclear.

Methods

A case–control cross-sectional study was conducted on 180 women, including 90 patients with non-metastatic breast cancer and 90 healthy controls. Clinical and anthropometric data were collected. Serum estrogen levels were measured using ELISA, and stool samples were analyzed for *Lactobacillus* isolation. Statistical analyses evaluated differences in estrogen levels and their association with *Lactobacillus* species.

Results

Serum estrogen levels were significantly lower in breast cancer patients compared to healthy controls. ROC analysis demonstrated high discriminatory performance of estrogen levels in distinguishing patients from controls. No significant association was identified between specific *Lactobacillus* species and circulating estrogen levels.

Conclusion

Circulating estrogen levels are altered in non-metastatic breast cancer and may serve as a complementary biomarker. The lack of association with individual *Lactobacillus* species underscores the complexity of the gut microbiota–estrogen–breast cancer axis, warranting further mechanistic and large-scale studies.

Keywords: Breast cancer, Microbiota, *Lactobacillus*, Estrogen

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List of abbreviation

BC	Breast cancer
BMI	Body mass index
E1	Estrone
E2	Estradiol
E3	Estriol
ER	Estrogen receptor
ERE	Estrogen response elements
ERα	Estrogen Receptor alpha
ERβ	Estrogen Receptor beta
HER2	Human epidermal growth factor receptor 2
HC	Hip circumference
HDL	High density lipoprotein
HOMA-IR	Homeostatic model assessment for insulin resistance
HR	Hormone receptor
LAB	Lactic acid bacteria
LDL	Low density lipoprotein
MUAC	Mid upper arm circumference
SHBG	Sex hormone-binding globulin
TC	Total cholesterol
TG	Triglycerides
Tis	Tumor in situ
WC	Waist circumference
WHR	Waist hip ration

I. Introduction

Cancer is the second greatest cause of death worldwide and a significant public health issue (Siegel et al., 2023). It is generally acknowledged that it results from oncogenic somatic mutations that arise in normal cells and cause them to divide uncontrollably (Warenius, 2023). The induction of sustained proliferative signaling (e.g., gain-of-function mutations in oncogenes), evasion of growth suppressive signals (e.g., loss-of-function mutations in tumor suppressors), and resistance against death signals (e.g., avoidance of apoptotic signaling and immune destruction) are well-defined hallmarks that drive this growth phenotype (Truskowski et al., 2023). Cancer is caused by a combination of genetic (inherited and somatic), epigenetic, viral, and environmental factors. Epigenetic modifications modify a gene's expression through different mechanisms than genetic alterations, which alter gene expression directly through direct mutations within the gene. It is also known that some viruses can cause cancer (Reagan, 2019).

Breast cancer is the most common and the most frequent malignancy in women and the leading cause of cancer-related deaths in women worldwide and is curable in 70-80% of patients with early stage, non-metastatic disease. It is the second most common cause of female death from cancers. Advanced breast cancer with distant organ metastasis is considered incurable with the currently available therapies. Many breast cancer cases occur in women with little or no known risk (Nandi et al., 2023).

For many years, estrogenic hormones have been solely thought to regulate sexual function. Estrogenic hormones elicit a wide range of biologic responses, not just those related to reproduction. Estrogens regulate the immune system, growth, neuronal function, and metabolism in addition to their non-reproductive roles (Ezhilarasan, 2020).

Estrogen is involved in female reproduction, as well as numerous other biological systems, it is also implicated in many different diseases and conditions including cancers, mainly breast cancer (Hamilton et al., 2017). The main uses of estrogen in a clinical setting are for hormone replacement therapy and contraception. Estrogens can be found in the

bloodstream free or bound to proteins, and they have a variety of biological effects (Kwa et al., 2016).

The microbiome represents a huge part of the human body, it is a big precursor of extra genes than the ones we already have in our bodies and it has incredibly diverse roles in health and disease. Numerous studies in the past decade have demonstrated how microbiome impacts various organ-specific cancers. In relation to breast cancer, recent research studies have shown that the intestinal flora is related to the occurrence and progression of breast cancer (Parida and Sharma, 2019).

The human gut is home to the majority of the microbiota. It has only recently been recognized that bacterial communities within a host may be an additional environmental factor linked to breast cancer in cases of sporadic breast cancers with unknown etiology (Ruo et al., 2021).

Research has revealed a distinct microbial community in breast tissue, which was previously believed to be sterile. Furthermore, unlike normal mammary gland tissue, breast tumors have their own unique microbial community, and intestinal flora may be the cause of all of them. The development of breast cancer may be influenced by a particular microbial community found in breast tissue. With an emphasis on the connection between the microbial community and breast cancer (Song et al., 2023).

Lactobacillus species are a small but important component of the human gut microbiota. Research has shown that these species are frequently linked to a variety of illnesses and are intimately related to human health. An increasing number of Lactobacillus species have been regularly linked to the human gastrointestinal tract in recent years. A few researches have documented the advantageous function of Lactobacillus species in terms of the harmony they can establish with the gut microbiome (Kong et al., 2020).

The project’s aim is to determine and confirm estrogen and Lactobacillus's function as a risk factor for the development and progression of breast cancer. Therefore, altering the gut microbiota may lead to the development of a novel therapeutic approach for the disease. We attempt to investigate the variables that disturb the typical composition of gut microbes as well as the part that dysbiosis plays in the emergence of breast cancer. The characterizations

of the gut and breast microbiomes are to be examined. Consequently, our hypothesis is that the human microbiota specially Lactobacillus is highly involved in the progression and development of breast cancer, and by insuring this it can help in coming up with new ways through modulating specific microbiota, we might control and/ or prevent women breast cancer.

II. Review of literature

2.1. Cancer

It is well known that genetic mutation is the commonality linking all cancers across stage, location, and triggering event. The origins of these genetic abnormalities, however, are often diverse, multifactorial, and not well understood between patients, tumor types, and during disease progression. Environmental influences, epigenetic changes, random mutations, and systemic alterations, such as inflammation and metabolic dysfunction. most commonly initiate genetic mutations (known as "acquired" somatic mutations), with only 10% of cancers stemming from inherited genetic mutations (known as "germline" mutations)(Reagan, 2019) .

2.2. Breast cancer

Breast cancer (BC) is the most common malignancy in females in Egypt and it accounts for 32 % of cancers among Egyptian women (Ibrahim et al., 2014). Infectious agents are known to be the third most common risk factor for developing cancer overall, after tobacco use and obesity, accounting for 15–20% of cases of breast cancer (Marongiu et al., 2021).Genetics account for less than 10% of breast cancers and as many as 70% of breast cancers occur in women at seemingly-average risk. While the biological reasons remain poorly understood, approximately 70% of all breast cancers are estrogen receptor positive subtype (Harbeck et al., 2019).Breast cancer affects one in eight women in their lifetime. Though diet, age and genetic predisposition are established risk factors, the majority of breast cancers have unknown etiology (Xuan et al., 2014).

