University of Kerbala
College of Medicine
Department of Chemistry and Biochemistry
Clinical Biochemistry Program (M.Sc.)

BRCA1 and *BARD1* Gene Polymorphisms, *BRCA1* Promoter Methylation, and Related Protein Levels in Women with Breast Cancer of Karbala City

by

Shireen Adil Abid

B.Sc. Chemistry (University of Diyala, 2022)

A Thesis Submitted to the Faculty of the
COLLEGE OF MEDICINE OF THE UNIVERSITY OF KERBALA

In Partial Fulfillment of the Requirements

for the Degree of

Master of Science in Clinical Chemistry

Supervised by

Prof. Dr. Rana M. Hameed, *Ph.D.*
Assist. Prof. Dr. Mohammed F. Alqanbar, *M.D.*, *F.I.C.M.S. (Histopath.)*

2025 AD **1446 AH**

بسم الله الرحمن الرحيم

وَفِي أَنفُسِكُمْ

أَفَلَا تُبْصِرُونَ

(سورة الذاريات، الآية الحادية والعشرون)

*****

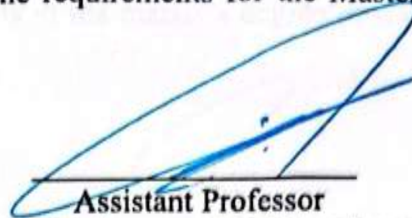
In the Name of Allah, the Most Gracious, the Most Merciful

“And in yourselves. Then will you not see?”

(Surah Adh-Dhariyat, 21)

Supervisors' Approval

We, the undersigned, certify that the thesis entitled **"*BRCA1* and *BARD1* Gene Polymorphisms, *BRCA1* Promoter Methylation, and Related Protein Levels in Women with Breast Cancer of Karbala City"** has been prepared under our supervision at the Department of Chemistry and Biochemistry, College of Medicine, University of Kerbala in partial fulfillment of the requirements for the Master of Science (M.Sc.) degree in Clinical Chemistry.



Professor (full)
Dr. Rana M. Hameed
M.Sc.& Ph.D. Biochemistry
Department of Chemistry & Biochemistry,
College of Medicine, University of Kerbala,
Karbala, Iraq

Assistant Professor
Dr. Mohammed F. Alqanbar
M.B.Ch.B., M.Sc. Clinical biochemistry &
FIBMS (Histopathology)
Department of Pathology,
College of Medicine, University of Kerbala,
Karbala, Iraq

Department's Note(s)

In view of the available recommendations, I hereby forward this M.Sc. thesis for the debate by the Examining Committee.

Professor (full)
Dr. Rana M. Hameed
Head of the Department of Chemistry and Biochemistry.
College of Medicine
University of Kerbala (Karbala, Iraq)

/ 06 / 2025

Scanned with CamScanner

Examining Committee's Approval

We, the undersigned, the examining committee of the thesis entitled "***BRCA1* and *BARD1* Gene Polymorphisms, *BRCA1* Promoter Methylation, and Related Protein Levels in Women with Breast Cancer of Karbala City**" have reviewed it, and examined the postgraduate student "**Shireen Adil Abid**" in the thesis content.

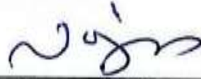
In our opinion, the thesis meets the standards of the master's degree of science (M.Sc.) degree in **Clinical Chemistry.**

Lecturer
Dr. Karrar Kadhim Mohsin
Committee member
Imam Hussain Oncology & Hematology Center, Karbala Health Department (Karbala, Iraq)
Date: / 09 / 2025

Assistant Professor
Dr. Maher Abbood Mukheef
Committee member
College of Medicine, University of Kerbala, Karbala, Iraq
Date: / 09 / 2025

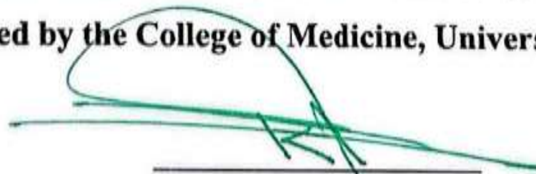
Professor (full)
Dr. Rana M. Hameed
Committee member & supervisor
College of Medicine, University of Kerbala, Karbala, Iraq
Date: / 09 / 2025

Assistant Professor
Dr. Mohammed F. Alqanbar
Committee member & supervisor
College of Medicine, University of Kerbala, Karbala, Iraq
Date: 15 / 09 / 2025



Professor (full)
Dr. Narjis Hadi Al-Saadi
Committee's chairman
College of Science, University of Kerbala (Karbala, Iraq)
/ 09 / 2025

Approved by the College of Medicine, University of Kerbala



Professor (full)
Dr. Khalid Khalil Alarji
Dean of the College of Medicine, University of Kerbala
Karbala, Iraq
/ 09 / 2025

Scanned with CamScanner

Dedication

To my brother, who gave his life fighting darkness so others could live in light..

To my grandmother, whose healing hands soothed countless children and whose wisdom lives on...

To my family and friends, who never let me fall..

This work is my love and gratitude made visible.

Acknowledgment

This work exists because of extraordinary people who believed in it. Prof. Dr. Rana M. Hameed, my supervisor, turned confusion into clarity and doubt into determination. Her patience never wavered, even when mine did. Prof. Dr. Mohammed F. Alqanbar's wisdom shaped this research in ways I'm still discovering. Dr. Hussein A. Abid made statistics fascinating rather than frightening, while Dr. Shukrya H. Alwan transformed bureaucratic mountains into manageable hills.

The teams at Khadijah Specialized Hospital for Women and Imam Hussein Medical City treated this project as their own. Dr. Karrar Almosawy, the Head of the Oncology and Hematology Center, guided me through sample collection and provided great support during patients' recruitment alongside Dr. Ahmad Majli, Dr. Hawra'a Al-Ghanimi, and Dr. Zaid Khudhair. The Early Breast Cancer Detection Unit team, Dr. Atheel S. Al-Nasrawi, Dr. Maha Sahib, Dr. Anwar Al-Hashimi, and Ms. Ruqaiyah A. Obaid, provided irreplaceable support. The laboratory staff handled each sample with remarkable care: Mr. Ghazwan Saeed Hamza, Mr. Naseem H. Al-Mosawi, Ms. Ibtihal Dakheel, Mr. Haider Dhiyaa, and Mrs. Iman Hamza.

Dr. Hamid K. Ali from the University of Bilad Alrafidain bridged possibility and reality through his expertise in genetic analysis. My sister Nisreen A. Abid, a biologist, helped with sample collection and documentation. The Biostatistics Unit and Blood Bank Division provided both data and encouragement on difficult days.

To everyone who contributed a word, a moment, or a gesture—you helped shape not just this thesis, but the researcher I've become. This work carries pieces of all of you within its pages.

Summary

The current thesis presents the first comprehensive molecular characterization of breast cancer in women from Karbala governorate, Iraq, addressing a critical knowledge gap in a population with high disease incidence (80 per 100,000). Breast cancer remains the most prevalent female malignancy globally, affecting 1.4-2.3 million women annually, with Iraq reporting 8,708 cases in 2023 alone. While international research has established the significance of *BRCA1*/*BARD1* genetic variants, epigenetic modifications, and molecular biomarkers in breast cancer, no systematic investigation had been conducted in the Iraqi population. Thus, this study aimed to evaluate the associations between genetic polymorphisms (*BRCA1* rs4986850 and *BARD1* rs1048108), *BRCA1* promoter methylation, protein expression levels, and miR-498 expression with breast cancer risk and clinical outcomes in this understudied population. A case-control study was conducted, to attain this goal, recruiting 188 women (90 breast cancer patients and 98 healthy controls) from Karbala between November 2024 to June 2025. The analysis included genetic polymorphisms (BRCA1 rs4986850 and BARD1 rs1048108), BRCA1 promoter methylation, protein levels (BRCA1 and BARD1), and microRNA-498 expression using PCR, sequencing, ELISA, and real-time PCR techniques.

The demographic analysis revealed significant risk factors unique to this population, including low education levels (47.8% of patients had only elementary education), extensive hormone replacement therapy use (65.6% vs 8.2% in controls), and younger age at first childbirth (20.5 vs 26.0 years). Clinically, patients presented with advanced disease, as 81.1% of tumors exceeded 20mm and 65.6% showed lymph node involvement, despite 86.7% being hormone receptor-positive. The genetic analysis yielded a novel protective finding: the *BARD1* rs1048108 AA genotype conferred 66% reduced breast cancer risk (OR=0.34, p=0.014), with the A allele providing 37% protection. *BRCA1* promoter methylation was significantly elevated in patients (17.8% vs 13.3%, p=0.043), accompanied by dramatic reductions

in both BRCA1 (4.194 vs 7.887 ng/ml) and BARD1 (3.991 vs 10.053 ng/ml) protein levels. Notably, BARD1 protein demonstrated exceptional diagnostic accuracy with 93.6% area under the curve, 88.5% sensitivity, and 95.8% specificity.

The molecular correlation analysis revealed critical disruptions in cancer patients. The normal positive correlation between BRCA1 and BARD1 proteins (ρ=0.464) was lost in cancer patients, indicating compromised DNA repair mechanisms. Conversely, a novel correlation emerged between miR-498 (which was upregulated 3-fold in patients) and BRCA1 protein exclusively in cancer cases (ρ=0.475), suggesting aberrant regulatory networks. Biochemical analyses confirmed systemic effects, including elevated inflammatory markers (hs-CRP: 8.18 vs 2.00 mg/L) and reversed albumin/globulin ratios. These findings establish population-specific molecular signatures that differ from global patterns, highlighting the importance of localized research for effective cancer management.

Based on these discoveries, several evidence-based recommendations emerge for improving breast cancer outcomes in Karbala. Implementation of screening programs incorporating BARD1 protein testing should target women over 40, particularly those with limited education or hormone therapy exposure. The exceptional diagnostic performance of BARD1 protein, measurable through simple blood tests, makes it particularly suitable for resource-limited settings. Genetic counseling services must address the protective BARD1 variant while public health campaigns should urgently address the exceptionally high hormone replacement therapy use. Treatment protocols require adaptation for the predominantly hormone-positive, locally advanced presentations characteristic of this population. This pioneering study transforms understanding of breast cancer in Iraqi women, providing crucial molecular data that can guide targeted interventions, improve early detection, and ultimately reduce the substantial disease burden in this high-incidence region.

Keywords: *BRCA1 protein, breast neoplasms, DNA methylation, microRNAs, single nucleotide polymorphism, tumor biomarkers.*

Table *of* Contents

Summary I
List of figures VI
List of tables VIII
List of abbreviations IX

Introduction 1
1.1. Introduction 1
1.2. Aims and objectives 7

Review of Literature 8
2.1. Breast cancer 8
2.1.1. Definition and diagnostic criteria 8
2.1.2. Epidemiology 10
2.1.3. Pathophysiology 11
2.1.4. Genetics and epigenetics 15
2.2. Breast cancer gene 1 (*BRCA1*) 17
2.2.1. Structure and function 17
2.2.2. Mutations and polymorphisms 19
2.2.3. Methylation 23
2.2.4. Protein expression 26
2.3. BRCA1-associated ring domain protein 1 (BARD1) 29
2.3.1. Structure and function 29
2.3.2. Mutations and polymorphisms 32
2.3.3. Protein expression 34
2.4. microRNA-498 36
2.4.1. Structure and function 36
2.4.2. Role in cancer 38

Materials and Methods 40
3.1. Materials 40
3.1.1. Chemicals, reagents and kits 40
3.1.2. Devices, tools, equipment and software 41
3.2. Study design and setting 42
3.3. Study participants 43
3.3.1. Participants 43
3.3.2. Inclusion and exclusion criteria 43
3.3.3. Sample size calculation 44
3.4. Data collection 45
3.4.1. Demographic and clinical data 45
3.4.2. Anthropometric measurements 46
3.4.3. Life style and environmental factors assessment 46
3.4.4. Reproductive and hormonal history 47
3.5. Sample collection and processing 47
3.5.1. Blood sample collection 47
3.5.2. Sample storage and handling 48
3.6. Laboratory analysis 48
3.6.1. Biochemical parameters 48

3.5.2. Inflammatory, oxidative and tumor biomarkers 49
3.5.3. BRCA1 and BARD1 protein 52
3.7. Molecular, genetic and epigenetic analysis 54
3.7.1. DNA extraction and quantification 54
3.7.1.1. DNA extraction from whole blood 54
3.7.1.2. DNA quality assessment (agarose gel electrophoresis) 55
3.7.1.3. DNA quantification and purity assessment 56
3.7.1.2. DNA storage 57
3.7.2. Polymerase chain reaction 57
3.7.3. Sanger sequencing 59
3.7.4. DNA methylation 60
3.8. miRNA expression analysis 61
3.8.1. RNA extraction 61
3.8.2. miRNA-498 expression 62
3.9. Biostatistical analyses 64

Results and Discussion 65
4.1. Results 65
4.1.1. Study population characteristics 65
4.1.2. Clinical, pathological and laboratory characteristics 67
4.1.3. Genetic polymorphism analysis 79
4.1.4. Epigenetic analysis 84
4.1.5. Protein expression analysis 86
4.1.6. miRNA expression analysis 88
4.1.7. Correlation analysis 90
4.1.8. ROC curve analysis 93
4.2. Discussion 95
4.2.1. Study population characteristics 95
4.2.2. Clinical, pathological and laboratory characteristics 95
4.2.3. Genetic polymorphism analysis 100
4.2.4. Epigenetic analysis 102
4.2.5. Protein expression analysis 103
4.2.6. miRNA expression analysis 104
4.2.7. Correlation analysis 106
4.2.8. ROC curve analysis 107

Conclusions and Recommandations 108
5.1. Conclusions 108
5.2. Recommendations 109

References 110
Appendices 133
المُلخص (باللغة العربية) .... أ

List of figures

Figure	Caption	Page
2.1	Breast cancer molecular subtypes based on receptor expression profiles.	10
2.2	Age-standardized incidence rates of breast cancer among Iraqi females by provinces in 2023.	12
2.3	Overview of major molecular pathways in breast cancer development.	13
2.4	Major epigenetic regulatory modifications in cancer pathogenesis.	17
2.5	*BRCA1* gene structure and protein interaction network.	20
2.6	DNA methylation dynamics and transcriptional consequences.	25
2.7	*BRCA1* gene expression and disruption mechanisms.	29
2.8	*BARD1* gene organization, protein structure and function.	31
2.9	BRCA1-BARD1 heterodimer complex structure and function.	32
2.10	Overview of RNA classification based on functional properties.	37
2.11	Schematic representation of microRNA biogenesis.	38
3.1	Schematic presentation of study design and protocols.	46
3.2	Standard curves for inflammatory cytokine quantification by ELISA.	51
3.3	Standard curve for 8-hydroxy-2'-deoxyguanosine (8-OHdG) quantification using competitive ELISA.	52
3.4	Standard curve for BRCA1 protein quantification using sandwich ELISA.	54
3.5	Standard curve for BARD1 protein quantification using competitive ELISA.	54
3.6	Agarose gel electrophoresis analysis of genomic DNA quality and integrity.	57
4.1	Age distribution histograms comparing controls and breast cancer patients.	68
4.2	Hormone receptor status and molecular subtype distribution in breast cancer patients.	74
4.3	Sankey diagram showing the flow from individual hormone receptor status (ER, PR, HER2) to molecular subtype classification in breast cancer patients.	76
4.4	Comparison of inflammatory and tumor biomarkers between breast cancer patients and healthy controls.	79
4.5	PCR optimization for *BRCA1* and *BARD1* amplification.	80
4.6	PCR amplification of target SNP regions.	81
4.7	Sanger sequencing chromatograms of *BRCA1* rs4986850.	82
4.8	Sanger sequencing chromatograms of *BARD1* rs1048108.	82
4.9	Distribution of *BRCA1-BARD1* haplotype frequencies in breast cancer patients versus controls.	84
4.10	*BRCA1* methylation levels in control versus breast cancer patients.	85
4.11	*BRCA1* methylation analysis in breast cancer patients.	86
4.12	Comparison of BRCA1 and BARD1 protein concentrations between control and breast cancer patients.	87
4.13	Density distributions of BRCA1 and BARD1 protein concentrations.	88
4.14	BRCA1 and BARD1 protein concentrations in sporadic vs non-sporadic breast cancer	88
4.15	BRCA1 and BARD1 protein concentrations in Luminal A vs Luminal B breast cancer subtypes.	89
4.16	miR-498 expression analysis comparing between control subjects and breast cancer patients.	90

4.17	miR-498 expression by receptor status (ER, PR, HER2).	90
4.18	Scatter plots showing correlations between four molecular biomarkers in the overall study population (controls and patients)	92
4.19	Scatter plots showing correlations between four molecular biomarkers exclusively in breast cancer patients.	93
4.20	Receiver operating characteristic (ROC) curves for seven breast cancer biomarkers (CA 15-3, miR-498, IL-1β, IL-6, TNF-α, 8-OHdG, and hs-CRP, BRCA1, BARD1).	95

List of tables

Table	Title	Page
2.1	Some common *BRCA1* SNPs and their association with breast cancer risk	21
2.2	Some *BARD1* SNPs and their association with breast cancer risk.	33
3.1	Chemicals, reagents, kits, and solutions used in this study.	41
3.2	Devices, equipment, instruments, and software used in this study.	42
3.3	Primer sequences and characteristics.	59
3.4	PCR reaction components and conditions	59
4.1	Baseline demographic characteristics of study population.	68
4.2	Reproductive history and hormonal factors.	70
4.3	Lifestyle factors and environmental exposures.	71
4.4	Histopathological features of breast cancer cases.	73
4.5	Molecular subtypes by clinical parameters.	75
4.6	Comprehensive biochemical parameters comparison	78
4.7	Genotype and allele frequencies of *BRCA1* (rs4986850) and *BARD1* (rs1048108) single nucleotide polymorphism	83
4.8	Diagnostic accuracy of breast cancer biomarkers.	94

List of abbreviations

Abbreviation	Full term
8-OHdG	8-hydroxy-2'-deoxyguanosine
A/G ratio	Albumin/Globulin ratio
ADH	Atypical ductal hyperplasia
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANK	Ankyrin
APC	Adenomatous polyposis coli
ASR	Age-standardized rate
AST	Aspartate aminotransferase
AUC	Area under the curve
BARD1	BRCA1-associated RING domain protein 1
BCG	Bromocresol green
BMI	Body mass index
BRCA1	Breast cancer gene 1
BRCA2	Breast cancer gene 2
BRCT	BRCA1 C-terminal
CA 15-3	Cancer antigen 15-3
CDH1	Cadherin 1
CHEK2	Checkpoint kinase 2
CI	Confidence interval
CpG	Cytosine-phosphate-guanine
CstF-50	Cleavage stimulation factor 50
CtIP	CtBP-interacting protein
DCIS	Ductal carcinoma in situ
DDR	DNA damage response
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DSB	Double-strand break
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-mesenchymal transition
EOR	Excess odds ratio
ER	Estrogen receptor
FEA	Flat epithelial atypia
GLDH	Glutamate dehydrogenase
GSK3β	Glycogen synthase kinase 3 beta
HER2	Human epidermal growth factor receptor 2
HR	Hazard ratio / Homologous recombination
HRT	Hormone replacement therapy
hs-CRP	High-sensitivity C-reactive protein
HWE	Hardy-Weinberg equilibrium
IGF	Insulin-like growth factor
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
IQR	Interquartile range

LDH	Lactate dehydrogenase
LOH	Loss of heterozygosity
MAPK	Mitogen-activated protein kinase
MDM2	Mouse double minute 2 homolog
miRNA/miR	MicroRNA
mRNA	Messenger RNA
MRE11	Meiotic recombination 11
MS-PCR	Methylation-specific PCR
mTOR	Mechanistic target of rapamycin
NBS1	Nijmegen breakage syndrome 1
NCBI	National Center for Biotechnology Information
NES	Nuclear export signal
NF-κB	Nuclear factor kappa B
NLS	Nuclear localization signal
OR	Odds ratio
PALB2	Partner and localizer of BRCA2
PARP	Poly(ADP-ribose) polymerase
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3-kinase
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
qRT-PCR	Quantitative real-time PCR
RAD50	RAD50 double strand break repair protein
RAD51	RAD51 recombinase
RAS	Rat sarcoma
RB	Retinoblastoma
RING	Really interesting new gene
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
SD	Standard deviation
SNP	Single nucleotide polymorphism
STK11	Serine/threonine kinase 11
TAE	Tris-acetate-EDTA
TMB	3,3',5,5'-Tetramethylbenzidine
TNBC	Triple-negative breast cancer
TNF-α	Tumor necrosis factor-alpha
TNM	Tumor, Node, Metastasis
TP53	Tumor protein p53
UTR	Untranslated region
UV	Ultraviolet
VUS	Variant of uncertain significance
WHO	World Health Organization

Chapter one

INTRODUCTION

Introduction

1.1. Introduction

Breast cancer constitutes the most significant cancer burden among women globally, with an estimated 1.4-2.3 million new cases and 459,000-685,000 deaths annually, accounting for 25.1% of all female cancers worldwide (Ghoncheh *et al*., 2016; Youlden *et al*., 2012, 2014). The disease shows marked geographic differences, with higher incidence rates in developed countries but greater mortality in less developed regions, reflecting differences in healthcare infrastructure and access to treatment (Ghoncheh *et al*., 2016; Zaidi & Dib, 2019). Five-year survival rates vary dramatically from 12% in parts of Africa to nearly 90% in the United States, Australia, and Canada, attributed to factors including early detection capabilities, access to treatment, and cultural barriers (Hortobagyi *et al*., 2005; Youlden *et al*., 2014). The global burden is projected to increase, particularly in Asia, due to increasingly westernized lifestyles and demographic transitions (Bray *et al*., 2004; Elhawary *et al*., 2025).

In Iraq, breast cancer represents a substantial public health challenge, with 8,708 cases among females in 2023, constituting 34.8% of all female cancers and achieving an age-standardized rate of 65.5/100,000 (Iraqi Cancer Board, 2023). The disease shows significant regional variations within Iraq, with the highest ASR in Erbil (92.3/100,000), followed by Baghdad (83.8/100,000), Karbala (80/100,000), Al-Najaf (71.4/100,000), and Al-Basrah (69/100,000). The mean age at diagnosis is 53.7 years with a median of 53 years, showing peak incidence in the 50-70 age group (Iraqi Cancer Board, 2023). Iraq's cancer incidence has increased dramatically from 31.5/100,000 in 1976 to 99.4/100,000 in 2023, reflecting demographic changes, lifestyle modifications, and improved detection capabilities (Iraqi Cancer Board, 2023). This rising trend, combined with morphological analysis revealing infiltrating duct carcinoma as the predominant type (92.2%), underscores the need for comprehensive breast cancer control strategies tailored to the Iraqi population's

specific epidemiological patterns and healthcare infrastructure (Iraqi Cancer Board, 2023).

Building upon the epidemiological patterns observed in Iraq and globally, understanding the molecular genetics of breast cancer offers vital understandings into disease mechanisms and potential therapeutic targets. The genetic landscape of breast cancer comprises both hereditary and sporadic forms, with approximately 5-10% of cases attributed to inherited genetic mutations (Ergül & Sazci, 2001; Pavelić & Gall-Trošelj, 2001). Hereditary breast cancer predominantly results from mutations in specific tumor suppressor genes, particularly *BRCA1* and *BRCA2*, which normally function as guardians of genomic stability through their role in DNA damage repair (Buchholz *et al*., 1999; Paul, 2014). Furthermore, additional genes including *TP53*, *ATM*, *PTEN*, *CHEK2*, *PALB2*, and *CDH1* contribute to breast cancer susceptibility, with approximately 20% of hereditary cases attributed to *BRCA1* and *BRCA2* mutations alone (Sokolova *et al*., 2023).

The difference between hereditary and sporadic breast cancer extends beyond mere genetic etiology to include different molecular pathways and clinical behaviors. Tumors arising in individuals with *BRCA1* or *BRCA2* germline mutations typically exhibit more extensive genetic defects and follow a more aggressive progression pathway compared to their sporadic counterparts (Ingvarsson, 1999; Pavelić & Gall-Trošelj, 2001). These hereditary tumors show impaired repair mechanisms for DNA damage, leading to increased genomic instability and accumulation of additional mutations (Buchholz *et al*., 1999; Paul, 2014). Moreover, recent advances in genomics and bioinformatics, including DNA-chip technology, have enhanced our understanding of these molecular differences, revealing that disturbances in cell proliferation, genetic stability, and cell death pathways precede tumorigenesis (Ingvarsson, 1999; E. Y. H. P. Lee, 1995). This molecular heterogeneity has significant implications for treatment strategies, as evidenced by the efficacy of PARP inhibitors specifically in *BRCA*-mutated cancers (Sun *et al*., 2021). In addition, polygenic risk arising from multiple low-penetrance alleles has been reported as a recognized contributor to breast cancer susceptibility, which draws attention to the

complex interplay between various genetic factors in disease development (Torabi Dalivandan *et al*., 2021).

Following the understanding of breast cancer's molecular genetics, *BRCA1* was as a critical tumor suppressor gene encoding a 1,863 amino acid protein that maintains genomic stability through multiple domains including an N-terminal RING finger domain, nuclear localization signals, and BRCT domains essential for DNA repair and cell cycle regulation (Paul, 2014; Sokolova *et al*., 2023). *BRCA1* mutations, particularly in the RING and BRCT domains, confer dramatically increased breast cancer risk, with carriers facing 45-87% lifetime risk compared to 12% in the general population, while specific polymorphisms like D693N (rs4986850) show selective association with triple-negative breast cancer (OR=2.31, 95% CI: 1.08-4.93) despite no overall breast cancer risk (Durocher, 1996; Ricks-Santi *et al*., 2013). Complementing BRCA1's function, BARD1 (BRCA1-associated RING domain protein 1) forms an obligate heterodimer through its N-terminal RING domain interaction with BRCA1, creating an E3 ubiquitin ligase complex crucial for DNA damage response (Cimmino *et al*., 2018; Lee *et al*., 2014). The *BARD1* Pro24Ser polymorphism (rs1048108) has been reported to have cancer-specific effects, providing protection against breast cancer in Chinese populations (OR=0.562 for TT genotype, 95% CI: 0.355-0.891) while increasing uveal melanoma risk, which highlights the complex tissue-specific nature of these genetic variants (Liu *et al*., 2013; Mukhana *et al*., 2023). Clinically, these findings emphasize the importance of comprehensive genetic assessment, as *BRCA1*/*BARD1* variants not only influence cancer risk but also affect treatment decisions, with *BRCA1* mutation carriers showing enhanced sensitivity to PARP inhibitors and platinum-based chemotherapy, while *BARD1* variants may serve as biomarkers for risk stratification and therapeutic selection in personalized breast cancer management (Mukhana *et al*., 2023; Sokolova *et al*., 2023).

In addition to genetic alterations in *BRCA1* and *BARD1*, epigenetic mechanisms, particularly DNA methylation, represent an alternative pathway for *BRCA1* inactivation in sporadic breast cancers. DNA methylation involves the addition of

methyl groups to cytosine residues in CpG islands within gene promoters, leading to transcriptional silencing without altering the DNA sequence itself (Li *et al*., 2015; Shestakova, 2016). *BRCA1* promoter hypermethylation occurs in 8.7% to 31% of sporadic breast cancers, with this epigenetic silencing being particularly prevalent in triple-negative breast cancers (TNBCs) where it accounts for 16% of cases (Singh *et al*., 2011; Yamashita *et al*., 2015). The effect of *BRCA1* methylation on gene expression is deep, as methylated tumors show significantly reduced *BRCA1* mRNA and protein levels, effectively phenocopying germline *BRCA1* mutations and compromising DNA repair capacity (Rice *et al*., 2000; Saelee *et al*., 2015). Clinically, *BRCA1* methylation correlates with aggressive tumor characteristics including higher nuclear grade, lymphovascular invasion, and specific histopathologic subtypes such as medullary and mucinous carcinomas, while also showing associations with decreased estrogen receptor and p27 expression alongside increased p21 levels (Esteller, 2000; Niwa *et al*., 2000). The prognostic significance of this epigenetic alteration is exemplified by Yamashita *et al*. (2015), who found that *BRCA1* promoter methylation in TNBC patients was associated with significantly shorter overall survival and aggressive features , while Rice *et al*. (2000) reported that the three breast cancer specimens with the lowest *BRCA1* expression levels all harbored aberrant promoter methylation, establishing a direct correlation between methylation status and gene silencing.

When it comes to protein-level alterations, BRCA1 and BARD1 protein expression patterns provide useful information on breast cancer biology and clinical outcomes. BRCA1 protein expression analysis tells that loss or aberrant cytoplasmic localization occurs frequently in breast cancers, with most cases showing low or undetectable levels, particularly in advanced grade 3 disease (Hedau *et al*., 2015; Rakha *et al*., 2008). This altered BRCA1 expression correlates significantly with aggressive tumor characteristics including high nuclear grade, larger tumor size, and notably, an inverse correlation with CD44+ cancer stem cell phenotype, suggesting a link between BRCA1 dysfunction and tumor-initiating cell populations (Issac *et al*., 2021; Madjd *et al*., 2011). Complementing these findings, BARD1 protein shows

paradoxical overexpression in breast cancers, with aberrant cytoplasmic localization replacing the normal nuclear pattern observed in healthy tissues (Chen *et al*., 2019; Wu *et al*., 2006). The oncogenic BARD1 isoforms, particularly BARD1β and BARD1δ, show increased expression in malignant tissues and contribute to cancer development through dominant-negative effects on the BRCA1-BARD1 complex (Śniadecki *et al*., 2020). Clinical correlations reveal that reduced BRCA1 expression associates with shorter disease-free intervals, increased recurrence rates, and poorer overall survival, while maximal BARD1 expression correlates with poor differentiation, large tumor size, and short disease-free survival (Chen *et al*., 2019; Wu *et al*., 2006). The diagnostic and prognostic potential of these proteins is exemplified by Rakha *et al*. (2008), who demonstrated that altered BRCA1 expression patterns could group patients into distinct prognostic groups, while other researchers found that cytoplasmic BARD1 expression in breast cancer patients correlated with a median disease-free survival of only 2.5 years compared to 5.5 years in patients with normal expression, establishing these proteins as promising biomarkers for clinical assessment and management (Wu *et al*., 2006).

Expanding beyond protein expression patterns, microRNAs (miRNAs) are a key layer of post-transcriptional regulation in breast cancer, functioning as small non-coding RNAs that modulate gene expression by binding to complementary sequences in target mRNAs (Khalife *et al*., 2020; Yang & Liu, 2020). These 18-25 nucleotide molecules act as either oncogenes or tumor suppressors, influencing fundamental cellular processes including proliferation, apoptosis, metastasis, and tumor stemness, with their dysregulation being a hallmark of breast cancer initiation and progression (Mu *et al*., 2021; Subramanian & Sinha, 2024). Among these regulatory molecules, miR-498 reported as a significant oncogenic player, particularly in triple-negative breast cancer (TNBC), where it promotes proliferation and migration by directly targeting the 3' untranslated region of PTEN mRNA, thereby reducing PTEN tumor suppressor protein levels and enhancing cell cycle progression (Chai *et al*., 2018). The broader landscape of miRNA dysregulation in breast cancer shows distinct expression patterns, with studies identifying 28 differentially expressed miRNAs

showing excellent diagnostic performance (AUC >0.9), including upregulated oncomiRs (miR-21, miR-141, miR-200c) and downregulated tumor suppressors (miR-10b, miR-99a, miR-100, miR-125b, miR-143, miR-145) (Kim, 2021; Muñoz *et al*., 2023). The biomarker potential of miRNAs is particularly promising due to their stability in circulation and tissue specificity, with circulating miRNAs offering minimally invasive diagnostic and prognostic capabilities, as shown by Chai *et al.* (2018) who showed that suppressing miR-498 overexpression significantly impaired TNBC cell proliferation and migration.

Despite the extensive global research on breast cancer genetics, epigenetics, and molecular biomarkers, a critical knowledge gap exists regarding these molecular alterations in the Iraqi population, particularly in Karbala governorate where breast cancer shows high incidence rates (125.4/100,000) second only to Erbil. While international studies have established the significance of *BRCA1*/*BARD1* polymorphisms, epigenetic modifications, protein expression patterns, and miRNA dysregulation in breast cancer development and prognosis, none of these molecular markers have been systematically investigated in Karbala's population, leaving clinicians without population-specific data to guide risk assessment and treatment decisions. This absence of local molecular epidemiological data is particularly concerning given that genetic variants often show population-specific effects, as evidenced by the differential impact of *BARD1* SNPs across ethnic groups and the varying frequencies of *BRCA1* methylation between populations. Furthermore, the current diagnostic scenery in Iraq faces significant challenges, with traditional histopathological methods and limited biomarker panels failing to capture the molecular heterogeneity of breast cancer, particularly in distinguishing aggressive subtypes like triple-negative breast cancer that require targeted therapeutic approaches. The integration of multiple molecular markers including *BRCA1* and *BARD1* polymorphisms, *BRCA1* promoter methylation status, protein expression patterns, and miRNA profiles such as miR-498 could transform breast cancer diagnosis and prognosis in Karbala, enabling early detection, accurate risk stratification, and personalized treatment strategies. Therefore, there is an urgent need

for comprehensive molecular studies in the Karbala population to establish region-specific biomarker profiles and validate international findings in the local context to improve clinical outcomes for Iraqi women facing this devastating disease.

1.2. Aims and objectives

The general objective of the current study, is to evaluate the association of genetic polymorphisms (*BRCA1* rs4986850 and *BARD1* rs1048108), epigenetic alterations (*BRCA1* promoter methylation), protein expression patterns (BRCA1 and BARD1), and microRNA profiles (miR-498) with breast cancer risk and clinical outcomes in women from Karbala governorate, Iraq. However, the specific objectives are:

1. To determine the frequency and distribution of *BRCA1* (rs4986850) and BARD1 (rs1048108) polymorphisms in breast cancer patients and healthy controls from Karbala population.
2. To assess *BRCA1* promoter methylation status in breast cancer blood samples and evaluate its correlation with protein levels, histopathological subtypes, and clinical parameters including tumor grade, stage, hormone receptor status, and miRNA-498 expression in breast cancer patients and healthy controls.
3. To analyze BRCA1 and BARD1 protein levels in blood samples using ELISA and determine their association with clinicopathological characteristics in breast cancer patients and healthy controls.
4. To quantify miR-498 expression levels in serum samples using real-time PCR and evaluate its correlation with protein levels and assess its diagnostic accuracy in breast cancer patients and healthy controls.

Chapter two

LITERATURE REVIEW

Review of Literature

2.1. Breast cancer

2.1.1. Definition and diagnostic criteria

Breast cancer is the most common malignancy affecting women worldwide, characterized by the abnormal growth of cells in breast tissue that can spread to other parts of the body (Harbeck *et al*., 2019). The disease typically originates in either the ducts that carry milk (ductal carcinoma) or the glands that produce milk (lobular carcinoma) (Harbeck *et al*., 2019). Early breast cancer is considered curable when contained within the breast or only spread to nearby lymph nodes, with cure rates of approximately 70-80% in these cases (Harbeck *et al*., 2019).

Typically, the diagnosis begins with the triple assessment approach that combines clinical examination, radiological evaluation (mammography, ultrasonography), and histopathological analysis (Jan *et al*., 2010; Karim *et al*., 2020). The clinical assessment involves systematic evaluation of breast morphology and associated changes (Lohani *et al*., 2024). Furthermore, the imaging protocol typically incorporates mammography and ultrasonography, while magnetic resonance imaging is indicated in select clinical scenarios (Beremauro & Girio-Fragkoulakis, 2022). Accordingly, histopathological examination via tissue biopsy provides definitive diagnosis and, hence, enables determination of critical diagnostic and prognostic biomarkers including hormone receptor status (estrogen and progesterone receptors), HER2 status, and histological grade (Rashmi *et al*., 2022). As a result, these molecular markers are instrumental in classifying the disease into distinct subtypes that inform therapeutic strategies. Thus, contemporary clinical practice employs five primary surrogate subtypes based on these biological parameters (Figure 2.1): luminal A-like, normal-like (HER2-negative and low Ki-67 levels), luminal B-like (HER2-positive/negative and high Ki-67 levels), HER2-positive (non-luminal or HER2-enriched), and triple-negative breast cancer (Rashmi *et al*., 2022).

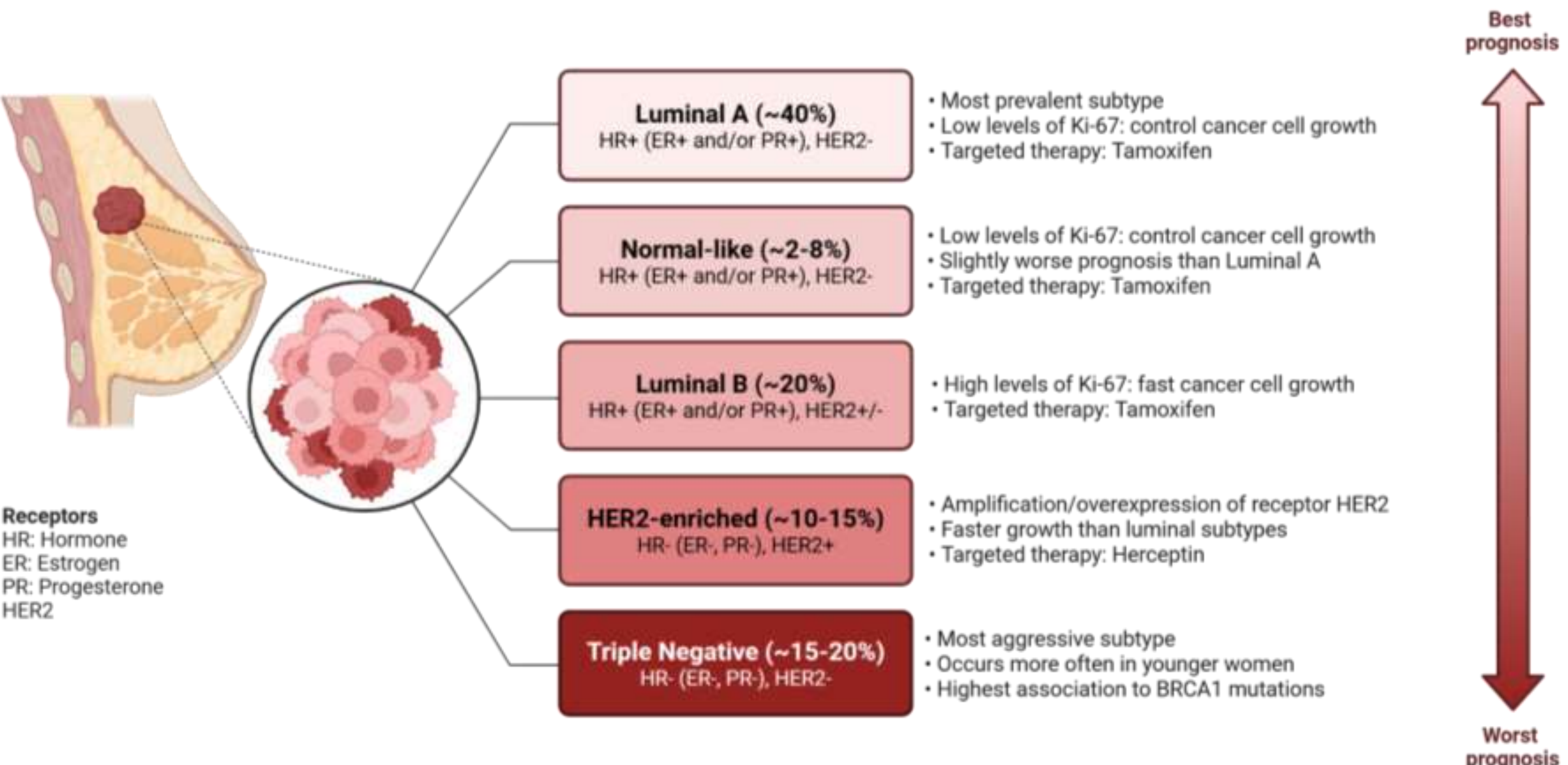


Figure 2.1. The five breast cancer molecular subtypes based on receptor expression profiles are: Luminal A-like (ER+/PR+/HER2-/Ki-67↓), Normal-like (ER+/PR+/HER2-/Ki-67↓), Luminal B-like (HER2± with ↑Ki-67), HER2-enriched (ER-/PR-/HER2+), and Triple-negative (ER-/PR-/HER2-). Receptor status determines therapeutic options including endocrine therapy (tamoxifen) for ER-positive tumors and HER2-targeted therapy (trastuzumab/Herceptin) for HER2-positive tumors. Prognostic outcomes vary by subtype. Adapted from Dai et al. (2015), De Cicco *et al*. (2019), Hashmi *et al*. (2019) Larsen *et al*. (2013) and Rashmi *et al.* (2022). Created using bioRender.com by Anna Lazaratos and Erin Marshall.

2.1.2. Epidemiology

The global distribution of breast cancer shows marked geographic and socioeconomic variations, with higher incidence rates generally observed in developed regions (Giaquinto *et al*., 2024). Age-adjusted incidence rates vary substantially between countries, influenced by differences in reproductive patterns, lifestyle factors, and screening practices. Most developed nations have experienced declining mortality rates over recent decades, primarily attributed to enhanced early detection programs and therapeutic advances, while developing regions continue to face rising mortality trends due to limited healthcare resources and delayed diagnosis (Cope, 2013; Rivera-Franco & Leon-Rodriguez, 2018; Youlden *et al*., 2012).

In the Iraqi context, detailed governorate-level analysis of 2023 (Figure 2.2) reveals distinct geographic patterns in cancer distribution (Iraqi Cancer Board, 2023). The highest age-standardized rates (ASR) are observed in Erbil (92.3/100,000), followed by Baghdad (83.8/100,000) and Karbala (80/100,000). A notable difference exists among governorates, with Najaf (71.4/100,000) and Basrah (69/100,000) also showing elevated rates. To conclude, this suggests probable regional variations in mammography screening access, risk factors, and early detection programs throughout Iraqi governorates, justifying targeted breast cancer interventions in high-incidence areas.

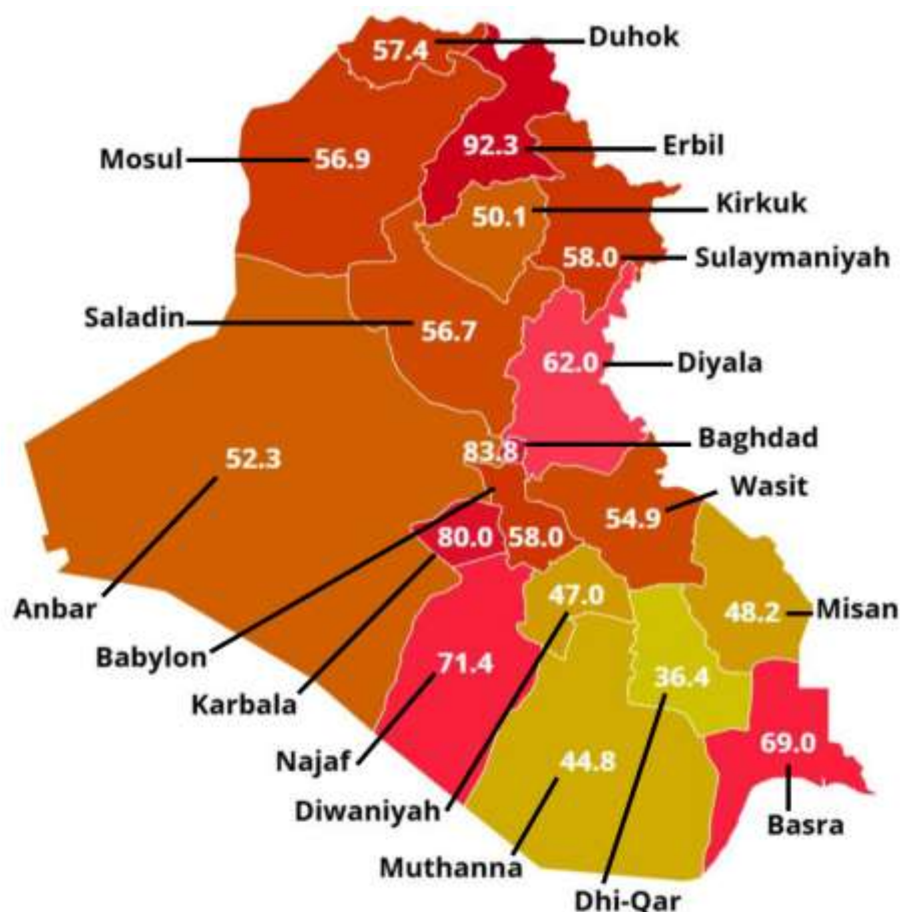


Figure 2.2. Age-standardized incidence rates of breast cancer among Iraqi females by provinces in 2023, data extracted from the annual report of the Iraqi Cancer Board (2023).

2.1.3. Pathophysiology

The exact mechanism initiating breast cancer development remains unknown, but research has revealed it follows two major different molecular pathways of progression (Figure 2.3), primarily related to estrogen receptor (ER) expression, tumor grade, and proliferation rates (Bombonati & Sgroi, 2011). The first pathway, known as the low-grade-like pathway, is characterized by specific genetic alterations including gain of chromosome 1q, loss of 16q, and infrequent amplification of chromosome 17q12 (Kao & Pollack, 2006). This pathway typically displays a gene expression signature associated with ER-positive phenotype, showing diploid or near-diploid karyotypes and low tumor grade, corresponding mainly to luminal A and to some extent luminal B subtypes (Yu *et al*., 2006). At the cellular level, breast cancer

can evolve through either a clonal evolution model, where mutations and epigenetic changes accumulate in tumor cells with the most fit cells surviving, or through a cancer stem cell model, where only precursor cancer cells drive and sustain progression (Campbell & Polyak, 2007; Polyak, 2007). The molecular heterogeneity is further complicated by the fact that cancer stem cells may also evolve in a clonal fashion (Campbell & Polyak, 2007).

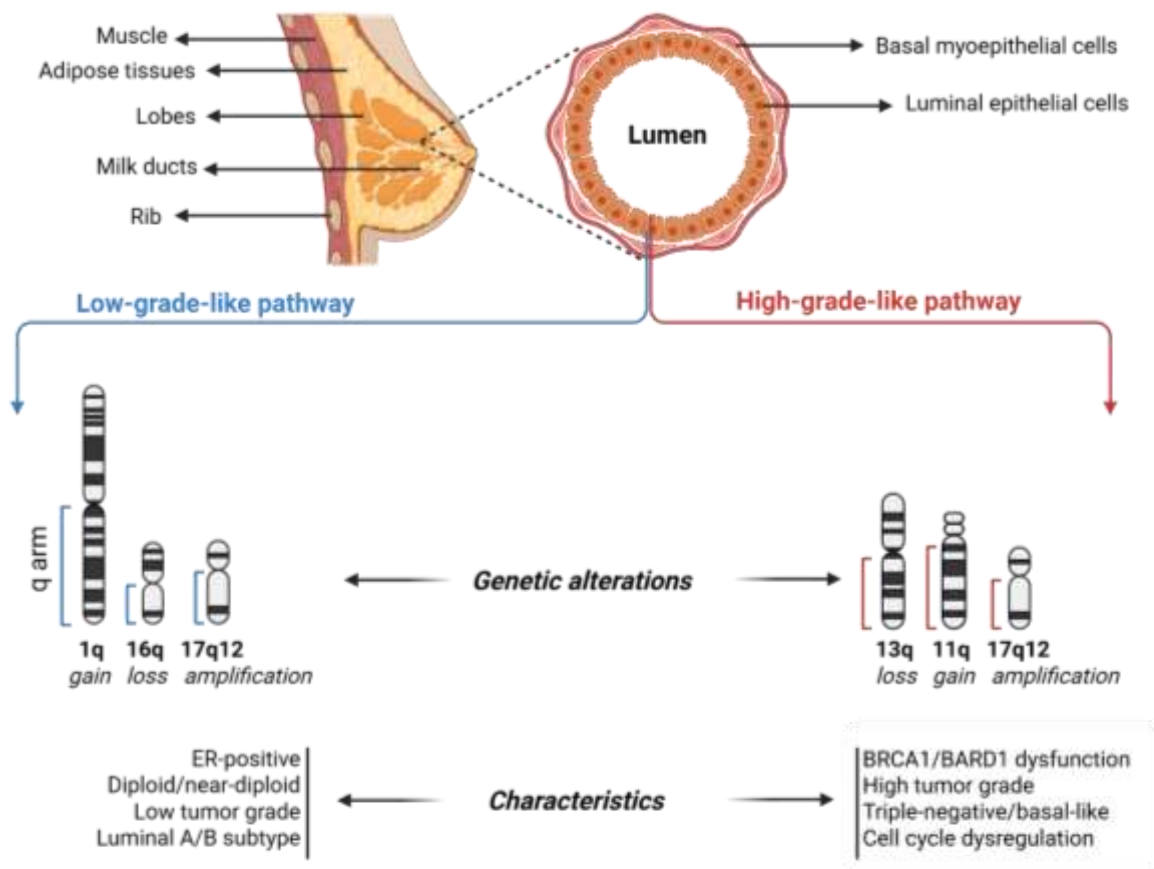


Figure 2.3. Overview of the two major molecular pathways in breast cancer development, the low-grade-like pathway (left, blue) is characterized by ER-positive phenotype, diploid karyotype, and specific chromosomal alterations (gain of 1q, loss of 16q), primarily resulting in luminal A/B subtypes. The high-grade-like pathway (right, red) shows *BRCA1*/*BARD1* dysfunction, complex chromosomal changes (loss of 13q, gain of 11q13, amplification of 17q12), and predominantly results in triple-negative/basal-like phenotypes. Created using bioRender.com.

The second major molecular pathway, known as the high-grade-like pathway, is critically influenced by *BRCA1* (breast cancer susceptibility gene 1) and its interacting partners, particularly *BARD1* (Gorodetska *et al*., 2019; Hashizume *et al*.,

2001; Roy *et al*., 2012a). *BRCA1*, located on chromosome 17q21, functions as an important tumor suppressor gene whose protein is integrally involved in DNA double-strand break repair through homologous recombination (Caestecker & Van de Walle, 2013; Zhang & Powell, 2005). This pathway is characterized by distinct genetic alterations including loss of chromosome 13q, gain of chromosomal region 11q13, amplification of 17q12 (containing *ERBB2*), and an expression signature dominated by genes involved in cell cycle regulation and cellular proliferation (Huang *et al*., 2018; Johnson *et al*., 2002; Kao & Pollack, 2006; Karlsson *et al*., 2011; Kauraniemi *et al*., 2001; Schuuring *et al*., 1992; Shiu *et al*., 2014; Sotiriou & Pusztai, 2003; Tsukamoto *et al*., 1996; Witus *et al*., 2021).

Approximately 10% of breast cancers are hereditary, with germline *BRCA1* mutations conferring a 72% cumulative risk of developing breast cancer by age 80 (Apostolou & Fostira, 2013; Kuchenbaecker *et al*., 2017). *BRCA1*-associated tumors typically display high-grade features, with 75-80% exhibiting a triple-negative/basal-like phenotype (Badowska-Kozakiewicz & Budzik, 2016; Turner & Reis-Filho, 2006). Epigenetic alterations, particularly promoter hypermethylation of *BRCA1*, can lead to silencing of this critical tumor suppressor even in the absence of genetic mutations, representing an important alternate mechanism of *BRCA1* inactivation in sporadic breast cancers (Yamashita *et al*., 2015).

The complex relationship between *BRCA1* and *BARD1* plays a key role in breast cancer development and progression (Hawsawi *et al*., 2022). *BARD1* (BRCA1-associated RING domain protein 1) forms a heterodimer with *BRCA1* through their respective RING finger domains, creating a complex (heterodimer) that exhibits E3 ubiquitin ligase activity essential for tumor suppression (Wang *et al*., 2023). This BRCA1-BARD1 complex is necessary for multiple cellular functions, including DNA damage repair, cell cycle checkpoint control, and maintenance of genomic stability (Shabbeer *et al*., 2013). When either protein accordingly is conceded through genetic polymorphisms or other alterations, it can disrupt their heterodimer formation and subsequent tumor suppressor functions.

Thus, the clinical consequences of these molecular pathways are significant, tumors with dysfunctional BRCA1-BARD1 pathways often display increased sensitivity to specific treatment lines, particularly poly adenosine diphosphate-ribose polymerase (PARP) inhibitors, which exploit synthetic lethality in cells with compromised homologous recombination repair (Gelmon *et al*., 2011; Litton *et al*., 2018; Robson *et al*., 2017). Moreover, epigenetic modifications, including aberrant methylation patterns in the *BRCA1* promoter region, can lead to transcriptional silencing, affecting protein levels and ultimately contributing to breast cancer development, particularly in cases where genetic mutations are absent but protein expression is nonetheless compromised (Rice *et al*., 2000).

Recent advances in understanding the complex interplay between genetic and epigenetic alterations have revealed that BRCA1-BARD1 pathway dysfunction can manifest through multiple mechanisms beyond just genetic mutations (Jimenez-Johnson, 2008; Stratton & Rahman, 2008). These include variations in protein expression levels, alterations in post-translational modifications, and changes in subcellular localization of these proteins (Bombonati & Sgroi, 2011). Studies have shown that promoter methylation status of *BRCA1* can help as a biomarker for breast cancer risk and prognosis, particularly in specific populations (Panagopoulou *et al*., 2024). The methylation patterns, combined with protein expression levels, provide vital information into the functional status of the BRCA1-BARD1 tumor suppressor pathway (Harbeck *et al*., 2019; Matros *et al*., 2005; Tarapara & Shah, 2025). This understanding has important clinical implications for both risk assessment and therapeutic decision-making, as patients with compromised BRCA1-BARD1 pathway function may benefit from specific therapeutic strategies, such as platinum-based chemotherapy or PARP inhibitors (Imyanitov, 2021; Kahán, 2020; Tung & Garber, 2018). Furthermore, the assessment of *BRCA1* promoter methylation, alongside BRCA1 and BARD1 protein levels, may offer potential as a non-invasive diagnostic tool, particularly relevant for populations where genetic testing may not be readily available or cost-effective.

2.1.4. Genetics and epigenetics

Breast cancer's genetic landscape is characterized by both hereditary and sporadic forms, with approximately 5-10% of cases being hereditary in nature (Apostolou & Fostira, 2013; Isaacs & Rebbeck, 2007; Wittersheim *et al*., 2015). The genetic architecture involves three distinct categories of susceptibility genes: high-penetrance, moderate-penetrance, and low-penetrance variants (Stratton & Rahman, 2008). High-penetrance genes, primarily *BRCA1* and *BRCA2*, account for 21-40% of hereditary cases and confer a significantly elevated lifetime risk of developing breast cancer (Chen & Parmigiani, 2007; Jimenez-Johnson, 2008). *BRCA1*, as mentioned earlier, located on chromosome 17q21 and plays a crucial role in DNA double-strand break repair through homologous recombination, while *BRCA2* is essential for maintaining genomic stability (Gudmundsdottir & Ashworth, 2006). Other rare but important high-penetrance genes include *PTEN*, *TP53*, *CDH1*, and *STK11*, each associated with specific cancer predisposition syndromes (Razack & Prabhuswamimath, 2024; Shiovitz & Korde, 2015). The inheritance of pathogenic mutations in these genes follows an autosomal dominant pattern, with variable penetrance influenced by both genetic and environmental factors (Chen & Parmigiani, 2007).

The genetic complexity of breast cancer extends beyond high-penetrance genes to include moderate-penetrance genes such as *CHEK2*, *BRIP1*, *ATM*, and *PALB2*, which confer approximately a twofold increase in breast cancer risk *(Hollestelle et al*., 2010; Vega, 2013). These genes typically function within the DNA damage response and repair pathways, often interacting with *BRCA1* and *BRCA2* (Vega, 2013). Complementing these genetic alterations are important epigenetic modifications that play a significant role in breast cancer development and progression (Hollestelle *et al*., 2010; Vega, 2013). DNA methylation, particularly in the promoter regions of tumor suppressor genes like *BRCA1*, represents a key epigenetic mechanism that can silence gene expression without altering the DNA sequence (Han, 2010; Yamashita *et al*., 2015). This methylation process is reversible and has been reported as both a potential biomarker and therapeutic target (Szyf,

2000; Urban *et al*., 2024). Histone modifications, including acetylation and methylation, further contribute to the epigenetic regulation of gene expression in breast cancer, affecting chromatin structure and accessibility (Messier *et al*., 2016). These epigenetic alterations are particularly significant in sporadic breast cancers, which include approximately 90% of cases, where they can mimic the effects of genetic mutations (Figure 2.4) by disrupting normal gene function through transcriptional silencing (Karsli-Ceppioglu *et al*., 2014; Lee & Muller, 2010).

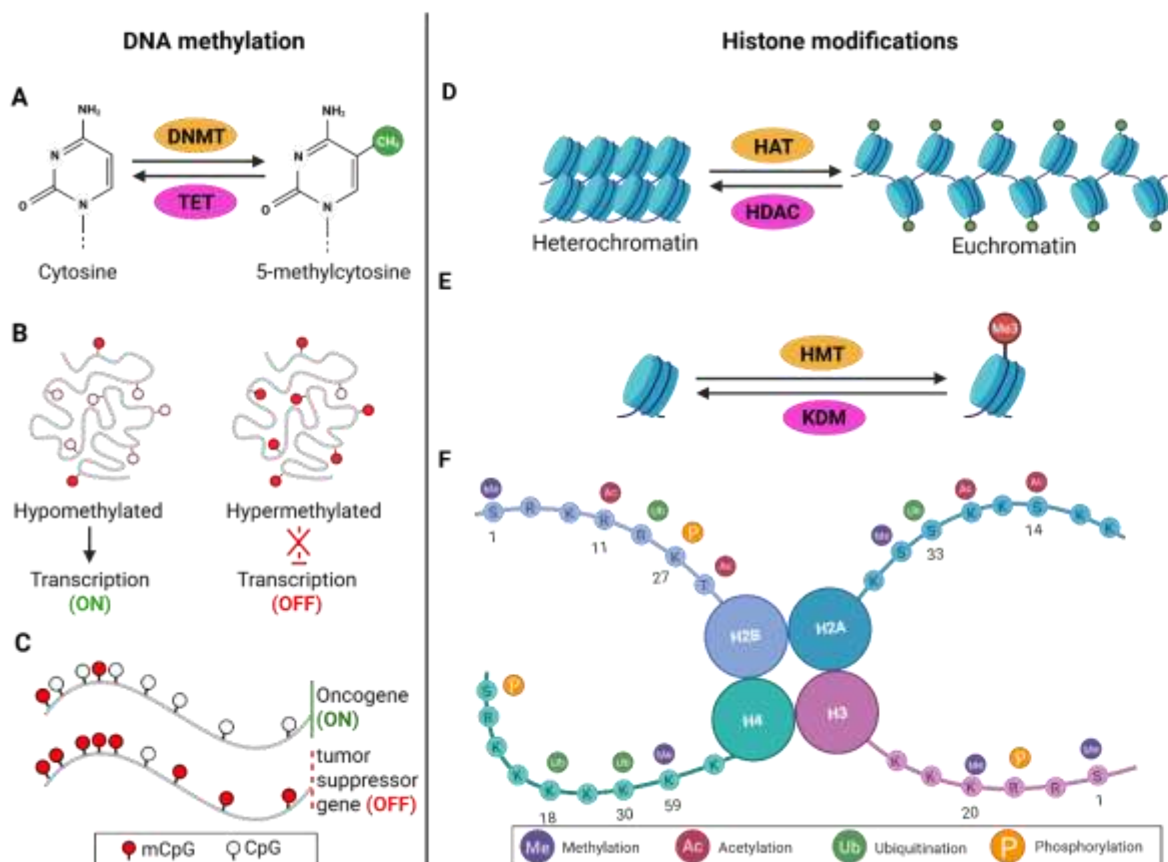


Figure 2.4. The two major classes of epigenetic regulatory modifications (mechanisms) in cancer pathogenesis (DNA methylation and histone modifications): A) reversible conversion between cytosine and 5-methylcytosine mediated by DNA methyltransferases (DNMTs, methylation) and ten-eleven translocation (TET) enzymes (demethylation), B) differential gene expression based on methylation status, hypomethylation doesn't stop the transcription while hypermethylation silences genes, C) cancer-associated methylation abnormalities showing hypermethylation-induced silencing of tumor suppressor genes and hypomethyl-ation-driven activation of oncogenes, D) dynamic acetylation regulated by the histone acetyltransferases (HATs) and histone deacetylases (HDACs) disturbing the chromatin accessibility, E) methylation dynamics controlled by histone methyltransferases (HMTs) and lysine demethylases (KDMs) with context-dependent effects on transcription, and F) distribution of multiple post-translational modifications (methylation, acetylation, ubiquitination, phosphorylation) across the N-terminal tails of core histones H2A, H2B, H3, and H4, collectively forming the histone marks that directs chromatin states and gene expression programs. Adapted from Thakur *et al*. (2022), and created using bioRender.com.

The relationship between *BRCA1* promoter methylation and *BARD1* connections is a critical area in breast cancer pathogenesis that remains incompletely understood. The *BRCA1* promoter methylation has been reported as a significant epigenetic modification that can lead to transcriptional silencing, effectively reducing BRCA1 protein levels even in the absence of genetic mutations (Miyamoto, 2002; Rice *et al*., 2000). This epigenetic silencing can disrupt the important BRCA1-BARD1 heterodimer formation, which is essential for their tumor suppressor functions including DNA repair, cell cycle checkpoint control, and maintenance of genomic stability (Esteller, 2000; Rice, 2000; Rice *et al*., 2000; Yamashita *et al*., 2015). Recent studies have shown that altered *BRCA1* promoter methylation patterns can vary significantly across different populations and may serve as both a prognostic marker and a predictor of treatment response (Panagopoulou *et al*., 2024). The relationship between *BRCA1* methylation status and BARD1 protein levels appears to be complex and bidirectional, with evidence suggesting that compensatory mechanisms may exist when *BRCA1* function is compromised through epigenetic silencing, potentially involving upregulation of alternative DNA repair pathways or post-translational modifications of BARD1 (Irminger-Finger & Leung, 2002; Tarsounas & Sung, 2020). Still, the complex relationships between genetic polymorphisms, epigenetic modifications, and protein expression levels, particularly in Karbala population are not investigated yet.

2.2. Breast cancer gene 1 (*BRCA1*)

2.2.1. Structure and function

The *BRCA1* gene has a complex and well-conserved structural organization essential for its tumor suppressor function. Located on chromosome 17q21, *BRCA1* contains 24 exons (Figure 2.5) that encode a large nuclear protein of 1863 amino acids with a molecular weight of 220 kDa (Ashworth, 2013). The protein's structure features several critical functional domains, with the most important being the highly conserved RING domain at the N-terminus (amino acids 24-64) and the BRCA1 C-

terminal (BRCT) domains (encoded by exons 16-24) (Clark *et al*., 2012). The RING domain exhibits E3 ligase activity and forms a key heterodimer with BARD1 (BRCA1-associated RING domain protein 1), which is essential for BRCA1's stability and function (Wang *et al*., 2023). Between these terminal domains lies a nuclear localization sequence (NLS) region and a large central region containing a coiled-coil domain. The coiled-coil domain is particularly important as it facilitates binding with PALB2 (Partner and Localizer of BRCA2), which serves as a molecular scaffold in forming the BRCA1-PALB2-BRCA2 complex (Roy *et al*., 2012b).

BRCA1 protein works as a pleiotropic DNA damage response protein with multiple critical cellular functions (Figure 2.5), primarily involved in maintaining genomic stability. The protein operates through several distinct mechanisms: it plays a vital role in DNA double-strand break repair through homologous recombination, where it partners with BRCA2 and RAD51 to orchestrate precise DNA repair processes (Wu *et al*., 2010). Through its E3 ubiquitin ligase activity, BRCA1 also regulates the cell cycle at multiple checkpoints, particularly during the G2/M transition, ensuring cells with damaged DNA don't proceed through division (Vázquez-Arreguín *et al*., 2018; Wang *et al*., 2023). The protein shows extraordinary versatility in its interactions, forming at least four different complexes in cells through its BRCT domain: the BRCA1/RAP80/Abraxas complex, which helps prevent excessive DNA end resection; the BRCA1/BACH1 complex, vital for DNA damage-induced G2/M checkpoint control; the BRCA1/PALB2/BRCA2 complex, essential for homologous recombination; and the BRCA1/CtIP complex, which promotes DNA end resection (Wang *et al*., 2007). Additionally, BRCA1 participates in chromatin remodeling and transcriptional regulation, particularly through its interaction with RNA polymerase II and various transcription factors (Bochar *et al*., 2000; L. Starita, 2003; Ye *et al*., 2001).

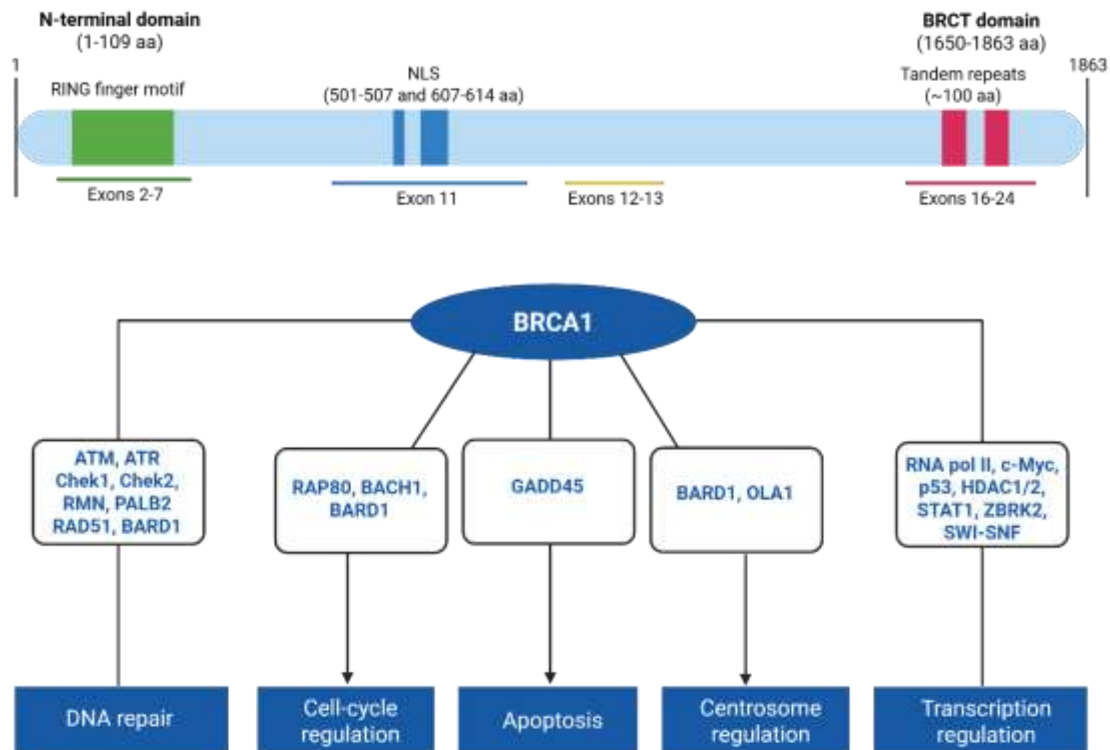


Figure 2.5. *BRCA1* gene structure and protein interaction network: A) gene structure with 24 exons spanning ~81 kb, the exon 11 is the largest coding region. Functional domains are color-coded and mapped to specific exons: RING domain (green, exons 2-7), nuclear localization signals (blue, exons 11 mainly), serine cluster domain (yellow, exons 12-13), and BRCT repeats (pink, exons 16-24). B) BRCA1 protein and its key binding partners organized by functional categories. Partially adapted from Lavoro *et al*. (2022), and created using bioRender.com.

2.2.2. Mutations and polymorphisms

BRCA1 mutations show notable diversity in their manifestations and molecular characteristics, with current databases documenting over 2,900 distinct pathogenic variants (Clark *et al*., 2012; Fu *et al*., 2022; Li *et al*., 2022). These mutations predominantly cluster in the highly conserved functional domains, the RING domain (exons 2-7) and BRCT domain (exons 15-24), significantly impacting protein functionality (Clark *et al*., 2012). The mutation spectrum includes several distinct categories, with the majority promoting frameshifts that result in missense or non-functional protein products (Linger & Kruk, 2010; Sharma *et al*., 2018). Structurally, *BRCA1*'s genetic architecture contains a high density of repeated elements that facilitate Alu-mediated genomic rearrangements, leading to significant structural

variations (Godet & Gilkes, 2017; Walsh *et al*., 2006). These rearrangements can span from less than 1 kilobase to more than 250 kilobases, often going undetected by conventional screening techniques (Song *et al*., 2018). The molecular mechanisms underlying these mutations include intron mutations, missense mutations, nonsense mutations, and frameshift mutations, with each type having distinct implications for protein function (Fu *et al*., 2022; Tudini *et al*., 2018). In germline mutation carriers, a "second hit" frequently occurs through somatic mutation of the wild-type allele, leading to complete loss of functional BRCA1 protein, a classic example of Knudson's two-hit hypothesis in tumor suppressor genes (Godet & Gilkes, 2017; Knudson, 1971; Li *et al*., 2024). The location and type of mutation can significantly influence both cancer risk and therapeutic response, with mutations in key functional domains generally associated with higher penetrance and more severe phenotypes (Rebbeck *et al*., 2015). Below, in Table 2.1, some common *BRCA1* SNPs were listed.

Table 2.1. Some common *BRCA1* SNPs and their association with breast cancer risk.

SNP ID	Amino acid change	Association with breast cancer	Population	Reference
rs16942	Lys1183Arg	Decreased risk in *BRCA1* mutation carriers (HR 0.86, 95% CI 0.77-0.95, *p*= 0.003)	*BRCA1* mutation carriers (CIMBA consortium)	Cox *et al.* (2011)
rs1799950	Gln356Arg	Decreased risk (protective effect, Arg356 homozygotes more frequent in controls, *p*= 0.01)	General UK population	Dunning *et al.* (1997)
rs16941	Pro871Leu	No association	General UK population	Dunning *et al.* (1997)
rs799917	Pro871Leu	No association, but showed trend toward increased risk in *BRCA1* carriers (OR 1.55) and decreased risk in non-carriers (OR 0.84)	Caucasian women with breast cancer (WECARE Study)	Figueiredo *et al.* (2011)
rs8176318	3'UTR variant (non-coding)	Increased risk in African Americans (homozygous A/A: OR 9.48, 95% CI	African American	Pelletier *et al.* (2011)

SNP ID	Amino acid change	Association with breast cancer	Population	Reference
		1.01-88.80, *p*= 0.04); increased risk for triple negative breast cancer in African American women (A/A: OR 12.19, 95% CI 1.29-115.21, *p*= 0.02)	women with breast cancer	
rs2736098	None (synonymous)	Decreased risk (protective effect, OR<1, *p*-value= 9.30E-04)	Polish women	Ledwoń *et al.* (2013)
rs13281615	N/A (gene desert)	Increased risk (*p*-value ≤0.0197)	Polish women	Ledwoń *et al.* (2013)
rs1219648	N/A (intronic)	Increased risk (*p*-value= 1.62E-05)	Polish women	Ledwoń *et al.* (2013)
rs2981582	N/A (intronic)	Increased risk (*p*-value= 1.46E-05)	Polish women	Ledwoń *et al.* (2013)
rs1799966	None (intronic variant, located in intron 5)	No significant association	Polish women with *BRCA1* mutations carriers	Jakubowska *et al.* (2010)
rs5820483	None (intron 10 variant)	Decreased breast cancer risk in *BRCA1* pathogenic variant carriers (protective effect, HR 0.84, $p= 7.5 \times 10^{-4}$).	*BRCA1* pathogenic variant carriers from the CIMBA consortium	Ruiz de Garibay *et al.* (2021)
Various 3'UTR SNPs[1]	3'UTR variant (non-coding)	Five rare haplotypes containing these 3'UTR variants were found in 9.50% of breast cancer chromosomes vs 0.11% of control chromosomes (p = 0.0001); highest prevalence in triple negative breast cancer (14.85%)	Breast cancer patients (European American and African American)	Pelletier *et al.* (2011)
rs80357164	Cys39Gly	Significantly increased risk (OR: 12.24, 95% CI: 1.53-98.05, *p*=0.018)	Greenlandic Inuit women	Wielsøe *et al.*, (2018)
rs1060915	None (synonymous variant)	No significant association	Italian breast cancer patients	Seymour *et al.* (2008)
rs16940	Missense variant	No significant association	Italian breast cancer patients	Seymour *et al.* (2008)

[1]Three 3'UTR variants (rs12516, rs8176318, rs3092995) were identified as part of rare *BRCA1* haplotypes.

The clinical manifestations and functional consequences of *BRCA1* mutations demonstrate significant variability depending on their specific molecular characteristics. Mutations targeting the RING domain frequently compromise E3 ligase activity and disrupt crucial protein-protein interactions, particularly with BARD1, while alterations in the BRCT domain interfere with phospho-protein binding and DNA damage response pathways (Nelson & Holt, 2010). An important molecular feature is the existence of alternative splicing variants, with at least six documented forms including *BRCA1* exon 1a, exon 1b, exon 1c, BRCA1a (Δ11q, Δ11), BRCA1b (Δ9,10), and BRCA1-IRIS. These splice variants can produce proteins with different molecular weights and, in some cases, exhibit functions that differ from or even oppose those of the complete transcript (Lixia *et al*., 2007; Orban, 2003). Walker and colleagues' comprehensive analysis of 77 published studies, examining 235 *BRCA1* splicing assays, revealed that certain exon boundary variants may retain partial functionality through naturally occurring in-frame RNA isoforms (Walker *et al*., 2013). To conclude, this molecular complexity has significant implications for genetic counseling and treatment decisions, as the specific mutation type and location can influence both cancer risk assessment and therapeutic response prediction.

The *BRCA1* D693N variant (rs4986850), which results in an aspartic acid to asparagine substitution at codon 693 and located in exon 11, represents a significant missense mutation that results in an aspartic acid to asparagine substitution at codon 693 (Choi *et al*., 2009). This variant has gained particular attention due to its position within a highly conserved region of *BRCA1* and its complex impact on protein function. Multiple population studies have investigated its clinical significance, yielding complex and sometimes contradictory findings (Bhatti *et al*., 2008; Brown *et al*., 2016; Choi *et al*., 2009; Durocher, 1996; Johnson *et al*., 2007; Shanazarov *et al*., 2023; Svyatova *et al*., 2023). While initial studies classified it as "Benign", recent research has revealed its potential role as an effect modifier, particularly in the context of DNA damage response (Menke *et al*., 2021). Notably, Bhatti *et al*. (2008) demonstrated that carriers of the variant allele exhibited significantly increased breast

cancer risk when exposed to diagnostic radiation (EOR/unit dose score= 9.4, 95% CI: 1.4-25.7), suggesting a gene-environment interaction. The variant has also been associated with triple negative breast cancer (Ricks-Santi *et al*., 2013; Shanazarov *et al*., 2023), with an odds ratio of 2.31 (95% CI: 1.08-4.93), and shows interactions with hormone therapy use among postmenopausal women (*p* for interaction= 0.01). The variant's association with cancer risk appears to be population-specific, with varying frequencies and penetrance across different ethnic groups occurring at 2.7% in the Kazakh population (Svyatova *et al*., 2023) compared to a global minor allele frequency of 3.4% (ClinVar) (Bhatti *et al*., 2008; Durocher, 1996; Ricks-Santi *et al*., 2013; Shanazarov *et al*., 2023; Svyatova *et al*., 2023). However, there remains a critical gasp in understanding its precise molecular mechanisms, particularly in Middle Eastern populations where genetic studies have been limited.

2.2.3. Methylation

DNA methylation is an important epigenetic mechanism that can silence gene expression without altering the DNA sequence itself (Chera *et al*., 2024). In breast cancer, methylation of the *BRCA1* gene promoter region appears as a significant area of study, with research showing varying rates of methylation across different populations (Birgisdottir *et al*., 2006; Q. Li *et al*., 2015; Niwa *et al*., 2000; Yamashita *et al*., 2015). The methylation specifically targets the CpG islands within the *BRCA1* α promoter region, particularly at sites within nucleotides -150 to +32 relative to the main transcription start site of *BRCA1* (Al-Moghrabi, 2017; Mancini *et al*., 1998; Moore *et al*., 2013). Studies have showed methylation frequencies ranging from 13-40% in sporadic breast cancer cases, though some populations show markedly higher rates up to 80-85% in certain Asian cohorts (Butcher & Rodenhiser, 2007; Harahap *et al*., 2018; Oubaddou *et al*., 2023; Yamashita *et al*., 2015). This epigenetic modification typically occurs through the addition of methyl groups to CpG islands in the promoter region (Figure 2.6), leading to transcriptional silencing of the *BRCA1* gene (Jovanovic *et al*., 2010). The methylation process involves DNA

methyltransferases (DNMTs) adding methyl groups to cytosine residues in CpG dinucleotides, creating 5-methylcytosine (Lopomo & Coppedè, 2018). Research has shown that *BRCA1* promoter methylation is often mutually exclusive with *BRCA1* mutations, suggesting it serves as an alternative mechanism for *BRCA1* inactivation in sporadic breast cancers (Wei *et al*., 2005). This methylation pattern has been particularly associated with specific clinicopathological features, including triple-negative status, higher histological grade, and younger age at diagnosis (Birgisdottir *et al*., 2006; Wong *et al*., 2011; Zhang & Long, 2015).

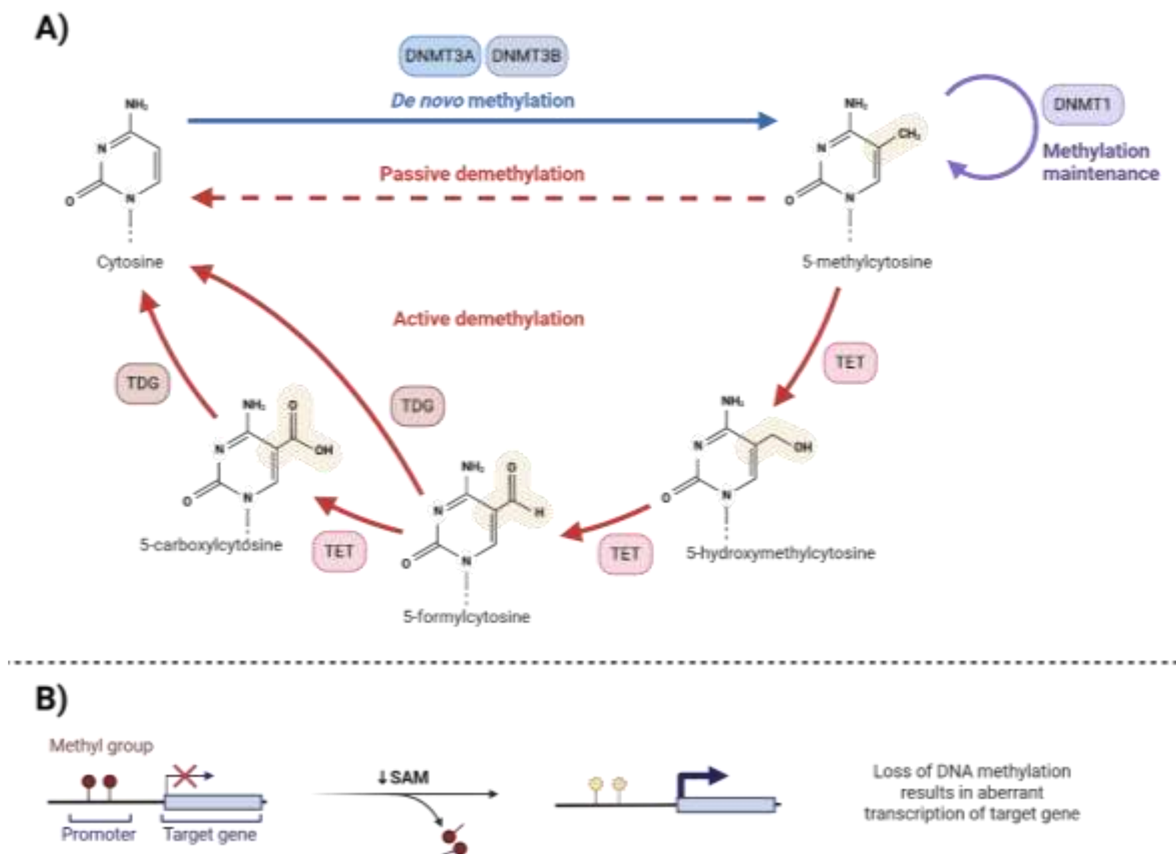


Figure 2.6. DNA methylation dynamics and transcriptional consequences: A) enzymatic regulation of DNA methylation showing DNMT-mediated methylation and ten-eleven translocation (TET)-mediated demethylation of cytosine residues, and B) impact of methylation loss on gene expression, showing how hypomethylation leads to aberrant transcriptional activation of normally silenced genomic regions. Created with BioRender.

The functional consequences of *BRCA1* promoter methylation include markedly reduced or absent *BRCA1* protein expression, confirmed through immunohisto-chemical studies (Kumar *et al*., 2017; Miyamoto, 2002; Niwa *et al*., 2000). Tumors with *BRCA1* methylation display a "BRCA-like" phenotype similar to familial *BRCA1*-mutated cancers, including high tumor grade, triple-negative status, and increased genomic instability (Daniels *et al*., 2016). Studies have shown that *BRCA1* methylation frequently co-occurs with allelic imbalance at the *BRCA1* locus and elevated frequencies of *TP53* mutations (Birgisdottir *et al*., 2006). This epigenetic modification acts as one of "Knudson's hits" in sporadic breast tumorigenesis, where promoter methylation of one allele often accompanies deletion of the other allele (Sharma *et al*., 2014; Stefansson *et al*., 2012; Xu *et al*., 2013).

The clinical significance and therapeutic consequences of *BRCA1* promoter methylation have been well-established. Tumors with *BRCA1* methylation tend to be more sensitive to DNA-damaging agents like platinum compounds and PARP inhibitors (Sharma *et al*., 2014; Stefansson *et al*., 2020; Xu *et al*., 2013). The methylation status has also shown significant correlations with mRNA expression levels, where higher methylation typically corresponds to decreased *BRCA1* transcription (Li *et al*., 2015). Regarding prognosis, Xu *et al*. (2009) found that *BRCA1* methylation was significantly associated with increased breast cancer-specific mortality (age-adjusted HR 1.71; 95% CI: 1.05-2.78) and higher all-cause mortality (age-adjusted HR 1.49; 95% CI: 1.02-2.18). Furthermore, the distribution of *BRCA1* methylation varies across breast cancer subtypes. Matros *et al*. (2005) found that methylation was particularly prevalent in medullary (67%) and mucinous (55%) subtypes, which are histological patterns commonly observed in hereditary *BRCA1*-mutated cancers. The methylation status occurs more frequently in invasive cancers compared to *in situ* carcinomas and is more common among premenopausal cases (Esteller, 2000; Xu *et al*., 2009). *BRCA1* methylation is more prevalent in tumors with at least one node involved and with tumor sizes greater than 2 cm (Matros *et al*., 2005).

Detection methods for *BRCA1* methylation have evolved from qualitative techniques like methylation-specific PCR (MS-PCR) to quantitative approaches including real-time PCR analysis and newer platforms like MassARRAY that offer precise methylation quantification (Pal & Anderson, 2018; Quann *et al*., 2015; Rodriguez *et al*., 2022; Snell *et al*., 2008). These methodological advances are important for starting accurate methylation thresholds and understanding the relationship between methylation levels and clinical outcomes.

2.2.4. Protein expression

BRCA1 protein expression patterns in breast tissue offer important information about its role in both normal breast development and cancer progression. In normal breast tissue, BRCA1 shows strong, uniform nuclear expression within the parenchymal cells of ductal and lobular regions (Figure 2.7), where it functions as a critical tumor suppressor (Issac *et al*., 2021; Madjd *et al*., 2011; Rakha *et al*., 2008). However, in malignant breast tissues, BRCA1 expression exhibits notable heterogeneity and is frequently diminished compared to normal tissue (Dimitrov *et al*., 2001; Yoshikawa *et al*., 1999). Studies in multiple research cohorts have consistently shown that approximately 15-30% of sporadic breast cancers display complete loss of nuclear BRCA1 expression, while an additional 30-45% show reduced expression levels (Rakha *et al*., 2008). This altered expression manifests through various patterns: some cases show complete absence of nuclear staining, others demonstrate weak or reduced nuclear presence, and many exhibit abnormal cytoplasmic localization. Importantly, the subcellular distribution of BRCA1 protein has been reported as a significant factor, with normal cells showing predominantly nuclear localization while cancer cells often display both nuclear and cytoplasmic patterns or, in some cases, predominantly cytoplasmic expression (Chen *et al*., 1995; Mahmoud *et al*., 2017).

The clinical and prognostic significance of BRCA1 protein expression patterns has been comprehensively documented through multiple immunohistochemical

studies. In a large-scale study by Rakha *et al*. (2008) examining 1940 cases, 15% of breast cancers showed complete loss of nuclear expression, while 39% demonstrated reduced expression. Their analysis revealed that altered BRCA1 expression significantly correlated with high-grade tumors and early-onset disease. In the same context, Madjd *et al*. (2011) found in their study of 156 cases that 57% of breast tumors showed either absent (15%) or reduced (42%) nuclear expression of BRCA1, with a significant association with high-grade breast tumors. More recent research by Issac *et al*. (2021) examining 110 cases found that 19% showed reduced BRCA1 immunoreactivity, with a notable correlation with lymphovascular emboli ($p<0.05$). Regarding hormone receptor status, Hedau *et al*. (2015) demonstrated in their cohort that reduced BRCA1 expression showed significant correlation with negative hormone receptor status and was more prevalent in post-menopausal patients, appearing in 57.5% of these cases. These studies collectively establish that diminished BRCA1 expression serves as a marker for more aggressive disease characteristics and could potentially guide therapeutic decisions.

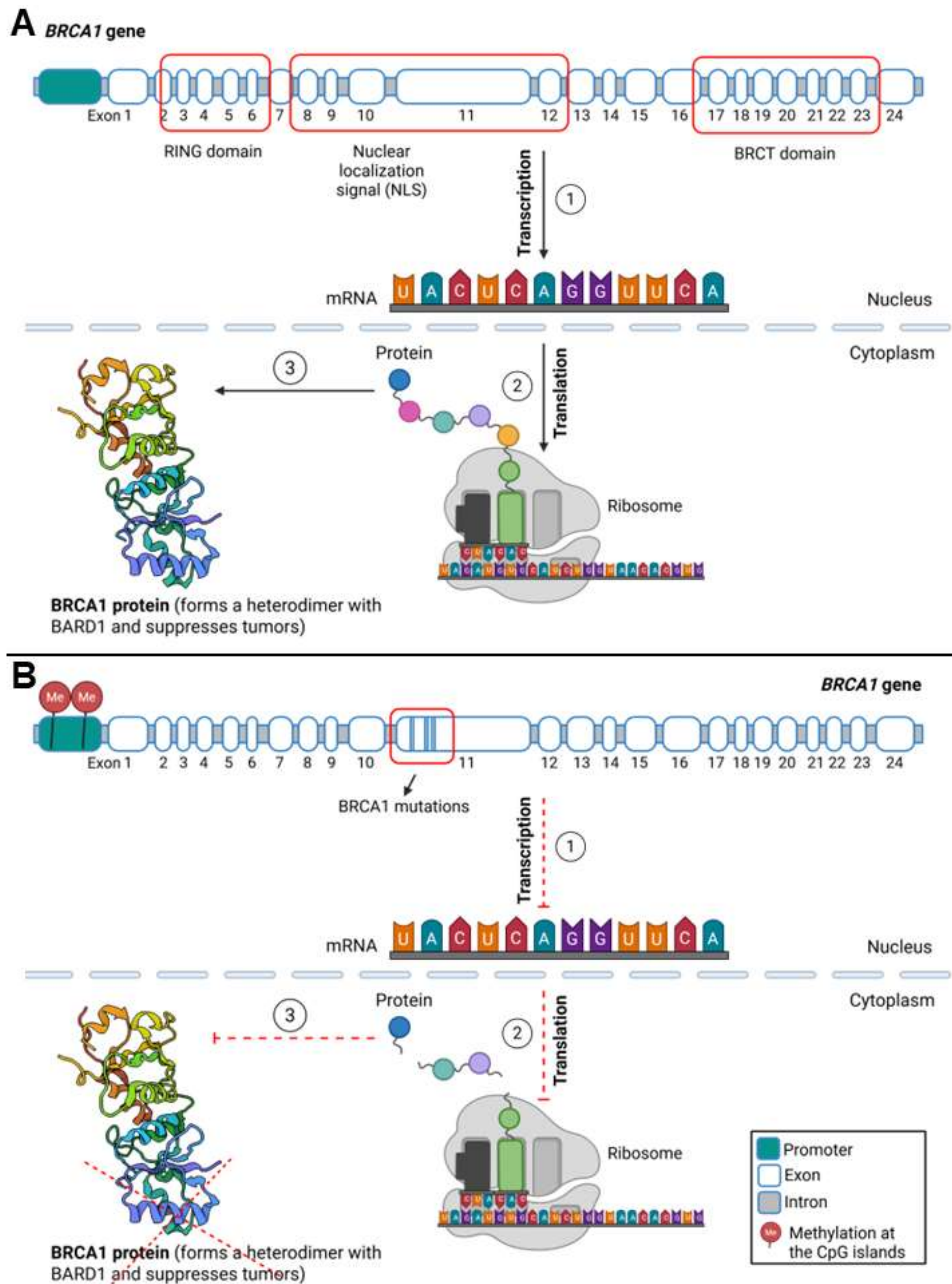


Figure 2.7. *BRCA1* gene expression and disruption mechanisms: A) normal *BRCA1* expression pathway showing nuclear transcription to mRNA, nuclear export, ribosomal translation, and mature protein production.; B) impaired *BRCA1* expression due to mutations and promoter methylation, resulting in reduced or absent protein synthesis (Xu *et al*., 2009).