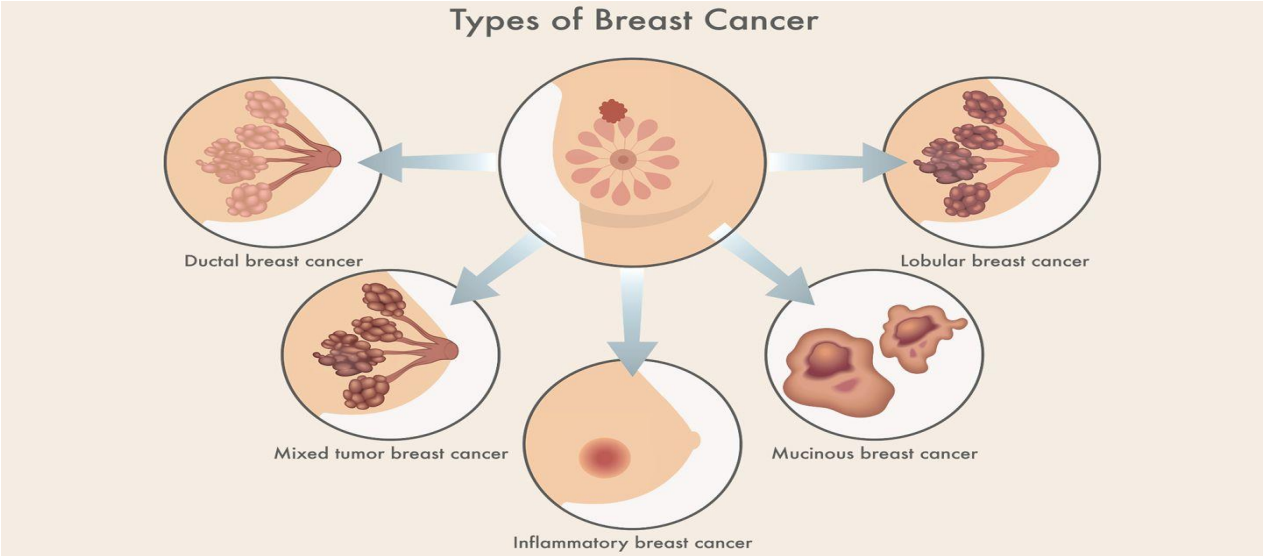

Figure 1. The different types of breast cancer (nature reviews).

Breast cancer can start in the tissue that lies between the lobules, the ducts, or any other part of the breast. Depending on how invasive the cancer is in relation to the primary tumor sites, there are several distinct types of breast cancer within the broad category of diverse breast carcinomas. Due to their disparate prognoses and treatment implications, the various subtypes must be distinguished from one another. Given the striking similarities between the molecular progression of breast cancer and normal development. These subtypes were basal-like, human epidermal growth factor receptor 2 (HER2)-enriched, luminal A and luminal B (which express the estrogen receptor) (without ER expression) (Harbeck et al., 2019).

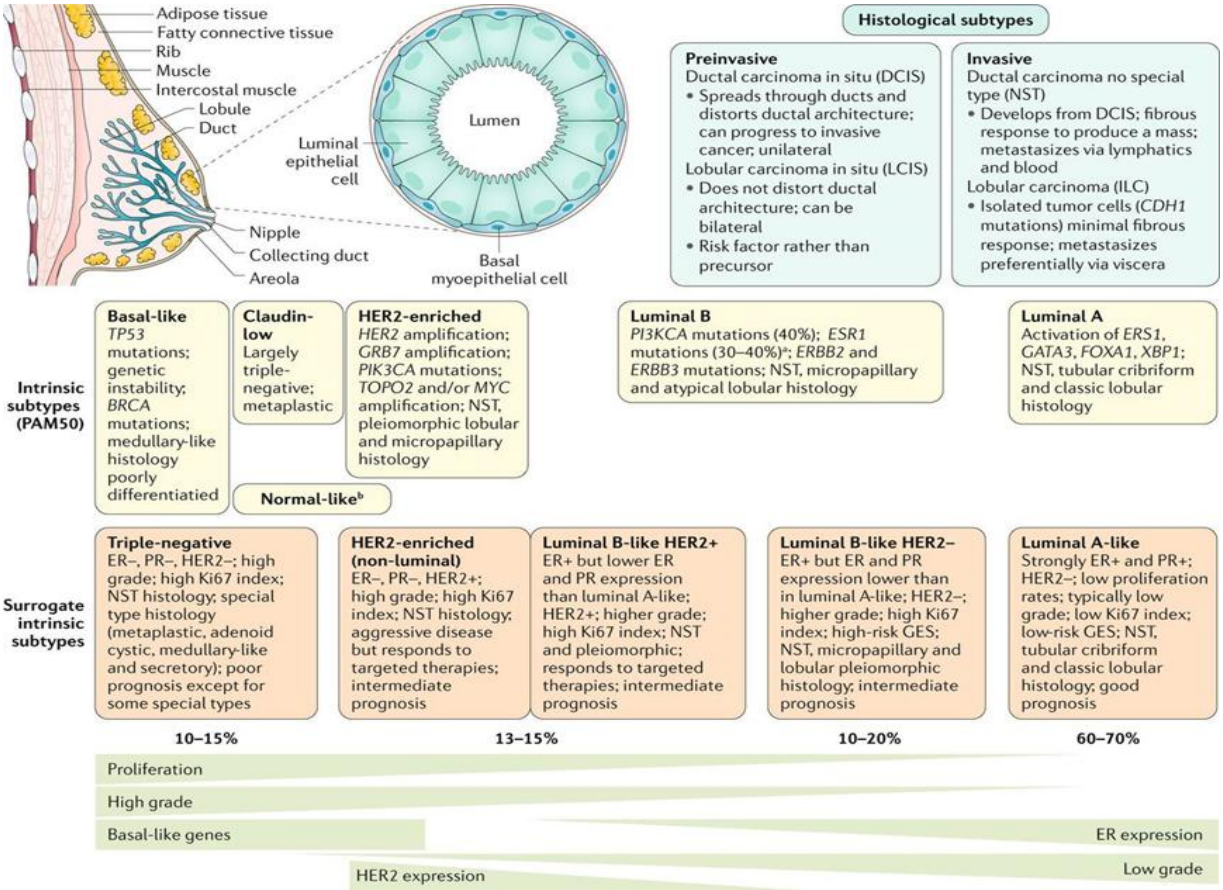

Figure 2. The different forms of breast cancer (Harbeck et al., 2019).

The two main pillars of breast cancer management are systemic therapy and locoregional treatment; treatment choices are heavily influenced by the molecular and histological features of the disease (Harbeck et al., 2019).

2.3. Estrogen

Estrogen is a steroid hormone that is connected to the female reproductive system and is in charge of the development of female sexual characteristics. Estrogen is often referred to as estrone, estradiol, and estriol (Delgado and Lopez-Ojeda, 2023).