2.3. BRCA1-associated ring domain protein 1 (BARD1)

2.3.1. Structure and function

BARD1 (BRCA1-Associated RING Domain 1) is a complex and highly conserved protein with a unique structural architecture that enables its diverse cellular functions (Russi *et al*., 2022). The protein consists of 777 amino acids (Figure 2.8) in humans and contains three key conserved domains that define its structure and interactions (Irminger-Finger *et al*., 2016). At its N-terminus, BARD1 features a RING finger domain (residues 46-90) that is specifically homologous to BRCA1's RING domain and serves as the primary interface for BRCA1 binding (Brzovic, Meza, et al., 2001). The most distinctive structural feature of BARD1 is the presence of three ankyrin (ANK) repeats (residues 427-525) located in the central region, which are notably absent in BRCA1 and represent the most evolutionarily conserved portion of the protein (Ayi *et al*., 1998; Irminger-Finger *et al*., 1998). These ANK repeats are followed by two tandem BRCT domains (residues 616-777) at the C-terminus, which like the RING domain, share homology with BRCA1 (Bork *et al*., 1997). The protein also contains important regulatory elements, including a nuclear export signal (NES) at residues 102-120 and six predicted nuclear localization signals (NLS) distributed near its functional domains (Jefford *et al*., 2004; Schüchner *et al*., 2005). As established through structural studies by Wu *et al*. (1996), Brzovic *et al*. (2001), and Irminger-Finger *et al*. (1998), this precise arrangement of domains is unique to BARD1 among all known proteins and enables its specific interactions with binding partners, particularly its ability to form stable heterodimers with BRCA1 through coordinated binding of their respective RING domains (Meza *et al*., 1999).

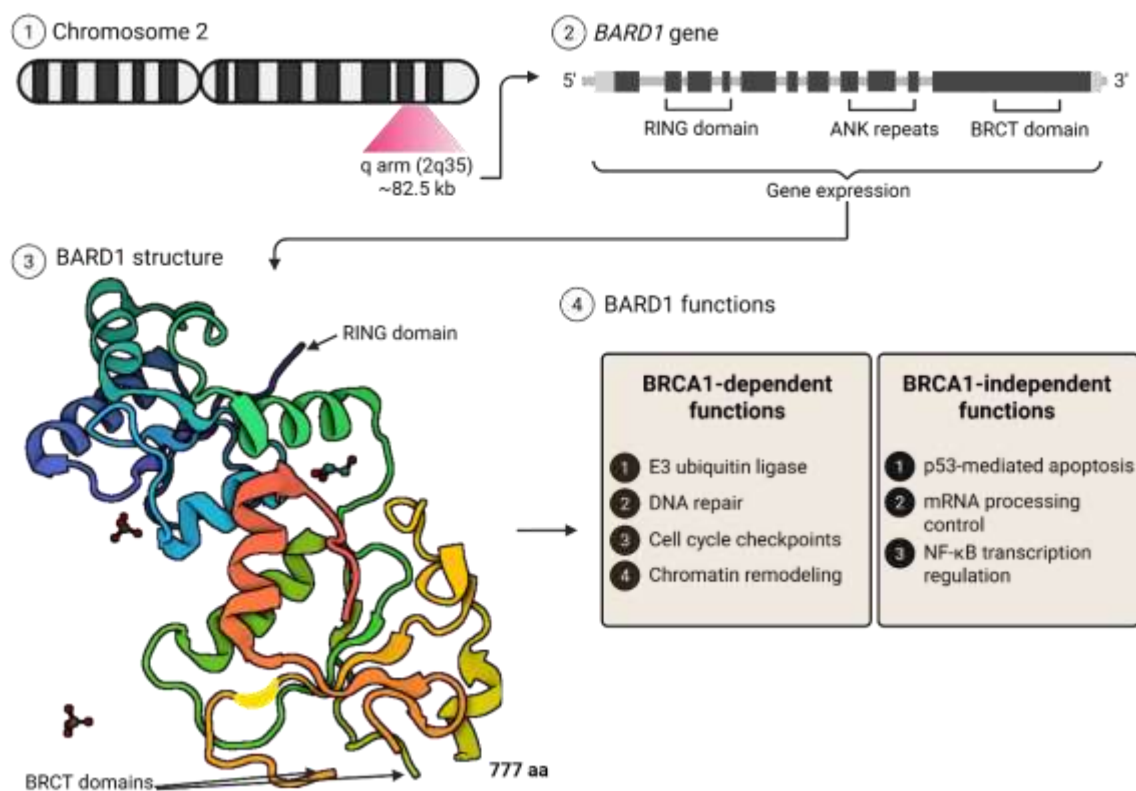


Figure 2.8. *BARD1* gene organization, protein structure and function: 1) the *BARD1* gene spans 82.5 kb on the reverse strand of chromosome 2q35, 2) containing 11 exons, which its 3) translation initiates in exon 1 and terminates in exon 11, producing a 777-amino acid protein (PDB: 3FA2, https://www.rcsb.org/). The yielded protein functions are summarized in 4. Coding regions are shown as filled boxes, while 5' and 3' UTRs are depicted as gray boxes. Domain encoding is indicated: RING finger (exons 2-4), nuclear export signal (exon 5), ankyrin repeats (exons 8-10), and both BRCT domains (exon 11), (Birrane et al., 2007). Created using bioRender.com.

Functionally, BARD1 operates through both BRCA1-dependent and independent pathways to maintain genomic stability and regulate cell cycle progression (Figure 2.9). In its primary role, BARD1 forms a stable heterodimer with BRCA1 through their RING domains, creating an active E3 ubiquitin ligase complex that is essential for DNA damage response and repair (Hashizume *et al*., 2001). This complex targets several key proteins for ubiquitination, including histone H2A and H2AX, RNA polymerase II, and the Aurora kinases, thereby regulating transcription, DNA repair, and cell cycle progression (Bosse *et al*., 2012; Chen *et al*., 2002; Kleiman *et al*., 2005; Mallery, 2002; Ryser *et al*., 2009; Starita *et al*., 2005; Xia *et al*., 2003). As explained by Hashizume *et al*. (2001), the ubiquitin ligase activity is specifically dependent on the BARD1-BRCA1 heterodimer formation, as neither protein alone exhibits

significant activity. Beyond its BRCA1-dependent functions, BARD1 has been reported as an independent regulator of cellular processes, particularly through its distinctive ANK repeats. BARD1 independently interacts with p53 through these ANK repeats, promoting apoptotic responses to cellular stress and stabilizing p53 protein levels (Feki *et al*., 2005; Irminger-Finger *et al*., 2001). The protein also binds to the polyadenylation factor CstF-50, inhibiting mRNA processing during DNA damage responses (Kleiman & Manley, 1999), and interacts with NF-κB to modulate transcriptional activity (Dechend *et al*., 1999). These diverse functions make BARD1 a critical guardian of genomic stability, with its disruption leading to severe consequences including embryonic lethality between days E7.5 and E8.5 in mouse models and increased cancer susceptibility (McCarthy *et al*., 2003).

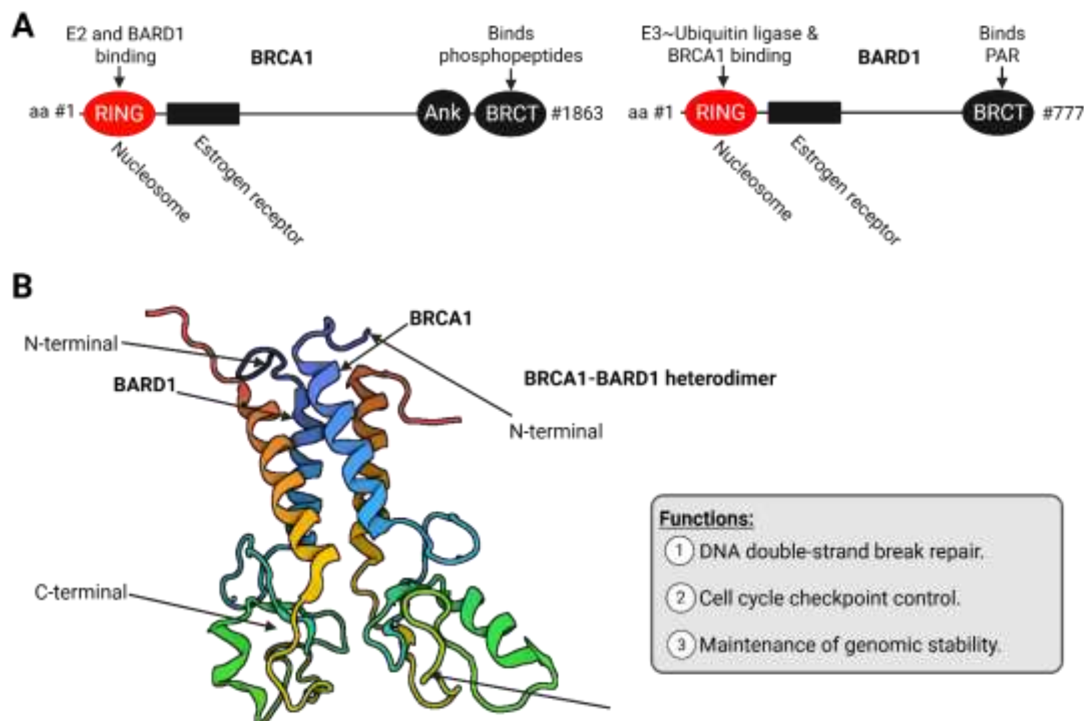


Figure 2.9. BRCA1-BARD1 heterodimer complex structure and function: A) BRCA1 and BARD1 structures and their domains, especially the RING domain through which the hetero-dimer formation initiated and E3 ubiquitin ligase with activity essential for DNA repair, cell cycle control, and genomic stability; B) the 3D structure of the heterodimer (PDB: 1JM7, https://www.rcsb.org/) with its domains (Brzovic *et al*., 2001). Created using bioRender.com.

2.3.2. Mutations and polymorphisms

Studies have revealed a diverse spectrum of pathogenic *BARD1* mutations, with over 69 distinct variants identified across breast and ovarian cancer patients (Suszynska & Kozlowski, 2020). The mutations are distributed throughout the coding sequence but show particular clustering in two key regions: one overlapping the RING-finger domain and another spanning the ankyrin repeat and BRCT I domains. The most frequently recurring mutations include Q564*, E652Vfs*69, S551*, R112*, and R641*, with Q564* being notably prevalent in cases with family history of breast/ovarian cancer (Suszynska & Kozlowski, 2020). Population studies indicate that *BARD1* mutation carriers have approximately 2.9-fold increased risk of developing breast cancer, classifying it as a moderate-risk gene (Suszynska & Kozlowski, 2020). The risk appears to be higher for triple-negative breast cancer (OR=3.6) and bilateral breast cancer (OR=5.1), suggesting BARD1 plays a particularly important role in these aggressive subtypes (Suszynska *et al*., 2019). In Table 2.2, several BARD1 common SNPs associated with breast cancer are listed.

Table 2.2. Some *BARD1* SNPs and their association with breast cancer risk.

SNP ID	Amino acid change	Association with breast cancer	Population	Reference
rs28997575	Leu359/ Pro365del	Increased risk by 3.475-fold (OR= 3.475, 95% CI = 1.302–9.276, *p*= 0.013)	Han Chinese women with early-onset breast cancer	Wu *et al.* (2022)
rs2229571	Arg378Ser	Decreased risk (protective effect: GC: 72.6% risk reduction; OR= 0.274, 95% CI= 0.134–0.562, *p*=0.001; CC: 82.8% risk reduction (aOR1 = 0.172, 95% CI = 0.076–0.392, *p*=0.001)	Han Chinese women with early-onset breast cancer	Wu *et al.* (2022)
rs2070094	Val507Met	GA genotype showed protective but not statistically significant after adjustment (OR= 0.834, *p*>0.580)	Han Chinese women with early-onset breast cancer	Wu *et al.* (2022)

SNP ID	Amino acid change	Association with breast cancer	Population	Reference
rs1048108	Pro24Ser	In the study by Wu *et al*. (2022): No significant association. In Liu *et al*. (2013): Decreased effect (protective effect, OR: 0.66, $p<0.05$)	Han Chinese women with early-onset breast cancer	Wu *et al*. (2022), Liu et al. (2013)
rs3738888	Arg658Cys	No significant association	Han Chinese women with early-onset breast cancer	Wu *et al*. (2022)

Recent large-scale analyses have found important variant-specific differences in cancer risk, with certain mutations conferring significantly higher risk than the overall gene-specific estimates. For instance, three recurrent variants (R112*, S551*, and Q564*) are associated with odds ratios exceeding 5.0, suggesting they may be particularly deleterious (Capasso *et al*., 2009). The functional impact of these variants appears to be mediated through various mechanisms, some disrupt the BRCA1-BARD1 heterodimer formation, while others affect splicing regulation or protein stability (Alshatwi *et al*., 2012; Capasso *et al*., 2009). Notably, while *BARD1* mutations clearly influence breast cancer susceptibility, their role in ovarian cancer risk remains controversial, with most studies showing no significant association (OR=1.36, p=0.17). The exception is the Q564* variant, which shows evidence of elevated ovarian cancer risk in European populations, the matter confirms the importance of population-specific risk assessment (Suszynska & Kozlowski, 2020).

Research indicates that rs1048108 may have a protective effect against certain cancers. In studies of Han Chinese women, the variant was associated with a significantly decreased risk of breast cancer, with heterozygous carriers showing approximately 30% lower risk compared to non-carriers (Liu *et al*., 2013). However, the findings around breast cancer risk have been inconsistent across different populations. A more recent study by Wu *et al*. (2022) in Han Chinese women found that while rs1048108 showed a trend toward protection (30.6% decreased risk for

heterozygous carriers and 43.8% for homozygous carriers), these associations did not reach statistical significance after adjustment for confounding factors. These conflicting results show that while some studies suggest a protective role, others have found no significant association between rs1048108 and breast cancer susceptibility.

The most compelling evidence for rs1048108's impact comes from neuroblastoma research (Lee *et al*., 2014). Multiple studies, including Lee's *et al.* (2014) large genome-wide association study, found that this variant is significantly associated with high-risk neuroblastoma susceptibility. The rs1048108 variant was identified through linkage disequilibrium analysis (r^2= 0.841 with rs6744811) and pathway-based analysis as a candidate causal SNP contributing to neuroblastoma susceptibility. This association was validated across multiple independent cohorts, providing strong evidence for its role in neuroblastoma predisposition (Capasso *et al*., 2009; Capasso *et al*., 2013).

The obvious inconsistency in findings between breast cancer and neuroblastoma studies suggests that rs1048108's effects may be tissue-specific or influenced by other genetic and environmental factors. The variant is located in exon 1 of *BARD1* and results in a proline to serine amino acid change, classified as potentially deleterious, which could potentially affect protein function differently in various cellular contexts. However, more research is needed to fully understand the molecular mechanisms underlying these associations and to resolve the seemingly contradictory findings across different cancer types and populations.

2.3.3. Protein expression

BARD1 protein expression exhibits complex spatiotemporal patterns across different tissues and developmental stages. Under normal physiological conditions, BARD1 predominantly localizes to the nucleus where it forms a heterodimer with BRCA1, though cytoplasmic expression has also been documented in specific cellular contexts (Irminger-Finger *et al*., 2016). The protein's expression is tightly regulated throughout the cell cycle, with peak levels observed during S-phase when

DNA repair mechanisms are most active (Cimmino *et al*., 2017). Studies have shown that BARD1 expression is particularly high in tissues with elevated proliferation rates, including the developing embryo, regenerating liver tissue, and rapidly dividing epithelial cells (Irminger-Finger *et al*., 2012). This expression pattern aligns with BARD1's critical roles in maintaining genomic stability, cell cycle regulation, and DNA damage response pathways, as showed through extensive functional studies (Sporn *et al*., 2011).

In the context of breast cancer, BARD1 protein expression patterns undergo significant alterations that correlate with disease progression and clinical outcomes. Multiple studies have demonstrated that while full-length BARD1 typically shows reduced expression in breast cancer tissues compared to normal breast epithelium, there is a marked upregulation of specific BARD1 isoforms, particularly in aggressive subtypes of breast cancer (Wu *et al*., 2006; Zhang *et al*., 2012). Notably, research has shown that aberrant cytoplasmic localization of BARD1 protein is associated with poor prognosis in breast cancer patients, suggesting a potential role for BARD1 mislocalization in disease progression (Thai, 1998). Recent evidence by Śniadecki *et al*. (2020) draws attention to the oncogenic role of certain isoforms (BARD1β and BARD1δ) in breast cancer development, with BARD1β isoform detection proposed as a potential screening test for high-risk patients. Furthermore, Weber-Lassalle *et al*. (2019) reported that germline loss-of-function variants in BARD1 are significantly associated with early-onset familial breast cancer (OR= 12.04 for diagnosis <40 years). This area represents a significant gap in current knowledge, particularly regarding the mechanisms controlling BARD1 isoform expression and the development of clinical screening tests for BARD1 variants. Moreover, the association between BARD1 levels and various genotypes yet to be elucidated.

2.4. microRNA-498

2.4.1. Structure and function

MicroRNA-498 (miR-498) belongs to the class of small non-coding RNA molecules (Figure 10), with a length of 19-25 nucleotides (Chai *et al*., 2018). Its mature sequence functions as a post-transcriptional regulator by binding to specific sites within the 3' untranslated regions (3'UTR) of target messenger RNAs (mRNAs) (Chai *et al*., 2018; Li & Jiang, 2020). The structure of miR-498 includes a distinctive seed region spanning nucleotides 2-8 from the 5' end, which is important for target recognition and binding. This seed sequence enables miR-498 to form complementary base pairs with its target mRNAs, particularly at positions 833-855 of the 3'UTR in genes like *PTEN* and positions 168-189 in genes like *BRCA1* (Chai *et al*., 2018; Matamala *et al*., 2016). The mature miR-498 is processed from a larger precursor molecule through a series of enzymatic steps involving Drosha and Dicer, ultimately incorporating into the RNA-induced silencing complex (RISC) to execute its regulatory functions (Li & Jiang, 2020). The secondary structure of miR-498's precursor forms a characteristic stem-loop configuration, which is essential for its proper processing and maturation into the functional form (Li & Jiang, 2020).

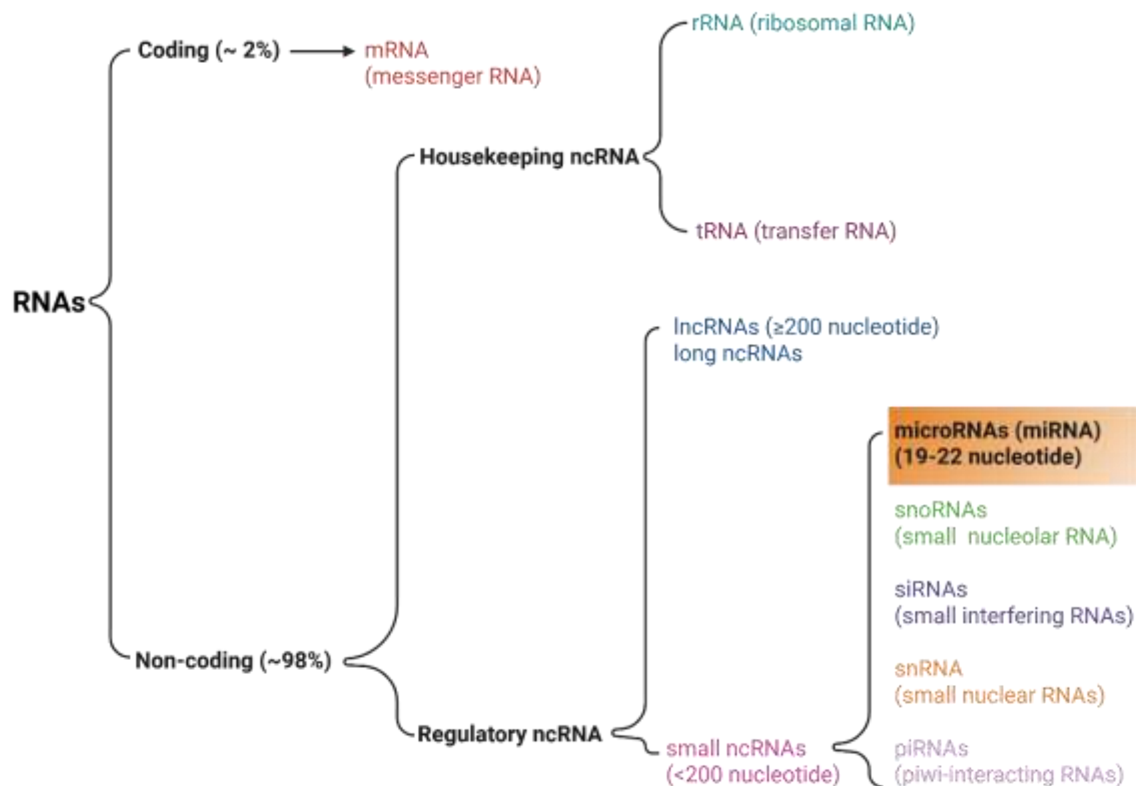


Figure 2.10. Overview of RNA classification based on functional properties. RNAs in eukaryotic cells divide into coding mRNAs and various non-coding RNA families. Non-coding RNAs include constitutively expressed housekeeping molecules (rRNAs for ribosome assembly, tRNAs for translation) and regulatory molecules that control gene expression. Regulatory ncRNAs span two size classes: long ncRNAs (>200 nucleotides) and small ncRNAs including miRNAs, with specialized regulatory functions. Adapted from Inamura (2017), and created using bioRender.com.

MicroRNA-498 plays complex and tissue-specific roles in gene regulation and cellular processes (Chai *et al*., 2018; Raheem *et al*., 2024). In cancer biology, and as explained in Figure 2.11, it functions primarily by binding to and regulating key tumor-related genes through direct interaction with their 3'UTR regions (Chai *et al*., 2018; Matamala *et al*., 2016). The research shows that miR-498 can simultaneously target multiple genes involved in cell proliferation, migration, and survival pathways (Chai *et al*., 2018; Li & Jiang, 2020). It has been clearly shown to directly regulate both tumor suppressors and oncogenes, including *PTEN* and *BRCA1* through complementary binding sites in their mRNA (Chai *et al*., 2018; Matamala *et al*., 2016). Once bound to its target, miR-498 can either inhibit translation or promote mRNA degradation, depending on the degree of sequence complementarity and cellular context (Chai *et al*., 2018; Li & Jiang, 2020). In triple-negative breast cancer cells, miR-498 has been demonstrated to enhance cell proliferation and migration

through its regulatory effects on the PI3K/Akt pathway via *PTEN* suppression (Chai *et al*., 2018). The protein levels of its target genes can be reduced by 30-50% through this regulatory mechanism, highlighting its significant impact on gene expression and cellular phenotype (Chai *et al*., 2018; Matamala *et al*., 2016).

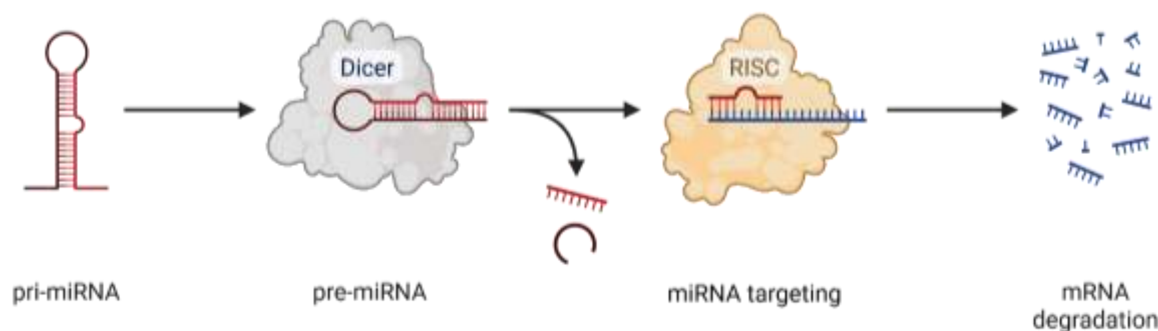


Figure 2.11. Schematic representation of microRNA biogenesis. The pathway illustrates: 1) pri-miRNA transcription and nuclear processing by Drosha-DGCR8; 2) pre-miRNA export through Exportin-5/RAN-GTP; 3) cytoplasmic processing by Dicer-TRBP; 4) mature miRNA incorporation into AGO2-containing RISC; and 5) target mRNA regulation (degradation). Figure designed using BioRender.com based on template by A. Finkbeiner and W. Jiang.

2.4.2. Role in cancer

Based on the comprehensive literature review, the miR-498 appears as a critical regulatory molecule with complex and context-dependent roles in cancer pathogenesis. Studies have demonstrated that miR-498 primarily functions as a tumor suppressor across multiple cancer types, including colorectal, lung, and ovarian cancers, where its expression is frequently downregulated compared to normal tissues (Gopalan *et al*., 2015; Li & Jiang, 2020). The tumor-suppressive effects of miR-498 are mediated through its ability to target and regulate several key oncogenic pathways (Li & Jiang, 2020). Specifically, miR-498 has been shown to directly target genes involved in cell proliferation, invasion, and metastasis, such as *FOXO3*, *BMI-1*, *HMGA2*, and *ZEB2*. In hepatocellular carcinoma, miR-498 suppresses tumor growth by targeting *FOXO3*, while in non-small cell lung cancer, it inhibits cell growth and metastasis by targeting *HMGA2* (Li & Jiang, 2020). The expression levels of miR-498 have also been correlated with clinical outcomes, where lower expression often

indicates poor prognosis and more aggressive disease characteristics (Chai *et al*., 2018; Li & Jiang, 2020).

In the context of breast cancer, miR-498 has shown particularly interesting roles that require further investigation. Matamala *et al*. (2016) found that miR-498 was significantly downregulated in triple-negative breast cancer and could potentially regulate *BRCA1* expression. Their research identified miR-498 as one of the few microRNAs that could directly target the 3'UTR of *BRCA1* mRNA, suggesting its probable role in breast cancer pathogenesis. Chai *et al*. (2018) further elucidated that miR-498 could promote proliferation and migration in breast cancer cells by targeting the tumor suppressor *PTEN*, which indicates a complex regulatory network. Remarkably, Leivonen *et al*. (2014) identified miR-498 as one of several microRNAs essential for HER2-positive breast cancer cell growth, suggesting its potential role in different breast cancer subtypes. However, there remains a significant knowledge gap in understanding the precise mechanisms through which miR-498 functions in breast cancer, particularly in relation to *BRCA1* and its associated pathways.

Chapter three

MATERIALS & METHODS

Materials and Methods

3.1. Materials

3.1.1. Chemicals, reagents and kits

All chemicals, solutions, reagents, and kits used in the current thesis work are presented in Table 3.1. All chemicals were of analytical grade.

Table 3.1. Chemicals, reagents, kits, and solutions used in this study.

No.	Material	Company	Country
1	Agarose LE	Intron Biotechnology	Korea
2	RedSafe nucleic acid staining solution	Intron Biotechnology	Korea
3	1kb DNA Ladder Plus	GDSBio	China
4	Safe-Green 100bp Opti-DNA Marker	Applied Biological Materials (abm)	Canada
5	GoTaq® G2 Green Master Mix	Promega Corporation	USA
6	10X TAE (Tris Acetate EDTA) buffer	Promega Corporation	USA
7	AddPrep Genomic DNA Extraction Kit	Addbio Inc.	Korea
8	Wizard SV Gel and PCR Clean-Up System	Promega Corporation	USA
9	PAXgene Blood RNA tubes	PreAnalytiX (Qiagen/BD)	Switzerland
10	Human BRCA1 ELISA Kit	MyBioSource Inc.	USA
11	Human BARD1 ELISA Kit	MyBioSource Inc.	USA
12	Human IL-1β ELISA Kit	Abcam	UK
13	Human IL-6 ELISA Kit	Abcam	UK
14	Human TNF-α ELISA Kit	Abcam	UK
15	Human 8-OHdG ELISA Kit	Abcam	UK
16	EDTA blood collection tubes (K2 EDTA)	BD Vacutainer	USA
17	Plain blood collection tubes (serum)	BD Vacutainer	USA
18	Proteinase K solution (20 mg/mL)	Sigma-Aldrich	USA
19	Absolute ethanol (molecular grade)	Sigma-Aldrich	USA
20	Nuclease-free water	Thermo Fisher Scientific	USA
21	DNase I, RNase-free	Thermo Fisher Scientific	USA
22	RNase inhibitor (40 U/µL)	Applied Biosystems	USA
23	dNTP Mix (10 mM each)	Thermo Fisher Scientific	USA
24	SYBR Green Master Mix	Applied Biosystems	USA
25	TaqMan Universal Master Mix	Applied Biosystems	USA
26	TMB substrate solution	Sigma-Aldrich	USA

27	Sulfuric acid stop solution (2N H2SO4)	Sigma-Aldrich	USA
28	Custom oligonucleotide primers	Macrogen Inc.	Korea
29	RNase A solution (10 mg/mL)	Sigma-Aldrich	USA
30	Reverse transcriptase enzyme	Promega Corporation	USA
31	Bisulfite conversion reagents	Zymo Research	USA
32	miRNA-specific stem-loop RT primers	Integrated DNA Technologies	USA
33	Universal reverse primer for miRNA	Integrated DNA Technologies	USA

3.1.2. Devices, tools, equipment and software

The equipment, devices, and software used in the current thesis work are all presented in the Table 3.2.

Table 3.2. Devices, equipment, instruments, and software used in this study

No.	Item	Model/Version	Company	Country
1	Microcentrifuge	Microfuge 16	Beckman Coulter	Germany
2	Dry bath incubator	AccuBlock	Labnet International	UK
3	Thermal cycler	Optimus 96G	QLab Solutions	UK
4	Horizontal gel electrophoresis system	multiSUB Choice	Cleaver Scientific	UK
5	Analytical balance	PFB 300-3	Kern & Sohn GmbH	Germany
6	UV gel documentation system	GelStudio	Analytik Jena	UK
7	Micropipettes (0.5-10, 10-100, 100-1000 µL)	Pipetman	Gilson	France
8	Clinical chemistry analyzer	Cobas c311	Roche Diagnostics	Germany
9	Immunoassay analyzer	Cobas e411	Roche Diagnostics	Germany
10	Microplate reader	ELx800	BioTek Instruments	USA
11	Real-time PCR system	StepOnePlus	Applied Biosystems	USA
12	UV-Vis spectrophotometer	NanoDrop 2000	Thermo Fisher Scientific	USA
13	Wall-mounted stadiometer	222	Seca GmbH	Germany
14	Digital scale	813	Seca GmbH	Germany
15	Ultra-low temperature freezer (-80°C)	MDF-U76VC	Panasonic Healthcare	Japan
16	Laboratory freezer (-20°C)	TSX2320FD	Thermo Fisher Scientific	USA
17	Laboratory refrigerator (2-8°C)	TSX2304RD	Thermo Fisher Scientific	USA

18	Microwave oven	R-209	Sharp Corporation	Japan
19	Vortex mixer	Vortex-Genie 2	Scientific Industries	USA
20	Liquid nitrogen dewar (35L)	LD35	Taylor-Wharton	USA
21	Refrigerated centrifuge	5810R	Eppendorf	Germany
22	Water bath	WB-22	PolyScience	USA
23	pH meter	SevenEasy	Mettler Toledo	Switzerland
24	Magnetic stirrer	PC-420D	Corning	USA
27	Microsoft Office Apps	365	Microsoft Corporation	USA
29	BioRender	Web-based	BioRender.com	Canada
30	R statistical software	3.6.0	R Foundation for Statistical Computing	Austria
31	RStudio Desktop	2023.06.1+524	RStudio PBC	USA
32	G*Power	3.1.9.7	Heinrich-Heine-Universität	Germany
33	ggplot2 (R package)	3.3.5	Hadley Wickham	New Zealand
34	NCBI BLAST	Web-based	National Center for Biotechnology Information	USA
39	SnapGene Viewer	5.3	GSL Biotech LLC	USA
40	Mendeley	1.19.4	Elsevier	USA

3.2. Study design and setting

This case-control study was conducted from November 2024 to June, 2025 to evaluate genetic, epigenetic, and biochemical biomarkers associated with breast cancer risk in Iraqi women. The study recruited participants from multiple healthcare facilities in Iraq, including Imam Hussain Medical City (the Cancer's Early Diagnosis Unit of Karbala, and Imam Hussain Oncology and Hematology Center in Karbala), and Al-Andalus Specialized Hospital in Baghdad. The study protocol was reviewed and approved by the Postgraduate Committee at the Department of Chemistry and Biochemistry of the College of Medicine of the University of Kerbala (No.: D/6/6658, Date: 01-12-2024; see appendices: supplemental Figure 1), and the Ethics Committee of the Health Directorate of Karbala (No.: 3986, Date: 03-11-2024; see appendices: supplemental Figure 2), and was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. Written informed consent (see appendices, supplemental figure 3) was obtained from all participants

prior to enrollment, after providing them with comprehensive information about the study objectives, procedures, potential risks and benefits, and their right to withdraw at any time without affecting their medical care.

3.3. Study participants

3.3.1. Participants

The study population consisted of 188 Iraqi women, divided into two groups: 90 patients with histopathologically confirmed breast cancer and 98 healthy controls. The patient group included women newly diagnosed with primary breast cancer who had not yet received any form of cancer treatment, including chemotherapy, radiation therapy, or hormonal therapy. All participants were residents of Karbala governorate or surrounding regions. The control group consisted of healthy women with no personal history of cancer or chronic inflammatory diseases, recruited from the same geographical area and matched for age to minimize confounding factors.

3.3.2. Inclusion and exclusion criteria

For the patient group, inclusion criteria were: 1) female sex; 2) age 18 years or older; 3) histopathologically confirmed diagnosis of primary breast cancer (ductal or lobular carcinoma); 4) newly diagnosed cases who had not received chemotherapy, radiation therapy, or hormonal therapy prior to blood sample collection; 5) Iraqi nationality with residence in the study area for at least 5 years; and 6) ability to provide informed consent. Patients who had undergone surgical removal of the breast mass (lumpectomy or mastectomy) were included if blood samples were collected prior to surgery or if surgery was the only treatment received before enrollment. For the control group, inclusion criteria included: 1) female sex; 2) age-matched to cases (±5 years); 3) no personal history of any type of cancer; 4) Iraqi nationality with residence in the study area for at least 5 years; and 6) ability to provide informed consent.

Exclusion criteria for both groups were: 1) pregnancy or lactation at the time of enrollment; 2) previous history of any malignancy other than the current breast cancer diagnosis for the patient group; 3) presence of chronic inflammatory diseases (rheumatoid arthritis, inflammatory bowel disease, or systemic lupus erythematosus); 4) chronic liver or kidney disease; 5) current use of immunosuppressive medications; 6) blood transfusion within the past 3 months; 7) active infection or fever within 2 weeks prior to enrollment; and 8) psychiatric conditions that would impair the ability to provide informed consent. Additional exclusion criteria for patients included receipt of neoadjuvant chemotherapy, radiation therapy, or hormonal therapy prior to sample collection, presence of bilateral breast cancer, or diagnosis of inflammatory breast cancer.

3.3.3. Sample size calculation

The sample size was calculated using G*Power software version 3.1.9.7 (Faul *et al*., 2007) based on the primary objective of detecting differences in genetic polymorphism frequencies between breast cancer patients and healthy controls. For genetic association studies, we assumed a minor allele frequency of 15% in controls based on previous studies. To detect a clinically meaningful odds ratio of 2.0 or higher (equivalent to a 15% difference in allele frequencies between groups), with a two-sided significance level (α) of 0.05 and statistical power (1-β) of 80%, the minimum required sample size was calculated as 85 participants per group. For secondary analyses involving continuous variables (biochemical markers, protein expression levels), assuming a medium effect size (Cohen's d = 0.5) based on previous cancer biomarker studies, a sample size of 64 would provide 80% power. To account for potential incomplete data, genotyping failures (estimated at 5%), and to ensure adequate power for subgroup analyses (molecular subtypes, menopausal status), we aimed to recruit 90 participants per group (180 total). The study design and protocols are described in Figure 3.1.

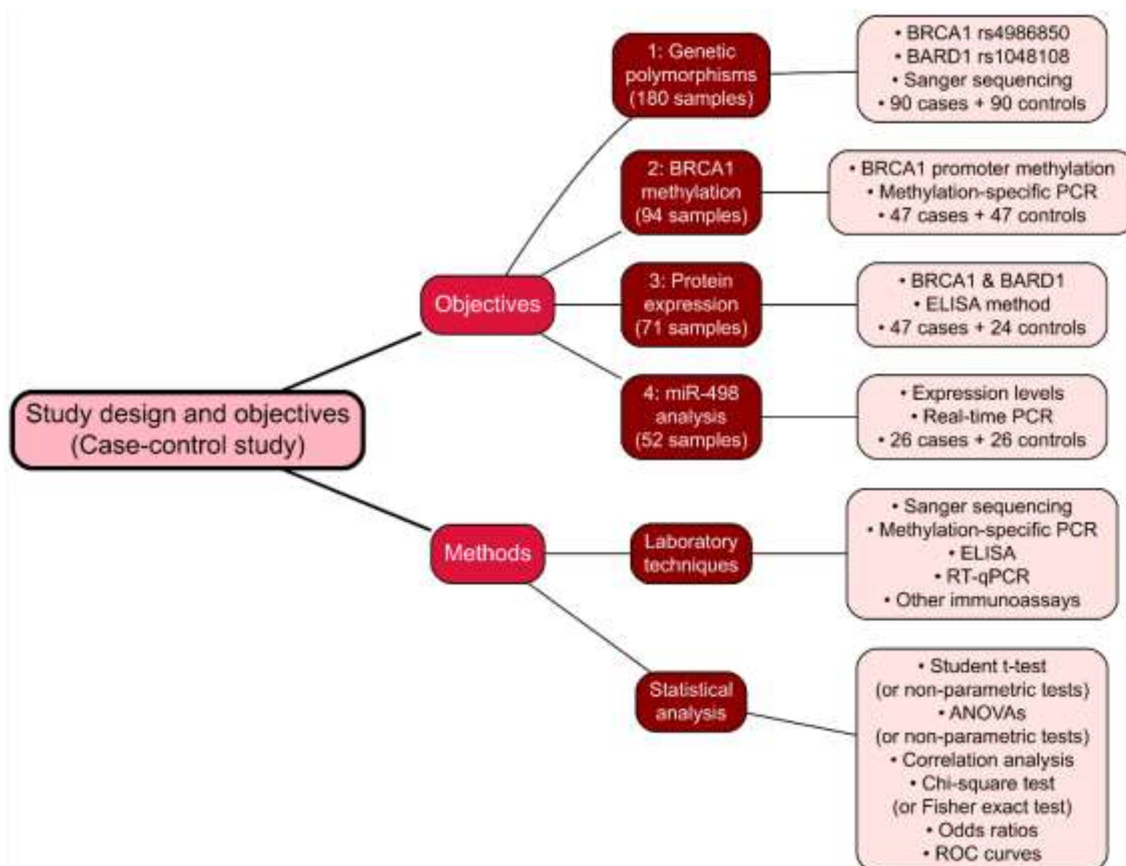


Figure 3.1. Schematic presentation of study design and protocols.

3.4. Data collection

3.4.1. Demographic and clinical data

A comprehensive structured questionnaire was developed and administered through face-to-face interviews (see appendices, supplemental Figure 4). The questionnaire collected detailed demographic information including age, marital status (single, married, divorced, widowed), educational level (uneducated, elementary school, secondary school, or higher education), and current occupation (housewife, employee, or other). For patients, clinical data were extracted from medical records and pathology reports, including tumor characteristics (histological type, grade, size), receptor status (ER, PR, HER2), TNM staging, and treatments received (if any). Family history of cancer was documented with specific attention to the affected family member and degree of relationship. Previous breast-related medical history was recorded, including any benign breast tumors, prior radiological examinations, breast biopsies, and their pathological results.

3.4.2. Anthropometric measurements

Following the demographic interview, standardized anthropometric measurements were obtained using calibrated equipment. Height was measured to the nearest 0.1 cm using a wall-mounted stadiometer with participants standing barefoot in an upright position. Weight was measured to the nearest 0.1 kg using a digital scale (Seca, Germany) with participants wearing light clothing and no shoes. Body Mass Index (BMI) in kg/m^2 was calculated using the formula:

$$\boldsymbol{BMI} = \frac{\boldsymbol{Weight}\ in\ kilograms}{\boldsymbol{Height}\ in\ meters\ sqaured}$$

The gained BMI values were used to categorize the participants according to WHO classification: underweight (<18.5), normal weight (18.5-24.9), overweight (25.0-29.9), and obese (≥30.0) kg/m^2. All measurements were taken twice, and the average was recorded to minimize measurement error.

3.4.3. Life style and environmental factors assessment

The questionnaire comprehensively evaluated various lifestyle factors known to influence breast cancer risk. Dietary habits were assessed through questions about the frequency of consumption of fruits, vegetables, red meat, canned meat, and processed meat, with response options ranging from daily, weekly, monthly, rarely, to never. The use of dietary supplements was recorded as yes/no with specification of supplement types if applicable. Smoking status was categorized as never, current, or former smoker, while alcohol consumption was recorded as yes/no, respecting the cultural context of the study population. Environmental exposure history included questions about occupational or residential exposure to chemicals or radiation, with participants asked to specify the type and duration of exposure. As well, the duration of residence in Karbala governorate was recorded in years to assess potential environmental risk factors specific to the region.

3.4.4. Reproductive and hormonal history

Because of the importance of reproductive factors in breast cancer etiology, detailed reproductive history was collected through the questionnaire. This included age at menarche, menopausal status (pre- or post-menopausal), and age at menopause for post-menopausal women. Complete obstetric history included the number of pregnancies, number of successful births, and age at first successful birth. Breastfeeding practices were also recorded including whether the participant had ever breastfed (yes/no) and the total duration of lactation across all children in months. Exogenous hormone exposure was assessed through questions about oral contraceptive use (ever/never use and duration in years) and hormone replacement therapy use (yes/no with duration in months). These reproductive variables have been consistently identified as important risk modifiers for breast cancer in numerous epidemiological studies and were collected using standardized definitions to ensure comparability with international literature (Anderson et al., 2014; Collaborative Group on Hormonal Factors in Breast Cancer, 2012).

3.5. Sample collection and processing

3.5.1. Blood sample collection

Blood samples were collected from all participants following an overnight fast of at least 8 hours to minimize dietary influences on biochemical parameters. Venipuncture was performed using standard aseptic techniques between 8:00 and 10:00 AM to control for circadian variations in biomarker levels (Aguilar-Arnal & Sassone-Corsi, 2015; Kanabrocki *et al*., 2002; Scheiermann *et al*., 2013). A total of 10 mL of venous blood was drawn from the antecubital vein using a 21-gauge needle into different collection tubes according to the intended analyses: 2.5 mL in EDTA-coated tubes for genetic analyses and complete blood count, 2.5 mL in plain tubes without anticoagulant for serum separation, and 5 mL in PAXgene Blood RNA tubes (PreAnalytiX, Switzerland) for miRNA analysis. The PAXgene tubes were

immediately inverted 8-10 times to ensure complete mixing of blood with the RNA stabilization reagent and then flash-frozen in liquid nitrogen within 2 minutes of collection to preserve RNA integrity. All samples were labeled with unique identification codes to maintain participant confidentiality throughout the analytical process.

3.5.2. Sample storage and handling

After collection, blood samples underwent immediate processing to ensure optimal preservation of biomolecules. Samples in plain tubes were allowed to clot at room temperature for 30 minutes, then centrifuged at 3000 rpm for 10 minutes at 4°C to separate serum. The resulting serum was carefully aliquoted into Eppendorf tubes to avoid repeated freeze-thaw cycles, which can compromise biomarker stability. EDTA blood samples for DNA extraction were aliquoted into 1 mL portions and stored at -20°C for short-term storage (up to one week) before transfer to -80°C for long-term preservation. The PAXgene tubes containing samples for miRNA analysis, which had been immediately preserved in liquid nitrogen at the collection site, were transported in liquid nitrogen dry shippers to the laboratory and subsequently stored at -80°C until RNA extraction. All frozen samples were maintained in a continuously monitored ultra-low temperature freezer with an alarm system and backup power supply to prevent temperature fluctuations.

3.6. Laboratory analysis

3.6.1. Biochemical parameters

All biochemical analyses were performed on serum samples using automated clinical chemistry analyzers. Liver function tests included measurement of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin, and lactate dehydrogenase (LDH) using standard enzymatic methods on a Cobas c311 analyzer (Roche Diagnostics, Germany). Metabolic parameters including serum glucose were determined using the hexokinase method.

Kidney function assessment included serum urea measurement by the urease-GLDH method and serum creatinine by the Jaffe kinetic method. Electrolyte panel analysis included total calcium, ionized calcium, phosphate, sodium, potassium, chloride, and magnesium using ion-selective electrode and colorimetric methods. Serum proteins were analyzed using the biuret method for total protein and bromocresol green (BCG) dye-binding method for albumin, with globulin calculated by subtraction and the albumin/globulin (A/G) ratio subsequently determined. All assays were performed according to manufacturer's instructions with appropriate quality control measures, including daily calibration and use of normal and pathological control sera. The inter-assay coefficients of variation (CV) were maintained below 5% for all parameters.

3.5.2. Inflammatory, oxidative and tumor biomarkers

Inflammatory cytokines were quantified using enzyme-linked immunosorbent assay (ELISA) techniques. Interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) were measured using commercial ELISA kits (Abcam, UK) following manufacturer's protocols. Samples with CV of greater than 10% were re-analyzed. Standard curves for each cytokine were fitted using four-parameter logistic (4PL) regression according to standard immunoassay practices (Findlay & Dillard, 2007) and exhibited excellent linearity with R^2 values of 0.952 for IL-1β, 0.968 for IL-6, and 0.975 for TNF-α (Figure 3.2).

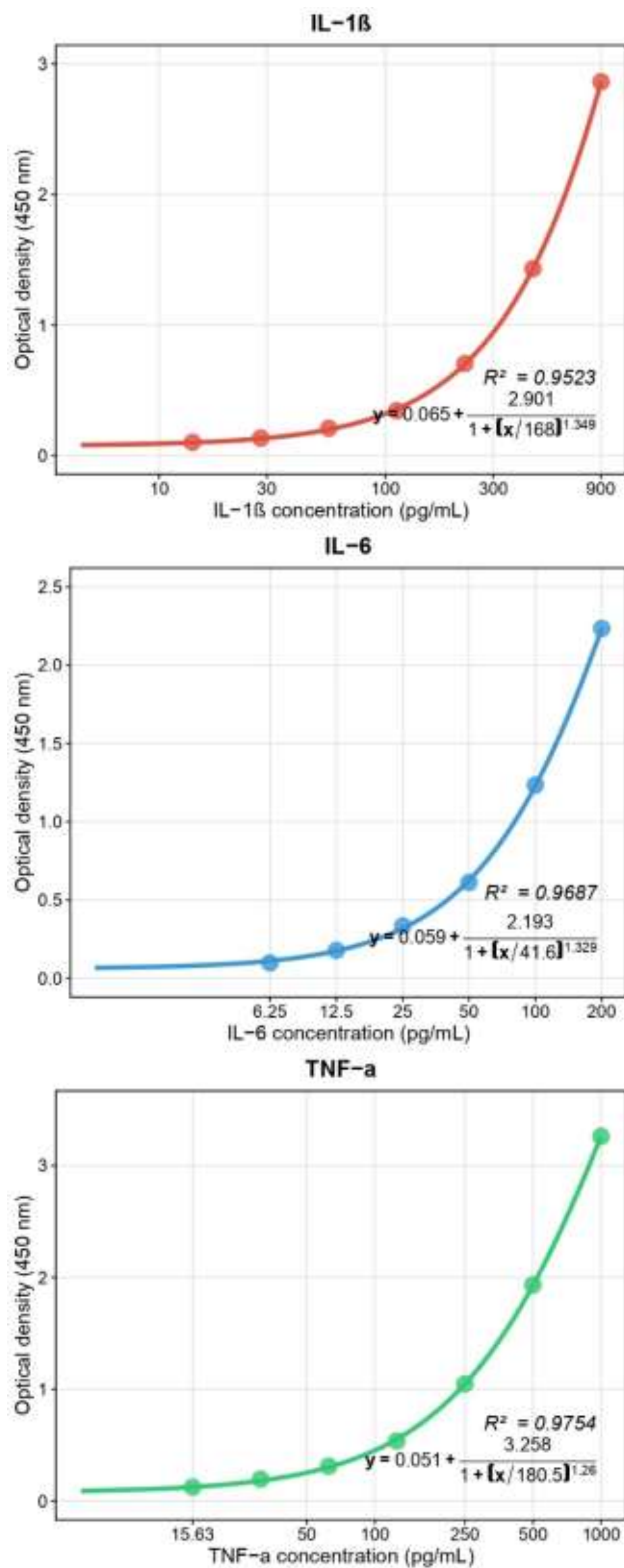


Figure 3.2. Standard curves for inflammatory cytokine quantification by ELISA. Four-parameter logistic regression curves for: A) interleukin-1β (IL-1β), B) interleukin-6 (IL-6), and C) tumor necrosis factor-alpha (TNF-α). Standards ranged from 14.06 to 900 pg/mL for IL-1β, 6.25 to 200 pg/mL for IL-6, and 15.63 to 1000 pg/mL for TNF-α with optical density measured at 450 nm. Each point represents the mean of duplicate measurements.

High-sensitivity C-reactive protein (hs-CRP) was determined using a particle-enhanced immunoturbidimetric assay on the Cobas c311 analyzer, with a detection limit of 0.1 mg/L. For oxidative stress assessment, 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels were measured as a biomarker of oxidative DNA damage using a competitive ELISA kit (Abcam, UK), with all samples diluted 1:2 in assay buffer to fall within the standard curve range (standard curve fitted with 4PL regression, Figure 3.3). The tumor marker cancer antigen 15-3 (CA 15-3) was quantified using an electrochemiluminescence immunoassay on a Cobas e411 analyzer (Roche Diagnostics, Germany) with a detection range of 0.6-300 U/mL. All ELISA procedures were performed at room temperature (20-25°C), and optical densities were read at 450 nm using a microplate reader (BioTek ELx800, USA). Standard curves were generated using 4PL regression for each plate with R^2 values ranging from 0.9468 to 0.9754, and samples with coefficients of variation >10% between duplicates were re-analyzed.

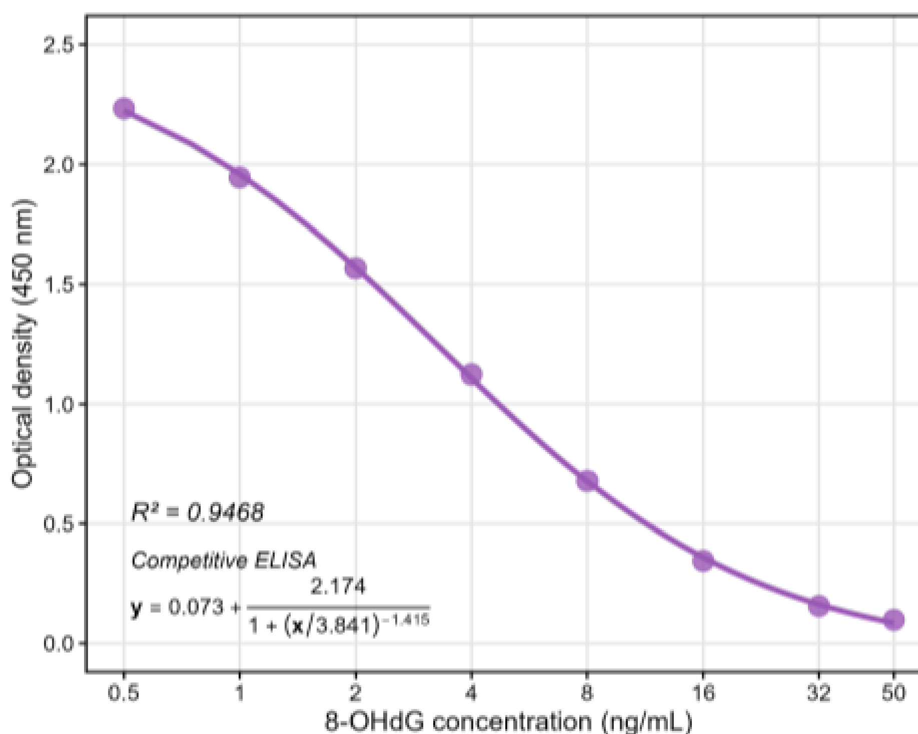


Figure 3.3. Standard curve for 8-hydroxy-2'-deoxyguanosine (8-OHdG) quantification using competitive ELISA. The logarithmic scale on the x-axis displays concentrations in ng/mL. The inverse relationship between analyte concentration and optical density is characteristic of competitive immunoassays. All samples were diluted 1:2 to fall within the linear range.

3.5.3. BRCA1 and BARD1 protein

Serum concentrations of BRCA1 and BARD1 proteins were determined using commercial sandwich ELISA kits specific for human targets. BRCA1 protein was quantified using the Human BRCA1 ELISA Kit (catalog #MBS2020774, MyBioSource, USA) with a detection range of 0.156-10 ng/mL and sensitivity of 0.053 ng/mL. BARD1 protein levels were measured using the Human BARD1 ELISA Kit (catalog #MBS7236583, MyBioSource, USA), a competitive ELISA with a detection range of 0.5-10 ng/mL and sensitivity of 0.1 ng/mL. For both assays, serum samples were diluted 1:2 with sample dilution buffer provided in the kits. The BRCA1 assay protocol involved a 2-hour incubation with samples, followed by sequential incubations with biotin-labeled detection antibody (1 hour) and HRP-streptavidin conjugate (30 minutes), with washing steps between each incubation. The BARD1 competitive assay required a single 1-hour incubation of samples with HRP-conjugated BARD1, followed by washing and substrate addition. For both assays, TMB substrate was added and color development was monitored, with reactions stopped by addition of sulfuric acid stop solution when appropriate color intensity was achieved (10-25 minutes). Absorbance was measured immediately at 450 nm, and protein concentrations were calculated from standard curves using 4PL regression (Figures 3.4, 3.5), as recommended for immunoassays (Findlay & Dillard, 2007). Quality control included blank wells, positive controls, and verification that all standard curve R^2 values from 4PL fitting exceeded 0.95. Any samples yielding values outside the detection range were appropriately diluted and re-assayed.

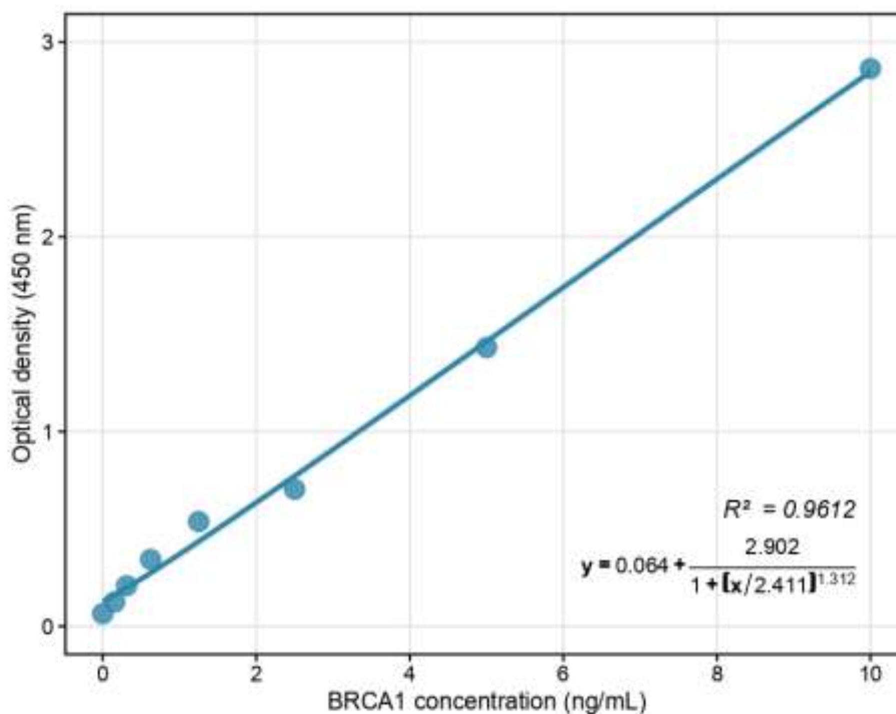


Figure 3.4. Standard curve for BRCA1 protein quantification using sandwich ELISA. The curve represents the four-parameter logistic regression model fitted to standard concentrations. Each point represents the mean optical density at 450 nm of duplicate measurements. The detection range was 0.313-20 ng/mL with a sensitivity of 0.188 ng/mL.

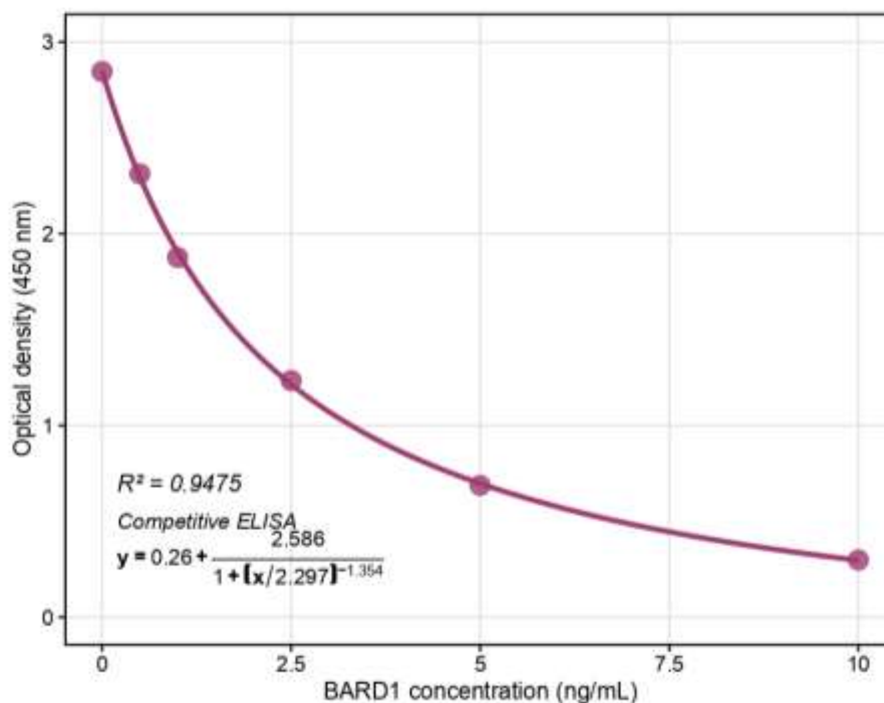


Figure 3.5. Standard curve for BARD1 protein quantification using competitive ELISA. The inverse relationship between BARD1 concentration and optical density at 450 nm is characteristic of competitive immunoassays. Each point represents the mean of duplicate measurements. The curve was fitted using four-parameter logistic regression. Detection limit: 0.1 ng/mL.

3.7. Molecular, genetic and epigenetic analysis

3.7.1. DNA extraction and quantification

3.7.1.1. DNA extraction from whole blood

Genomic DNA was extracted from peripheral blood samples using the AddPrep Genomic DNA Extraction Kit (Cat. No. 10023, Addbio, Korea). All procedures were performed at room temperature unless otherwise specified. The extraction protocol consisted of the following major steps:

A. Sample lysis and protein digestion: Blood samples (200 µL) were transferred to sterile 1.5 mL microcentrifuge tubes and mixed with an equal volume of Lysis Solution. Then, Proteinase K (20 µL at 20 mg/mL) was added to achieve complete protein digestion. The mixture was vortexed thoroughly and incubated at 56°C in a dry microtubes incubator (ae, UK) until complete lysis was achieved, with intermittent vortexing every 10 minutes to enhance cell disruption. Following incubation, tubes were briefly centrifuged at 13,000 rpm for 10 seconds using a Beckman Coulter Microfuge IB (Germany) to collect condensate from the tube lid.

B. DNA binding and precipitation: The lysate was mixed with 200 µL of Binding Solution by pulse-vortexing for 15 seconds, followed by incubation at 56°C for 10 minutes to optimize DNA binding conditions. Subsequently, 200 µL of absolute ethanol was added and mixed by pulse-vortexing for 15 seconds to precipitate nucleic acids. The resulting mixture was briefly centrifuged to ensure complete mixing and collection of the sample.

C. Column-based purification: The entire lysate was carefully transferred to a spin column assembled with a 2.0 mL collection tube, avoiding contact with the column rim to prevent contamination. Initial binding was achieved by centrifugation at 13,000 rpm for 1 minute. The column was then subjected to two sequential washing steps: first with 500 µL of Washing Solution 1, followed by 500 µL of Washing Solution 2, each followed by centrifugation at 13,000 rpm for 1 minute. Flow-through was discarded after each step. To ensure complete removal of residual ethanol, which

could interfere with downstream applications, an additional drying centrifugation at 13,000 rpm for 1 minute was performed.

D. DNA elution: The spin column was transferred to a new sterile 1.5 mL microcentrifuge tube. Elution Solution (100-200 µL, depending on the desired final concentration) was carefully added to the center of the column membrane. After incubation at room temperature for 1 minute to allow DNA rehydration, genomic DNA was eluted by centrifugation at 13,000 rpm for 1 minute. The eluted DNA was immediately placed on ice for quality assessment or stored at -20°C.

3.7.1.2. DNA quality assessment (agarose gel electrophoresis)

DNA integrity was evaluated using agarose gel electrophoresis performed on a Cleaver electrophoresis system (UK). Agarose gels (0.7% w/v) were prepared by dissolving 0.7 g of agarose LE (Cat. No. 32034-50, Intron, Korea) in 100 mL of 1X TAE buffer (prepared from 10X TAE buffer, Cat. No. V4271, Promega, USA). The mixture was heated in a microwave oven until complete dissolution, cooled to approximately 50°C, and supplemented with 5 µL of RedSafe nucleic acid staining solution (Cat. No. 21141, Intron, Korea). The gel was cast and allowed to polymerize for 30 minutes at room temperature.

DNA samples (5 µL) were loaded directly into wells, as the extraction kit's elution buffer contained tracking dyes. A 1kb DNA Ladder Plus (Cat. No. M1191, GDSBio, China) was included as a molecular weight marker. Electrophoresis was performed at 90V (constant voltage) for 45 minutes in 1X TAE running buffer. DNA bands were visualized under UV light (320 nm) using a UVP documentation system (Analytik Jena, UK). High-quality genomic DNA appeared as a single, high molecular weight band near the well origin without visible smearing, indicating minimal degradation (Figure 3.6).

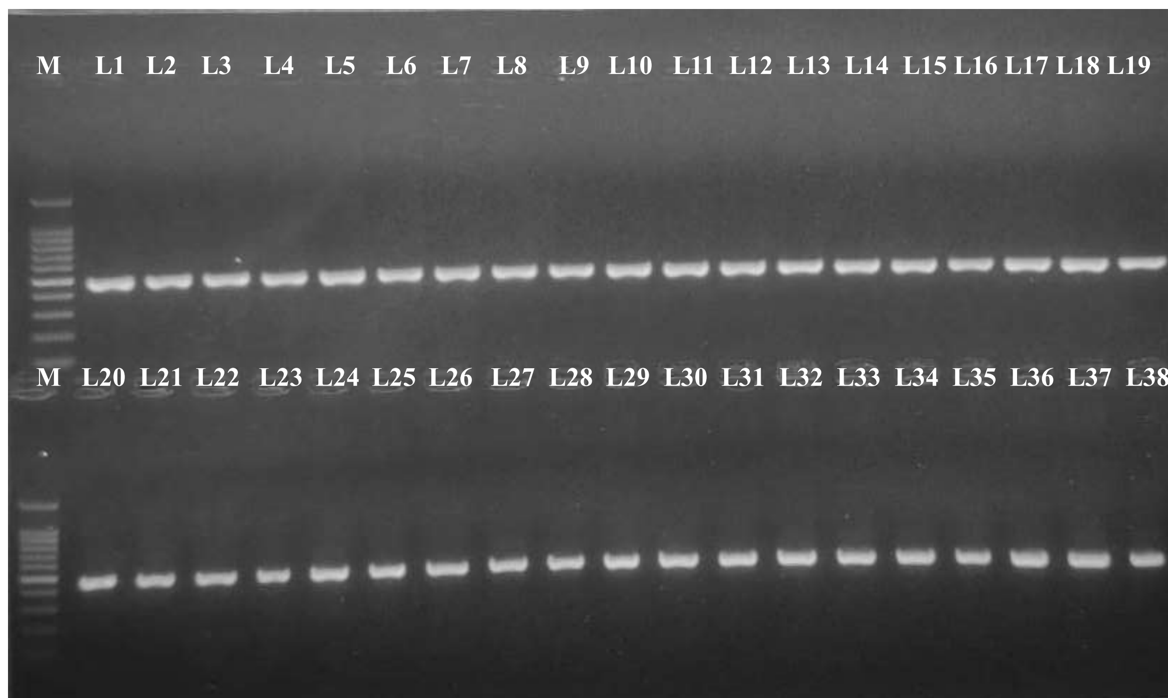


Figure 3.6. Agarose gel electrophoresis analysis of genomic DNA quality and integrity. Genomic DNA samples from breast cancer patients (lanes 1-19) and healthy controls (lanes 20-38) were separated on a 0.7% agarose gel containing RedSafe nucleic acid stain. Electrophoresis was performed at 90V for 45 minutes in 1X TAE buffer. M: 1000bp DNA Ladder Plus molecular weight marker. High molecular weight bands near the loading wells indicate intact genomic DNA with minimal degradation. The gel was visualized under UV light (320 nm) using a UVP documentation system.

3.7.1.3. DNA quantification and purity assessment

A. Spectrophotometric analysis: DNA concentration and purity were determined using UV spectrophotometry. Absorbance measurements were taken at 260 nm (A260) for DNA quantification, 280 nm (A280) for protein contamination assessment, and 230 nm (A230) for evaluation of organic contaminants. DNA concentration was calculated using the formula:

$$\textbf{\textit{DNA concentration}}_{(ng/\mu L)} = A_{260} \times 50 \times \textit{dilution factor}$$

where 50 ng/µL is the extinction coefficient for double-stranded DNA at $A_{260} = 1.0$

B. Quality control parameters: Strict quality control criteria were applied to ensure the suitability of extracted DNA for downstream molecular analyses. DNA samples were considered acceptable only when they met all of the following parameters: a minimum concentration of 20 ng/µL with an optimal range of 50-200 ng/µL to ensure sufficient material for multiple analyses; an A_{260}/A_{280} ratio between

1.7 and 1.9, indicating minimal protein contamination; an A_{260}/A_{230} ratio between 2.0 and 2.2, demonstrating the absence of significant organic contaminants such as phenol or guanidine salts; and the presence of a single, intact high molecular weight band on agarose gel electrophoresis without visible smearing or degradation products. Samples failing to meet any of these stringent criteria were excluded from further analysis, and DNA re-extraction was performed using fresh blood aliquots from the same participants to maintain the integrity of the study cohort.

3.7.1.2. DNA storage

Qualified DNA samples were aliquoted into multiple tubes to minimize freeze-thaw cycles. Working aliquots (20 µL) were stored at -20°C for immediate use (within 6 months), while stock solutions were maintained at -80°C for long-term storage. All samples were labeled with unique identifiers, extraction date, concentration, and quality parameters. A detailed DNA inventory was maintained in both electronic and physical formats for sample tracking and quality assurance purposes.

3.7.2. Polymerase chain reaction

Polymerase chain reaction (PCR) amplification was performed to target specific genomic regions containing single nucleotide polymorphisms (SNPs) of interest. Two genes were selected for analysis: *BRCA1* (rs4986850 in exon 10) and *BARD1* (rs1048108 in exon 1). Gene-specific primers were designed using the NCBI reference sequences NG_005905.2 for *BRCA1* and NG_012047.3 for *BARD1* (Table 3.3). All primers were synthesized by Macrogen (Korea) and received in lyophilized form. Upon arrival, primers were reconstituted in nuclease-free water to create stock solutions at 100 µM concentration, which were stored at -20°C. Working solutions were prepared by diluting the stock to 10 µM for routine use.

Table 3.3. Primer sequences and characteristics.

SNP	Target gene	Primer	5'-3' sequence	PCR size
rs4986850	*BRCA1*	F	CAGTAGAAATCTAAGCCCACCTAAT	461 bp
		R	CCTGAGTGCCATAATCAGTACC	
rs1048108 (set 1)	*BARD1*	F	CCCTGCGAGTCCCTATTT	454 bp
		R	GAAGGAGGAAACGGAAGATTTG	
rs1048108 (set 2)	*BARD1*	F	GGCAAGTTTCAGCCTCCA	533 bp
		R	GAAGGAGGAAACGGAAGATTTG	

Note: All the mentioned primers were for Homo sapiens species and designed for this study, not cited. The accession numbers were: 1) NG_005905.2 for BRCA1 and 2) NG_012047.3 for BARD1.

PCR amplification was carried out using GoTaq® G2 Green Master Mix (Cat. No. M7822, Promega, USA), which contained high-quality Taq DNA polymerase, deoxynucleotides, reaction buffer, and an inert green tracking dye allowing direct gel loading. Each 50 µL reaction mixture consisted of 25 µL of 2X GoTaq® G2 Green Master Mix, 4 µL each of forward and reverse primers (10 µM), 4 µL of genomic DNA template (40 ng), and 13 µL of nuclease-free water (Table 3.4). PCR amplification was performed using an Optimus 96G thermal cycler (QLS, UK) with the following cycling parameters: initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 1 minute, with a final extension step at 72°C for 5 minutes.

Table 3.4. PCR reaction components and conditions.

A. Reaction mixture components		
Components	**Concentration**	**Volume (50 µl)**
GoTaq® G2 Green Master Mix	1X	25 µl
Forward primer	10 µM/µl	4 µl
Reverse primer	10 µM/µl	4 µl
ddH_2O	-	13 µl
DNA	40 ng	4 µl

B. Cycling conditions			
Phase	**Tm (°C)**	**Time**	**Cycles**
Initial denaturation	94 °C	5 min	1X
Denaturation	94 °C	30 sec	35X
Annealing	56 °C	30 sec	
Extension	72 °C	1 min	
Final extension	72 °C	5 min	1X

The quality and specificity of PCR products were verified by agarose gel electrophoresis. PCR products (5 µL) were loaded directly onto 1-2% agarose gels prepared in 1X TAE buffer and supplemented with RedSafe nucleic acid staining solution. Safe-Green 100bp Opti-DNA Marker (Cat. No. G473, abm, Canada) was used as a molecular size standard. Electrophoresis was conducted at 90V for 45 minutes, and amplified products were visualized under UV light (320 nm) using a UVP documentation system (Analytik Jena, UK). The expected amplicon sizes were 461 bp for *BRCA1* exon 10, 454 bp for *BARD1* exon 1 (primer set 1), and 533 bp for *BARD1* exon 1 (primer set 2).

3.7.3. Sanger sequencing

Following successful amplification, PCR products were prepared for Sanger sequencing to identify genetic variants within the target regions. Prior to sequencing, all PCR products underwent purification using the Wizard SV Gel and PCR Clean-Up System (Cat. No. A9281, Promega, USA) to remove excess primers, nucleotides, and other contaminants that could interfere with sequencing reactions. The purification process involved binding of DNA to a silica membrane under high-salt conditions, washing to remove impurities, and elution of purified DNA in nuclease-free water.

Purified PCR products were quantified spectrophotometrically and adjusted to the appropriate concentration as specified by the sequencing service provider. Samples were then submitted to Macrogen (Korea) for bidirectional Sanger sequencing using the same primers employed for PCR amplification. This approach ensured complete coverage of the target regions and allowed for confirmation of any identified variants through analysis of both forward and reverse sequence traces.

Sequence analysis was performed using a systematic bioinformatics approach. Raw sequence chromatograms were initially evaluated for quality, with particular attention to peak resolution and signal strength. High-quality sequences were then compared to reference genome sequences using the NCBI BLASTn server

(https://blast.ncbi.nlm.nih.gov/ Blast.cgi). For variant calling and annotation, sequences were aligned against the human reference genome, specifically using accession numbers NG_005905.2 for *BRCA1* and NG_012047.3 for *BARD1*. Moreover, nucleotide sequences were translated into amino acid sequences using the BLASTx server (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to assess the potential functional impact of any identified variants. Multiple sequence alignments were performed using ClustalW (https://www.ebi.ac.uk/Tools/msa/clustalo/) to compare sequences across all samples and identify polymorphic sites. Any discrepancies between forward and reverse sequences were resolved by manual inspection of the chromatograms.

3.7.4. DNA methylation

DNA methylation analysis of the *BRCA1* promoter region was performed to evaluate epigenetic modifications that may influence gene expression and contribute to breast cancer susceptibility. A total of 94 genomic DNA samples that met the quality criteria established in section 3.7.1.3 were randomly selected for methylation analysis. The analysis focused specifically on CpG islands within the *BRCA1* promoter region, as aberrant methylation of these regulatory elements has been implicated in transcriptional silencing and disease pathogenesis.

Prior to methylation analysis, genomic DNA samples underwent bisulfite treatment to convert unmethylated cytosines to uracil while leaving methylated cytosines unchanged. This chemical modification allows for the differentiation between methylated and unmethylated cytosines through subsequent PCR amplification and sequencing. The bisulfite conversion was performed using standardized protocols to ensure complete conversion while minimizing DNA degradation.

Following bisulfite treatment, methylation-specific PCR (MSP) was conducted using four specifically designed primer sets targeting different regions within the *BRCA1* promoter. These primers were designed to amplify bisulfite-converted DNA

and distinguish between methylated and unmethylated sequences based on the retention or conversion of cytosine residues. PCR amplification was performed using conventional PCR methodology with optimized conditions for each primer set to ensure specific amplification of the target regions.

The methylation status of the *BRCA1* promoter was determined through direct sequencing of the bisulfite-PCR products. Amplified products were purified and submitted for Sanger sequencing to obtain high-resolution methylation data at individual CpG sites. Sequence analysis involved comparison of bisulfite-treated sequences with reference sequences to identify the methylation status of each cytosine residue within the CpG dinucleotides. The percentage of methylation at each CpG site was calculated based on the ratio of cytosine to thymine peaks in the sequencing chromatograms. Samples were categorized based on their methylation status (methylated vs. unmethylated) and methylation percentage values were recorded for quantitative analysis.

3.8. miRNA expression analysis

3.8.1. RNA extraction

Total RNA, including small RNA species, was extracted from peripheral blood samples to analyze miRNA expression profiles. A subset of 52 samples (26 from breast cancer patients and 26 from healthy controls) was selected for miRNA expression analysis. Blood samples were collected in PAXgene Blood RNA tubes. RNA extraction was performed using a column-based purification method optimized for the isolation of total RNA including miRNAs from whole blood samples.

Prior to extraction, blood samples were incubated with lysis buffer to ensure complete cell disruption and release of intracellular RNA. The lysis buffer contained guanidine isothiocyanate to immediately inactivate RNases and preserve RNA integrity. Following cell lysis, samples were treated with proteinase K to digest proteins and remove potential RNase contamination. The lysate was then mixed with

ethanol to create appropriate binding conditions for RNA adsorption to the silica-based spin column membrane.

The RNA-containing lysate was transferred to spin columns and centrifuged to allow RNA binding to the membrane. Sequential washing steps were performed using wash buffers containing ethanol to remove residual proteins, genomic DNA, and other contaminants while retaining RNA on the column. An on-column DNase I digestion step was included to eliminate any residual genomic DNA contamination that could interfere with downstream miRNA analysis. Following the wash steps, the column was dried by centrifugation to remove residual ethanol, and total RNA was eluted using RNase-free water preheated to 60°C to improve recovery of small RNA species.

RNA quality and concentration were assessed immediately after extraction. RNA purity was evaluated spectrophotometrically by measuring absorbance ratios at A_{260}/A_{280} (acceptable range: 1.8-2.1) and A_{260}/A_{230} (acceptable range: 1.8-2.2). RNA integrity was assessed using agarose gel electrophoresis, where intact RNA showed clear 28S and 18S ribosomal RNA bands with a 2:1 intensity ratio. RNA concentration was determined using the formula:

$$\textbf{\textit{RNA concentration (ng/µL)}} = A_{260} \times 40 \times \textit{dilution factor}$$

Only RNA samples meeting all quality criteria were used for subsequent miRNA expression analysis. Extracted RNA was aliquoted to avoid repeated freeze-thaw cycles and stored at -80°C until further use.

3.8.2. miRNA-498 expression

Expression analysis of miRNA-498 was performed using quantitative real-time PCR (qRT-PCR) to determine its levels in 26 breast cancer patients compared to 26 healthy controls. Prior to qRT-PCR, total RNA underwent reverse transcription using miRNA-specific stem-loop primers designed for both miR-498 and miR-16. This specialized primer design enhanced the specificity and sensitivity of miRNA

detection by extending the short miRNA sequence and providing a stable template for subsequent amplification.

Reverse transcription was carried out using 100 ng of total RNA in a 15 µL reaction containing miRNA-specific stem-loop RT primer, reverse transcriptase enzyme, RNase inhibitor, dNTPs, and reaction buffer. The RT reaction was performed with the following conditions: 16°C for 30 minutes to allow primer annealing, 42°C for 30 minutes for cDNA synthesis, and 85°C for 5 minutes to inactivate the reverse transcriptase. The resulting cDNA was diluted and used as template for real-time PCR amplification.

Quantitative PCR was performed using SYBR Green-based detection methods in a real-time PCR system. Each 20 µL reaction contained diluted cDNA template, miRNA-specific forward primers for miR-498 and miR-16, universal reverse primer, and real-time PCR master mix. The amplification protocol consisted of initial denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 60 seconds. Fluorescence data were collected during the extension phase of each cycle. A dissociation curve analysis was performed after amplification to verify the specificity of PCR products.

For normalization of miRNA expression data, miR-16 was used as an endogenous reference gene due to its stable expression in blood samples and widespread use in miRNA studies. The selection of miR-16 as a normalizer was based on its consistent expression across both breast cancer patients and healthy controls. Technical replicates were performed in triplicate for 15% of randomly selected samples to assess reproducibility and calculate intra-assay CV, following protocols for resource-optimization in large-scale studies (Bustin *et al*., 2009; Ståhlberg *et al*., 2004). The threshold cycle (Ct) values were determined automatically by the instrument software using the second derivative maximum method. Relative expression levels of miR-498 were calculated using the $2^{-\Delta\Delta Ct}$ (fold change) method (Livak & Schmittgen, 2001), where $\Delta Ct = Ct_{(miR\text{-}498)} - Ct_{(miR\text{-}16)}$ and $\Delta\Delta Ct = \Delta Ct_{(sample)} - \Delta\Delta Ct_{(calibrator)}$.

3.9. Biostatistical analyses

All statistical analyses were performed using R programming (version 3.6.0+, Austria) implemented through RStudio Desktop (version 2025.05.1+513, USA) on a Windows operating system. Descriptive statistics were calculated for all variables, with continuous data presented as means ± standard deviations or medians with interquartile ranges based on normality distribution assessed by Shapiro-Wilk test, while categorical variables were expressed as frequencies and percentages. For comparison of continuous variables between two groups (breast cancer patients vs. controls), independent sample t-test was used for normally distributed data and Mann-Whitney U test for non-parametric data. Chi-square test or Fisher's exact test (when expected cell counts were less than 5) were used to analyze associations between categorical variables, including genotype distributions and allele frequencies. Hardy-Weinberg equilibrium was assessed using chi-square goodness-of-fit test (Wigginton *et al*., 2005) to verify the genetic equilibrium of the studied polymorphisms in the control population. Odds ratios (OR) with 95% confidence intervals (CI) were calculated to estimate the risk associated with different genotypes and alleles using logistic regression models, with adjustments for potential confounding factors including age, BMI, and menopausal status. For miRNA expression analysis, the $2^{-\Delta\Delta Ct}$ values were log-transformed (Livak & Schmittgen, 2001) to achieve normal distribution, and differences between groups were analyzed using independent t-test, while Pearson or Spearman correlation coefficients were calculated to assess relationships between miR-498 expression levels and clinical parameters. Analysis of variance (ANOVA) or Kruskal-Wallis test was used for comparisons among multiple groups, followed by appropriate post-hoc tests (Tukey HSD or Dunn's test) for pairwise comparisons. The association between BRCA1 promoter methylation status and clinical-pathological features was evaluated using chi-square test, and methylation percentages were compared between groups using Mann-Whitney U test. Multiple testing corrections were applied using Benjamini-Hochberg false discovery rate method where appropriate (Benjamini & Hochberg, 1995). All statistical tests were two-tailed, and *p*-values less than 0.05 were considered statistically significant, with data visualization performed using ggplot2 (Wickham, 2011) and other relevant R packages to generate publication-quality figures including box plots, bar charts, and correlation matrices.

Chapter four

RESULTS & DISCUSSION

Results and Discussion

4.1. Results

4.1.1. Study population characteristics

The current study included 188 participants, who were distributed into 90 patients and 98 controls; furthermore, their baseline demographic characteristics are presented in Table 4.1. The median age was recorded as 43.0 years (IQR: 38.0-58.0) for controls and 50.0 years (IQR: 42.0-57.0) for patients; however, no significant difference was detected between the two groups (p>0.05). In addition, the age distribution patterns are illustrated in Figure 4.1, which shows similar age distributions between groups. In similar context, body mass index values were documented at 27.4 kg/m^2 (IQR: 25.4-32.7) for controls and 29.7 kg/m^2 (IQR: 25.8-35.8) for patients, yet statistical significance was not achieved (p>0.05). For family history of breast cancer, a highly significant difference was observed between groups (p<0.001), with 26.7% of patients reporting a positive family history compared to only 5.1% of controls, indicating a strong familial clustering in the patient population. Regarding marital status, the majority of participants were identified as married in both groups, with 87.8% in controls and 81.1% in patients; additionally, single individuals were 4.1% of controls and 4.5% of patients, while divorced participants were found only in the control group (2.0%). Moreover, widows comprised 6.1% of controls and 14.4% of patients. In terms of educational background, a significant difference was established between groups (p<0.05), whereby elementary school education was reported by 33.7% of controls compared to 47.8% of patients; conversely, higher education was attained by 23.5% of controls versus only 6.7% of patients. Secondary school education was reported by 36.7% of controls and 22.2% of patients, while uneducated participants constituted 6.1% of controls and 23.3% of patients. As for occupational status, housewives were predominant in both groups, comprising 75.5% of controls and 83.3% of patients; meanwhile, employees constituted 24.5% and 16.7% of the control and patient groups, respectively, though this difference was not statistically significant (p>0.05).

Table 4.1. Baseline demographic characteristics of study population (N= 188).

Variables	Controls (n=98)	Patients (n=90)	*p*-value
Age (years)	43.0 (38.0-58.0)	50.0 (42.0-57.0)	0.089
BMI (kg/m^2)	27.4 (25.4-32.7)	29.7 (25.8-35.8)	0.083
Family history			
Yes/No	5 (5.1%)/93 (94.9%)	24 (26.7%)/66 (73.3%)	<0.001
Marital status			0.134
Divorced	2 (2.0%)	0 (0.0%)	
Married	86 (87.8%)	73 (81.1%)	
Single	4 (4.1%)	4 (4.5%)	
Widow	6 (6.1%)	13 (14.4%)	
Educational level			
Elementary school	33 (33.7%)	43 (47.8%)	<0.001
Secondary school	36 (36.7%)	20 (22.2%)	
Higher education	23 (23.5%)	6 (6.7%)	
Uneducated	6 (6.1%)	21 (23.3%)	
Occupation			0.211
Employee	24 (24.5%)	15 (16.7%)	
Housewife	74 (75.5%)	75 (83.3%)	

Data are presented as median (IQR) for non-normally distributed continuous variables and n (%) for categorical variables. Normality was assessed using Shapiro-Wilk test. Mann-Whitney U test, chi-square test (or Fisher's exact test) were used for statistical assessment of data.

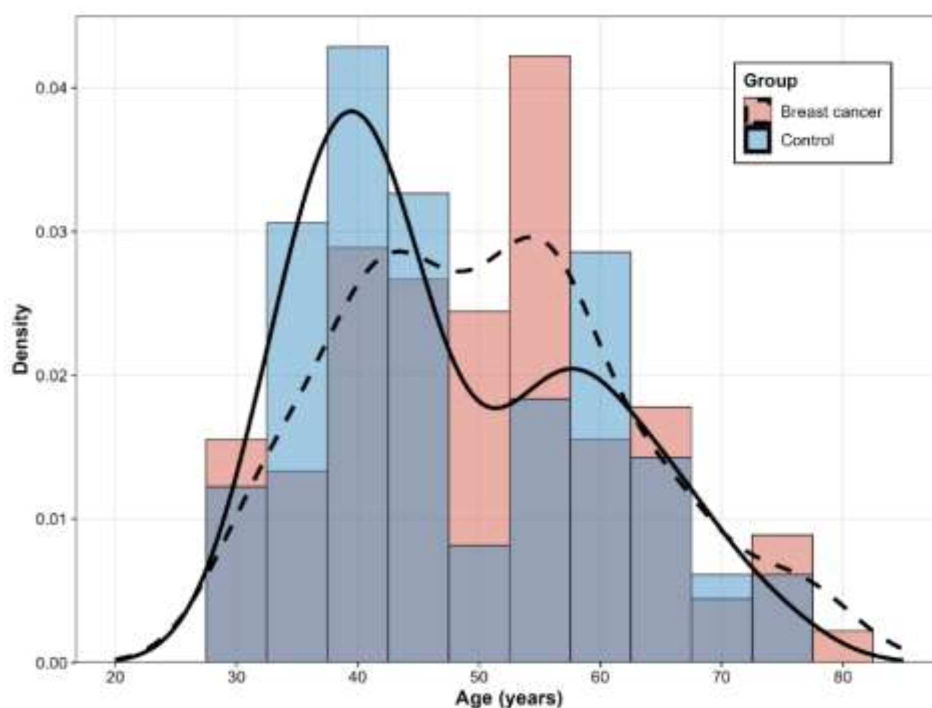


Figure 4.1. Age distribution histograms comparing controls and breast cancer patients. The histograms show the frequency distribution of age with overlaid kernel density curves (black lines). Blue bars represent controls (n= 98) and red bars represent breast cancer patients (n= 90). Each

histogram uses a bin width of 5 years. The distributions show similar age patterns between groups with slightly higher mean age in the breast cancer group (50.6±12.1 years) compared to controls (47.7±11.8 years), though this difference was not statistically significant.