Estrogen plays a key role in regulating breast growth during adolescence and breast maturation during pregnancy in order to facilitate lactation. The main factor in the formation of the ductal component of the breast, as well as the growth of connective tissue and fat deposition, is estrogen, which plays a significant role in breast development. Additionally, it indirectly elaborates in the lobuloalveolar component by stimulating the secretion of prolactin and enhancing the expression of progesterone receptors in the breasts. They can finish the lobuloalveolar growth during pregnancy by combining progesterone, estrogen, and prolactin (Al-Shami et al., 2023).

The aromatase enzyme transforms androgen into estrogen, which is essential for maintaining blood sugar levels, immune system strength, bone and cardiovascular health, fertility, and brain function. But practically all human pathologies, from infectious to autoimmune, metabolic, and degenerative, are centered around estrogen. Acute and chronic illnesses have been related to both hypo- and hyper-estrogen levels (Patel et al., 2018).

Estrogen works through its two distinct nuclear receptors, Estrogen Receptor alpha (ERα) and Estrogen Receptor beta (ERβ). Estrogen works through several possible cellular mechanisms to mediate its biological responses (Hamilton et al., 2017).

Estrogen’s mode of action is either free or bound to albumin or sex hormone-binding globulin (SHBG) proteins, estrogen enters the bloodstream. Estrogen that is not bound to proteins has the ability to freely and unrestrictedly diffuse into cells. When estrogen binds to either an alpha-or beta-estrogen receptor in the cell cytoplasm, the physiological response of the cell to estrogen is initiated. The activated estrogen-estrogen receptor complex then enters the cell nucleus and binds to nucleotide sequences called estrogen response elements (ERE) to trigger DNA transcription and a physiological reaction. The body's levels of estrogen hormone are controlled by the negative feedback that estrogen has on the pituitary and hypothalamus (Delgado and Lopez-Ojeda, 2023).

Three types of endogenous estrogen are produced by the female body naturally, and they serve a variety of physiological purposes. A steroid hormone called 17β estradiol (E2) is made from cholesterol. With the highest affinity for estrogen receptors, ovarian E2 is the most potent form of estrogen produced. In the periphery, less potent metabolites of E2 are estriol (E3) and esttrone (E1). The production of E1, E2, and E3 is also significantly influenced by the aromatization of the A rings of androgenic precursors, such as testosterone or androstenedione. In women who are not yet menopausal, E2 is the most common form of estrogen (Abou-Ismail et al., 2020).

2.4. Microbiota

The term "microbiota" has its roots in the early 1900s. A variety of microorganisms, such as bacteria, yeasts, and viruses, have been discovered to coexist in the human body's many organ systems (gut, skin, lung, and oral cavity). The microbiota's makeup varies from place to place (Hou et al., 2022).

A multitude of factors, including host characteristics, environmental pollutants, geographic location, bacterial infections, antibiotic treatment, lifestyle choices, surgical procedures, and age, influence the composition of the gut microbiota. Despite being relatively stable once established, the adult gut microbiota composition remains consistent (Plaza-Diaz and Álvarez-Mercado, 2023).

Numerous essential processes are carried out by the gut microbiota, such as vitamin synthesis, dietary compound metabolism, and defense against the growth and systemic infiltration of gut pathogens. Any disruption to this delicate balance could result in dysbiosis, a disorder known as impaired microbiota that has been connected to a number of human diseases, including breast cancer (Lauby-Secretan et al., 2015). The Human Microbiome Project (2008–2013) shows that the microbiome varies clearly between organs and between individuals, suggesting that it may be a factor in the development of disease. The development and aggressiveness of cancers have also been linked to the makeup of the human microbiota (Ruo et al., 2021).

The impact of microbiota on human health and illness is a relatively new and quickly developing field of study. Diseases and microbes have been related (Xuan et al., 2014). These microbiotas have a commensurate, mutually beneficial relationship with the human host. The host's digestive tract provides the microbiota with a nutrient niche. The microbiota safeguards

against pathogens, supports immune system development, aids in the breakdown of dietary toxins and carcinogens, produces essential amino acids and vitamins, aids in the absorption of minerals, and helps with nutrient reclamation from food. Since the baby is delivered by vaginal canal, the mother's gut microbiota is passed down to the infant. The intestinal microbiota is altered later in development by variables that are both independent of host decisions, like age and genetics, and dependent on host choices, like nutrition (Shapira et al., 2013).

Recent research points to the active involvement of local and distant microbiota in the development, progression, and prognosis of breast cancer. By causing genetic instability, starting DNA damage and the proliferation of the damaged offspring, generating a positive immune response, inducing metabolic dysregulation, and altering the body's response to therapy, a dysbiotic microbiota causes the body to the development of cancer. Microbiota can be communities of bacteria, fungi, protists, Archaea and viruses that inhabit the human body (Plaza-Diaz and Álvarez-Mercado, 2023).

The goal of thoroughly characterizing the microbiota in various body parts under various medical conditions—including breast cancer—has been pursued recently. Bacterial microbes can affect homeostasis through interacting with the metabolic processes of the host (Ruo et al., 2021).

2.4.1. Lactobacillus One of the largest groups of lactic acid bacteria (LAB) are the rod-shaped, facultatively anaerobic, Gram-positive Lactobacillus spp. They are devoid of both catalase and oxidase, and they have the ability to hydrolyze esculin and ferment carbohydrates. Pickles and dairy products are two examples of fermented foods from which Lactobacillus spp. can be isolated. In addition, Lactobacillus species are common bacteria that inhabit the gastrointestinal system and the female genital tract. They produce lactic acid, which helps to partially inhibit the growth of pathogenic microorganisms in these areas. Furthermore, these are typical microbiological species found in the human mouth, which have been linked to dental caries. There are currently 253 species in the genus Lactobacillus, whose names have been legally published. Common Lactobacillus species vary according to the types of animals they feed on, including herbivores, carnivores, and omnivores (Kong et al., 2020).

Beijerinck proposed the genus Lactobacillus in 1901, and it contains non-spore-forming, facultatively anaerobic, gram-positive, fermentative microorganisms. The genus is included in the family Lactobacillaceae, order Lactobacillales, class Bacilli, phylum Firmicutes, which also includes the genera Lactobacillus, Paralactobacillus, and Pediococcus. The closest family members are the Leuconostocaceae, which includes the genera Convivina, Fructobacillus, Leuconostoc, Oenococcus, and Weissella. The level of genetic diversity present in the genus Lactobacillus as it is currently defined is becoming more widely acknowledged, and it far surpasses that of most bacterial genera and even families. Nonetheless, the various phylogroups within the genus are made up of species whose physiological and phylogenetic diversity is comparable to that of other bacterial genera (Zheng et al., 2020).

Despite being a relatively small component of the human colonic microbiota, the proportions of Lactobacillus are often positively or negatively associated with chronic conditions and human disease. New insights into the significance of this genus for human health have been brought about by recent discoveries on Lactobacillus species in studies on the microbiomes of humans and animals, as well as by the growing body of knowledge regarding probiotics and other ingested lactobacilli (Heeney et al., 2018).