4.1.2. Clinical, pathological and laboratory characteristics

The reproductive history and hormonal factors of study participants are detailed in Table 4.2. The age at menarche was identical between groups, with a median of 13.0 years (IQR: 13.0-14.0) for both controls and patients ($p>0.05$). However, menopause status differed significantly between groups ($p<0.05$), as post-menopausal women comprised 43.9% of controls versus 52.2% of patients; moreover, among post-menopausal women, the median age at menopause was 46.0 years (IQR: 45.0-48.0) in controls and 48.0 years (IQR: 45.0-50.0) in patients ($p<0.05$). Regarding reproductive characteristics, significant differences were observed in all pregnancy-related variables; specifically, the median number of pregnancies was 3.0 (IQR: 2.0-5.0) for controls compared to 6 (IQR: 3.0-7.0) for patients ($p<0.05$). Also, the number of successful births was documented as 3.0 (IQR: 2.0-4.0) in controls versus 4.0 (IQR: 3.0-5.0) in patients ($p<0.05$). Furthermore, age at first birth was significantly different, with controls experiencing first childbirth at a median age of 26.0 years (IQR: 24.0-28.0) compared to 20.5 years (IQR: 18.0-24.0) in patients ($p<0.05$). In terms of breastfeeding practices, natural lactation was reported by 89.8% of controls and 83.3% of patients, though this difference was not statistically significant ($p>0.05$); nevertheless, among women who breastfed, lactation duration was markedly different, with controls breastfeeding for a median of 7.0 months (IQR: 6.0-7.0) compared to 18.0 months (IQR: 11.5-24.0) in patients ($p<0.05$). Contraceptive pill use was reported in 44.9% of controls and 53.3% of patients, yet this difference did not reach statistical significance ($p>0.05$). On the other hand, hormonal replacement therapy (HRT) use showed a highly significant difference ($p<0.05$), being reported by only 8.2% of controls compared to 65.6% of patients.

Table 4.2. Reproductive history and hormonal factors

Variables	Controls (n=98)	Patients (n=90)	*p*-value
Age at menarche (years)	13.0 (13.0-14.0)	13.0 (13.0-14.0)	0.688
Menopause status			<0.001
Pre-menopause	55 (56.1%)	43 (47.8%)	
Post-menopause	43 (43.9%)	47 (52.2%)	
Age at menopause (years)*	46.0 (45.0-48.0)	48.0 (45.0-50.0)	0.049
Number of pregnancies	3.0 (2.0-5.0)	6.0 (3.0-7.0)	<0.001
Number of successful births	3.0 (2.0-4.0)	4.0 (3.0-5.0)	<0.001
Age at first birth (years)	26.0 (24.0-28.0)	20.5 (18.0-24.0)	<0.001
Natural lactation			0.365
No	10 (10.2%)	15 (16.7%)	
Yes	88 (89.8%)	75 (83.3%)	
Lactation duration (months)**	7.0 (6.0-7.0)	18.0 (11.5-24.0)	<0.001
Contraceptive pill use			0.313
No	54 (55.1%)	42 (46.7%)	
Yes	44 (44.9%)	48 (53.3%)	
HRT use			<0.001
No	90 (91.8%)	31 (34.4%)	
Yes	8 (8.2%)	59 (65.6%)	

Data are presented as median (IQR) for non-normally distributed continuous variables, and n (%) for categorical variables. Normality was assessed using Shapiro-Wilk test. Mann-Whitney U test, Chi-square test (or Fisher's exact test) were used for statistical assessment of data. **Abbreviation:** HRT, hormonal replacement therapy. *Among post-menopausal women only, **Among women who had successful births, ***Among women who breastfed.

In addition to reproductive factors, the lifestyle factors and environmental exposures of the study participants are comprehensively presented in Table 4.3. Regarding smoking habits, the majority of participants were non-smokers in both groups, with 95.9% of controls and 87.8% of patients reporting no smoking history; although a higher proportion of smokers was observed among patients (12.2% vs. 4.1%), this difference did not reach statistical significance ($p>0.05$). Furthermore, alcohol consumption was completely absent in both groups, as 100% of participants reported no alcohol use. In terms of dietary patterns, fruit consumption showed

significant differences between groups (p<0.05); specifically, daily fruit intake was reported by 72.4% of controls and 71.1% of patients, while monthly consumption was exclusively observed among patients (4.4%). In addition, vegetable consumption patterns differed significantly (p<0.05), whereby daily intake was markedly higher among controls (95.9%) compared to patients (81.1%); on the contrary, weekly vegetable consumption was reported by only 3.1% of controls versus 17.8% of patients.

The consumption of various meat products also revealed different patterns between the study groups. Red meat consumption demonstrated significant differences (p<0.05), as daily intake was reported by 3.1% of controls compared to 7.8% of patients; moreover, 11.1% of patients reported no red meat consumption versus only 1.0% of controls. Although canned meat consumption showed no significant difference (p>0.05), processed meat consumption patterns were significantly different (p<0.05); notably, daily processed meat intake was observed only among patients (2.2%), while monthly consumption was also exclusive to the patient group (5.6%). Regarding dietary supplement use, no significant difference was detected between groups, with 50.0% of controls and 42.2% of patients reporting supplement intake (p>0.05). Similarly, chemical or radiation exposure was reported by 5.1% of controls and 7.8% of patients, though this difference was not statistically significant (p>0.05). Finally, the duration of residence in Karbala was comparable between groups, with means of 37.4±14.2 years for controls and 37.8±19.1 years for patients (p>0.05), indicating similar environmental exposure duration for both populations.

Table 4.3. Lifestyle factors and environmental exposures.

Variables	Controls (n=98)	Patients (n=90)	*p*-value
Smoking status			
No	94 (95.9%)	79 (87.8%)	0.074
Yes	4 (4.1%)	11 (12.2%)	
Alcohol consumption			
No	98 (100.0%)	90 (100.0%)	—
Yes	0 (0.0%)	0 (0.0%)	
Fruit consumption			

Variables	Controls (n=98)	Patients (n=90)	*p*-value
Daily	71 (72.4%)	64 (71.1%)	<0.001
Weekly	26 (26.5%)	22 (24.4%)	
Monthly	0 (0.0%)	4 (4.4%)	
Rarely	1 (1.0%)	0 (0.0%)	
Vegetable consumption			
Daily	94 (95.9%)	73 (81.1%)	<0.001
Weekly	3 (3.1%)	16 (17.8%)	
Monthly	0 (0.0%)	1 (1.1%)	
Rarely	1 (1.0%)	0 (0.0%)	
Red meat consumption			
Daily	3 (3.1%)	7 (7.8%)	0.010
Weekly	67 (68.4%)	48 (53.3%)	
Monthly	26 (26.5%)	23 (25.6%)	
Rarely	1 (1.0%)	2 (2.2%)	
No	1 (1.0%)	10 (11.1%)	
Canned meat consumption			
No	68 (69.4%)	69 (76.7%)	0.086
Rarely	26 (26.5%)	12 (13.3%)	
Monthly	2 (2.0%)	4 (4.4%)	
Weekly	2 (2.0%)	3 (3.3%)	
Daily	0 (0.0%)	2 (2.2%)	
Processed meat consumption			
No	69 (70.4%)	68 (75.6%)	0.003
Rarely	27 (27.6%)	11 (12.2%)	
Monthly	0 (0.0%)	5 (5.6%)	
Weekly	2 (2.0%)	4 (4.4%)	
Daily	0 (0.0%)	2 (2.2%)	
Dietary supplements use			
No	49 (50.0%)	52 (57.8%)	0.279
Yes	49 (50.0%)	38 (42.2%)	
Chemical/radiation exposure			
No	93 (94.9%)	83 (92.2%)	0.652
Yes	5 (5.1%)	7 (7.8%)	
Years lived in Karbala	37.00 (30.00-45.00)	39.00 (23.25-51.75)	0.852

Data are presented as n (%) for categorical variables and median (IQR) for continuous variables. Chi-square test (or Fisher's exact test) were used for categorical data comparisons. For the years lived in Karbala: Welch's t-test was used to compare the means.

The histopathological characteristics of breast cancer cases are presented in Table 4.4 and Figure 4.2. Among the 90 patients examined, ductal carcinoma was identified as the predominant tumor type, accounting for 93.3% of cases, whereas lobular carcinoma comprised only 6.7%. With respect to tumor grading, grade 2 tumors were most frequently observed in 52.2% of patients; furthermore, grade 3 tumors were detected in 36.7% of cases, while grade 1 tumors represented the smallest proportion at 11.1%. The T staging distribution showed that T2 tumors were most common at 40.0%; additionally, T1, T3, and T4 stages each constituted 18.9%, 22.2%, and 18.9% of cases respectively. Regarding nodal involvement, N0 status was documented in 34.4% of patients; however, positive nodal involvement was present in the majority, with N1 comprising 32.2%, N2 representing 20.0%, and N3 accounting for 13.3% of cases. Distant metastasis was uncommon, as M0 status was recorded in 96.7% of patients, while only 3.3% presented with M1 disease. In terms of hormone receptor expression, estrogen receptor (ER) positivity was observed in 86.7% of cases, whereas 13.3% were ER-negative; likewise, progesterone receptor (PR) positivity was detected in 80.0% of patients compared to 20.0% who were PR-negative. Finally, HER2 status assessment showed that 58.9% of tumors were HER2-negative, while 41.1% demonstrated HER2 positivity.

Table 4.4. Histopathological features of breast cancer participants (n= 90).

Histopathological features	**Value**	
	n	**%**
Tumor type		
Ductal carcinoma	84	93.3%
Lobular carcinoma	6	6.7%
Tumor grade		
G 1	10	11.1%
G2	47	52.2%
G3	33	36.7%
T stage		
T1	17	18.9%
T2	36	40.0%
T3	20	22.2%
T4	17	18.9%
N stage		
N0	31	34.4%

Histopathological features	Value	
	n	%
N1	29	32.2%
N2	18	20.0%
N3	12	13.3%
M stage		
M0	87	96.7%
M1	3	3.3%
ER status		
Negative	12	13.3%
Positive	78	86.7%
PR status		
Negative	18	20.0%
Positive	72	80.0%
HER2 status		
Negative	53	58.9%
Positive	37	41.1%

Data presented for breast cancer patients only (n=90). Categorical variables presented as frequencies and percentages.

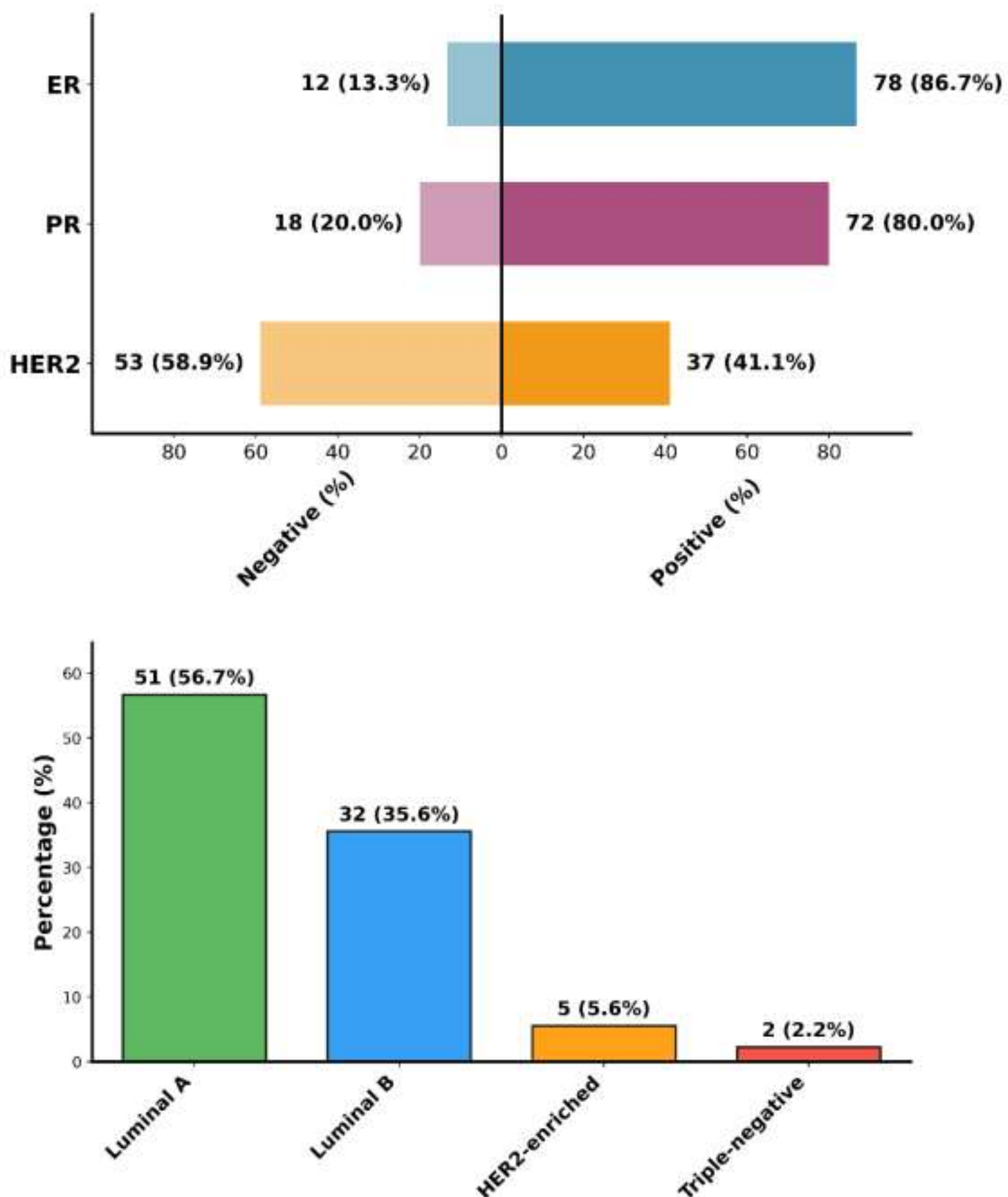


Figure 4.2. Hormone receptor status and molecular subtype distribution in breast cancer patients (n=90). Top: Butterfly chart showing ER (+ve= 78), PR (+ve= 72), and HER2 receptor (+ve= 37) positivity rates. Bottom: Distribution of molecular subtypes based on receptor combinations, luminal A (n= 51), luminal B (n= 32), HER2-enriched (n= 5), and triple-negative (n= 2).

The distribution of molecular subtypes according to clinical parameters is displayed in Table 4.5, while Figure 4.3 shows the flow from individual receptor status to subtype classification. With 90 patients with complete receptor data, Luminal A was the most prevalent subtype across various clinical categories.

Regarding tumor grade, Luminal A comprised 60.0% of grade 1 and 63.8% of grade 2 tumors, but decreased to 45.5% in grade 3 tumors; conversely, Luminal B varied from 40.0% in grade 1 to 29.8% in grade 2 and 42.4% in grade 3 tumors. HER2-enriched and triple-negative subtypes were rarely found in lower grades and slightly increased in grade 3 tumors. However, these differences across grades were not statistically significant (p>0.05). With respect to T stage, Luminal A was most frequent in T1 tumors (76.5%) and decreased to 41.7% in T2 tumors, then increased again in T3 (65.0%) and T4 tumors (58.8%); meanwhile, Luminal B showed highest prevalence in T2 tumors (47.2%). Nonetheless, subtype distribution across T stages showed no significant association (p>0.05). Similarly, nodal status revealed no significant relationship with molecular subtypes (p=0.098), although Luminal A was notably more common in N1 cases (76.0%) and N3 cases (70.0%), while showing equal frequency with Luminal B in N0 cases (48.4% each). Regarding metastatic status, Luminal A comprised 56.3% and Luminal B 35.6% of M0 cases, while among the three M1 cases, two were Luminal A and one was Luminal B; however, this difference was not significant (p>0.05). Finally, histological type analysis revealed that ductal carcinomas were distributed as 54.8% Luminal A, 36.9% Luminal B, 6.0% HER2-enriched, and 2.4% triple-negative, whereas lobular carcinomas were predominantly Luminal A (83.3%), though this association was not statistically significant (p>0.05). The Sankey diagram in Figure 4.5 visually confirms the predominance of Luminal A subtype among ER-positive tumors, demonstrating the strong relationship between hormone receptor positivity and luminal subtype classification.

Table 4.5. Molecular subtypes by clinical parameters.

Parameter	Luminal A, n= 51	Luminal B, n= 32	HER2-enriched, n=5	Triple-negative, n=2	*p*-value
Tumor grade					
G1	6 (60.0%)	4 (40.0%)	0 (0.0%)	0 (0.0%)	0.726
G2	30 (63.8%)	14 (29.8%)	2 (4.3%)	1 (2.1%)	
G3	15 (45.5%)	14 (42.4%)	3 (9.1%)	1 (3.0%)	

Parameter	Luminal A, n= 51	Luminal B, n= 32	HER2-enriched, n=5	Triple-negative, n=2	*p*-value
T stage					
T1	13 (76.5%)	4 (23.5%)	0 (0.0%)	0 (0.0%)	0.492
T2	15 (41.7%)	17 (47.2%)	3 (8.3%)	1 (2.8%)	
T3	13 (65.0%)	5 (25.0%)	1 (5.0%)	1 (5.0%)	
T4	10 (58.8%)	6 (35.3%)	1 (5.9%)	0 (0.0%)	
N stage					
N0	15 (48.4%)	15 (48.4%)	1 (3.2%)	0 (0.0%)	0.098
N1	19 (76.0%)	5 (20.0%)	1 (4.0%)	0 (0.0%)	
N2	6 (42.9%)	5 (35.7%)	2 (14.3%)	1 (7.1%)	
N3	7 (70.0%)	2 (20.0%)	1 (10.0%)	0 (0.0%)	
M stage					
M0	49 (56.3%)	31 (35.6%)	5 (5.7%)	2 (2.3%)	0.993
M1	2 (66.7%)	1 (33.3%)	0 (0.0%)	0 (0.0%)	
Histo-type					
Ductal	46 (54.8%)	31 (36.9%)	5 (6.0%)	2 (2.4%)	0.560
Lobular	5 (83.3%)	1 (16.7%)	0 (0.0%)	0 (0.0%)	

Data presented as n (%) within each clinical parameter category. The *p*-values calculated using chi-square test (or Fisher's exact test). Only patients with complete receptor status data included.

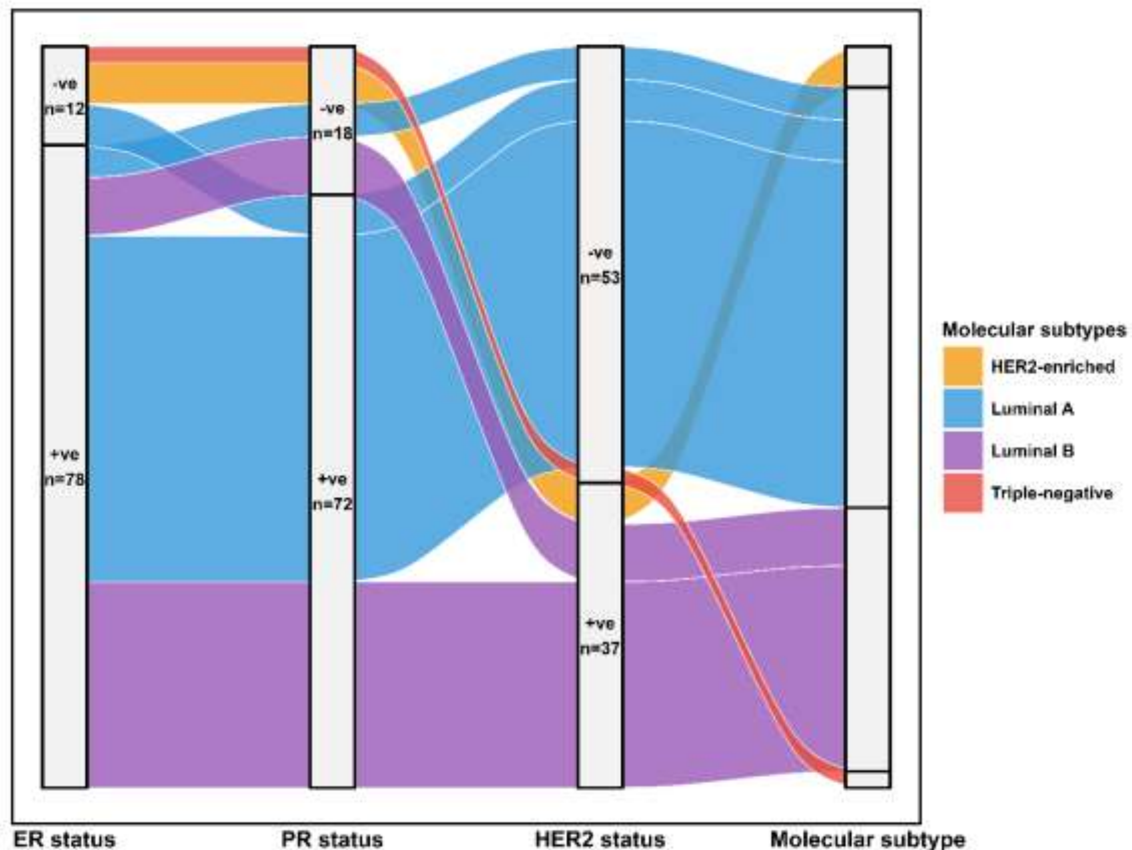


Figure 4.3. Sankey diagram showing the flow from individual hormone receptor status (ER, PR, HER2) to molecular subtype classification in breast cancer patients (n=90). Width of flows represents the number of patients. Luminal A predominates among ER-positive tumors.

The comprehensive biochemical parameters comparison between controls and breast cancer patients is presented in Table 4.6 and Figure 4.4. Among liver function tests, alkaline phosphatase (ALP) levels were significantly elevated in patients compared to controls (75.29±29.08 vs 64.95±23.85 U/L, $p<0.05$); similarly, total bilirubin was significantly higher in patients (0.98±0.41 vs 0.81±0.32 mg/dL $p<0.05$). However, AST and ALT levels showed no significant differences between groups ($p>0.05$). Regarding metabolic parameters, glucose levels were significantly increased in patients (102.46±21.27 vs 96.20±18.17 mg/dL, $p<0.05$). Kidney function assessment revealed that serum urea was markedly elevated in patients compared to controls (35.53±14.08 vs 28.37±11.15 mg/dL, $p<0.05$), while serum creatinine showed no significant difference ($p>0.05$). Electrolyte analysis demonstrated no significant differences between groups for calcium, phosphate, sodium, potassium, chloride, or magnesium levels (all $p>0.05$). Furthermore, inflammatory cytokines including IL-1β, IL-6, and TNF-α showed trends toward higher levels in patients, though these differences did not reach statistical significance ($p>0.05$). The tumor marker CA 15-3 and oxidative stress marker 8-OHdG also showed non-significant elevations in patients ($p>0.05$). Most notably, protein metabolism parameters revealed striking differences: albumin levels were significantly decreased in patients (3.74±0.50 vs 4.33±0.40 g/dL, $p<0.05$), while globulin levels were significantly increased (3.32±0.51 vs 2.91±0.35 g/dL, $p<0.05$). Consequently, the albumin/globulin (A/G) ratio was significantly reduced in patients (1.16±0.28 vs 1.51±0.25, $p<0.05$). Additionally, total protein levels were slightly but significantly lower in patients (7.06±0.58 vs 7.24±0.47 g/dL, $p<0.05$). Finally, high-sensitivity C-reactive protein (hs-CRP) showed the most dramatic difference, being markedly elevated in patients compared to controls (8.18 [4.68-12.24] vs 2.00 [1.04-2.73] mg/L, $p<0.05$).

Table 4.6. Comprehensive biochemical parameters comparison (N= 188).

Parameter	Control (n= 98)	Patients (n= 90)	*p*-value
AST (U/L)	25.47 ± 9.85	26.69 ± 8.86	0.372
ALT (U/L)	22.88 ± 10.29	24.83 ± 10.15	0.192
ALP (U/L)	64.95 ± 23.85	75.29 ± 29.08	0.009
Total bilirubin (mg/dL)	0.81 ± 0.32	0.98 ± 0.41	0.002
LDH (U/L)	184.90 ± 52.83	198.56 ± 62.72	0.110
Glucose (mg/dL)	96.20 ± 18.17	102.46 ± 21.27	0.032
Serum urea (mg/dL)	28.37 ± 11.15	35.53 ± 14.08	<0.001
Serum creatinine (mg/dL)	0.89 ± 0.25	0.95 ± 0.25	0.143
Total calcium (mg/dL)	9.66 ± 0.58	9.56 ± 0.66	0.278
Ionized calcium (mg/dL)	4.85 ± 0.36	4.80 ± 0.34	0.314
Phosphate (mg/dL)	3.57 ± 0.60	3.70 ± 0.62	0.126
Sodium (mEq/L)	139.93 ± 3.56	140.72 ± 3.75	0.143
Potassium (mEq/L)	4.16 ± 0.52	4.29 ± 0.49	0.071
Chloride (mEq/L)	102.33 ± 3.80	102.76 ± 3.46	0.422
Magnesium (mg/dL)	2.01 ± 0.25	2.07 ± 0.24	0.078
IL-1β (pg/mL)	2.60 ± 1.10	2.88 ± 1.28	0.116
IL-6 (pg/mL)	3.21 ± 1.48	3.51 ± 1.50	0.169
TNF-α (pg/mL)	4.98 ± 1.99	5.39 ± 2.32	0.196
CA 15-3 (U/mL)	14.66 ± 5.81	15.94 ± 6.22	0.149
8-OHdG (ng/mL)	2.74 ± 0.90	2.90 ± 1.06	0.286
Albumin (g/dL)	4.33 ± 0.40	3.74 ± 0.50	<0.001
Total protein (g/dL)	7.24 ± 0.47	7.06 ± 0.58	0.026
hs-CRP (mg/L)	2.00 (1.04-2.73)	8.18 (4.68-12.24)	<0.001
Globulin (g/dL)	2.91 ± 0.35	3.32 ± 0.51	<0.001
A/G ratio	1.51 ± 0.25	1.16 ± 0.28	<0.001

Data are presented as mean ± SD or median (IQR) based on normality. For statistical comparisons, t-test for normal data, Mann-Whitney U for non-normal data. IQR: Interquartile Range (25th to 75th percentile).

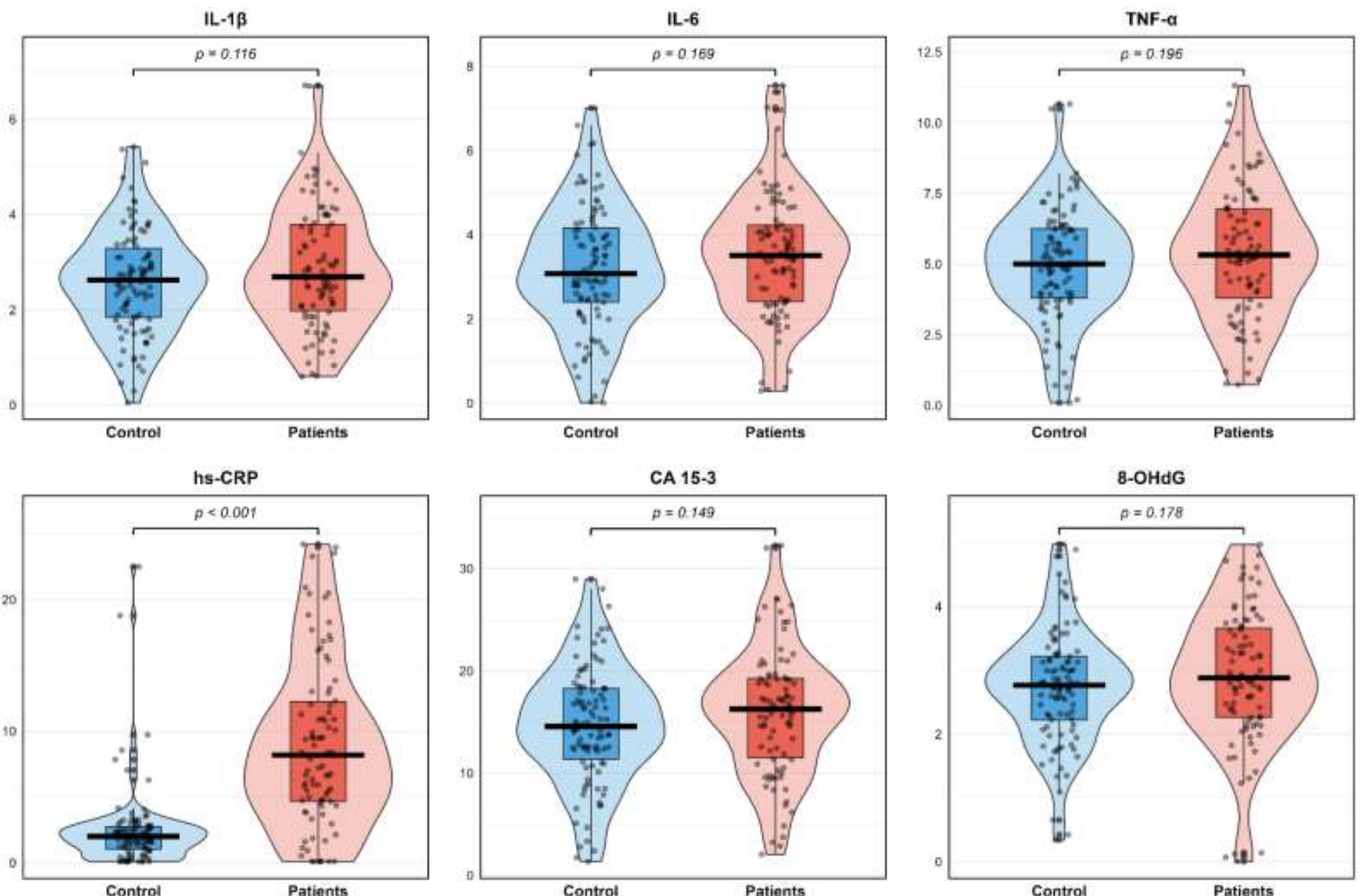


Figure 4.4. Comparison of inflammatory and tumor biomarkers between breast cancer patients (n= 90) and healthy controls (n= 98). The boxes represent the interquartile range (IQR) with the median shown as a horizontal line. Whiskers extend to 1.5 times the IQR, with outliers shown as individual points. The violin plots display the kernel density estimation of the data distribution. Statistical comparisons were performed using Mann-Whitney U test or Student's t-test based on data distribution (normality test of Shapiro-Wilk.

4.1.3. Genetic polymorphism analysis

The SNP analysis was performed using conventional PCR followed by Sanger sequencing to detect single nucleotide polymorphisms in *BRCA1* (rs4986850) and *BARD1* (rs1048108) genes. Initially, optimization experiments were conducted using two annealing temperatures (54°C and 56°C) for three primer sets targeting these polymorphisms (Figure 4.6). The optimal annealing temperature of 56°C was selected based on the clear and specific amplification bands observed.

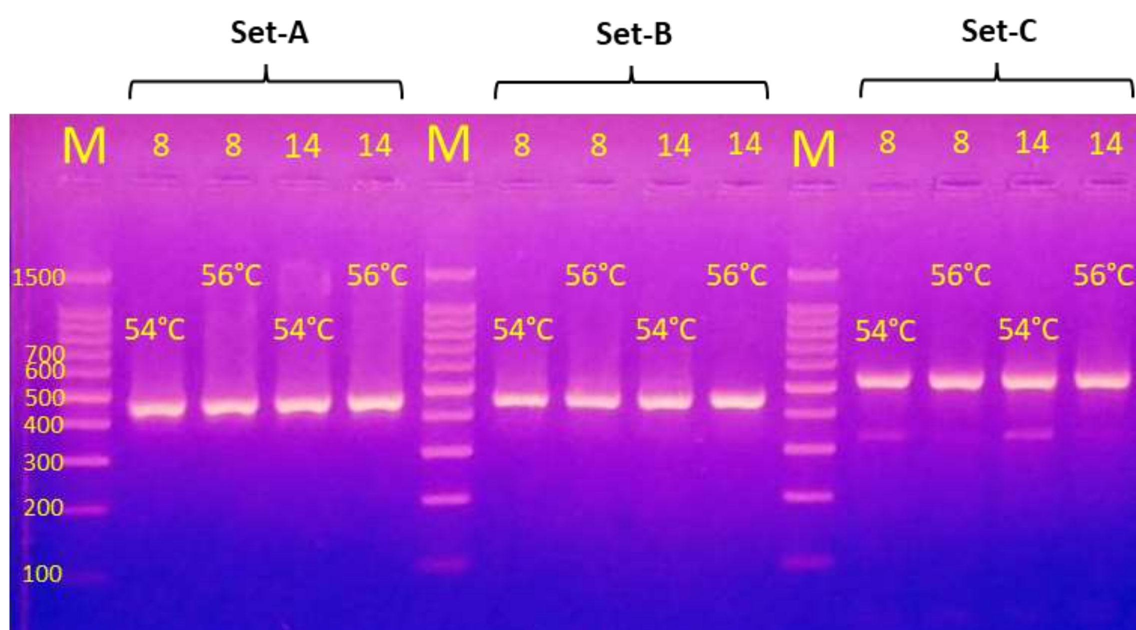


Figure 4.5. PCR optimization for *BRCA1* and *BARD1* amplification. Agarose gel electrophoresis (1.5%) showing PCR products at two annealing temperatures (54°C and 56°C). Set A: *BRCA1* rs4986850 (461 bp); Set B: *BARD1* rs1048108 (454 bp); Set C: *BARD1* rs1048108 alternative primers (533 bp). M: DNA ladder (marker). Samples 8 and 14 were randomly selected and tested for each condition. Electrophoresis conditions: 90V for 45 minutes with RedSafe staining.

For *BRCA1* rs4986850 detection, PCR amplification yielded the expected 461 bp product in all tested samples, as visualized on 1.5% agarose gel electrophoresis with RedSafe staining (Figures 4.6:A). Similarly, *BARD1* rs1048108 amplification produced the anticipated 454 bp fragment across all patient and control samples (Figures 4.6:B). The consistent amplification patterns indicated successful DNA extraction and PCR conditions for subsequent sequencing analysis.

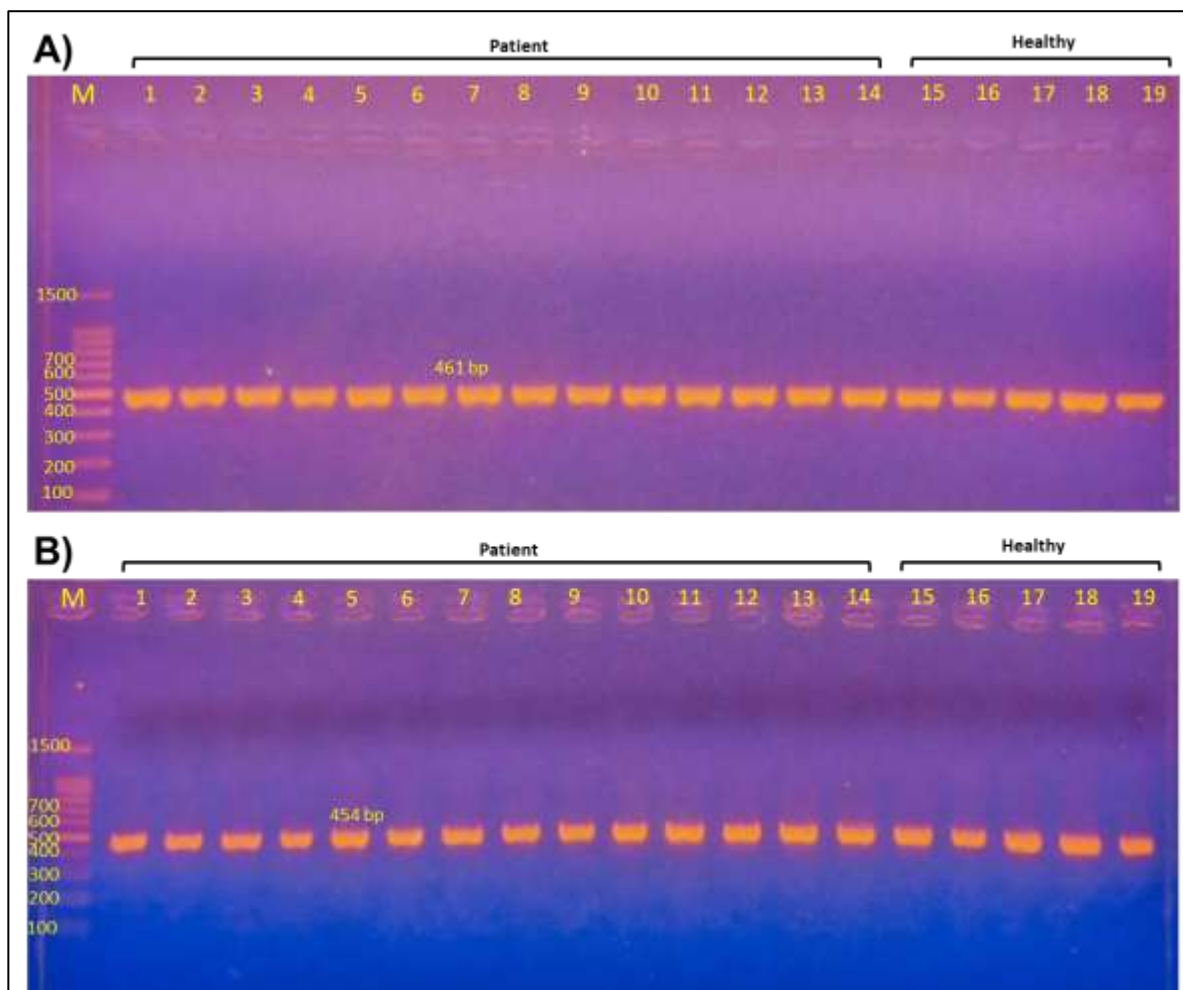


Figure 4.6. PCR amplification of target SNP regions: A) *BRCA1* rs4986850 amplification in patient samples (lanes 1-14) and healthy controls (lanes 15-19) showing the expected 461 bp product, B) *BARD1* rs1048108 amplification in patient samples (lanes 1-14) and healthy controls (lanes 15-19) showing the expected 454 bp product. M: DNA ladder (marker). All gels were 1.5% agarose with RedSafe staining, run at 90V for 45 minutes.

Sanger sequencing showed the genotype distribution for both polymorphisms. For *BRCA1* rs4986850, chromatogram analysis identified the wild-type CC genotype in the majority of samples (Figure 4.7:A), while the heterozygous CT genotype was detected in less frequency and TT in only one sample (Figure 4.7B, Table 4.6). Multiple sequence alignment against the reference *BRCA1* sequence (NG_005905) confirmed these variations. It's also important to mention that BLAST analysis identified several other clinically significant variants aren't mentioned in this work to keep only answering the research questions.

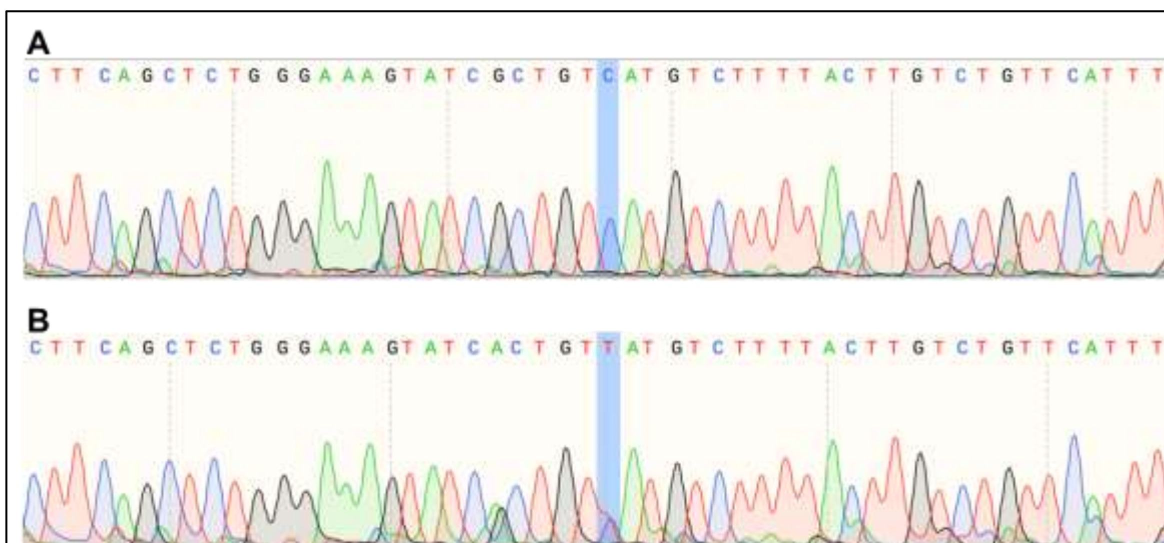


Figure 4.7. Sanger sequencing chromatograms of *BRCA1* rs4986850: A) wild-type CC genotype showing homozygous cytosine at the SNP position (blue shaded), B) heterozygous CT genotype displaying overlapping cytosine and thymine peaks at the SNP position (arrow). Sequencing was performed by Macrogen, Korea.

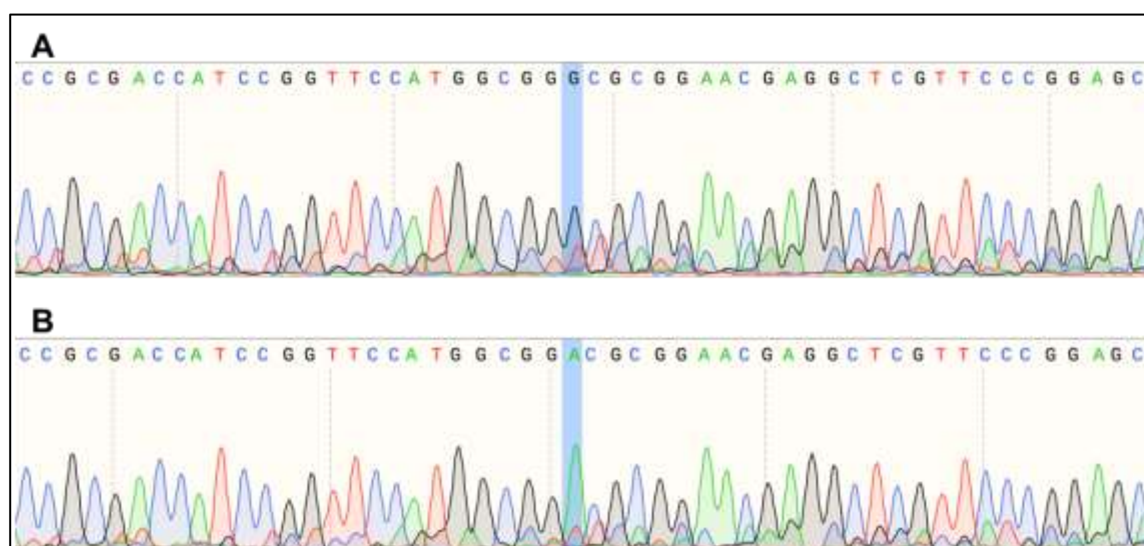


Figure 4.8. Sanger sequencing chromatograms of *BARD1* rs1048108: A) Wild-type GG genotype showing homozygous cytosine at the SNP position (blue shaded), B) Heterozygous GA genotype displaying overlapping cytosine and thymine peaks at the SNP position (arrow). Sequencing was performed by Macrogen, Korea.

The detailed genotype and allele frequencies of *BRCA1* (rs4986850) and *BARD1* (rs1048108) polymorphisms are presented in Table 4.7. For the *BRCA1* polymorphism, the CC genotype was identified as the most prevalent in both groups, being detected in 85.6% of controls and 75.6% of patients. The heterozygous CT

genotype was observed in 14.4% of controls and 23.3% of patients, yielding an odds ratio of 1.82 (95% CI: 0.80-4.29, $p>0.05$). The homozygous TT genotype was found exclusively in one patient (1.1%) and was absent in controls. Regarding allele distribution, the C allele was predominant, comprising 92.8% in controls and 87.2% in patients, while the T allele frequency was recorded as 7.2% in controls and 12.8% in patients (OR=1.88, 95% CI: 0.88-4.19, $p>0.05$). For the *BARD1* polymorphism, the genotype distribution showed more variation; the GG genotype was observed in 35.6% of controls and 37.8% of patients. The heterozygous GA genotype was detected in 33.3% of controls versus 51.1% of patients (OR=1.44, 95% CI: 0.70-2.97, $p<0.05$). Notably, the homozygous AA genotype was significantly less frequent in patients (11.1%) compared to controls (31.1%), demonstrating a protective effect (OR=0.34, 95% CI: 0.13-0.86, $p<0.05$). The allele frequency analysis revealed that the G allele comprised 52.2% in controls and 63.3% in patients, whereas the A allele was significantly reduced in patients (36.7%) compared to controls (47.8%), with an odds ratio of 0.63 (95% CI: 0.41-0.99, $p<0.05$). All genotype distributions were confirmed to be in Hardy-Weinberg equilibrium, as evidenced by non-significant *p*-values for both polymorphisms in controls and patients.