2.5. Breast cancer and Estrogen

During female puberty, estrogen is in charge of the development of mammary gland tissue as well as parenchymal and stromal changes in breast tissue. In addition, estrogen plays a role in the development of the mammary ducts during puberty and pregnancy as well as in the secretion of breast milk during the postpartum period (Delgado and Lopez-Ojeda, 2023). The majority of breast cancers are estrogen dependent (McNamara et al., 2017).

Estrogen is known to have a causal role in the genesis of HR-positive breast cancer and to be crucial in the initiation and stimulation of cancerous growth (Kwa et al., 2016). Women who developed postmenopausal breast cancer had a significant approximate 15% increase in estrogens compared to those who did not develop the disease, according to a meta-analysis of six prospective studies. The highest serum estrogen levels were linked to a doubling of the risk of breast cancer, according to a second, more recent meta-analysis of nine prospective studies. Higher estrogen levels in women were linked to an increased risk of postmenopausal breast cancer, according to another study analyzing data from the Nurses' Health Study.

Given that 70% of breast cancers are of the estrogen-receptor positive subtype, endogenous estrogen is clearly a significant contributor to breast cancer, particularly after menopause. The main location of estrogen production prior to menopause is the ovaries, and circulating estrogen has endocrine effects on the bones, brain, and immune system (Parida and Sharma, 2019).

2.6. Breast cancer and microbiota

The microbial community compositions of cancer patients' breasts and those of healthy ones were found to differ strikingly. Differences in microbiome were also apparent between benign and malignant disease. These microbes are capable of producing numerous secondary metabolites that can act as signaling mediators effecting breast cancer progression. These microbes may change how the body reacts to treatment, which could have an impact on toxicity, anti-tumor effects, pharmacokinetics, and drug metabolism. In conclusion, the human breast contains a diverse community of microbes that can interact with host cells to trigger downstream signaling pathways and modulate different aspects of breast cancer growth and metastatic progression. It is obvious that there are other risk factors for the development of breast cancer. One such factor that has received a lot of attention recently is the "Human Microbiome". Many cancer types have been linked to the human microbiome as a major factor in both cancer incidence and progression. Breast tissue was once thought to be sterile and devoid of any microbial population, but numerous investigations have shown that the breast has a unique local microbiota, and a dysbiosis in this community structure is assumed to contribute to and cause carcinogenesis (Parida and Sharma, 2019).

Microbial dysbiosis characterizes the breast cancer, both in the breast tissue and in the gut. It is unclear how the breast microbiome contributes to the development of cancer. On the other hand, it has been demonstrated that the gut microbiota produces or alters metabolites that travel through the bloodstream to far-off locations, like the breast, where they alter breast cancer cell activity. These metabolites seem to be significant components of the microenvironment surrounding the tumor. The disease's aggressive behavior, grade, and function may all be altered by the breast microbiome (Hieken et al., 2016).

As early as 1990, a study comparing the gut microbiomes of seven healthy women and eleven women with breast cancer revealed a correlation between altered gut microbiome and breast cancer. According to this study, patients with breast cancer had higher concentrations

of Clostridium, Enterobacterium, Lactobacilli, Bacteroides, and Escherichia coli (Parida and Sharma, 2019).

2.7. Estrogen and microbiota

One of the most prominent roles of the human microbiome is the regulation of steroid hormone metabolism since endogenous estrogens are the most important risk factor in breast cancer development especially in postmenopausal women. The regulation of estrogen metabolism by microbes has been known for over a decade now and yet our understanding in this regard is limited due to the complexity of the microbiome that can vary based on diet, body mass index, ethnicity, race, age, occupation, disease status, and antibiotic usage (Parida and Sharma, 2019).

The liver is where estrogens are first metabolized and where they are conjugated along with their metabolites. By being metabolically converted into water-soluble molecules and excreted in the urine or bile into the feces, conjugated estrogens are removed from the body. Bacterial species in the gut with beta-glucuronidase activity (components of the "estrobolome") have the ability to deconjugate the conjugated estrogens excreted in the bile, which results in the reabsorption of estrogen into the bloodstream. Target tissues, such as the breast, are affected by circulating estrogens, which promote cellular growth and proliferation. The estrobolome influences the excretion and circulation of estrogens by regulating their enterohepatic circulation. On the other hand, substances that alter the functional activity of the estrobolome, like antibiotics, other medications, and food, can also affect its composition. The modulation of systemic estrogens by the intestinal microbiome plays a significant role in the regulation of estrogen circulation and reabsorption. Theoretically, an estrobolome rich in deconjugation-promoting enzymes would enhance the reabsorption of free estrogen and raise the relative total estrogen burden, which could raise the risk of developing hormone-driven cancers like breast cancer. In turn, host characteristics like age and ethnicity as well as environmental influences throughout life like nutrition, alcohol consumption, and antibiotic use may have an impact on the bacterial composition of the estrobolome and apply selective pressures to bacterial populations (Kwa et al., 2016).

III. Aim

The project's aim is to study the Estrogen level and microbial interaction in Breast cancer and determine the function of the microbiome and/or its metabolites in the regulation of the development of breast cancer.

IV. Objectives

4.1. The association between microbiome metabolites and breast cancer among Egyptian women.

4.2. The Path-physiology of breast cancer and its relation to microenvironment of tumor cells.

4.3. The Link of interaction between gut and breast microbiome and tumorigenesis.

4.4. The correlation between breast and gut microbiota with anti-cancer therapy.

V. Materials and methodology

5.1 Study design:

This study was a case-control cross sectional study. This study was also conducted and reported in accordance with the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines for case–control studies. Compliance with these reporting standards was ensured throughout the study design, data collection, analysis, and presentation of results to improve transparency, reproducibility, and overall scientific credibility.

5.2 Study place:

The study was conducted at Baheya Breast Hospital in collaboration with the National Research Centre and Misr University for Science and Technology. The study took place in Egypt among Egyptian population of women.

5.3 Sample population:

- samples were obtained from 180 women, of which 90 of them have non-metastatic breast cancer and the other 90 are healthy control women.
- Participants underwent physical examinations and clinical assessments to gain all the possible data about the patients and their disease.
- Patients information and data were collected from their records, these data included other factors that may affect the study and must be put into consideration, as blood pressure diseases -hypertension or hypotension-, diabetes, genetic disorders, mental and physical illnesses that might interfere with the results of this research.
- Medical records were obtained and reviewed as well from patients' records, this included all the required data about patients' cancers; their types and stages as well as diagnosis, treatment history and the development and progression of the disease.

5.4 Sample size justification

The sample size was determined to provide adequate statistical power to detect meaningful differences between women with non-metastatic breast cancer and healthy control women. A total of 180 participants were included, with 90 cases and 90 controls, using a 1:1 case–control ratio to enhance comparability between groups and minimize selection bias. This sample size was considered sufficient based on previous comparable case-control studies investigating microbiota composition, hormonal levels, and anthropometric parameters in breast cancer populations. The chosen sample size allows for reliable estimation of group differences and associations while accounting for potential variability in biological and clinical measurements. All eligible participants who met the inclusion and exclusion criteria during the study period were consecutively recruited until the required sample size was reached.

5.4 Inclusion criteria

For women patients' sample: -

- Women with breast cancer
- Non-metastatic breast cancer
- Women of child bearing age between 25 to 45

For healthy control women: -

- Women with no disorders or known chronic diseases that could affect the microbiota concentration as hypertension, diabetes or bleeding tendency.
- Normal mammography.

5.5 Exclusion criteria for both patients and control group

- Pregnant or lactating women
- Women with any other disease or condition that might interfere with the study assessments
- Women who use contraceptives

5.6 Anthropometric assessments:

Anthropometric parameters compromise body weight, height, waist, body mass index (BMI) calculation, hip and mid upper arm circumferences, waist

circumference (WC), hip circumference (HC), waist hip ration (WHR). WHR and body composition were measured. All measurements were taken 3 times on the left side of the body then the mean of the 3 values were used. Body weight and height were measured to the nearest 0.1 kg and 0.1 com respectively. A stadiometer was used for measuring height where every woman stands with her back leaned against it. BMI was calculated as weight in kilograms divided by height in meters square. WC was measured with light clothing at a level midway between the lower rib margin and the iliac crest. HC was measured at the level of the widest circumference over the buttocks (at the greater trochanter). Hence, WHR was calculated. Mid upper arm circumference (MUAC) was measured at the midway between the olecranon process and acromion on the upper arm with the elbow flexed 90 degrees. Skin fold thickness of biceps, triceps, subscapular, suprailiac and abdominal skin fold thickness were measured using Holtain caliper (Ltd, Bryberian, Crymmych, Pemborkeshire).

5.7 Laboratory investigation

- **Blood sample from both cases:**

Complete blood picture (leukocyte, platelet, granulocyte/lymphocyte ratio) was obtained from the women records at Baheya hospital.

- **Stool samples from both samples:**

Stool samples were analyzed for microbiome characterization. Stool specimens were collected, kept in sterilized caps at +4°c and then delivered to PCR laboratory within 4 hours.

5.8 Methods for measuring estrogen levels in serum.

Venous blood samples were drawn under aseptic conditions in sterile tubes. Tubes were centrifuged and about 2mL of serum was collected, stored at -20ºC for serum estrogen level determination.

Enzyme-linked immunoassays (ELISAs) were used in clinical diagnosis, research, and monitoring to measure the levels of circulating estrogens. It depends on the process of an antigen (estrogen) attaching to particular antibodies; in ELISA, this interaction is detected by incubating the substrate(s) with a known ability to produce a detectable product.

5.8 Lactobacillus isolation

Stool samples were collected from 90 healthy women at Baheya Hospital, Egypt. All procedures involving human subjects were approved by the Ethics Committees of Baheya Hospital, Egypt (reference number:). The samples were collected in a sterile tube containing Man-Rogosa-Sharpe (MRS) broth. Stool samples were kept in an anaerobic box on ice during transportation. For isolation, the stool samples were diluted with normal saline (0.85% NaCl) and cultured on MRS agar containing 0.05% l-cysteine and CaCO3 for 24–48 h at 37 °C in an anaerobic chamber (Jamyuang et al., 2019).

5.9 Ethical aspect

Privacy and confidentiality of subjects:

The patient entire data recorded were highly confidential by working staff. -Patient laboratory samples were discarded after performing labs required and will not be further used for any purpose - We informed the patient by the laboratory results and the importance of the follow up with positive results of virus infection to can avoid the cardiovascular risk factors.

Risks and benefits

-Risks: there were mild risks that might have occurred to patients, including infection while blood sample withdrawn so all aseptic precautions will be addressed. *Risk ratio= impact of event (injection infection) x probability (occurrence inside NRC lab.) = >5 is mild risk and the action needed (Requires medical attention)

-Benefits: the project aimed to help and improve health and in breast cancer Egyptian women.

-Risk benefit ratio: however, there were minimal risks, potential benefits exceed any expected risks, wherever these risks were under control and continuously evaluated for prevention.

5.10. Statistical analysis

Statistical analysis was done using SPSS (Statistical Package for the Social Science for Windows) version 20.0. Mean and standard deviation were used to describe quantitative

data and for comparison, unpaired t test and one-way ANOVA test was used. Categorical variables were presented by frequency counts, and intergroup comparisons were analyzed by a chi-square test. Odds ratio (OR) with 95% confidence intervals were calculated. Correlation was done using Pearson correlation coefficient. Logistic and linear regression analyses were used to examine the relationships between different variables. The level of significance is considered at $p < 0.05$.

VI. RESULTS

The results provide insights into the differences in basic characteristics, clinico-pathological data, estrogen concentrations, and the association between estrogen levels and Lactobacillus species among the studied groups.

6.1. Basic Characteristics of the Studied Groups

The basic characteristics of the healthy controls (n=48) and the malignant group (n=47) were analyzed. The mean age of the healthy controls was 57.34±16.83 years, while the malignant group had a mean age of 59.98±8.82 years. There was no significant difference in age between the two groups (p=0.33). Hypertension was significantly more prevalent in the malignant group (44.7%) compared to the healthy controls (14.5%) with a p-value of 0.002. The incidence of diabetes was higher in the malignant group (25.5%) compared to the healthy controls (12.5%), though not statistically significant (p=0.17). The body mass index (BMI) showed no significant difference between the two groups. Family history, menopausal status, and hormonal contraception use also did not show significant differences between the groups as shown in table (1)

Table 1: Basic characteristics of the studied groups

Studied variable	**Healthy controls N= 48 No (%)**	**Malignant group N=47 No (%)**	**P- value**
Age			
Mean± SD	57.34± 16.83	59.98± 8.82	
Median	58.0	60.0	0.33
Min-max	39-67	36 -77	
Hypertension	7 (14.5)	21 (44.7)	0.002
Diabetes	6 (12.5)	12 (25.5)	0.17
BMI			0.15
Mean± SD	31.26± 6.140	32.9± 5.42	
Median	30.0	32.34	
Min-max	22 -51	21.64- 47.87	
Family history			
Positive	10 (20.8)	14 (29.8)	0.44
Menopausal status			
Pre			
Post	12 (25.0) 36 (75.0)	9 19.1 38 80.9	0.49
Hormonal contraception using	4 (8.3)	3 (6.4)	0.71

6.2. Clinic-Pathological Data of Breast Cancer Group

In the non-metastatic breast cancer group, the T stage distribution was as follows: Tis (5.1%), T1 (12.8%), T2 (53.8%), T3 (17.9%), and T4 (10.3%). The N stage was predominantly N0 (65%), followed by N1 (25%), N2 (7.5%), and N3 (2.5%). Tumor laterality was nearly evenly split between left (46.8%) and right (48.9%) sides, with a small percentage being bilateral (4.3%). The most common site of the tumor was the upper outer quadrant (UOQ) at 51.0%. The biopsy results showed that invasive ductal carcinoma (IDC) was the most prevalent type at 64.3%, followed by other types including DCIS (11.9%) and others (14.3%). Grade distribution indicated that the majority were grade 2 (78.9%). Estrogen receptor (ER) positivity was found in 87.5% of cases, progesterone receptor (PR) positivity in 78.9%, and all cases were HER2 negative as shown in table (2)