Table 4.7. Genotype and allele frequencies of *BRCA1* (rs4986850) and *BARD1* (rs1048108) single nucleotide polymorphism.

Gene/SNP	Genotype/ allele	Controls n(%)	Patients n(%)	OR (95% CI)	*p*-value
BRCA1 (rs4986850)	CC	77 (85.6%)	68 (75.6%)	1.00 (reference)	—
	CT	13 (14.4%)	21 (23.3%)	1.82 (0.80-4.29)	0.131
	TT	0 (0.0%)	1 (1.1%)	∞ (0.03-∞)	0.473
	C allele	167 (92.8%)	157 (87.2%)	1.00 (reference)	—
	T allele	13 (7.2%)	23 (12.8%)	1.88 (0.88-4.19)	0.113
BARD1 (rs1048108)	GG	32 (35.6%)	34 (37.8%)	1.00 (reference)	—
	GA	30 (33.3%)	46 (51.1%)	1.44 (0.70-2.97)	0.312
	AA	28 (31.1%)	10 (11.1%)	0.34 (0.13-0.86)	0.014
	G allele	94 (52.2%)	114 (63.3%)	1.00 (reference)	—
	A allele	86 (47.8%)	66 (36.7%)	0.63 (0.41-0.99)	0.042

Hardy-Weinberg Equilibrium (HWE) test: *BRCA1* Controls p=0.561, Patients p=0.808; *BARD1* Controls p=0.123, Cases p=0.064. Abbreviations: OR, odds ratio; CI, confidence interval. The *p*-values calculated using Fisher's exact test. Reference genotypes: *BRCA1* (CC), *BARD1* (GG).

The analysis of BRCA1-BARD1 haplotype frequencies revealed significant associations with breast cancer risk among 180 participants (90 cases, 90 controls) (Figure 4.9). The overall haplotype distribution differed significantly between groups ($\chi^2 = 19.005$, $p= 0.004$). The C/C-G/A haplotype was more prevalent in breast cancer patients (n= 37, 41.1%) compared to controls (n= 24, 26.7%), while the C/C-A/A haplotype showed a strong protective effect, occurring much less frequently in cases (n= 5, 5.6%) versus controls (n= 23, 25.6%) with an odds ratio of 0.14 ($p<0.001$). The C/C-G/G haplotype was slightly more common in controls (n= 30, 33.3%) than cases (n= 26, 28.9%). These findings suggest that specific BRCA1-BARD1 haplotype combinations may influence breast cancer susceptibility in the population of Karbala.

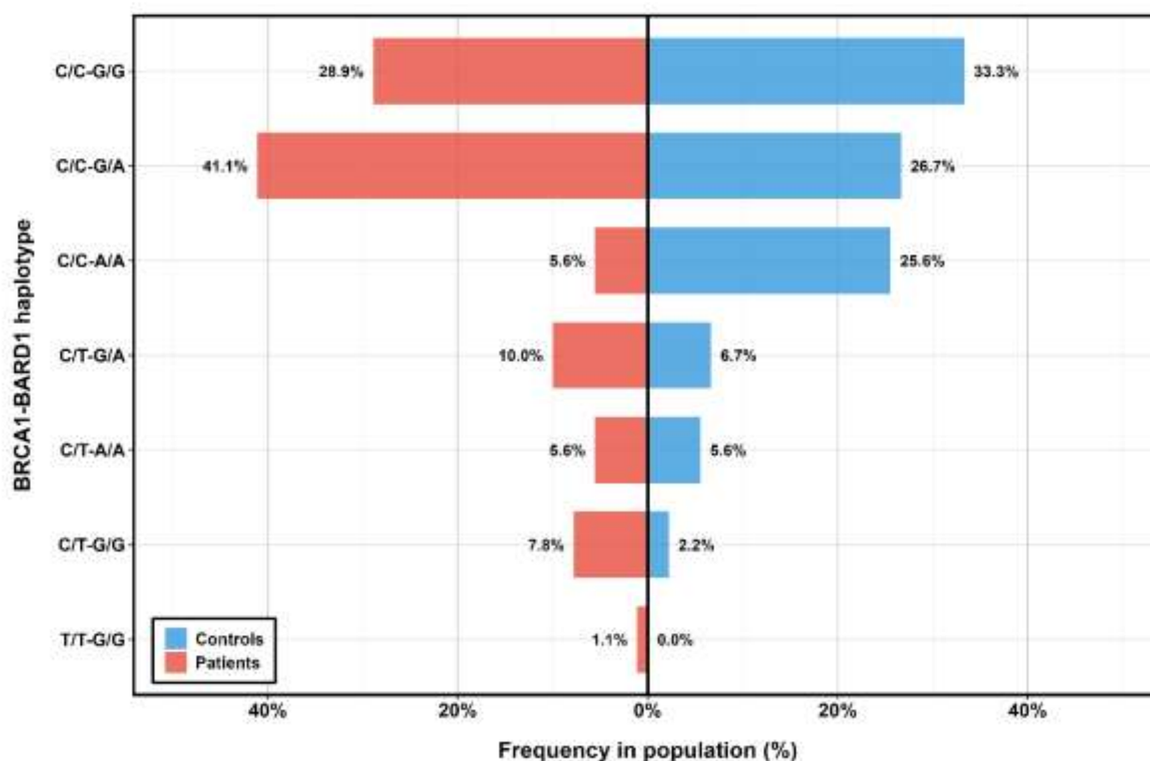


Figure 4.9. Distribution of *BRCA1-BARD1* haplotype frequencies in breast cancer patients versus controls. Each haplotype represents a combination of *BRCA1* rs4986850 and *BARD1* rs1048108 genotypes. Control frequencies are displayed above the baseline (blue bars) and patient frequencies below (red bars). Values indicate the percentage frequency in each group. The most common haplotype C/C-G/A shows a frequency of 26.7% in controls and 41.1% in patients.

4.1.4. Epigenetic analysis

The methylation analysis revealed a statistically significant difference between control subjects and breast cancer patients (Figure 4.10). Patients demonstrated higher methylation levels with a median of 17.8% (IQR: 10.7-45.0%) compared to controls who showed a median of 13.3% (IQR: 8.0-19.3%), with this difference reaching statistical significance (Mann Whitney's U test, $p<0.05$).

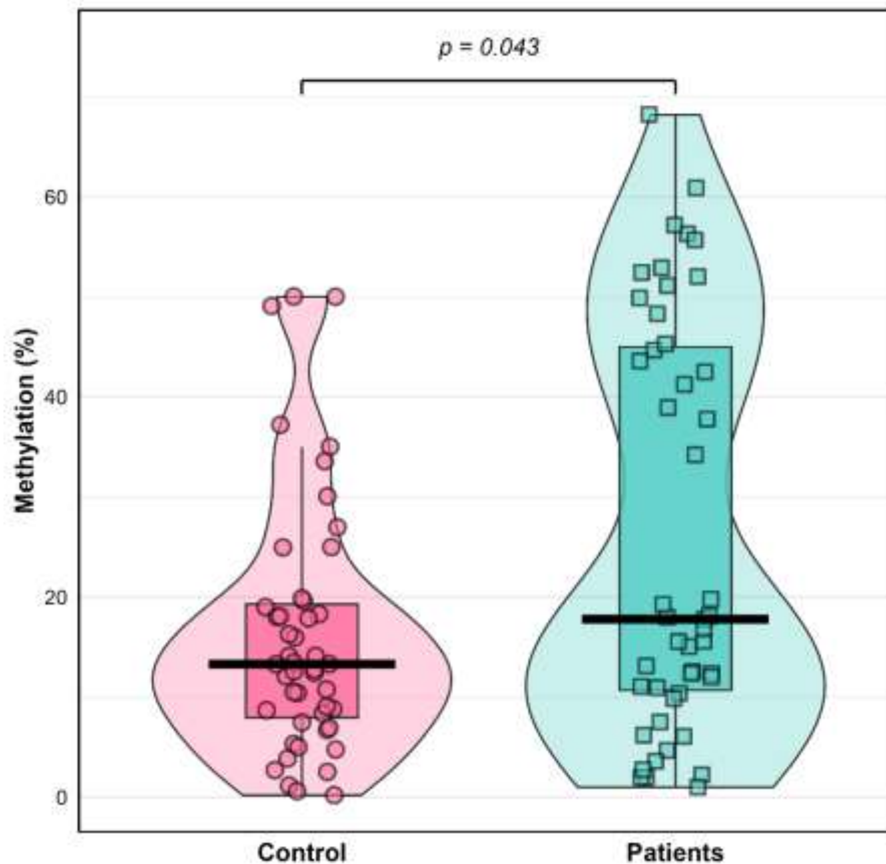


Figure 4.10. *BRCA1* methylation percentages in control (n= 47) versus breast cancer patients (n= 47). Box plots show median, quartiles, and individual data points. Pink represents control group, teal represents patients.

Further subgroup analysis of the patient cohort revealed no significant methylation differences based on cancer type or molecular subtypes (Figure 4.11). When comparing sporadic versus non-sporadic breast cancers based on family history (upper plot), no significant difference was observed, with sporadic cases showing a median methylation of 15.6% (IQR: 10.4-41.3%) and non-sporadic cases 19.6%

(IQR: 12.1-52.7%, *p*>0.0.05). Similarly, analysis across the four molecular subtypes (lower plot): Luminal A, Luminal B, HER2-enriched, and Triple-negative, showed no significant differences in methylation levels (Kruskal-Wallis test, *p*>0.05).

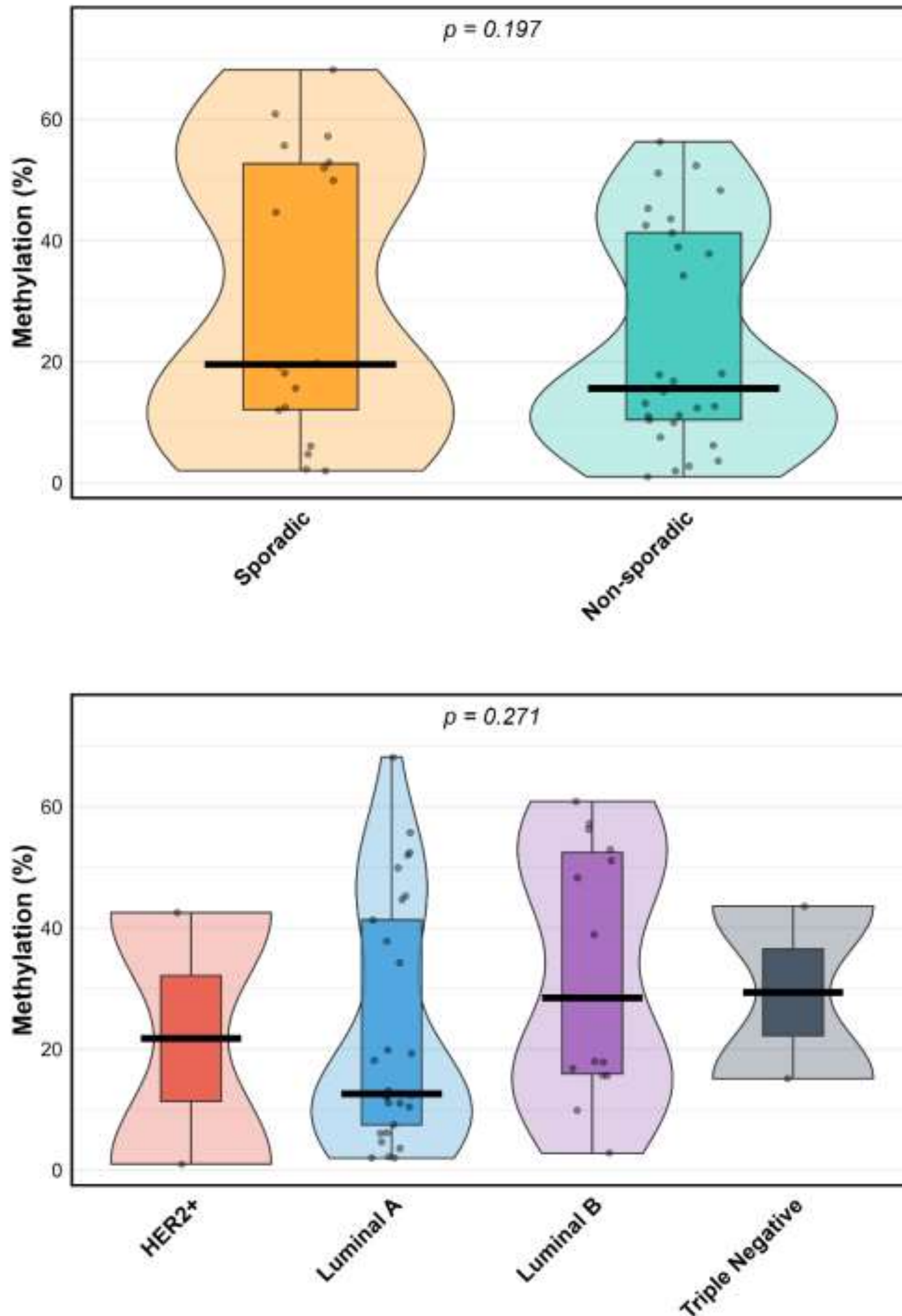


Figure 4.11. *BRCA1* methylation analysis in breast cancer patients: A) comparison between sporadic (family history positive) and non-sporadic (family history negative) cases, B) distribution across molecular subtypes based on ER, PR, and HER2 receptor status. Data presented as box plots with individual points. Statistical significance assessed by Mann-Whitney U test or Kruskal-Wallis test.

4.1.5. Protein expression analysis

The analysis of BRCA1 and BARD1 protein concentrations revealed significant differences between control subjects and breast cancer patients (Figure 4.12). Both proteins showed markedly reduced levels in patients compared to controls, with BRCA1 concentrations decreasing from a median of 7.887 ng/ml (IQR: 6.394-10.078) in controls to 4.194 ng/ml (IQR: 3.313-5.128) in patients ($p<0.05$). Similarly, BARD1 concentrations decreased from 10.053 ng/ml (IQR: 8.476-11.057) in controls to 3.991 ng/ml (IQR: 3.111-4.915) in patients ($p<0.05$). The density distributions (Figure 4.13) clearly illustrate the distinct separation between the two groups, with the blue regions representing higher protein concentrations in healthy controls and the red areas showing the lower concentrations characteristic of breast cancer patients.

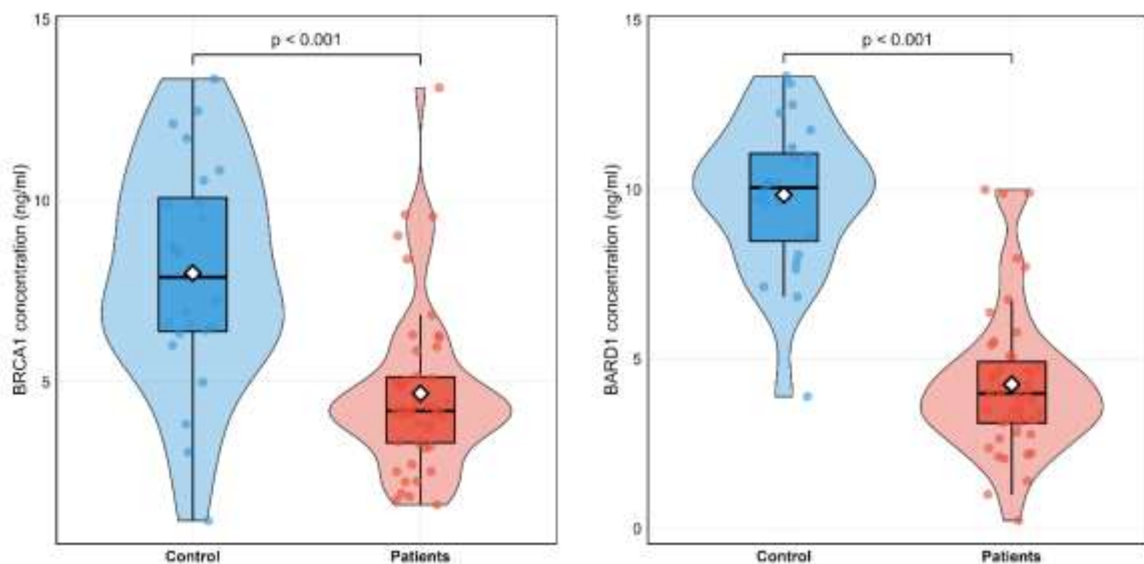


Figure 4.12. Comparison of BRCA1 and BARD1 protein concentrations between control and breast cancer patients.

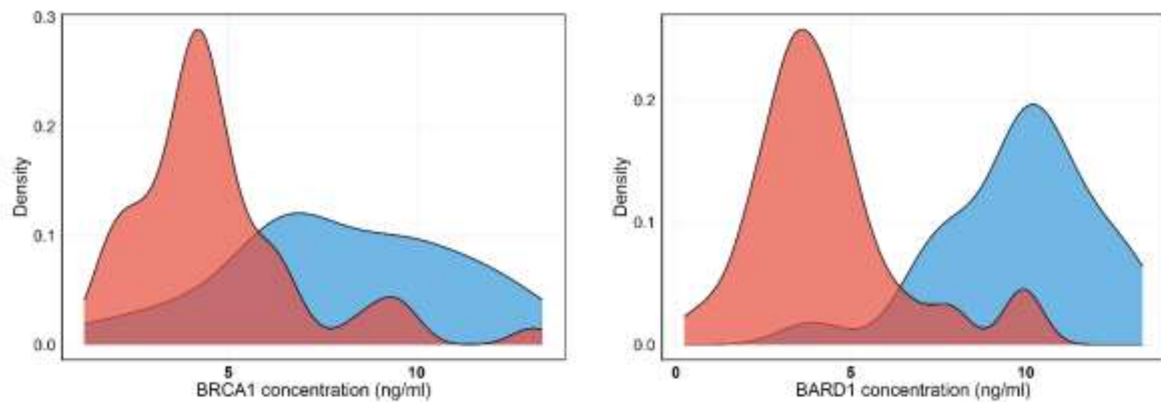


Figure 4.13. Density distributions of BRCA1 and BARD1 protein concentrations. The red area represent the patients and blue region represent the healthy controls.

Deeper analysis of the patients showed interesting patterns based on cancer type and molecular subtypes. When comparing sporadic versus non-sporadic breast cancers (Figure 4.14), BARD1 showed significantly higher concentrations in sporadic cases (median: 4.485 ng/ml) compared to non-sporadic cases (median: 3.116 ng/ml, $p<0.05$), while BRCA1 levels showed no significant difference between these groups ($p>0.05$). Analysis of molecular subtypes (Figure 4.15) revealed no significant differences between Luminal A and Luminal B subtypes for either BRCA1 ($p>0.05$) or BARD1 ($p>0.05$) protein concentrations.

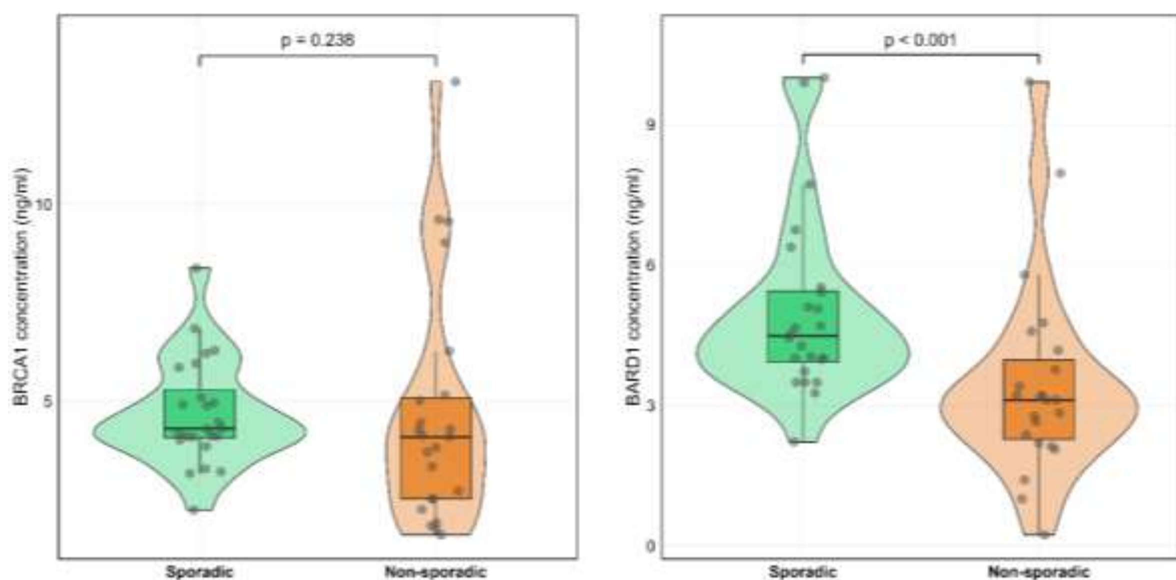


Figure 4.14. BRCA1 and BARD1 protein concentrations in sporadic vs non-sporadic breast cancer.

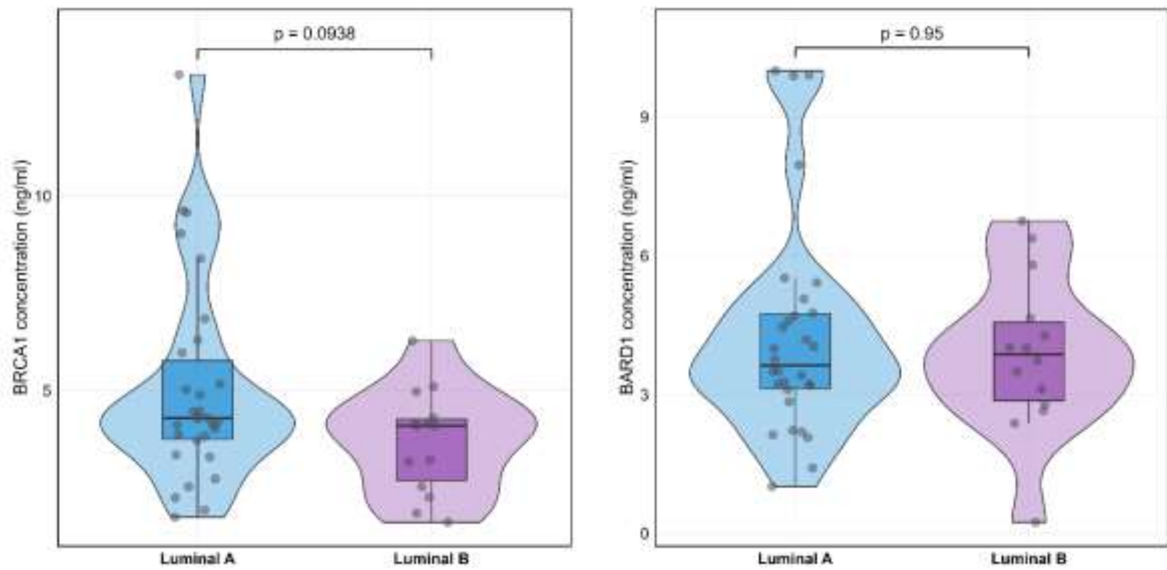


Figure 4.15. BRCA1 and BARD1 protein concentrations in Luminal A vs Luminal B breast cancer subtypes.

4.1.6. miRNA expression analysis

The analysis of miR-498 expression revealed significantly elevated levels in breast cancer patients compared to healthy controls (Figure 4.16). Patients had a marked upregulation with a median fold change of 2.423 (IQR: 1.726-5.868) compared to controls who showed a median of 0.796 (IQR: 0.424-2.327), with this difference being highly significant ($p<0.001$).

Deeper analysis of miR-498 expression by receptor status revealed no significant associations (Figure 4.17). Expression levels were similar between ER-positive and ER-negative tumors (median 2.378 vs 2.632, $p=0.886$), PR-positive and PR-negative tumors (median 2.378 vs 25.178, $p=0.302$), and HER2-positive and HER2-negative tumors (median 2.448 vs 2.423, $p=0.882$).

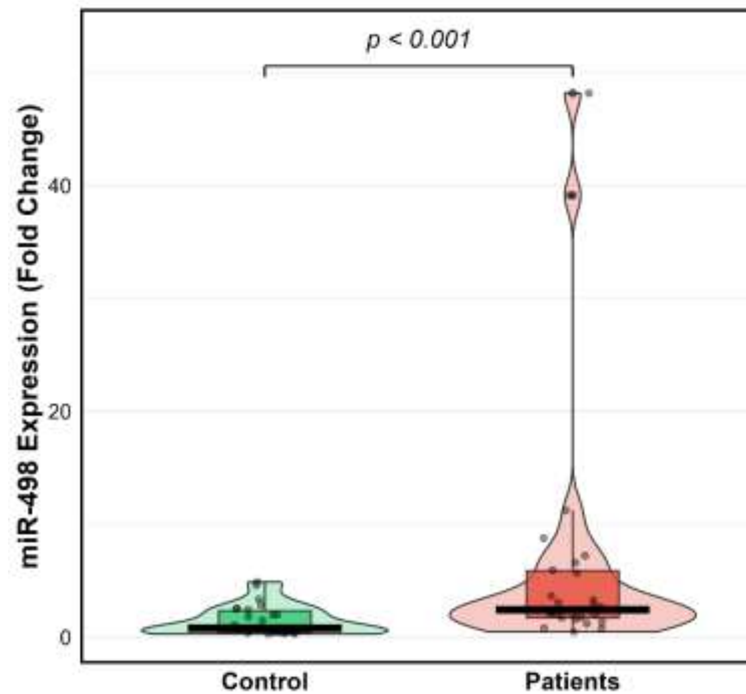


Figure 4.16. miR-498 expression analysis comparing between control subjects and breast cancer patients. Statistical significance assessed by Mann-Whitney U test or Kruskal-Wallis test.

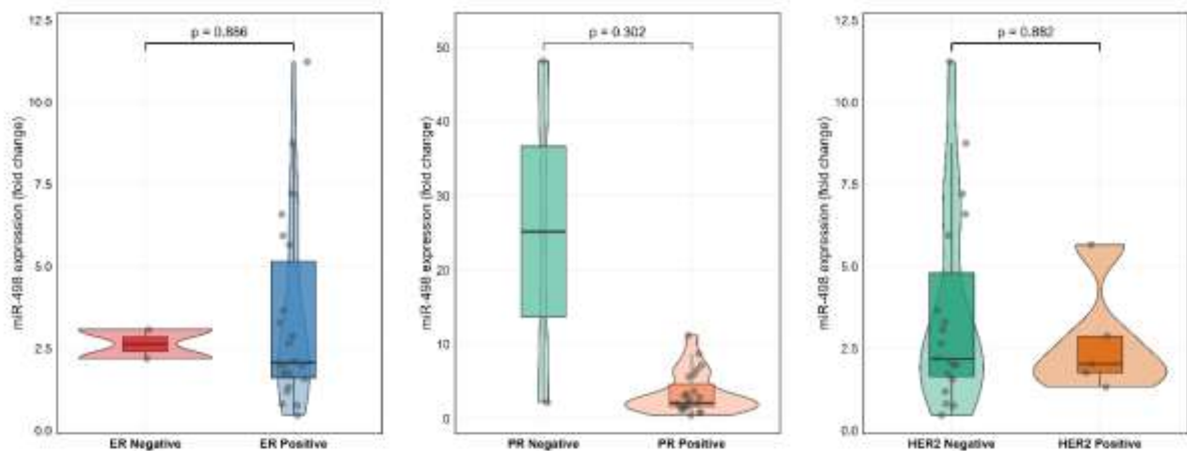


Figure 4.17. miR-498 expression by receptor status (ER, PR, HER2). Statistical significance assessed by Mann-Whitney U test.

4.1.7. Correlation analysis

The correlation analysis of molecular biomarkers showed interesting patterns of associations when examining the overall study cohort versus patients alone (Figures 4.18 and 4.19). In the overall cohort including both controls and patients (Figure 4.18), the most notable finding was a significant moderate positive correlation between BRCA1 and BARD1 protein concentrations ($\rho = 0.464$, $p < 0.05$), consistent with their known functional partnership in DNA repair pathways. Additionally, a significant negative correlation was observed between miR-498 expression and BARD1 protein levels ($\rho = -0.337$, $p < 0.05$), suggesting that increased miR-498 expression may be associated with decreased BARD1 protein abundance. No significant correlations were found between BRCA1 methylation and other biomarkers, including miR-498 expression ($\rho = 0.218$, $p > 0.05$), BRCA1 protein ($\rho = 0.030$, $p > 0.05$), or BARD1 protein ($\rho = -0.180$, $p > 0.05$).

When the analysis was restricted to breast cancer patients only (Figure 4.19), the correlation patterns shifted notably. The moderate positive correlation between BRCA1 and BARD1 proteins observed in the overall cohort was lost in the patient-only analysis ($\rho = 0.172$, $p > 0.05$), suggesting that the relationship between these proteins may be disrupted in breast cancer. Conversely, a new significant positive correlation appeared between miR-498 expression and BRCA1 protein levels ($\rho = 0.475$, $p < 0.05$) exclusively in patients, which was not present in the overall cohort. The correlation between miR-498 and BARD1 protein, which was significant in the overall analysis, became non-significant in patients ($\rho = 0.057$, $p > 0.05$).

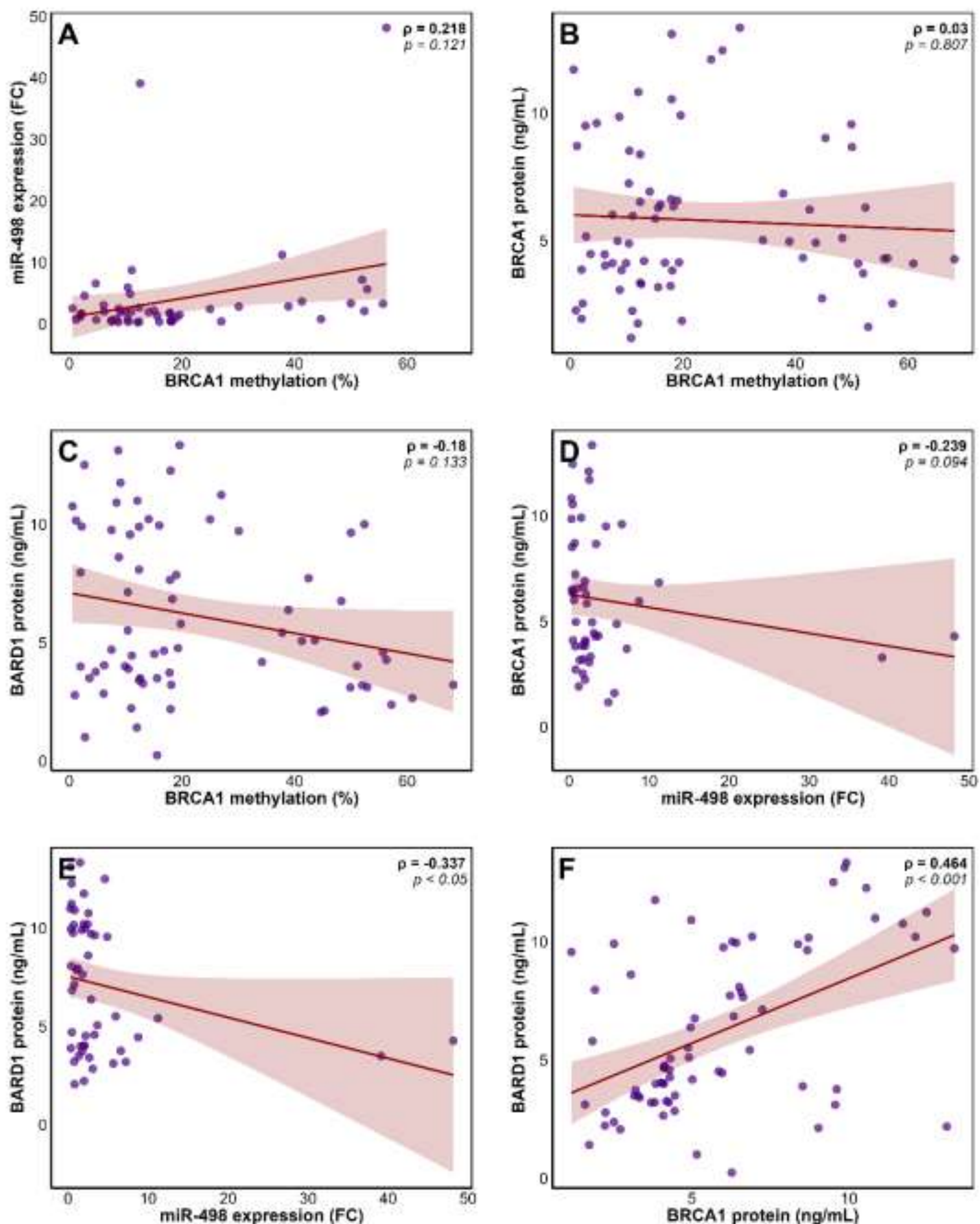


Figure 4.18. Scatter plots showing correlations between four molecular biomarkers in breast cancer study participants including both controls and patients (Breast cancer) from Karbala, Iraq. Each panel represents a unique biomarker pair: A) BRCA1 methylation vs miR-498 expression, B) BRCA1 methylation vs BRCA1 protein, C) BRCA1 methylation vs BARD1 protein, D) miR-498 expression vs BRCA1 protein, E) miR-498 expression vs BARD1 protein, and F) BRCA1 protein vs BARD1 protein. Purple dots represent individual subjects, with dark red regression lines showing linear relationships. Correlation coefficients (r for Pearson or ρ for Spearman, based on normality testing) and p-values are displayed in each panel. Statistical significance was set at $p < 0.05$.

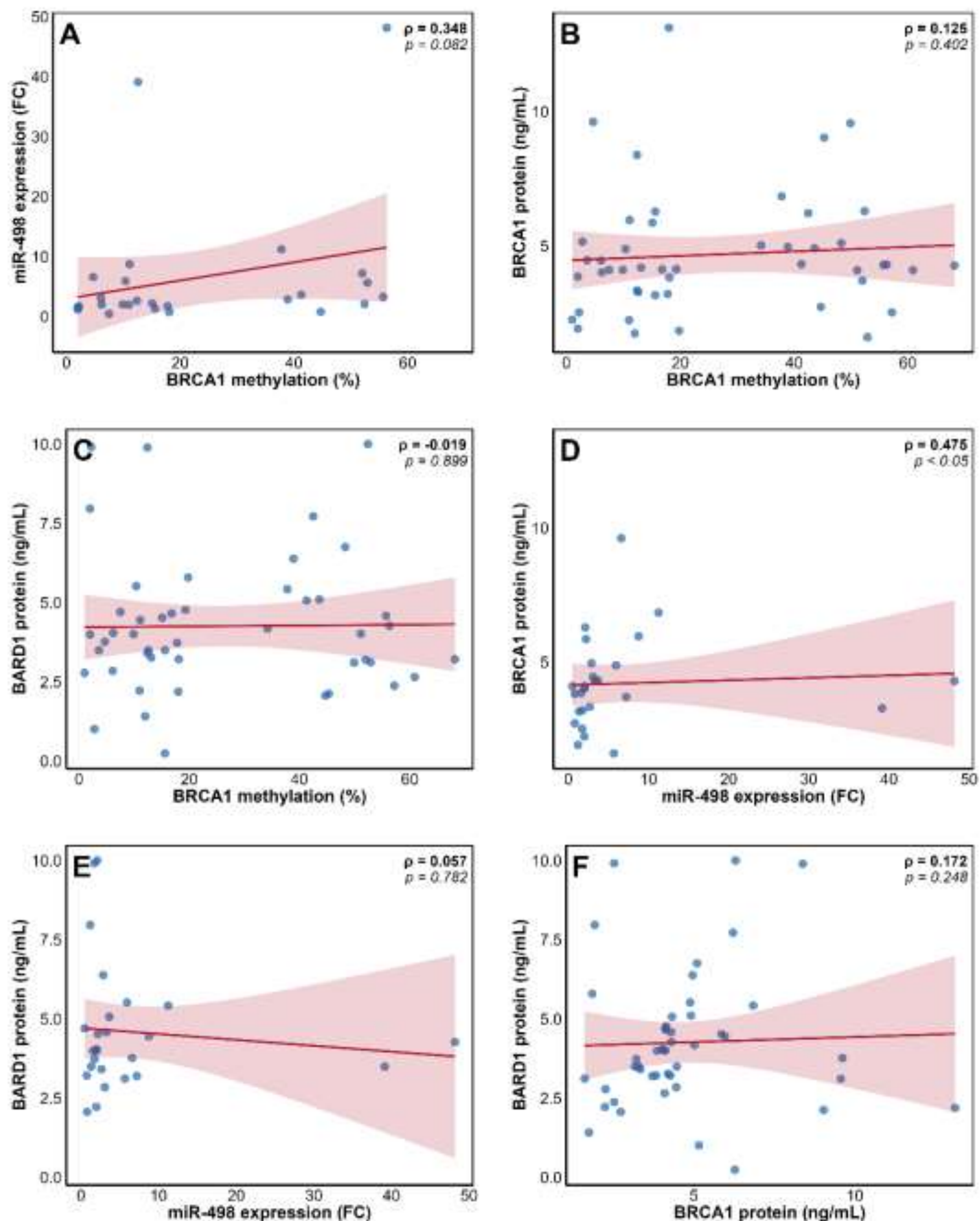


Figure 4.19. Scatter plots showing correlations between four molecular biomarkers exclusively in Breast cancer patients from Karbala, Iraq. Panel arrangement and biomarker pairs are identical to Figure 1: A) BRCA1 methylation vs miR-498 expression, B) BRCA1 methylation vs BRCA1 protein, C) BRCA1 methylation vs BARD1 protein, D) miR-498 expression vs BRCA1 protein, E) miR-498 expression vs BARD1 protein, and F) BRCA1 protein vs BARD1 protein. Blue dots represent individual patients, with red regression lines showing linear relationships. Correlation method (Pearson or Spearman) was selected based on normality testing of each variable. This patients-only analysis allows for assessment of biomarker relationships specifically within the cancer population.

4.1.8. ROC curve analysis

The diagnostic performance of nine biomarkers for breast cancer detection was evaluated using receiver operating characteristic (ROC) curve analysis (Table 4.7 and Figure 4.20). Among all tested biomarkers, BARD1 protein concentration had the highest diagnostic accuracy with an excellent area under the curve (AUC) of 0.936, followed by BRCA1 protein with an AUC of 0.862 (both $p<0.001$). Using an optimal cutoff value of 6.608 ng/ml, BARD1 protein showed high sensitivity (88.5%) and exceptional specificity (95.8%), resulting in excellent positive and negative predictive values (95.8% and 88.5%, respectively).

Among the other biomarkers, miR-498 expression, IL-6, and hs-CRP showed moderate diagnostic performance with AUC values of 0.766, 0.732, and 0.731, respectively (all $p<0.05$). While miR-498 demonstrated high sensitivity (88.5%) at a cutoff of 1.123 fold change, its specificity was relatively low (54.2%). In contrast, inflammatory markers IL-1β and TNF-α showed perfect specificity (100%) but very poor sensitivity (15.4%), making them unsuitable for screening purposes. The traditional tumor marker CA 15-3 and oxidative stress marker 8-OHdG showed poor diagnostic performance with AUC values near 0.5, indicating no better discrimination than chance.

Table 4.8. Diagnostic accuracy of breast cancer biomarkers.

Biomarker	**AUC (95% CI)**	***p*-value**	**Cutoff**	**Sensitivity (%)**	**Specificity (%)**
CA 15-3	0.612 (0.453-0.771)	0.160	14.840	69.2	58.3
miR-498	0.766 (0.635-0.897)	<0.001***	1.123	88.5	54.2
IL-1β	0.457 (0.290-0.624)	0.599	4.595	15.4	100.0
IL-6	0.732 (0.583-0.880)	0.001**	2.940	92.3	62.5
TNF-α	0.494 (0.327-0.660)	0.938	8.360	15.4	100.0
8-OHdG	0.503 (0.338-0.669)	0.969	2.885	53.8	62.5
hs-CRP	0.731 (0.575-0.887)	0.001**	3.590	76.9	87.5
BRCA1 protein	0.862 (0.745-0.980)	<0.001***	5.987	88.5	83.3
BARD1 protein	0.936 (0.867-1.000)	<0.001***	6.608	88.5	95.8

AUC= Area Under the Curve; CI = Confidence Interval. **Statistical significance:** ***$p<0.001$, **$p<0.01$, * $p<0.05$.

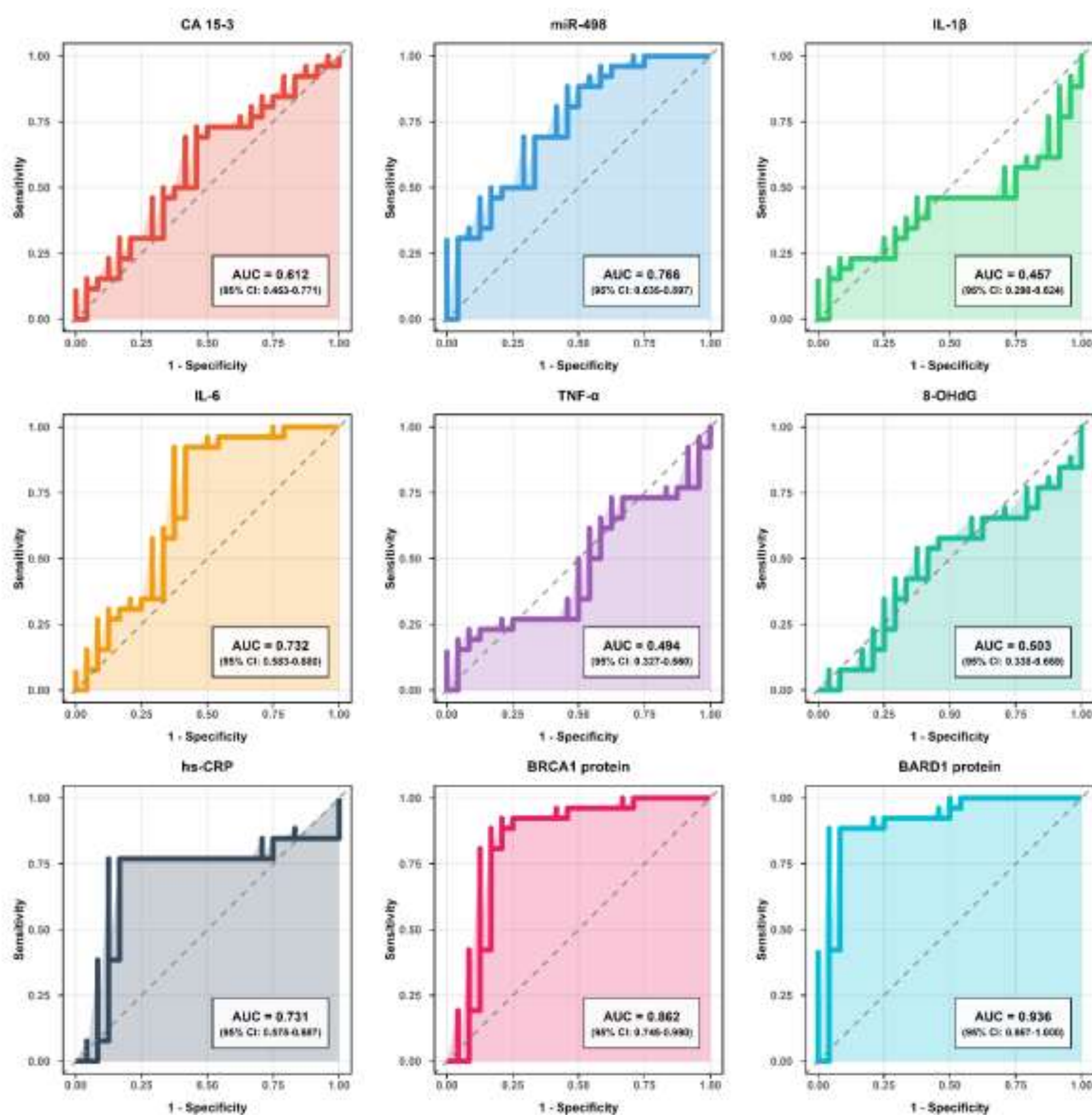


Figure 4.20. Receiver operating characteristic (ROC) curves for seven breast cancer biomarkers (CA 15-3, miR-498, IL-1β, IL-6, TNF-α, 8-OHdG, and hs-CRP, BRCA1, BARD1). Each panel displays the ROC curve with the area under the curve (AUC) and 95% confidence interval. The diagonal dashed line represents the reference line for a non-discriminatory test (AUC= 0.5).

4.2. Discussion

4.2.1. Study population characteristics

Although the median age of patients (50.0 years) fell within the typical range for breast cancer occurrence, it agrees with other Iraqi studies that reported mean ages between 47.89 and 51-59 years (Al-Fadhli *et al*., 2016; Alwan *et al*., 2019; Shwana *et al*., 2020). Furthermore, the trend toward higher BMI in patients, despite not reaching statistical significance, aligns with the findings by Molah Karim *et al*. (2015), who demonstrated a positive association between elevated BMI and breast cancer risk among Iraqi Kurdish women, particularly in postmenopausal populations. The predominance of married women in both groups corresponds to findings by Al-Fadhli *et al*. (2016) in Baghdad; however, the higher proportion of married women among controls (87.8%) compared to patients (81.1%) suggests that marriage may have a protective effect against breast cancer risk, possibly through better social support systems, improved healthcare access, or different reproductive and lifestyle factors (Falagas *et al*., 2007; Osborne *et al*., 2005).

Most notably, the significant educational disparity observed, with 47.8% of patients having only elementary education compared to 33.7% of controls, strongly corroborates multiple Iraqi studies that identified low education levels as a central risk factor for breast cancer (Al-Alwan, 2020; Alwan *et al*., 2017; Shwana *et al*., 2020). This association may be attributed to reduced health literacy, limited access to screening programs, and delayed healthcare seeking behavior among less educated women. Finally, the predominance of housewives in both groups, particularly among patients (83.3%), supports previous research linking unemployment and housewife status with increased breast cancer risk in Iraqi populations (Shwana *et al*., 2020), possibly due to reduced physical activity, limited social support networks, or economic constraints affecting healthcare access.

4.2.2. Clinical, pathological and laboratory characteristics

The identical age at menarche (13 years) between groups contrasts with Abedalrahman *et al*. (2019), who identified early menarche (<12 years) as a

significant risk factor among Iraqi women; however, this finding aligns with Roman Lay *et al*. (2020), who associated later menarche (≥13 years) with delayed menopause rather than direct cancer risk. And, the higher proportion of post-menopausal women among patients supports previous findings, while the mean age at menopause (48.1 years) corresponds closely with Dhia Al-Deen and Sadik (2009), who reported 47.96 years for Iraqi women. Most extraordinarily, the significantly higher number of pregnancies (6.0 vs. 3.0) and births among patients contradicts the protective effect of multiparity typically reported; however, this must be interpreted alongside the markedly younger age at first birth (20.5 vs 26.0 years), which aligns with Zangana and Garota's (2012) findings, who found that younger age at first birth decreased breast cancer risk in Kurdish women. This apparent paradox might be explained by the competing effects of early childbearing versus high parity in this population. The unexpected finding of longer breastfeeding duration among patients (18.0 vs. 7.0 months) challenges the established protective role of lactation; nevertheless, Zangana and Garota (2012) similarly found no association between breastfeeding and breast cancer risk in Kurdish women, suggesting population-specific factors may modify this relationship. The trend toward higher contraceptive use among patients, though not significant, supports Abedalrahman et al. (2019) findings, who found increased risk with ≥1 year of contraceptive use. Most dramatically, the extensive HRT use among patients (65.6% vs 8.2%) represents a major modifiable risk factor, particularly concerning given that Hagemans (2004) found that natural reproductive years alone account for significant variance in health outcomes without additional hormonal exposures. These findings suggest that the reproductive risk profile for breast cancer in Karbala women is characterized by early childbearing, high parity, and extensive exogenous hormone use, creating a distinct pattern that may require tailored prevention strategies.

The lifestyle and dietary patterns observed in this study reveal complex associations that both align with and differ from established breast cancer risk factors in Middle Eastern populations. The trend toward higher smoking prevalence among patients (12.2% vs. 4.1%), though not statistically significant, needs attention given

Mohammed *et al*. (2016) reported genetic variations among Iraqi smokers that may influence cancer susceptibility. The complete absence of alcohol consumption in both groups distinguishes this population from Western cohorts and reflects cultural and religious practices in Iraqi society (Al Ansari *et al*., 2022). The significantly lower daily vegetable consumption among patients (81.1% vs 95.9%) aligns with regional studies; Alsheridah and Akhtar (2018) demonstrated that infrequent fruit and vegetable intake increased colorectal cancer risk in Kuwait, while van Duijnhoven *et al*. (2009) found high consumption of fruits and vegetables protective against various cancers, particularly in non-smokers. This protective effect may extend to breast cancer through similar antioxidant and anti-inflammatory mechanisms. The meat consumption observations present a particularly concerning pattern; the higher daily red meat intake among patients (7.8% vs. 3.1%) agrees with Alsheridah and Akhtar (2018), who identified excessive red meat consumption as a significant cancer risk factor in Middle Eastern populations. Interestingly, Mahalhal and Ghafel (2021) found that Iraqi women with breast cancer consumed less red meat than normal, which contrasts with our findings of higher daily consumption but aligns with the subset who avoided red meat entirely (11.1% *vs*. 1.0%), suggesting heterogeneous dietary responses to cancer diagnosis. The exclusive presence of daily processed meat consumption among patients (2.2%) raises particular concern given the established links between processed meats and cancer risk. These dietary patterns, combined with significantly lower vegetable intake, mirror findings from neighboring Jordan where Arafa *et al*. (2011) identified similar dietary risk factors for colorectal cancer, suggesting common nutritional challenges across the region. The comparable environmental exposure duration between groups indicates that individual lifestyle choices, particularly dietary habits, may play a more critical role than ambient environmental factors in determining breast cancer risk in this population, emphasizing the potential for dietary interventions in cancer prevention strategies specific to Iraqi women.

The histopathological findings in this study align closely with previous reports from Iraq and highlight concerning patterns in breast cancer presentation. The

predominance of ductal carcinoma (93.3%) agrees with Al-Isawi (2016) and Mutar *et al*. (2019), who reported 81-88% ductal carcinoma rates among Iraqi patients. The tumor size distribution, with 81.1% of cases exceeding 20 mm and median size of 44.4 mm, shows late presentation patterns constantly reported across Iraqi studies. This advanced presentation is further evidenced by the staging distribution, where only 18.9% presented with T1 tumors, contrasting sharply with Western populations where early-stage detection is more common. The high prevalence of nodal involvement, with 65.6% showing positive nodes, mirrors findings from Abood (2018) and Mutar *et al*. (2019), who reported 40-68% of Iraqi patients presenting at stages III-IV. Particularly, the relatively low rate of distant metastasis (3.3%) suggests that while patients present with locally advanced disease, systemic spread remains uncommon at diagnosis. The hormone receptor profile observed, with 86.7% ER-positive and 80.0% PR-positive tumors, indicates a predominance of hormone-sensitive disease, consistent with Alwan *et al*. (2017), who identified Luminal A as the most common subtype (42-48%) among Iraqi patients. However, the 41.1% HER2 positivity rate appears higher than the 10-14% reported in previous Iraqi studies, possibly reflecting differences in testing methods or population characteristics specific to Karbala. The relatively moderate tumor grades, with 52.2% being grade 2, contrasts with Al-Isawi (2016), who noted higher grades in Western Iraq, which may suggest regional variations within the country. These findings collectively underline that breast cancer in Karbala presents with larger tumors, frequent nodal involvement, and predominantly hormone-sensitive characteristics, the matter draw attention to the urgent need for enhanced early detection programs to identify cases before they progress to advanced stages, as successful early detection initiatives have shown promise in reducing late-stage presentations among high-risk groups in Iraq.

The predominance of Luminal A subtype across various clinical parameters corresponds with Alwan *et al*. (2017), who reported 45% Luminal A among Iraqi patients, though our findings show even higher prevalence in certain categories. The lack of significant associations between molecular subtypes and clinical parameters

(tumor grade, stage, nodal status) contrasts with international studies; Liu *et al*. (2016) and Widodo *et al*. (2014) found significant differences in tumor characteristics among subtypes, with HER2-enriched and triple-negative tumors typically presenting with more aggressive features. The relatively low frequency of triple-negative cases (2-3%) in our cohort differs markedly from global reports of 15-25%, suggesting either population-specific biological differences or potential under-detection due to technical factors. The distribution pattern showing decreased Luminal A prevalence in grade 3 tumors (45.5% vs 60-63.8% in lower grades) aligns with Cheang *et al*. (2009) and Inic *et al*. (2014), who found that Luminal A tumors typically have lower proliferation rates and better differentiation. The high proportion of Luminal A in lobular carcinomas (83.3%) compared to ductal carcinomas (54.8%) reflects the known association between lobular histology and hormone receptor positivity. Interestingly, the equal distribution of Luminal A and B subtypes in node-negative disease (48.4% each) suggests that early-stage disease in this population includes both indolent and more aggressive hormone-positive tumors, while the marked predominance of Luminal A in N1 (76.0%) and N3 (70.0%) cases is unexpected and requires further investigation. The absence of significant associations between subtypes and staging parameters may be attributed to the relatively small sample size or the predominance of hormone-positive disease in this population. These findings draw attention to that while molecular subtyping provides valuable prognostic information, as noted by Turkoz *et al*. (2013) and Howlader *et al*. (2014), the subtype distribution and its clinical correlations may vary significantly across different populations, highlighting the importance of region-specific data for optimizing treatment strategies and understanding breast cancer biology in Iraqi women.

The significant elevation of liver parameters, particularly ALP and total bilirubin, aligns with previous Iraqi studies; Al-Mashhadani *et al*. (2015) and Aziz *et al*. (2023) reported similar increases in liver function parameters among breast cancer patients. Since our patients were newly diagnosed without prior treatment, these elevations likely reflect tumor-related metabolic changes or paraneoplastic effects rather than therapy-induced hepatotoxicity. The elevated glucose levels observed (102.46 vs.

96.20 mg/dL) agreed with Devi *et al*. (2015) findings, who found hyperglycemia in breast cancer patients, potentially indicating metabolic dysregulation or insulin resistance associated with malignancy itself. The significant increase in serum urea without corresponding creatinine elevation suggests mild renal dysfunction or increased protein catabolism from the tumor burden. Most notably, the dramatic alterations in protein metabolism—decreased albumin, increased globulin, and reduced A/G ratio—indicate significant changes in hepatic synthetic function and immune activation in response to the malignancy. This pattern has been consistently reported across multiple Iraqi studies and reflects the systemic inflammatory response to cancer. The marked elevation of hs-CRP (8.18 vs 2.00 mg/L) confirms the inflammatory state and corresponds with Bakheet *et al*. (2024), who documented increased inflammatory markers including CRP, IL-6, and TNF-α in Iraqi breast cancer patients. Although our cytokine levels showed non-significant trends toward elevation, the overall inflammatory profile is evident. The lipid profile alterations reported by Banger *et al*. (2019) and Ali (2014), showing increased cholesterol and triglycerides in untreated patients, were not assessed in our study but would complement these metabolic findings. Interestingly, the normal calcium and phosphate levels contrast with some reports suggesting mineral dysregulation in breast cancer (Abdelgawad *et al*., 2015). And, the non-significant elevation of CA 15-3, despite being a breast cancer marker, likely reflects early-stage disease in many patients (Keyhani *et al*., 2005). These comprehensive biochemical alterations in treatment-naïve patients underline the systemic nature of breast cancer, affecting multiple organ systems through tumor-host interactions, and draw attention to the importance of baseline metabolic and inflammatory assessment for optimal patient management in the Iraqi population.

4.2.3. Genetic polymorphism analysis

For *BRCA1* rs4986850 (D693N), no significant association with overall breast cancer risk was detected in this population, with the CT genotype showing only a non-significant trend toward increased risk (OR=1.82, 95% CI: 0.80-4.29, p=0.131).

The C allele was confirmed as the wild-type reference allele in the current SNP database, with the T allele representing the variant. This finding aligns with previous reports demonstrating no significant main effect on sporadic breast cancer risk in general populations (Durocher, 1996; Ricks-Santi *et al*., 2013). However, previous studies have identified associations with specific breast cancer subtypes, particularly triple-negative breast cancer (OR=2.31, 95% CI: 1.08-4.93), and significant interactions with hormone therapy use in postmenopausal women (Ricks-Santi *et al*., 2011; Ricks-Santi *et al*., 2013). The low frequency of the T allele observed in this study (7.2% in controls, 12.8% in cases) is consistent with frequencies reported in other populations (3-7%) (Durocher, 1996). The absence of TT homozygotes in controls and presence of only one in cases (1.1%) reflected the rarity of this variant. The lack of significant association may be attributed to the limited sample size (n=90 per group), which provided insufficient statistical power to detect modest effects, or to population-specific factors in the Iraqi population that may modulate the variant's effect on breast cancer susceptibility.

The genotyping results for *BARD1* rs1048108 revealed that the GG genotype represented the wild-type reference allele according to the SNP database used in this study, with recent updates confirming G as the ancestral allele. A significant protective effect was observed for the AA genotype (OR=0.34, 95% CI: 0.13-0.86, p=0.014) and A allele (OR=0.63, 95% CI: 0.41-0.99, p=0.042) against breast cancer in the Karbala population. These findings are consistent with protective effects reported in other populations, despite differences in allele nomenclature across databases and studies. Previous investigations have documented protective associations for this polymorphism in breast cancer, with studies in Chinese populations showing reduced risk (OR=0.562, 95% CI: 0.355-0.891) and European populations demonstrating similar protective effects (OR=0.65, 95% CI: 0.58-0.73), though these studies utilized different reference allele designations (Cimmino *et al*., 2018; Liu *et al*., 2013). The maintenance of Hardy-Weinberg equilibrium in the control population (p=0.123) supported the validity of the genotyping data, and the

allele frequency distribution (G allele: 52.2% in controls, 63.3% in cases) indicated a higher frequency of the wild-type allele among breast cancer patients.

4.2.4. Epigenetic analysis

The *BRCA1* promoter methylation analysis in this study revealed significantly elevated methylation levels in breast cancer patients compared to controls (17.8% vs 13.3%, p=0.043), which aligns with previous investigations demonstrating the role of *BRCA1* hypermethylation in breast carcinogenesis. These findings are consistent with Bosviel *et al*. (2012), who reported higher *BRCA1* promoter methylation in peripheral blood DNA of breast cancer patients compared to controls, and Daniels *et al*. (2016), who similarly observed elevated methylation levels in breast cancer patients. The use of peripheral blood DNA for methylation analysis in this study is particularly valuable as it provides a non-invasive biomarker that could potentially be utilized for early detection and risk assessment. The median methylation level of 17.8% observed in patients falls within the range reported in other studies examining blood-based *BRCA1* methylation, supporting the reliability of these findings. However, Anjum *et al*. (2014) demonstrated that blood-based DNA methylation signatures could predict sporadic breast cancer risk and survival, suggesting that the methylation differences observed in this study may have prognostic significance beyond their diagnostic value.

The absence of significant methylation differences across molecular subtypes (p=0.271) or between sporadic and non-sporadic cases (p=0.197) in this study contrasts with some previous reports but aligns with others. While Holm *et al*. (2010) found obvious methylation patterns between molecular subtypes, with luminal B tumors showing the highest methylation levels, other studies have reported similar findings to the current investigation. However, Hosny *et al*. (2016) group found no significant differences in *BRCA1* methylation levels based on molecular subtypes, and Bosviel *et al*. (2012) similarly reported no methylation differences between subtypes in peripheral blood DNA. The lack of association with molecular subtypes

in this study may be attributed to several factors: the use of peripheral blood rather than tumor tissue, which may not fully reflect tumor-specific methylation patterns; the relatively small sample size when divided into subgroups; or population-specific methylation patterns in the Iraqi cohort. Interestingly, while Stefansson *et al.* (2011) specifically associated *BRCA1* methylation with triple-negative breast cancer in tumor tissue, this association was not observed in the current blood-based analysis. These findings suggest that while *BRCA1* promoter methylation in peripheral blood is generally good biomarker for breast cancer presence, it may not be sensitive enough to distinguish between molecular subtypes or familial clustering patterns, which sheds light on the complex relationship between constitutional and tumor-specific methylation events.

4.2.5. Protein expression analysis

The analysis of BRCA1 and BARD1 protein concentrations showed marked reductions in breast cancer patients compared to controls, with both proteins showing approximately 47% and 60% decreases respectively. These findings are consistent with extensive literature documenting reduced expression of these tumor suppressor proteins in breast malignancies. Yoshikawa *et al.* (1999) reported reduced BRCA1 protein expression in Japanese sporadic breast carcinomas, with 79% of BRCA1-associated cases showing reduction or complete loss of nuclear BRCA1 expression. Also, Rio *et al.* (1999) quantified BRCA1 protein levels in sporadic breast carcinomas and found significant decreases compared to normal tissues, regardless of loss of heterozygosity status. The parallel reduction observed for BARD1 protein aligns with findings by Yoshikawa *et al.* (2000), who documented abnormal expression patterns of both BRCA1 and its interacting partner BARD1 in breast carcinomas. The considerable decrease in both proteins observed in this study (BRCA1: 7.887 to 4.194 ng/ml; BARD1: 10.053 to 3.991 ng/ml) reflects the disruption of the BRCA1-BARD1 heterodimer complex, which is necessary for DNA repair and tumor suppression functions.

The differential expression patterns observed between sporadic and non-sporadic cases provide important information on disease mechanisms. BARD1 concentrations were significantly higher in sporadic cases (4.485 ng/ml) compared to non-sporadic cases (3.116 ng/ml), while BRCA1 showed no such distinction. This contrasts with Wu *et al*. (2006), who reported aberrant BARD1 expression with cytoplasmic localization associated with poor prognosis, though their study focused on immunohistochemical expression rather than serum protein concentrations. The higher BARD1 levels in sporadic cases may reflect compensatory mechanisms or differential regulation pathways between hereditary and sporadic breast cancers. In a study, Magdinier *et al*. (1998) found that BRCA1 down-regulation in sporadic breast cancers occurs through mechanisms other than DNA methylation, suggesting complex regulatory processes that may differentially affect BRCA1 and BARD1 expression. The absence of significant differences between Luminal A and Luminal B subtypes for either protein (BRCA1 p=0.093; BARD1 p=0.95) suggests that the reduction in these tumor suppressors is a general feature of breast carcinogenesis rather than subtype-specific. These findings support the potential utility of BRCA1 and BARD1 protein levels as biomarkers for breast cancer detection, as suggested by Śniadecki *et al*. (2020) and Irminger-Finger *et al*. (2012), who explored BARD1 isoforms for cancer screening applications.

4.2.6. miRNA expression analysis

The analysis of miR-498 expression revealed a significant 3-fold upregulation in breast cancer patients compared to healthy controls (median fold change: 2.423 vs 0.796, p<0.001), is a novel finding that contrasts with previous literature reporting miR-498 downregulation in other cancer types. This upregulation in breast cancer patients suggests tissue-specific regulatory mechanisms, as Zhang *et al*. (2018) demonstrated that miR-498 primarily functions as a tumor suppressor in colorectal, lung, and ovarian cancers where it is frequently downregulated. The observed overexpression in breast cancer aligns with findings by Chai *et al*. (2018), who

showed that miR-498 could promote proliferation and migration in breast cancer cells by targeting the tumor suppressor PTEN, indicating its potential oncogenic role in breast tissue. The moderate effect size (r=0.498) observed in this study underscores the biological significance of this dysregulation. Interestingly, Matamala *et al*. (2016) reported miR-498 downregulation specifically in triple-negative breast cancer, suggesting that miR-498 expression patterns may vary across breast cancer subtypes or study populations. The ability of miR-498 to directly target BRCA1's 3'UTR, as demonstrated by Matamala *et al*. (2016), offers a probable mechanistic link to the reduced BRCA1 protein levels observed in the same patient cohort, supporting a regulatory axis between miR-498 upregulation and BRCA1 suppression.

The absence of significant associations between miR-498 expression and hormone receptor status (ER, PR, HER2) contrasts with established literature documenting subtype-specific miRNA signatures in breast cancer. Blenkiron *et al*. (2007) identified distinct miRNA expression profiles that could classify basal versus luminal tumor subtypes, while Lowery *et al*. (2009) showed that specific miRNA signatures could predict ER, PR, and HER2 receptor status. The lack of differential expression across receptor status in this study (ER: p=0.886, PR: p=0.302, HER2: p=0.882) suggests that miR-498 upregulation may represent a general feature of breast carcinogenesis rather than a subtype-specific alteration. This finding disagrees with findings by Radojicic *et al*. (2011), who identified specific miRNAs (miR-21, miR-210, miR-221) with distinct expression patterns in triple-negative breast cancer. The similar expression levels between sporadic and non-sporadic cases (p=0.623) indicate that miR-498 dysregulation occurs independently of hereditary factors. While Leivonen *et al*. (2014) identified miR-498 as essential for HER2-positive breast cancer cell growth, the current study found no association with HER2 status, possibly due to differences in methodology (tissue culture vs patient samples) or population characteristics. The relatively uniform expression across molecular subtypes, despite numerical differences (Luminal A: 2.657, Luminal B: 2.448, triple-negative: 2.189), suggests that miR-498 may serve as a general breast cancer biomarker rather than a subtype-specific marker, contrasting with findings by Søkilde

et al. (2019) who demonstrated that miRNA profiles could refine molecular subtype classification.

4.2.7. Correlation analysis

The correlation analysis showed distinct patterns of molecular biomarker interactions that differed markedly between the overall cohort and breast cancer patients, providing information into the disruption of normal regulatory networks in malignancy. The significant positive correlation between BRCA1 and BARD1 protein concentrations in the overall cohort (ρ= 0.464, p<0.05) aligns with their well-established functional partnership as heterodimeric tumor suppressors in DNA repair pathways. This finding is consistent with those by Myhre *et al*. (2013), who stated that certain cancer-related proteins show correlations between their expression levels that reflect their biological interactions. The negative correlation between miR-498 and BARD1 protein levels (ρ= -0.337, p<0.05) in the overall cohort supports the regulatory role of miRNAs in controlling protein expression, as documented by Petrovic *et al*. (2017), who showed that BRCA1 alone is targeted by up to 100 miRNAs in complex regulatory networks. The absence of significant correlations between BRCA1 methylation and other biomarkers suggests that methylation may operate through independent mechanisms rather than directly influencing protein levels or miRNA expression, contrasting with findings by Aure *et al*. (2021), who described crosstalk between miRNA expression and DNA methylation in hormone-dependent breast cancer phenotypes.

The loss of BRCA1-BARD1 protein correlation in breast cancer patients (ρ= 0.172, p>0.05) represents a critical finding indicating disruption of their normal heterodimeric partnership during carcinogenesis. This disruption may reflect the complex regulatory alterations that occur in breast cancer, where multiple mechanisms including miRNA dysregulation, methylation changes, and post-translational modifications can independently affect each protein. The emergence of a significant positive correlation between miR-498 and BRCA1 protein exclusively

in patients (ρ= 0.475, $p<0.05$) is particularly intriguing, as it contradicts the expected negative regulatory relationship. This paradoxical finding aligns with the miR-498 overexpression observed in patients and suggests potential feedback mechanisms or compensatory responses, similar to complex regulatory networks described by Enerly *et al*. (2011) in their integrated miRNA-mRNA analysis. The loss of miR-498-BARD1 correlation in patients further supports the notion of disrupted regulatory networks in cancer. These findings collectively suggest that breast cancer is characterized not only by altered expression levels of individual biomarkers but also by fundamental disruptions in their normal regulatory relationships, as supported by Moskwa *et al*. (2011) and Chang *et al*. (2011), who explained how miRNA-mediated regulation of *BRCA1* can impact DNA repair pathways and therapeutic sensitivity. The distinct correlation patterns between controls and patients highlight the importance of analyzing biomarker relationships in addition to individual expression levels for understanding breast cancer pathogenesis.

4.2.8. ROC curve analysis

The diagnostic performance of various biomarkers in our study showed BARD1 protein as the most promising candidate with exceptional accuracy (AUC=0.936, sensitivity 88.5%, specificity 95.8%), followed by BRCA1 protein (AUC=0.862, sensitivity 88.5%, specificity 83.3%). These findings align with the growing evidence supporting protein-based biomarkers in cancer detection, similar to Zhang *et al*. (2004) who identified novel protein biomarkers for early-stage ovarian cancer. The moderate performance of miR-498 (AUC=0.766, sensitivity 88.5%, specificity 54.2%) in our cohort is consistent with recent studies showing the diagnostic potential of circulating microRNAs, as demonstrated by Xie *et al*. (2016) who reported 88% sensitivity and 84% specificity for multiple miRNAs in breast cancer diagnosis. Interestingly, while inflammatory markers IL-6 and hs-CRP showed reasonable diagnostic accuracy (AUC=0.732 and 0.731 respectively), similar to the multianalyte panel approach described by Edgell *et al*. (2010), the traditional tumor marker CA

15-3 performed poorly (AUC=0.612), reinforcing findings by Zaleski *et al*. (2018) that CA 15-3 alone has limited diagnostic value and requires combination with other biomarkers. The superior performance of BARD1 and BRCA1 proteins compared to conventional markers suggests their potential as primary screening tools, particularly in populations like ours where late-stage presentation is common, offering a more accurate alternative to traditional approaches and supporting the trend toward multi-biomarker panels for improved cancer detection as advocated by multiple recent studies (Ali *et al*., 2022; Li *et al*., 2025).

Chapter five

CONCLUSIONS & RECOMMENDATIONS

Conclusions and Recommandations

5.1. Conclusions

The current study provides the first comprehensive molecular characterization of breast cancer in Karbala governorate, Iraq, and figures unique genetic, epigenetic, and protein expression patterns that distinguish this population. The combination of multiple biomarkers offers new opportunities for improving breast cancer diagnosis and management in the region. The specific evidence-based conclusions are:

1. Late-stage presentation is prevalent, with 65.6% showing nodal involvement at diagnosis.
2. *BARD1* rs1048108 A allele confers significant protection against breast cancer, particularly the AA genotype.
3. *BRCA1* promoter methylation is significantly elevated in patients compared to controls.
4. BRCA1 and BARD1 proteins are markedly reduced in breast cancer patients, with BARD1 showing exceptional diagnostic accuracy (AUC= 0.936).
5. miR-498 is significantly upregulated in patients (2.4-fold vs 0.8-fold in controls).
6. The BRCA1-BARD1 protein correlation is disrupted in cancer, indicating compromised DNA repair mechanisms.

5.2. Recommendations

The findings of the present study draw attention to the critical areas for intervention to improve breast cancer outcomes in Karbala. Implementation of these recommendations could significantly enhance early detection, diagnosis, and treatment strategies:

1. Establishing population-based screening programs targeting women over 40, particularly those with identified risk factors.
2. Investigating and validate BARD1 and BRCA1 protein deeper to assess their diagnostic accuracy in breast cancer early detection.
3. Improving genetic counseling services for *BARD1* and *BRCA1* polymorphism assessment in high-risk families.
4. Investigating and validate *BRCA1* methylation analysis to identify epigenetically silenced cases.
5. Launching public health campaigns addressing modifiable risk factors, especially hormone replacement therapy use.
6. Establishing a regional biobank for future research and validation studies.
7. Conducting prospective studies to validate biomarker prognostic value and treatment response.
8. Extend molecular studies to other Iraqi governorates for national comparison.

Chapter six

REFERENCES

References

Abdelgawad, I. A., El-Mously, R. H., Saber, M. M., Mansour, O. A., & Shouman, S. A. (2015). Significance of serum levels of vitamin D and some related minerals in breast cancer patients. *International Journal of Clinical and Experimental Pathology*, *8*(4), 4074–4082. http://www.ncbi.nlm.nih.gov/pubmed/26097595

Abedalrahman, S. K., Ali, B. M., Al-Khalidy, N. A. I., & Al-Hashimi, A. S. (2019). Risk factors of breast cancer among Iraqi women. *Journal of Contemporary Medical Sciences*, *5*(3), 149–153. https://doi.org/10.22317/jcms.v5i3.609

Abood, R. A. (2018). Breast cancer in Basra oncology center: A clinico- epidemiological analysis. *Asian Pacific Journal of Cancer Prevention*, *19*(10), 2943–2946. https://doi.org/10.22034/APJCP.2018.19.10.2943

Aguilar-Arnal, L., & Sassone-Corsi, P. (2015). Chromatin landscape and circadian dynamics: Spatial and temporal organization of clock transcription. *Proceedings of the National Academy of Sciences*, *112*(22), 6863–6870. https://doi.org/10.1073/pnas.1411264111

Al-Alwan, N. A. S. (2020). The actual practice of breast self-examination among sample of Iraqi patients with breast cancer. *AL-Kindy College Medical Journal*, *15*(2), 28–34. https://doi.org/10.47723/kcmj.v15i2.157

Al-Fadhli, U. M., Al-Shawi, A. F., & Al-Nuaimi, A. S. (2016). Assessment of sociodemographic characteristics in a sample of breast cancer patients in Baghdad. *Journal of the Faculty of Medicine Baghdad*, *58*(4), 357–360. https://doi.org/10.32007/jfacmedbagdad.584285

Al-Isawi, A. O. J. (2016). Breast cancer in western Iraq: Clinicopathological single institution study. *Advances in Breast Cancer Research*, *05*(02), 83–89. https://doi.org/10.4236/abcr.2016.52009

Al-Mashhadani, Z. I., Mukhlis, A. J. A., & Al-Faraji, A. S. (2015). Estimation of ALP, GPT and GOT activities in Iraqi patients female with breast cancer. *Ibn Al-Haitham Journal For Pure and Applied Sciences*, *25*(1).

Al-Moghrabi, N. M. (2017). BRCA1 promoter methylation in peripheral blood cells and predisposition to breast cancer. *Journal of Taibah University Medical Sciences*, *12*(3), 189–193. https://doi.org/10.1016/j.jtumed.2017.01.004

Al Ansari, M., Dawson, A., Room, R., Abdulzahra, M., & Conigrave, K. M. (2022). Alcohol and university students in Iraq: Attitudes, availability, and use. *Journal of Muslim Mental Health*, *16*(2). https://doi.org/10.3998/jmmh.224

Ali, F. T., Soliman, R. M., Hassan, N. S., Ibrahim, A. M., El-Gizawy, M. M., Mandoh, A. A. Y., & Ibrahim, E. A. (2022). Sensitivity and specificity of microRNA-204, CA125, and CA19.9 as biomarkers for diagnosis of ovarian cancer. *PLOS ONE*, *17*(8), e0272308. https://doi.org/10.1371/journal.pone.0272308

Ali, L. O. (2014). Study effect of breast cancer on some hematological and biochemical parameters in Babylon province, Iraq. *IOSR Journal of Pharmacy and Biological Sciences*, *9*(3), 20–24. https://doi.org/10.9790/3008-09352024

Alshatwi, A. A., Hasan, T. N., Syed, N. A., Shafi, G., & Grace, B. L. (2012). Identification of functional SNPs in BARD1 gene and in silico analysis of damaging SNPs: based on data procured from dbSNP database. *PloS One*, *7*(10), e43939. https://doi.org/10.1371/journal.pone.0043939

Alsheridah, N., & Akhtar, S. (2018). Diet, obesity and colorectal carcinoma risk: results from a national cancer registry-based middle-eastern study. *BMC Cancer*, *18*(1), 1227. https://doi.org/10.1186/s12885-018-5132-9

Alwan, N. A. S., Tawfeeq, F. N., Maallah, M. H., Sattar, S. A., & Saleh, W. A. (2017). The

stage of breast cancer at the time of diagnosis: Correlation with the clinicopathological findings among Iraqi patients. *Journal of Neoplasm*, *02*(03). https://doi.org/10.21767/2576-3903.100020

Alwan, N. A. S., Tawfeeq, F. N., & Mallah, N. A. G. (2019). Demographic and clinical profiles of female patients diagnosed with breast cancer in Iraq. *Journal of Contemporary Medical Sciences*, *5*(1), 14–19. https://doi.org/10.22317/jcms.v5i1.544

Alwan, N. A. S., Tawfeeq, F. N., & Muallah, F. H. (2017). Breast cancer subtypes among Iraqi patients: Identified ny their ER, PR and HER2 status. *Journal of the Faculty of Medicine Baghdad*, *59*(4), 303–307.

Anderson, K. N., Schwab, R. B., & Martinez, M. E. (2014). Reproductive risk factors and breast cancer subtypes: a review of the literature. *Breast Cancer Research and Treatment*, *144*(1), 1–10. https://doi.org/10.1007/s10549-014-2852-7

Anjum, S., Fourkala, E.-O., Zikan, M., Wong, A., Gentry-Maharaj, A., Jones, A., Hardy, R., Cibula, D., Kuh, D., Jacobs, I. J., Teschendorff, A. E., Menon, U., & Widschwendter, M. (2014). A BRCA1-mutation associated DNA methylation signature in blood cells predicts sporadic breast cancer incidence and survival. *Genome Medicine*, *6*(6), 47. https://doi.org/10.1186/gm567

Apostolou, P., & Fostira, F. (2013). Hereditary breast cancer: The Era of new susceptibility genes. *BioMed Research International*, *2013*, 1–11. https://doi.org/10.1155/2013/747318

Arafa, M. A., Waly, M. I., Jriesat, S., Al Khafajei, A., & Sallam, S. (2011). Dietary and lifestyle characteristics of colorectal cancer in Jordan: a case-control study. *Asian Pacific Journal of Cancer Prevention : APJCP*, *12*(8), 1931–1936.

Ashworth, A. (2013). BRCA1/BRCA2. In *Brenner's Encyclopedia of Genetics* (pp. 381–383). Elsevier. https://doi.org/10.1016/B978-0-12-374984-0.00171-6

Aure, M. R., Fleischer, T., Bjørklund, S., Ankill, J., Castro-Mondragon, J. A., Børresen-Dale, A.-L., Tost, J., Sahlberg, K. K., Mathelier, A., Tekpli, X., & Kristensen, V. N. (2021). Crosstalk between microRNA expression and DNA methylation drives the hormone-dependent phenotype of breast cancer. *Genome Medicine*, *13*(1), 72. https://doi.org/10.1186/s13073-021-00880-4

Ayi, T.-C., Tsan, J. T., Hwang, L.-Y., Bowcock, A. M., & Baer, R. (1998). Conservation of function and primary structure in the BRCA1-associated RING domain (BARD1) protein. *Oncogene*, *17*(16), 2143–2148. https://doi.org/10.1038/sj.onc.1202123

Aziz, Z., Sarhat, E., & Zaidan, Z. (2023). Estimation of serum ferroportin and liver enzymes in breast cancer patients. *Georgian Medical News*, *49*(339), 37–41. http://www.tandfonline.com/doi/full/10.3109/13880209.2011.553625

Badowska-Kozakiewicz, A. M., & Budzik, M. P. (2016). Immunohistochemical characteristics of basal-like breast cancer. *Współczesna Onkologia*, *6*, 436–443. https://doi.org/10.5114/wo.2016.56938

Banger, M., Bhise, M., & Khillare, S. (2019). Evaluation biochemical parameter in breast cancer. *International Journal of Biochemistry & Physiology*, *4*(3). https://doi.org/10.23880/IJBP-16000165

Benjamini, Y., & Hochberg, Y. (1995). Controlling the false discovery rate: A practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society Series B: Statistical Methodology*, *57*(1), 289–300. https://doi.org/10.1111/j.2517-6161.1995.tb02031.x

Beremauro, S., & Girio-Fragkoulakis, C. (2022). Imaging techniques in breast cancer. *Surgery (Oxford)*, *40*(2), 94–103. https://doi.org/10.1016/j.mpsur.2021.11.014

Bhatti, P., Struewing, J. P., Alexander, B. H., Hauptmann, M., Bowen, L., Mateus-Pereira,

L. H., Pineda, M. A., Simon, S. L., Weinstock, R. M., Rosenstein, M., Stovall, M., Preston, D. L., Linet, M. S., Doody, M. M., & Sigurdson, A. J. (2008). Polymorphisms in DNA repair genes, ionizing radiation exposure and risk of breast cancer in U.S. Radiologic technologists. *International Journal of Cancer*, *122*(1), 177–182. https://doi.org/10.1002/ijc.23066

Birgisdottir, V., Stefansson, O. A., Bodvarsdottir, S. K., Hilmarsdottir, H., Jonasson, J. G., & Eyfjord, J. E. (2006). Epigenetic silencing and deletion of the BRCA1gene in sporadic breast cancer. *Breast Cancer Research*, *8*(4), R38. https://doi.org/10.1186/bcr1522

Birrane, G., Varma, A. K., Soni, A., & Ladias, J. A. A. (2007). Crystal structure of the BARD1 BRCT domains. *Biochemistry*, *46*(26), 7706–7712. https://doi.org/10.1021/bi700323t

Blenkiron, C., Goldstein, L. D., Thorne, N. P., Spiteri, I., Chin, S.-F., Dunning, M. J., Barbosa-Morais, N. L., Teschendorff, A. E., Green, A. R., Ellis, I. O., Tavaré, S., Caldas, C., & Miska, E. A. (2007). MicroRNA expression profiling of human breast cancer identifies new markers of tumor subtype. *Genome Biology*, *8*(10), R214. https://doi.org/10.1186/gb-2007-8-10-r214

Bochar, D. A., Wang, L., Beniya, H., Kinev, A., Xue, Y., Lane, W. S., Wang, W., Kashanchi, F., & Shiekhattar, R. (2000). BRCA1 is associated with a human SWI/SNF-related complex: linking chromatin remodeling to breast cancer. *Cell*, *102*(2), 257–265. https://doi.org/10.1016/s0092-8674(00)00030-1

Bombonati, A., & Sgroi, D. C. (2011). The molecular pathology of breast cancer progression. *The Journal of Pathology*, *223*(2), 308–318. https://doi.org/10.1002/path.2808

Bork, P., Hofmann, K., Bucher, P., Neuwald, A. F., Altschul, S. F., & Koonin, E. V. (1997). A superfamily of conserved domains in DNA damage- responsive cell cycle checkpoint proteins. *The FASEB Journal*, *11*(1), 68–76. https://doi.org/10.1096/fasebj.11.1.9034168

Bosse, K. R., Diskin, S. J., Cole, K. A., Wood, A. C., Schnepp, R. W., Norris, G., Nguyen, L. B., Jagannathan, J., Laquaglia, M., Winter, C., Diamond, M., Hou, C., Attiyeh, E. F., Mosse, Y. P., Pineros, V., Dizin, E., Zhang, Y., Asgharzadeh, S., Seeger, R. C., Maris, J. M. (2012). Common variation at BARD1 results in the expression of an oncogenic isoform that influences neuroblastoma susceptibility and oncogenicity. *Cancer Research*, *72*(8), 2068–2078. https://doi.org/10.1158/0008-5472.CAN-11-3703

Bosviel, R., Garcia, S., Lavediaux, G., Michard, E., Dravers, M., Kwiatkowski, F., Bignon, Y.-J., & Bernard-Gallon, D. J. (2012). BRCA1 promoter methylation in peripheral blood DNA was identified in sporadic breast cancer and controls. *Cancer Epidemiology*, *36*(3), e177–e182. https://doi.org/10.1016/j.canep.2012.02.001

Bray, F., McCarron, P., & Parkin, D. M. (2004). The changing global patterns of female breast cancer incidence and mortality. *Breast Cancer Research*, *6*(6), 229. https://doi.org/10.1186/bcr932

Brown, C., McDonald, J., Abbas, M., Dunston, G., Kanaan, Y., & Ricks-Santi, L. (2016). Abstract B22: BRCA1 and BRCA2 mutational spectra in African American men with prostate cancer. *Cancer Epidemiology, Biomarkers & Prevention*, *25*(3_Supplement), B22–B22. https://doi.org/10.1158/1538-7755.DISP15-B22

Brzovic, P. S., Meza, J. E., King, M.-C., & Klevit, R. E. (2001). BRCA1 RING domain cancer-predisposing mutations. Structural consequences and effects on protein–protein interactions. *Journal of Biological Chemistry*, *276*(44), 41399–41406.

https://doi.org/10.1074/jbc.M106551200

Brzovic, P. S., Rajagopal, P., Hoyt, D. W., King, M.-C., & Klevit, R. E. (2001). Solution structure of the BRCA1/BARD1 RING-domain heterodimer. In *Worldwide Protein Data Bank* (Vol. 30, Issue 10, pp. 663–670). https://doi.org/10.2210/pdb1jm7/pdb

Buchholz, T. A., Weil, M. M., Story, M. D., Strom, E. A., Brock, W. A., & McNeese, M. D. (1999). Tumor suppressor genes and breast cancer. *Radiation Oncology Investigations*, *7*(2), 55–65. https://doi.org/10.62472.kjps.v15.i25.103-121

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J., & Wittwer, C. T. (2009). The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry*, *55*(4), 611–622. https://doi.org/10.1373/clinchem.2008.112797

Butcher, D. T., & Rodenhiser, D. I. (2007). Epigenetic inactivation of BRCA1 is associated with aberrant expression of CTCF and DNA methyltransferase (DNMT3B) in some sporadic breast tumours. *European Journal of Cancer*, *43*(1), 210–219. https://doi.org/10.1016/j.ejca.2006.09.002

Caestecker, K. W., & Van de Walle, G. R. (2013). The role of BRCA1 in DNA double-strand repair: Past and present. *Experimental Cell Research*, *319*(5), 575–587. https://doi.org/10.1016/j.yexcr.2012.11.013

Campbell, L. L., & Polyak, K. (2007). Breast tumor heterogeneity: Cancer stem cells or clonal evolution? *Cell Cycle*, *6*(19), 2332–2338. https://doi.org/10.4161/cc.6.19.4914

Capasso, M., Devoto, M., Hou, C., Asgharzadeh, S., Glessner, J. T., Attiyeh, E. F., Mosse, Y. P., Kim, C., Diskin, S. J., Cole, K. A., Bosse, K., Diamond, M., Laudenslager, M., Winter, C., Bradfield, J. P., Scott, R. H., Jagannathan, J., Garris, M., McConville, C., … Maris, J. M. (2009). Common variations in BARD1 influence susceptibility to high-risk neuroblastoma. *Nature Genetics*, *41*(6), 718–723. https://doi.org/10.1038/ng.374

Chai, C., Wu, H., Wang, B., Eisenstat, D. D., & Leng, R. P. (2018). MicroRNA-498 promotes proliferation and migration by targeting the tumor suppressor PTEN in breast cancer cells. *Carcinogenesis*, *39*(9), 1185–1196. https://doi.org/10.1093/carcin/bgy092

Chang, S., Wang, R.-H., Akagi, K., Kim, K.-A., Martin, B. K., Cavallone, L., Haines, D. C., Basik, M., Mai, P., Poggi, E., Isaacs, C., Looi, L. M., Mun, K. S., Greene, M. H., Byers, S. W., Teo, S. H., Deng, C.-X., & Sharan, S. K. (2011). Tumor suppressor BRCA1 epigenetically controls oncogenic microRNA-155. *Nature Medicine*, *17*(10), 1275–1282. https://doi.org/10.1038/nm.2459

Cheang, M. C. U., Chia, S. K., Voduc, D., Gao, D., Leung, S., Snider, J., Watson, M., Davies, S., Bernard, P. S., Parker, J. S., Perou, C. M., Ellis, M. J., & Nielsen, T. O. (2009). Ki67 index, HER2 status, and prognosis of patients with luminal B breast cancer. *Journal of the National Cancer Institute*, *101*(10), 736–750. https://doi.org/10.1093/jnci/djp082

Chen, A., Kleiman, F. E., Manley, J. L., Ouchi, T., & Pan, Z.-Q. (2002). Autoubiquitination of the BRCA1·BARD1 RING Ubiquitin Ligase. *Journal of Biological Chemistry*, *277*(24), 22085–22092. https://doi.org/10.1074/jbc.M201252200

Chen, S., & Parmigiani, G. (2007). Meta-analysis of BRCA1 and BRCA2 penetrance. *Journal of Clinical Oncology*. https://doi.org/10.1200/JCO.2006.09.1066

Chen, Y.-Z., Zuo, D., Ren, H.-L., Fan, S.-J., & Ying, G. (2019). Bioinformatics analysis of expression and alterations of BARD1 in breast cancer. *Technology in Cancer Research & Treatment*, *18*. https://doi.org/10.1177/1533033819892260

Chen, Y., Chen, C.-F., Riley, D. J., Allred, D. C., Chen, P.-L., Von Hoff, D., Osborne, C. K., & Lee, W.-H. (1995). Aberrant subcellular localization of BRCA1 in breast cancer. *Science*, *270*(5237), 789–791. https://doi.org/10.1126/science.270.5237.789

Chera, A., Stancu-Cretu, M., Zabet, N. R., & Bucur, O. (2024). Shedding light on DNA methylation and its clinical implications: the impact of long-read-based nanopore technology. *Epigenetics & Chromatin*, *17*(1), 39. https://doi.org/10.1186/s13072-024-00558-2

Choi, M., Scholl, U. I., Ji, W., Liu, T., Tikhonova, I. R., Zumbo, P., Nayir, A., Bakkaloğlu, A., Özen, S., Sanjad, S., Nelson-Williams, C., Farhi, A., Mane, S., & Lifton, R. P. (2009). Genetic diagnosis by whole exome capture and massively parallel DNA sequencing. *Proceedings of the National Academy of Sciences*, *106*(45), 19096–19101. https://doi.org/10.1073/pnas.0910672106

Cimmino, F., Avitabile, M., Diskin, S. J., Vaksman, Z., Pignataro, P., Formicola, D., Cardinale, A., Testori, A., Koster, J., de Torres, C., Devoto, M., Maris, J. M., Iolascon, A., & Capasso, M. (2018). Fine mapping of 2q35 high-risk neuroblastoma locus reveals independent functional risk variants and suggests full-length BARD1 as tumor-suppressor. *International Journal of Cancer*, *143*(11), 2828–2837. https://doi.org/10.1002/ijc.31822

Cimmino, F., Formicola, D., & Capasso, M. (2017). Dualistic role of BARD1 in cancer. *Genes*, *8*(12), 375. https://doi.org/10.3390/genes8120375

Clark, S. L., Rodriguez, A. M., Snyder, R. R., Hankins, G. D. V., & Boehning, D. (2012). Structure-function of the tumor suppressor BRCA1. *Computational and Structural Biotechnology Journal*, *1*(1), e201204005. https://doi.org/10.5936/csbj.201204005

Collaborative Group on Hormonal Factors in Breast Cancer. (2012). Menarche, menopause, and breast cancer risk: individual participant meta-analysis, including 118 964 women with breast cancer from 117 epidemiological studies. *The Lancet. Oncology*, *13*(11), 1141–1151. https://doi.org/10.1016/S1470-2045(12)70425-4

Cope, D. G. (2013). Breast cancer: Reviewing the past to give direction for the future. *Oncology Nursing Forum*, *40*(5), 425–428. https://doi.org/10.1188/13.ONF.425-428

Cox, D. G., Simard, J., Sinnett, D., Hamdi, Y., Soucy, P., Ouimet, M., Barjhoux, L., Verny-Pierre, C., McGuffog, L., Healey, S., Szabo, C., Greene, M. H., Mai, P. L., Andrulis, I. L., Thomassen, M., Gerdes, A.-M., Caligo, M. A., Friedman, E., Laitman, Y., … Perez-Segura, P. (2011). Common variants of the BRCA1 wild-type allele modify the risk of breast cancer in BRCA1 mutation carriers. *Human Molecular Genetics*, *20*(23), 4732–4747. https://doi.org/10.1093/hmg/ddr388

Dai, X., Li, T., Bai, Z., Yang, Y., Liu, X., Zhan, J., & Shi, B. (2015). Breast cancer intrinsic subtype classification, clinical use and future trends. *American Journal of Cancer Research*, *5*(10), 2929–2943.