Table 2:Clinico-pathological data of the breast cancer group

Studied variable	**Breast cancer patients N=47 No (%)**	
T stage		
Tis	2	5.1
T1	5	12.8
T2	21	53.8
T3	7	17.9
T4	4	10.3
N stage		
N0	26	65.0
N1	10	25.0
N2	3	7.5
N3	1	2.5
Laterality		
bilateral	2	4.3
Left	22	46.8
right	23	48.9
Site of the tumor:		
LIQ	3	6.4
LOQ	6	12.7
UIQ	3	6.4
UOQ	24	51.0
Retroareoral mass	3	6.4
Nipple	1	2.1

Biopsy (TCNB)		
IDC	27	64.3
ILC	2	4.8
mixed	2	4.8
others	6	14.3
DCIS	5	11.9
Grad		
1	2	5.3
2	30	78.9
3	6	15.8
ER		
Negative	5	12.5
Positive	35	87.5
PR	8	21.1
Negative	30	78.9
Positive		
HER2	47	100.0
Negative		
size of the tumor	4.80± 2.61	
Mean± SD		

6.3. Estrogen Concentrations Among Studied Groups

Estrogen concentrations were significantly lower in the non-metastatic group compared to the healthy controls. The mean estrogen level in the healthy controls was 32.40±12.821 pg/mL, while in the non-metastatic group it was 14.15±7.56 pg/mL. This difference was highly significant with a p-value of <0.001 as shown in table (3) and figure (3)

Table 3: Comparison of Estrogen concentration among studied groups

Studied variable	**Healthy controls N= 48**	**Non metastatic group N=47**	**P- value**
E2			
Mean± SD	32.40±12.821	14.15± 7.56	
Median	31.50	12.10	<0.001
Min- max	18-51	6- 38	

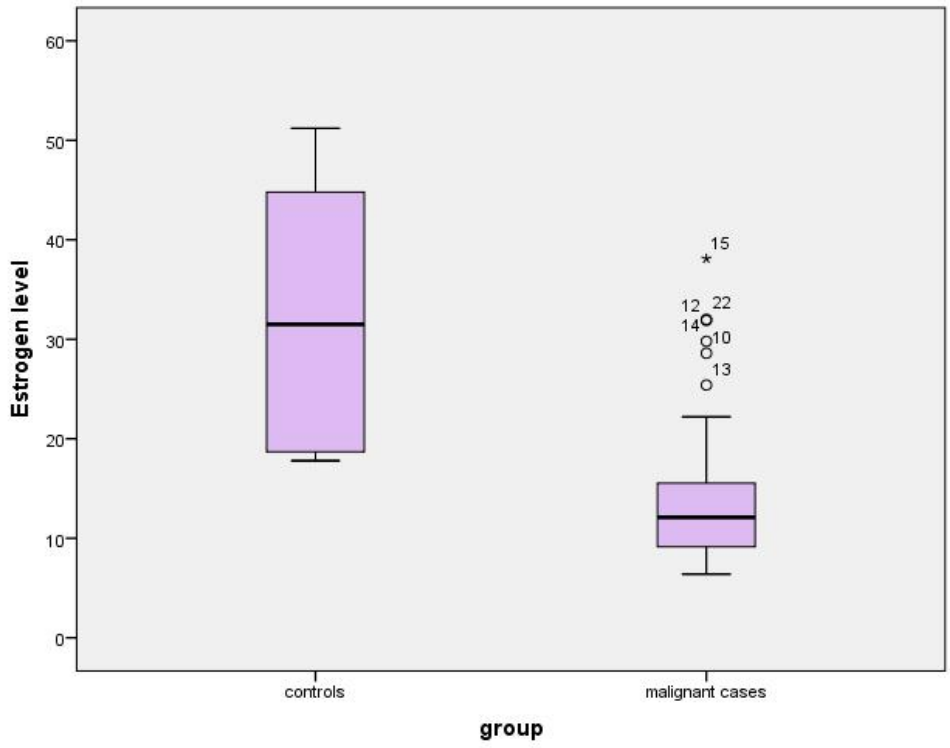

Figure 3: Serum level of Estrogen among non-metastatic malignent and healthy control group

6.4. Receiver Operating Characteristic Curve of Serum Estrogen

The receiver operating characteristic (ROC) curve analysis for serum estrogen levels between non-metastatic and healthy control groups showed a cut-off value of 17.5 pg/mL. The area under the curve (AUC) was 91.4% (95% CI: 85.6-97.2), with a sensitivity of 81.0% and specificity of 100.0% as shown in table (4) and figure (3)

Table 4: **Receiver operating characteristic curve of serum Estrogen between studied groups:**

Studied variable	Cut off	AUC (95% CI)	Sensitivity %	Specificity %
Non-metastatic Vs. healthy controls groups				
E2	17.5	91.4 (85.6-97.2)	81.0%	100.0%

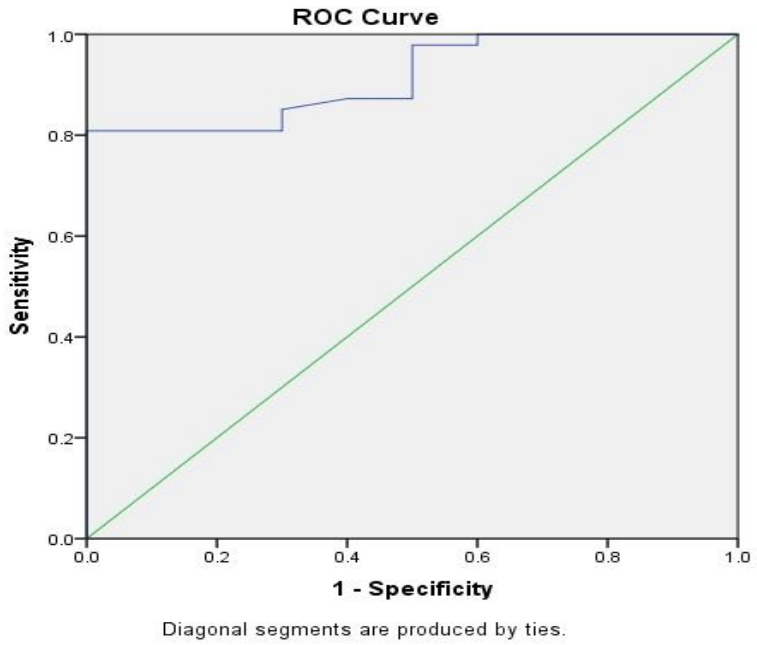

Figure 4:Receiver operating characteristic curves for prediction capacity of Estrogen between Non-metastatic Vs. healthy controls

6.5. Association Between Estrogen Levels and Isolated Lactobacillus Species

The association between estrogen levels and isolated Lactobacillus species was assessed. The mean estrogen levels among different Lactobacillus species showed no significant difference, with p-values above 0.05 across all comparisons. The species analyzed included Lactobacillus casei/paracasei/rhamnosus, Lactobacillus fermentum, Lactobacillus salivarius, and others

Figure 5: association between Estrogen levels and isolated lactobacillus species

	isolated lactobacillus species				**p- value**
	Lactobacillus casei/ paracasei/ rhamnosus	Lactobacillus fermentum	Lactobacillus fermentum	Lactobacillus salivarius	
Estrogen levels					
Mean± SD	14.8 ± 9.039	15.8 ± 10.6	18.7 ± 9.080	13.9 ± 6.317	0.68
Median	14.0	12.50	15.00	13.00	
Min- max	7- 30	6- 38	9- 32	6- 30	

VII. DISCUSSION

The present study provides important insights into the clinical, hormonal, and microbiological characteristics of women with non-metastatic breast cancer compared to healthy controls. A comprehensive interpretation of these findings requires integration with current evidence and an understanding of the biological mechanisms underlying estrogen regulation in breast cancer.

Age and menopausal status

No significant difference in age was observed between the malignant and healthy control groups, which is consistent with previous reports indicating that age alone is not an independent determinant of breast cancer occurrence but rather a cumulative risk factor (Qian et al., 2020). Although breast cancer risk increases with advancing age, particularly after menopause, the absence of significant differences in menopausal status between groups in the present study suggests that estrogen alterations observed in patients are unlikely to be solely attributable to menopausal transition. Nevertheless, menopausal status remains a critical determinant of endogenous estrogen levels and should be further stratified in future studies to refine hormonal interpretations.

Comorbidities and metabolic risk factors

The significantly higher prevalence of hypertension among breast cancer patients aligns with growing evidence linking cardio metabolic disorders to breast cancer risk and progression. Similarly, the higher, though non-significant, prevalence of diabetes in the malignant group supports previous findings suggesting that insulin resistance and chronic metabolic dysregulation may contribute to breast carcinogenesis (Xie et al., 2021). These conditions may also indirectly influence estrogen metabolism through alterations in adipose tissue function and systemic inflammation.

Tumor characteristics

The distribution of tumor stages, laterality, and anatomical site in this study reflects well-established patterns of breast cancer presentation. The predominance of invasive ductal carcinoma and grade 2 tumors is consistent with global epidemiological data and supports the representativeness of the study population (Wang et al., 2024).

Hormone receptor status

The high prevalence of ER- and PR-positive tumors observed in the malignant group is in agreement with the known hormonal dependency of the majority of breast cancers. This finding underscores the central role of estrogen signaling in tumor biology. The absence of HER2-positive cases may reflect the limited sample size or population-specific characteristics and does not diminish the relevance of the hormonal findings (Dijksterhuis et al., 2020).

Justification of low circulating estrogen levels in non-metastatic breast cancer

A key finding of the present study is the significantly lower serum estrogen levels in women with non-metastatic breast cancer compared to healthy controls. Although estrogen is a well-established risk factor for breast cancer initiation, reduced circulating estrogen levels in diagnosed patients have been reported in multiple studies and may be explained by several biological mechanisms (Malik et al., 2021).

First, estrogen action in breast cancer is largely mediated at the tissue level rather than reflected solely by circulating concentrations. Tumor cells can locally synthesize estrogen through increased aromatase activity, leading to enhanced intratumoral estrogen signaling despite lower systemic levels. This localized estrogen production may result in increased estrogen uptake and utilization by tumor tissue, thereby reducing measurable circulating estrogen levels.

Second, breast cancer development may induce systemic hormonal feedback mechanisms, including suppression of ovarian estrogen production or altered hypothalamic–pituitary–gonadal axis regulation. Additionally, inflammatory cytokines and metabolic changes associated with cancer may accelerate estrogen metabolism and clearance.

Third, many patients with breast cancer, even in non-metastatic stages, experience physiological stress and metabolic alterations that can influence hormone synthesis and degradation. Changes in liver function, adipose tissue distribution, and estrogen-binding proteins may further contribute to reduced circulating estrogen levels.

Importantly, estrogen levels are influenced by menstrual cycle phase, menopausal status, body mass index, and assay methodology. While these factors were partially controlled in the present study through inclusion and exclusion criteria, residual variability cannot be excluded and should be considered when interpreting hormonal measurements.

Diagnostic performance of estrogen levels

The ROC analysis demonstrated a high discriminatory ability of serum estrogen levels in differentiating non-metastatic breast cancer patients from healthy controls, with an AUC of 91.4%. This finding highlights the potential value of estrogen as a complementary biomarker rather than a standalone diagnostic tool. However, external validation in larger, multi-center cohorts is necessary to confirm its clinical utility and to account for confounding factors such as age, menopausal status, and body composition (Ravaioli, 2020).

Association with Lactobacillus species

The absence of significant differences in estrogen levels across different Lactobacillus species suggests that estrogen modulation in breast cancer is likely influenced by broader microbiome–host interactions rather than individual bacterial strains. While certain Lactobacillus species have been implicated in estrogen deconjugation and enterohepatic circulation, current evidence remains inconsistent. The complex relationship between gut microbiota, estrogen metabolism, and breast cancer risk likely involves microbial diversity, enzymatic activity, host genetics, and environmental factors (Hussain et al., 2021).

VIII. CONCLUSION

The study provided insights into differences in characteristics, clinic pathological data, estrogen levels, and the association between estrogen and Lactobacillus species in breast cancer patients versus healthy controls. The findings on age, comorbidities, tumor features, and hormone receptor status aligned with existing literature. A notable finding was significantly lower estrogen levels in the non-metastatic breast cancer group compared to healthy controls, consistent with estrogen's role as a risk factor. The diagnostic utility of serum estrogen levels was promising but needs further validation across larger, diverse cohorts. Interestingly, no significant association was found between estrogen levels and specific Lactobacillus species, highlighting the complex gut microbiome-estrogen-breast cancer interplay that requires more mechanistic investigation. Limitations included the relatively small sample size and lack of details on estrogen measurement methods. Future research should address these, explore underlying mechanisms of differential estrogen levels, and examine diagnostic/therapeutic implications. Overall, the study contributed to understanding breast cancer risk, diagnosis, and management by underscoring the importance of factors like estrogen levels and gut microbiome. Further research in this area could translate findings to improving clinical practice and patient outcome.

IX. REFERENCES

AL-SHAMI, K., AWADI, S., ALSHEIKH, A. M., AL-SHARIF, S., ALA'BERESHY, R., AL-EITAN, S. F., BANIKHALED, S. H., AL-QUDIMAT, A., AL-ZOUBI, R. M. & AL ZOUBI, M. S. 2023. Estrogens and the risk of breast cancer: A narrative review of literature. *Heliyon*.

DELGADO, B. J. & LOPEZ-OJEDA, W. 2023. Estrogen. *StatPearls.* Treasure Island (FL) ineligible companies. Disclosure: Wilfredo Lopez-Ojeda declares no relevant financial relationships with ineligible companies.: StatPearls Publishing

DIJKSTERHUIS, W. P., VERHOEVEN, R. H., MEIJER, S. L., SLINGERLAND, M., HAJ MOHAMMAD, N., DE VOS-GEELEN, J., BEEREPOOT, L. V., VAN VOORTHUIZEN, T., CREEMERS, G.-J. & VAN OIJEN, M. G. 2020. Increased assessment of HER2 in metastatic gastroesophageal cancer patients: a nationwide population-based cohort study. *Gastric Cancer,* 23**,** 579-590.

EZHILARASAN, D. 2020. Critical role of estrogen in the progression of chronic liver diseases. *Hepatobiliary Pancreat Dis Int,* 19**,** 429-434.

HAMILTON, K. J., HEWITT, S. C., ARAO, Y. & KORACH, K. S. 2017. Estrogen Hormone Biology. *Curr Top Dev Biol,* 125**,** 109-146.

HARBECK, N., PENAULT-LLORCA, F., CORTES, J., GNANT, M., HOUSSAMI, N., POORTMANS, P., RUDDY, K., TSANG, J. & CARDOSO, F. 2019. Breast cancer. *Nature Reviews Disease Primers,* 5**,** 66.

HEENEY, D. D., GAREAU, M. G. & MARCO, M. L. 2018. Intestinal Lactobacillus in health and disease, a driver or just along for the ride? *Curr Opin Biotechnol,* 49**,** 140-147.

HOU, K., WU, Z.-X., CHEN, X.-Y., WANG, J.-Q., ZHANG, D., XIAO, C., ZHU, D., KOYA, J. B., WEI, L. & LI, J. 2022. Microbiota in health and diseases. *Signal transduction and targeted therapy,* 7**,** 135.

HUSSAIN, T., MURTAZA, G., KALHORO, D. H., KALHORO, M. S., METWALLY, E., CHUGHTAI, M. I., MAZHAR, M. U. & KHAN, S. A. 2021. Relationship between gut microbiota and host-metabolism: Emphasis on hormones related to reproductive function. *Animal nutrition,* 7**,** 1-10.

IBRAHIM, A. S., KHALED, H. M., MIKHAIL, N. N., BARAKA, H. & KAMEL, H. 2014. Cancer incidence in egypt: results of the national population-based cancer registry program. *J Cancer Epidemiol,* 2014**,** 437971.

JAMYUANG, C., PHOONLAPDACHA, P., CHONGVIRIYAPHAN, N., CHANPUT, W., NITISINPRASERT, S. & NAKPHAICHIT, M. 2019. Characterization and probiotic properties of Lactobacilli from human breast milk. *3 Biotech,* 9**,** 1-11.

KONG, Y., OLEJAR, K. J., ON, S. L. W. & CHELIKANI, V. 2020. The Potential of Lactobacillus spp. for Modulating Oxidative Stress in the Gastrointestinal Tract. *Antioxidants,* 9**,** 610.

KWA, M., PLOTTEL, C. S., BLASER, M. J. & ADAMS, S. 2016. The intestinal microbiome and estrogen receptor–positive female breast cancer. *Journal of the National Cancer Institute,* 108**,** djw029.

LAUBY-SECRETAN, B., SCOCCIANTI, C., LOOMIS, D., BENBRAHIM-TALLAA, L., BOUVARD, V., BIANCHINI, F. & STRAIF, K. 2015. Breast-cancer screening--viewpoint of the IARC Working Group. *N Engl J Med,* 372**,** 2353-8.

MALIK, A., HAFEEZ, K., NAZAR, W., NAEEM, M., ALI, I., ALI, Q., MUJTABA, Z., RANA, M. & HAFEEZ, M. 2021. Assessment of controversial risk factors in development of breast cancer: a study from local population. *Biological and Clinical Sciences Research Journal,* 2021.

MARONGIU, L., LANDRY, J. J., RAUSCH, T., ABBA, M. L., DELECLUSE, S., DELECLUSE, H. J. & ALLGAYER, H. 2021. Metagenomic analysis of primary colorectal carcinomas and their metastases identifies potential microbial risk factors. *Molecular Oncology,* 15**,** 3363-3384.

MCNAMARA, K. M., OGURO, S., OMATA, F., KIKUCHI, K., GUESTINI, F., SUZUKI, K., YANG, Y., ABE, E., HIRAKAWA, H. & BROWN, K. A. 2017. The presence and impact of estrogen metabolism on

the biology of triple-negative breast cancer. *Breast cancer research and treatment,* 161**,** 213-227.
NANDI, D., PARIDA, S. & SHARMA, D. 2023. The gut microbiota in breast cancer development and treatment: The good, the bad, and the useful! *Gut Microbes,* 15**,** 2221452.
PARIDA, S. & SHARMA, D. 2019. The microbiome–estrogen connection and breast cancer risk. *Cells,* 8**,** 1642.
PLAZA-DIAZ, J. & ÁLVAREZ-MERCADO, A. I. 2023. The Interplay between Microbiota and Chemotherapy-Derived Metabolites in Breast Cancer. *Metabolites,* 13**,** 703.
QIAN, X., JIA, H., ZHANG, Y., MA, B., QIN, G. & WU, Z. 2020. Risk factors and prediction of second primary cancer in primary female non-metastatic breast cancer survivors. *Aging (Albany NY),* 12**,** 19628.
RAVAIOLI, S. 2020. Role of Androgen Receptor biomarker in breast cancer: a translational study from in situ to invasive carcinoma.
REAGAN, M. 2019. Causes of cancer: Genetic, epigenetic, viral, microenvironmental, and environmental contributions to cancer. *Cancer: Prevention, Early Detection, Treatment and Recovery***,** 53-74.
SHAPIRA, I., SULTAN, K., LEE, A. & TAIOLI, E. 2013. Evolving concepts: how diet and the intestinal microbiome act as modulators of breast malignancy. *International Scholarly Research Notices,* 2013.
SIEGEL, R. L., MILLER, K. D., WAGLE, N. S. & JEMAL, A. 2023. Cancer statistics, 2023. *CA Cancer J Clin,* 73**,** 17-48.
WANG, J., LI, B., LUO, M., HUANG, J., ZHANG, K., ZHENG, S., ZHANG, S. & ZHOU, J. 2024. Progression from ductal carcinoma in situ to invasive breast cancer: molecular features and clinical significance. *Signal Transduction and Targeted Therapy,* 9**,** 83.
WARENIUS, H. M. 2023. The essential molecular requirements for the transformation of normal cells into established cancer cells, with implications for a novel anti-cancer agent. *Cancer Rep (Hoboken),* 6**,** e1844.
XIE, Y., WANG, M., XU, P., DENG, Y., ZHENG, Y., YANG, S., WU, Y., ZHAI, Z., ZHANG, D. & LI, N. 2021. Association between antihypertensive medication use and breast cancer: a systematic review and meta-analysis. *Frontiers in pharmacology,* 12**,** 609901.
XUAN, C., SHAMONKI, J. M., CHUNG, A., DINOME, M. L., CHUNG, M., SIELING, P. A. & LEE, D. J. 2014. Microbial dysbiosis is associated with human breast cancer. *PloS one,* 9**,** e83744.