Daniels, S. L., Burghel, G. J., Chambers, P., Al-Baba, S., Connley, D. D., Brock, I. W., Cramp, H. E., Dotsenko, O., Wilks, O., Wyld, L., Cross, S. S., & Cox, A. (2016). Levels of DNA methylation vary at CpG sites across the BRCA1 promoter, and differ according to triple negative and “BRCA-like” status, in both blood and tumour DNA. *PLOS ONE*, *11*(7), e0160174. https://doi.org/10.1371/journal.pone.0160174

De Cicco, P., Catani, M. V., Gasperi, V., Sibilano, M., Quaglietta, M., & Savini, I. (2019). Nutrition and breast cancer: A literature review on prevention, treatment and recurrence. *Nutrients*, *11*(7), 1514. https://doi.org/10.3390/nu11071514

Dechend, R., Hirano, F., Lehmann, K., Heissmeyer, V., Ansieau, S., Wulczyn, F. G., Scheidereit, C., & Leutz, A. (1999). The Bcl-3 oncoprotein acts as a bridging factor between NF-κB/Rel and nuclear co-regulators. *Oncogene*, *18*(22), 3316–3323. https://doi.org/10.1038/sj.onc.1202717

Devi, L. I., Ralte, L., & Ali, M. A. (2015). Serum biochemical profile of breast cancer patients. *European Journal of Pharmaceutical and Medical Research*, *2*(6), 210–214.

Dhia Al-Deen, L., & Sadik, F. (2009). Age at natural menopause and factors influencing its timing in a sample of Iraqi women in Baghdad. *Iraqi Journal of Community Medicine*, *1*, 1–8.

Dimitrov, S. D., Matousková, E., & Forejt, J. (2001). Expression of BRCA1, NBR1 and NBR2 genes in human breast cancer cells. *Folia Biologica*, *47*(4), 120–127. http://www.ncbi.nlm.nih.gov/pubmed/11508855

Dunning, A. M., Chiano, M., Smith, N. R., Dearden, J., Gore, M., Oakes, S., Wilson, C., Stratton, M., Peto, J., Easton, D., Clayton, D., & Ponder, B. A. J. (1997). Common BRCA1 variants and susceptibility to breast and ovarian cancer in the general population. *Human Molecular Genetics*, *6*(2), 285–289. https://doi.org/10.1093/hmg/6.2.285

Durocher, F. (1996). Comparison of BRCA1 polymorphisms, rare sequence variants and/or missense mutations in unaffected and breast/ovarian cancer populations. *Human Molecular Genetics*, *5*(6), 835–842. https://doi.org/10.1093/hmg/5.6.835

Edgell, T., Martin-Roussety, G., Barker, G., Autelitano, D. J., Allen, D., Grant, P., & Rice, G. E. (2010). Phase II biomarker trial of a multimarker diagnostic for ovarian cancer. *Journal of Cancer Research and Clinical Oncology*, *136*(7), 1079–1088. https://doi.org/10.1007/s00432-009-0755-5

Elhawary, N. A., Ekram, S. N., Sembawa, H. A., Tashkandi, E., Bannani, S., Azher, Z. A., Abumansour, I. S., Almuqati, R. M., Attieh, R., Sindi, I. A., Almutrafi, M., Alsobahi, F., Alghamdi, G., & Elhawary, A. N. (2025). Descriptive epidemiology of female breast cancer around the world: incidence, mortality, and sociodemographic risks and disparities. *International Journal of Environmental Health Research*, 1–15. https://doi.org/10.1080/09603123.2025.2492826

Enerly, E., Steinfeld, I., Kleivi, K., Leivonen, S.-K., Aure, M. R., Russnes, H. G., Rønneberg, J. A., Johnsen, H., Navon, R., Rødland, E., Mäkelä, R., Naume, B., Perälä, M., Kallioniemi, O., Kristensen, V. N., Yakhini, Z., & Børresen-Dale, A.-L. (2011). miRNA-mRNA integrated analysis reveals roles for miRNAs in primary breast tumors. *PLoS ONE*, *6*(2), e16915. https://doi.org/10.1371/journal.pone.0016915

Ergül, E., & Sazci, A. (2001). Molecular genetics of breast cancer. *Turkish Journal of Medical Sciences*, *31*(1).

Esteller, M. (2000). Promoter hypermethylation and BRCA1 inactivation in sporadic breast and ovarian tumors. *Journal of the National Cancer Institute*, *92*(7), 564–569. https://doi.org/10.1093/jnci/92.7.564

Falagas, M. E., Zarkadoulia, E. A., Ioannidou, E. N., Peppas, G., Christodoulou, C., & Rafailidis, P. I. (2007). The effect of psychosocial factors on breast cancer outcome: a systematic review. *Breast Cancer Research*, *9*(4), R44. https://doi.org/10.1186/bcr1744

Faul, F., Erdfelder, E., Lang, A.-G., & Buchner, A. (2007). G*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behavior Research Methods*, *39*(2), 175–191. https://doi.org/10.3758/BF03193146

Feki, A., Jefford, C. E., Berardi, P., Wu, J.-Y., Cartier, L., Krause, K.-H., & Irminger-Finger, I. (2005). BARD1 induces apoptosis by catalysing phosphorylation of p53 by DNA-damage response kinase. *Oncogene*, *24*(23), 3726–3736. https://doi.org/10.1038/sj.onc.1208491

Figueiredo, J. C., Brooks, J. D., Conti, D. V., Poynter, J. N., Teraoka, S. N., Malone, K. E., Bernstein, L., Lee, W. D., Duggan, D. J., Siniard, A., Concannon, P., Capanu, M., Lynch, C. F., Olsen, J. H., Haile, R. W., & Bernstein, J. L. (2011). Risk of contralateral breast cancer associated with common variants in BRCA1 and BRCA2: potential modifying effect of BRCA1/BRCA2 mutation carrier status. *Breast Cancer Research*

and Treatment, *127*(3), 819–829. https://doi.org/10.1007/s10549-010-1285-1

Findlay, J. W. A., & Dillard, R. F. (2007). Appropriate calibration curve fitting in ligand binding assays. *The AAPS Journal*, *9*(2), E260–E267. https://doi.org/10.1208/aapsj0902029

Fu, X., Tan, W., Song, Q., Pei, H., & Li, J. (2022). BRCA1 and breast cancer: Molecular mechanisms and therapeutic strategies. *Frontiers in Cell and Developmental Biology*, *10*. https://doi.org/10.3389/fcell.2022.813457

Gelmon, K. A., Tischkowitz, M., Mackay, H., Swenerton, K., Robidoux, A., Tonkin, K., Hirte, H., Huntsman, D., Clemons, M., Gilks, B., Yerushalmi, R., Macpherson, E., Carmichael, J., & Oza, A. (2011). Olaparib in patients with recurrent high-grade serous or poorly differentiated ovarian carcinoma or triple-negative breast cancer: a phase 2, multicentre, open-label, non-randomised study. *The Lancet Oncology*, *12*(9), 852–861. https://doi.org/10.1016/S1470-2045(11)70214-5

Ghoncheh, M., Pournamdar, Z., & Salehiniya, H. (2016). Incidence and mrtality and epidemiology of breast cancer in the world. *Asian Pacific Journal of Cancer Prevention*, *17*(sup3), 43–46. https://doi.org/10.7314/APJCP.2016.17.S3.43

Giaquinto, A. N., Sung, H., Newman, L. A., Freedman, R. A., Smith, R. A., Star, J., Jemal, A., & Siegel, R. L. (2024). Breast cancer statistics 2024. *CA: A Cancer Journal for Clinicians*, *74*(6), 477–495. https://doi.org/10.3322/caac.21863

Godet, I., & Gilkes, D. M. (2017). BRCA1 and BRCA2 mutations and treatment strategies for breast cancer. *Integrative Cancer Science and Therapeutics*, *4*(1). https://doi.org/10.15761/ICST.1000228

Gopalan, V., Smith, R. A., & Lam, A. K. Y. (2015). Downregulation of microRNA-498 in colorectal cancers and its cellular effects. *Experimental Cell Research*, *330*(2), 423–428. https://doi.org/10.1016/j.yexcr.2014.08.006

Gorodetska, I., Kozeretska, I., & Dubrovska, A. (2019). BRCA genes: The role in genome stability, cancer stemness and therapy resistance. *Journal of Cancer*, *10*(9), 2109–2127. https://doi.org/10.7150/jca.30410

Gudmundsdottir, K., & Ashworth, A. (2006). The roles of BRCA1 and BRCA2 and associated proteins in the maintenance of genomic stability. *Oncogene*, *25*(43), 5864–5874. https://doi.org/10.1038/sj.onc.1209874

Hagemans, M. L. C. (2004). Indicators for the total duration of premenopausal endogenous estrogen exposure in relation to BMD. *Human Reproduction*, *19*(9), 2163–2169. https://doi.org/10.1093/humrep/deh381

Han, Z.-G. (2010). Epigenetic analysis in the search for tumor suppressor genes. *Epigenomics*, *2*(4), 489–493. https://doi.org/10.2217/epi.10.29

Harahap, W. A., Sudji, I. R., & Nindrea, R. D. (2018). BRCA1 promoter methylation and clinicopathological characteristics in sporadic breast cancer patients in Indonesia. *Asian Pacific Journal of Cancer Prevention : APJCP*, *19*(9), 2643–2649. https://doi.org/10.22034/APJCP.2018.19.9.2643

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*(1), 66. https://doi.org/10.1038/s41572-019-0111-2

Hashizume, R., Fukuda, M., Maeda, I., Nishikawa, H., Oyake, D., Yabuki, Y., Ogata, H., & Ohta, T. (2001). The RING heterodimer BRCA1-BARD1 is a ubiquitin ligase inactivated by a breast cancer-derived mutation. *Journal of Biological Chemistry*, *276*(18), 14537–14540. https://doi.org/10.1074/jbc.C000881200

Hashmi, A. A., Hashmi, K. A., Irfan, M., Khan, S. M., Edhi, M. M., Ali, J. P., Hashmi, S. K., Asif, H., Faridi, N., & Khan, A. (2019). Ki67 index in intrinsic breast cancer

subtypes and its association with prognostic parameters. *BMC Research Notes*, *12*(1), 605. https://doi.org/10.1186/s13104-019-4653-x

Hawsawi, Y. M., Shams, A., Theyab, A., Abdali, W. A., Hussien, N. A., Alatwi, H. E., Alzahrani, O. R., Oyouni, A. A. A., Babalghith, A. O., & Alreshidi, M. (2022). BARD1 mystery: tumor suppressors are cancer susceptibility genes. *BMC Cancer*, *22*(1), 599. https://doi.org/10.1186/s12885-022-09567-4

Hedau, S., Batra, M., Singh, U., Bharti, A., Ray, A., & Das, B. (2015). Expression of BRCA1 and BRCA2 proteins and their correlation with clinical staging in breast cancer. *Journal of Cancer Research and Therapeutics*, *11*(1), 158. https://doi.org/10.4103/0973-1482.140985

Hollestelle, A., Wasielewski, M., Martens, J. W. M., & Schutte, M. (2010). Discovering moderate-risk breast cancer susceptibility genes. *Current Opinion in Genetics & Development*, *20*(3), 268–276. https://doi.org/10.1016/j.gde.2010.02.009

Holm, K., Hegardt, C., Staaf, J., Vallon-Christersson, J., Jönsson, G., Olsson, H., Borg, Å., & Ringnér, M. (2010). Molecular subtypes of breast cancer are associated with characteristic DNA methylation patterns. *Breast Cancer Research*, *12*(3), R36. https://doi.org/10.1186/bcr2590

Hortobagyi, G. N., de la Garza Salazar, J., Pritchard, K., Amadori, D., Haidinger, R., Hudis, C. A., Khaled, H., Liu, M.-C., Martin, M., Namer, M., O'Shaughnessy, J. A., Shen, Z. Z., & Albain, K. S. (2005). The global breast cancer burden: Variations in epidemiology and survival. *Clinical Breast Cancer*, *6*(5), 391–401. https://doi.org/10.3816/CBC.2005.n.043

Hosny, M. M., Sabek, N. A., El-Abaseri, T. B., Hassan, F. M., & Farrag, S. H. (2016). Promoter Methylation Status of Breast Cancer Susceptibility Gene 1 and 17 Beta Hydroxysteroid Dehydrogenase Type 1 Gene in Sporadic Breast Cancer Patients. *International Journal of Breast Cancer*, *2016*, 1–12. https://doi.org/10.1155/2016/9545241

Howlader, N., Altekruse, S. F., Li, C. I., Chen, V. W., Clarke, C. A., Ries, L. A. G., & Cronin, K. A. (2014). US incidence of breast cancer subtypes defined by joint hormone receptor and HER2 status. *Journal of the National Cancer Institute*, *106*(5). https://doi.org/10.1093/jnci/dju055

Huang, S., Murphy, L., & Xu, W. (2018). Genes and functions from breast cancer signatures. *BMC Cancer*, *18*(1), 473. https://doi.org/10.1186/s12885-018-4388-4

Imyanitov, E. N. (2021). Cytotoxic and targeted therapy for BRCA1/2-driven cancers. *Hereditary Cancer in Clinical Practice*, *19*(1), 36. https://doi.org/10.1186/s13053-021-00193-y

Inamura, K. (2017). Major tumor suppressor and oncogenic non-coding RNAs: Clinical relevance in lung cancer. *Cells*, *6*(2), 12. https://doi.org/10.3390/cells6020012

Ingvarsson, S. (1999). Molecular genetics of breast cancer progression. *Seminars in Cancer Biology*, *9*(4), 277–288. https://doi.org/10.1006/scbi.1999.0124

Inic, Z., Zegarac, M., Inic, M., Markovic, I., Kozomara, Z., Djurisic, I., Inic, I., Pupic, G., & Jancic, S. (2014). Difference between luminal A and luminal B subtypes according to Ki-67, tumor size, and progesterone receptor negativity providing prognostic information. *Clinical Medicine Insights: Oncology*, *8*. https://doi.org/10.4137/CMO.S18006

Iraqi Cancer Board. (2023). *Iraqi Cancer Registry: Annual report 2023*.

Irminger-Finger, I., André, P.-A., Pilyugin, M., Hegedus, B., Laurent, G. J., & Bianco, A. (2012). Abstract B44: Development of a serum test for the detection of lung cancer based on oncogenic BARD1 isoform expression. *Clinical Cancer Research*,

18(3_Supplement), B44–B44. https://doi.org/10.1158/1078-0432.12AACRIASLC-B44

Irminger-Finger, I., & Leung, W.-C. (2002). BRCA1-dependent and independent functions of BARD1. *The International Journal of Biochemistry & Cell Biology*, *34*(6), 582–587. https://doi.org/10.1016/S1357-2725(01)00161-3

Irminger-Finger, I., Leung, W.-C., Li, J., Dubois-Dauphin, M., Harb, J., Feki, A., Jefford, C. E., Soriano, J. V., Jaconi, M., Montesano, R., & Krause, K.-H. (2001). Identification of BARD1 as Mediator between Proapoptotic Stress and p53-Dependent Apoptosis. *Molecular Cell*, *8*(6), 1255–1266. https://doi.org/10.1016/S1097-2765(01)00406-3

Irminger-Finger, I., Ratajska, M., & Pilyugin, M. (2016). New concepts on BARD1: Regulator of BRCA pathways and beyond. *The International Journal of Biochemistry & Cell Biology*, *72*, 1–17. https://doi.org/10.1016/j.biocel.2015.12.008

Irminger-Finger, I., Soriano, J. V., Vaudan, G., Montesano, R., & Sappino, A.-P. (1998). In vitro repression of Brca1-associated RING domain gene, Bard1, induces phenotypic changes in mammary epithelial cells. *The Journal of Cell Biology*, *143*(5), 1329–1339. https://doi.org/10.1083/jcb.143.5.1329

Isaacs, C., & Rebbeck, T. R. (2007). Hereditary Breast Cancer. In C. Isaacs & T. R. Rebbeck (Eds.), *Hereditary Breast Cancer*. CRC Press. https://doi.org/10.3109/9781420020366

Issac, R. M., Saldanha, P., Mathai, J. M., Mathews, R., Kumari, B., & Chacko, T. (2021). Potential role of BRCA1 protein expression as a prognostic tissue biomarker in breast carcinoma: an immunohistochemical and clinicopathologic study from South India. *Oncology in Clinical Practice*, *17*(5), 205–211. https://doi.org/10.5603/OCP.2021.0031

Jakubowska, A., Gronwald, J., Menkiszak, J., Górski, B., Huzarski, T., Byrski, T., Tołoczko-Grabarek, A., Gilbert, M., Edler, L., Zapatka, M., Eils, R., Lubiński, J., Scott, R. J., & Hamann, U. (2010). BRCA1-associated breast and ovarian cancer risks in Poland: no association with commonly studied polymorphisms. *Breast Cancer Research and Treatment*, *119*(1), 201–211. https://doi.org/10.1007/s10549-009-0390-5

Jan, M., Mattoo, J. A., Salroo, N. A., & Ahangar, S. (2010). Triple assessment in the diagnosis of breast cancer in Kashmir. *Indian Journal of Surgery*, *72*(2), 97–103. https://doi.org/10.1007/s12262-010-0030-7

Jefford, C. E., Feki, A., Harb, J., Krause, K.-H., & Irminger-Finger, I. (2004). Nuclear–cytoplasmic translocation of BARD1 is linked to its apoptotic activity. *Oncogene*, *23*(20), 3509–3520. https://doi.org/10.1038/sj.onc.1207427

Jimenez-Johnson, C. (2008). Understanding the genetics of breast cancer: A clinical overview. *The Internet Journal of Advanced Nursing Practice*, *10*(1). https://doi.org/10.5580/1106

Johnson, N., Fletcher, O., Palles, C., Rudd, M., Webb, E., Sellick, G., dos Santos Silva, I., McCormack, V., Gibson, L., Fraser, A., Leonard, A., Gilham, C., Tavtigian, S. V., Ashworth, A., Houlston, R., & Peto, J. (2007). Counting potentially functional variants in BRCA1, BRCA2 and ATM predicts breast cancer susceptibility. *Human Molecular Genetics*, *16*(9), 1051–1057. https://doi.org/10.1093/hmg/ddm050

Johnson, S. M., Shaw, J. A., & Walker, R. A. (2002). Sporadic breast cancer in young women: Prevalence of loss of heterozygosity at p53 , BRCA1 and BRCA2. *International Journal of Cancer*, *98*(2), 205–209. https://doi.org/10.1002/ijc.10197

Jovanovic, J., Rønneberg, J. A., Tost, J., & Kristensen, V. (2010). The epigenetics of breast cancer. *Molecular Oncology*, *4*(3), 242–254. https://doi.org/10.1016/j.molonc.2010.04.002

Kahán, Z. (2020). [Medical treatment options in BRCA-associated cancers]. *Magyar*

Onkologia, *64*(1), 13–24. https://doi.org/10.7150/ijbs.21635

Kanabrocki, E. L., Murray, D., Hermida, R. C., Scott, G. S., Bremner, W. F., Ryan, M. D., Ayala, D. E., Third, J. L. H. C., Shirazi, P., Nemchausky, B. A., & Hooper, D. C. (2002). Circadian variation in oxidative stress markers in healthy and type II diabetic men. *Chronobiology International*, *19*(2), 423–439. https://doi.org/10.1081/CBI-120002914

Kao, J., & Pollack, J. R. (2006). RNA interference-based functional dissection of the 17q12 amplicon in breast cancer reveals contribution of coamplified genes. *Genes, Chromosomes and Cancer*, *45*(8), 761–769. https://doi.org/10.1002/gcc.20339

Karim, M. O., Khan, K. A., Khan, A. J., Javed, A., Fazid, S., & Aslam, M. I. (2020). Triple assessment of breast lump: Should we perform core biopsy for every patient? *Cureus*, *12*(3), e7479. https://doi.org/10.7759/cureus.7479

Karlsson, E., Waltersson, M. A., Bostner, J., Pérez-Tenorio, G., Olsson, B., Hallbeck, A., & Stål, O. (2011). High-resolution genomic analysis of the 11q13 amplicon in breast cancers identifies synergy with 8p12 amplification, involving the mTOR targets S6K2 and 4EBP1. *Genes, Chromosomes and Cancer*, *50*(10), 775–787. https://doi.org/10.1002/gcc.20900

Karsli-Ceppioglu, S., Dagdemir, A., Judes, G., Ngollo, M., Penault-Llorca, F., Pajon, A., Bignon, Y.-J., & Bernard-Gallon, D. (2014). Epigenetic mechanisms of breast cancer: an update of the current knowledge. *Epigenomics*, *6*(6), 651–664. https://doi.org/10.2217/epi.14.59

Kauraniemi, P., Bärlund, M., Monni, O., & Kallioniemi, A. (2001). New amplified and highly expressed genes discovered in the ERBB2 amplicon in breast cancer by cDNA microarrays. *Cancer Research*, *61*(22), 8235–8240. http://www.ncbi.nlm.nih.gov/pubmed/11719455

Keyhani, M., Nasizadeh, S., & Dehghannejad, A. (2005). Serum CA15-3 measurement in breast cancer patients before and after mastectomy. *Archives of Iranian Medicine*, *8*(4), 263–266.

Khalife, H., Skafi, N., Fayyad-Kazan, M., & Badran, B. (2020). MicroRNAs in breast cancer: New maestros defining the melody. *Cancer Genetics*, *246–247*, 18–40. https://doi.org/10.1016/j.cancergen.2020.08.005

Kim, J. (2021). Identification of microRNAs as diagnostic biomarkers for breast cancer based on the cancer genome atlas. *Diagnostics*, *11*(1), 107. https://doi.org/10.3390/diagnostics11010107

Kleiman, F. E., & Manley, J. L. (1999). Functional interaction of BRCA1-associated BARD1 with polyadenylation factor CstF-50. *Science*, *285*(5433), 1576–1579. https://doi.org/10.1126/science.285.5433.1576

Kleiman, F. E., Wu-Baer, F., Fonseca, D., Kaneko, S., Baer, R., & Manley, J. L. (2005). BRCA1/BARD1 inhibition of mRNA 3′ processing involves targeted degradation of RNA polymerase II. *Genes & Development*, *19*(10), 1227–1237. https://doi.org/10.1101/gad.1309505

Knudson, A. G. (1971). Mutation and cancer: Statistical study of retinoblastoma. *Proceedings of the National Academy of Sciences*, *68*(4), 820–823. https://doi.org/10.1073/pnas.68.4.820

Kuchenbaecker, K. B., Hopper, J. L., Barnes, D. R., Phillips, K.-A., Mooij, T. M., Roos-Blom, M.-J., Jervis, S., van Leeuwen, F. E., Milne, R. L., Andrieu, N., Goldgar, D. E., Terry, M. B., Rookus, M. A., Easton, D. F., Antoniou, A. C., McGuffog, L., Evans, D. G., Barrowdale, D., Frost, D., … Olsson, H. (2017). Risks of breast, ovarian, and contralateral breast cancer for BRCA1 and BRCA2 mutation carriers. *JAMA*, *317*(23), 2402. https://doi.org/10.1001/jama.2017.7112

Kumar, M., Sahu, R. K., Goyal, A., Sharma, S., Kaur, N., Mehrotra, R., Singh, U. R., & Hedau, S. (2017). BRCA1 promoter methylation and expression - associations with ER+, PR+ and HER2+ subtypes of breast carcinoma. *Asian Pacific Journal of Cancer Prevention : APJCP*, *18*(12), 3293–3299. https://doi.org/10.22034/APJCP.2017.18.12.3293

Larsen, M. J., Kruse, T. A., Tan, Q., Lænkholm, A.-V., Bak, M., Lykkesfeldt, A. E., Sørensen, K. P., Hansen, T. v. O., Ejlertsen, B., Gerdes, A.-M., & Thomassen, M. (2013). Classifications within molecular subtypes enables identification of BRCA1/BRCA2 mutation carriers by RNA tumor profiling. *PLoS ONE*, *8*(5), e64268. https://doi.org/10.1371/journal.pone.0064268

Lavoro, A., Scalisi, A., Candido, S., Zanghì, G., Rizzo, R., Gattuso, G., Caruso, G., Libra, M., & Falzone, L. (2022). Identification of the most common BRCA alterations through analysis of germline mutation databases: Is droplet digital PCR an additional strategy for the assessment of such alterations in breast and ovarian cancer families? *International Journal of Oncology*, *60*(5), 58. https://doi.org/10.3892/ijo.2022.5349

Ledwoń, J. K., Hennig, E. E., Maryan, N., Goryca, K., Nowakowska, D., Niwińska, A., & Ostrowski, J. (2013). Common low-penetrance risk variants associated with breast cancer in Polish women. *BMC Cancer*, *13*(1), 510. https://doi.org/10.1186/1471-2407-13-510

Lee, E. Y. H. P. (1995). Tumor suppressor genes and their alterations in breast cancer. *Seminars in Cancer Biology*, *6*(3), 119–125. https://doi.org/10.1006/scbi.1995.0019

Lee, E. Y. H. P., & Muller, W. J. (2010). Oncogenes and tumor suppressor genes. *Cold Spring Harbor Perspectives in Biology*, *2*(10), a003236–a003236. https://doi.org/10.1101/cshperspect.a003236

Lee, Y. H., Kim, J.-H., & Song, G. G. (2014). Genome-wide pathway analysis in neuroblastoma. *Tumor Biology*, *35*(4), 3471–3485. https://doi.org/10.1007/s13277-013-1459-7

Leivonen, S.-K., Sahlberg, K. K., Mäkelä, R., Due, E. U., Kallioniemi, O., Børresen-Dale, A.-L., & Perälä, M. (2014). High-throughput screens identify microRNAs essential for HER2 positive breast cancer cell growth. *Molecular Oncology*, *8*(1), 93–104. https://doi.org/10.1016/j.molonc.2013.10.001

Li, C. M.-C., Cordes, A., Oliphant, M. U. J., Quinn, S. A., Thomas, M., Selfors, L. M., Silvestri, F., Girnius, N., Rinaldi, G., Zoeller, J. J., Shapiro, H., Tsiobikas, C., Gupta, K. P., Pathania, S., Regev, A., Kadoch, C., Muthuswamy, S. K., & Brugge, J. S. (2024). Brca1 haploinsufficiency promotes early tumor onset and epigenetic alterations in a mouse model of hereditary breast cancer. *Nature Genetics*, *56*(12), 2763–2775. https://doi.org/10.1038/s41588-024-01958-6

Li, J., Zhao, B., Huang, T., Qin, Z., & Wang, S. M. (2022). Human BRCA pathogenic variants were originated during recent human history. *Life Science Alliance*, *5*(5), e202101263. https://doi.org/10.26508/lsa.202101263

Li, Q., Wei, W., Jiang, Y., Yang, H., & Liu, J. (2015). Promoter methylation and expression changes of BRCA1 in cancerous tissues of patients with sporadic breast cancer. *Oncology Letters*, *9*(4), 1807–1813. https://doi.org/10.3892/ol.2015.2908

Li, W., & Jiang, H. (2020). Up-regulation of miR-498 inhibits cell proliferation, invasion and migration of hepatocellular carcinoma by targeting FOXO3. *Clinics and Research in Hepatology and Gastroenterology*, *44*(1), 29–37. https://doi.org/10.1016/j.clinre.2019.04.007

Li, X., Wang, F., Lin, F., Xie, B., Liu, Y., Xiao, Y., Qin, K., Li, W., & Zeng, Q. (2025). The diagnostic value of a breast cancer diagnosis model based on serum MiRNAs and serum

tumor markers. *World Journal of Surgical Oncology*, *23*(1), 109. https://doi.org/10.1186/s12957-025-03719-z

Linger, R. J., & Kruk, P. A. (2010). BRCA1 16 years later: risk-associated BRCA1 mutations and their functional implications. *The FEBS Journal*, *277*(15), 3086–3096. https://doi.org/10.1111/j.1742-4658.2010.07735.x

Litton, J. K., Rugo, H. S., Ettl, J., Hurvitz, S. A., Gonçalves, A., Lee, K.-H., Fehrenbacher, L., Yerushalmi, R., Mina, L. A., Martin, M., Roché, H., Im, Y.-H., Quek, R. G. W., Markova, D., Tudor, I. C., Hannah, A. L., Eiermann, W., & Blum, J. L. (2018). Talazoparib in patients with advanced breast cancer and a germline BRCA mutation. *New England Journal of Medicine*, *379*(8), 753–763. https://doi.org/10.1056/NEJMoa1802905

Liu, H., Zhang, H., Sun, X., He, Y., Li, J., & Guo, X. (2013). A cross-sectional study of associations between nonsynonymous mutations of the BARD1 gene and breast cancer in Han Chinese women. *Asia Pacific Journal of Public Health*, *25*(4_suppl), 8S-14S. https://doi.org/10.1177/1010539513497220

Liu, Z. F., Chen, C., Yao, X. L., & Sun, S. R. (2016). [Clinicopathological characteristics and prognosis of different molecular types of breast cancer]. *Zhonghua Yi Xue Za Zhi*, *96*(22), 1733–1737. https://doi.org/10.3760/cma.j.issn.0376-2491.2016.22.004

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods (San Diego, Calif.)*, *25*(4), 402–408. https://doi.org/10.1006/meth.2001.1262

Lixia, M., Zhijian, C., Chao, S., Chaojiang, G., & Congyi, Z. (2007). Alternative splicing of breast cancer associated gene BRCA1 from breast cancer cell line. *Journal of Biochemistry and Molecular Biology*, *40*(1), 15–21. https://doi.org/10.5483/bmbrep.2007.40.1.015

Lohani, K. R., Dhar, A., Chintamani, Jatoi, I., Kumar, S., & Srivastava, A. (2024). Asian Society of Mastology (ASOMA) Guide to Clinical Breast Assessment (CBA). *Indian Journal of Surgery*, *86*(2), 466–476. https://doi.org/10.1007/s12262-023-03881-2

Lopomo, A., & Coppedè, F. (2018). Epigenetic signatures in the diagnosis and prognosis of cancer. In *Epigenetic Mechanisms in Cancer* (pp. 313–343). Elsevier. https://doi.org/10.1016/B978-0-12-809552-2.00012-7

Lowery, A. J., Miller, N., Devaney, A., McNeill, R. E., Davoren, P. A., Lemetre, C., Benes, V., Schmidt, S., Blake, J., Ball, G., & Kerin, M. J. (2009). MicroRNA signatures predict oestrogen receptor, progesterone receptor and HER2/neureceptor status in breast cancer. *Breast Cancer Research*, *11*(3), R27. https://doi.org/10.1186/bcr2257

Madjd, Z., Karimi, A., Molanae, S., & Asadi-Lari, M. (2011). BRCA1 protein expression level and CD44(+) phenotype in breast cancer patients. *Cell Journal*, *13*(3), 155–162. http://www.ncbi.nlm.nih.gov/pubmed/23508738

Magdinier, F., Ribieras, S., Lenoir, G. M., Frappart, L., & Dante, R. (1998). Down-regulation of BRCA1 in human sporadic breast cancer; analysis of DNA methylation patterns of the putative promoter region. *Oncogene*, *17*(24), 3169–3176. https://doi.org/10.1038/sj.onc.1202248

Mahalhal, H. M., & Ghafel, H. H. (2021). Dietary habits of Iraqi women with breast cancer at oncology hospitals in Baghdad city: Comparative study. *Indian Journal of Forensic Medicine & Toxicology*, *15*(1), 8915–8940. https://doi.org/10.37506/ijfmt.v15i1.13748

Mahmoud, A. M., Macias, V., Al-alem, U., Deaton, R. J., Kadjaksy-Balla, A., Gann, P. H., & Rauscher, G. H. (2017). BRCA1 protein expression and subcellular localization in primary breast cancer: Automated digital microscopy analysis of tissue microarrays. *PLOS ONE*, *12*(9), e0184385. https://doi.org/10.1371/journal.pone.0184385

Mallery, D. L. (2002). Activation of the E3 ligase function of the BRCA1/BARD1 complex by polyubiquitin chains. *The EMBO Journal*, *21*(24), 6755–6762. https://doi.org/10.1093/emboj/cdf691

Mancini, D. N., Rodenhiser, D. I., Ainsworth, P. J., O'Malley, F. P., Singh, S. M., Xing, W., & Archer, T. K. (1998). CpG methylation within the 5′ regulatory region of the BRCA1 gene is tumor specific and includes a putative CREB binding site. *Oncogene*, *16*(9), 1161–1169. https://doi.org/10.1038/sj.onc.1201630

Matamala, N., Vargas, M. T., González-Cámpora, R., Arias, J. I., Menéndez, P., Andrés-León, E., Yanowsky, K., Llaneza-Folgueras, A., Miñambres, R., Martínez-Delgado, B., & Benítez, J. (2016). MicroRNA deregulation in triple negative breast cancer reveals a role of miR-498 in regulating BRCA1 expression. *Oncotarget*, *7*(15), 20068–20079. https://doi.org/10.18632/oncotarget.7705

Matros, E., Wang, Z. C., Lodeiro, G., Miron, A., Iglehart, J. D., & Richardson, A. L. (2005). BRCA1 promoter methylation in sporadic breast tumors: relationship to gene expression profiles. *Breast Cancer Research and Treatment*, *91*(2), 179–186. https://doi.org/10.1007/s10549-004-7603-8

McCarthy, E. E., Celebi, J. T., Baer, R., & Ludwig, T. (2003). Loss of Bard1, the heterodimeric partner of the Brca1 tumor suppressor, results in early embryonic lethality and chromosomal instability. *Molecular and Cellular Biology*, *23*(14), 5056–5063. https://doi.org/10.1128/MCB.23.14.5056-5063.2003

Menke, C., Nagaraj, C. B., Dawson, B., He, H., Tawde, S., & Wakefield, E. G. (2021). Understanding and interpretation of a variant of uncertain significance (VUS) genetic test result by pediatric providers who do not specialize in genetics. *Journal of Genetic Counseling*, *30*(6), 1559–1569. https://doi.org/10.1002/jgc4.1422

Messier, T. L., Gordon, J. A. R., Boyd, J. R., Tye, C. E., Browne, G., Stein, J. L., Lian, J. B., & Stein, G. S. (2016). Histone H3 lysine 4 acetylation and methylation dynamics define breast cancer subtypes. *Oncotarget*, *7*(5), 5094–5109. https://doi.org/10.18632/oncotarget.6922

Meza, J. E., Brzovic, P. S., King, M.-C., & Klevit, R. E. (1999). Mapping the functional domains of BRCA1. Interaction of the ring finger domains of BRCA1 and BARD1. *Journal of Biological Chemistry*, *274*(9), 5659–5665. https://doi.org/10.1074/jbc.274.9.5659

Miyamoto, K. (2002). Promoter hypermethylation and post-transcriptional mechanisms for reduced BRCA1 immunoreactivity in sporadic human breast cancers. *Japanese Journal of Clinical Oncology*, *32*(3), 79–84. https://doi.org/10.1093/jjco/hyf020

Mohammed, B. J., Al-Thwani, A. N., & Kannan, R. (2016). Demographic and genetic study for a sample of Iraqi smokers. *Cancer Biology*, *6*(4), 16–27.

Mohammed Bakheet, M., Mohssin Ali, H., & Jalil Talab, T. (2024). Evaluation of some proinflammatory cytokines and biochemical parameters in pre and postmenopausal breast cancer women. *Cytokine*, *179*(3), 156632. https://doi.org/10.1016/j.cyto.2024.156632

Molah Karim, S. A., Ali Ghalib, H. H., Rahim Fattah, F. H., Mohialdeen Gubari, M. I., & Baiz Majeed, A. (2015). Height, weight, and body mass index association with breast cancer risk in Iraqi Kurdish women. *Case Studies in Surgery*, *1*(1), 1–7. https://doi.org/10.5430/css.v1n1p1

Moore, L. D., Le, T., & Fan, G. (2013). DNA methylation and its basic function. *Neuropsychopharmacology*, *38*(1), 23–38. https://doi.org/10.1038/npp.2012.112

Moskwa, P., Buffa, F. M., Pan, Y., Panchakshari, R., Gottipati, P., Muschel, R. J., Beech, J., Kulshrestha, R., Abdelmohsen, K., Weinstock, D. M., Gorospe, M., Harris, A. L.,

Helleday, T., & Chowdhury, D. (2011). miR-182-mediated downregulation of BRCA1 impacts DNA repair and sensitivity to PARP inhibitors. *Molecular Cell*, *41*(2), 210–220. https://doi.org/10.1016/j.molcel.2010.12.005

Mu, H., Zhang, W., Qiu, Y., Tao, T., Wu, H., Chen, Z., & Xu, G. (2021). miRNAs as potential markers for breast cancer and regulators of tumorigenesis and progression (Review). *International Journal of Oncology*, *58*(5), 16. https://doi.org/10.3892/ijo.2021.5196

Mukhana, L., Aissa, A. A., Ahmed, A. A. M., Saakyan, S. V., Tsygankov, A. Y., Blagonravov, M. L., & Azova, M. M. (2023). Association of BARD1 and BRIP1 gene polymorphisms with the risk of uveal melanoma. *Bulletin of Experimental Biology and Medicine*, *175*(3), 399–403. https://doi.org/10.1007/s10517-023-05875-2

Muñoz, J. P., Pérez-Moreno, P., Pérez, Y., & Calaf, G. M. (2023). The role of microRNAs in breast cancer and the challenges of their clinical application. *Diagnostics*, *13*(19), 3072. https://doi.org/10.3390/diagnostics13193072

Mutar, M. T., Goyani, M. S., Had, A. M., & Mahmood, A. S. (2019). Pattern of presentation of patients with breast cancer in Iraq in 2018: A cross-sectional study. *Journal of Global Oncology*, *5*, 1–6. https://doi.org/10.1200/JGO.19.00041

Myhre, S., Lingjærde, O.-C., Hennessy, B. T., Aure, M. R., Carey, M. S., Alsner, J., Tramm, T., Overgaard, J., Mills, G. B., Børresen-Dale, A.-L., & Sørlie, T. (2013). Influence of DNA copy number and mRNA levels on the expression of breast cancer related proteins. *Molecular Oncology*, *7*(3), 704–718. https://doi.org/10.1016/j.molonc.2013.02.018

Nelson, A. C., & Holt, J. T. (2010). Impact of RING and BRCT domain mutations on BRCA1 protein stability, localization and recruitment to DNA damage. *Radiation Research*, *174*(1), 1–13. https://doi.org/10.1667/RR1290.1

Niwa, Y., Oyama, T., & Nakajima, T. (2000). BRCA1 expression status in relation to DNA methylation of the BRCA1 promoter region in sporadic breast cancers. *Japanese Journal of Cancer Research*, *91*(5), 519–526. https://doi.org/10.1111/j.1349-7006.2000.tb00976.x

Orban, T. I. (2003). Emerging roles of BRCA1 alternative splicing. *Molecular Pathology*, *56*(4), 191–197. https://doi.org/10.1136/mp.56.4.191

Osborne, C., Ostir, G. V., Du, X., Peek, M. K., & Goodwin, J. S. (2005). The influence of marital status on the stage at diagnosis, treatment, and survival of older women with breast cancer. *Breast Cancer Research and Treatment*, *93*(1), 41–47. https://doi.org/10.1007/s10549-005-3702-4

Oubaddou, Y., Oukabli, M., Fenniche, S., Elktaibi, A., Elochi, M. R., Al Bouzidi, A., Qmichou, Z., Dakka, N., Diorio, C., Richter, A., Bakri, Y., & Ameziane El Hassani, R. (2023). BRCA1 promoter hypermethylation in malignant breast tumors and in the histologically normal adjacent tissues to the tumors: Exploring its potential as a biomarker and its clinical significance in a translational approach. *Genes*, *14*(9), 1680. https://doi.org/10.3390/genes14091680

Pal, B., & Anderson, R. L. (2018). MiRNAs prognostic for basal and BRCA1 breast cancer. *Oncotarget*, *9*(87), 35717–35718. https://doi.org/10.18632/oncotarget.26297

Panagopoulou, M., Panou, T., Gkountakos, A., Tarapatzi, G., Karaglani, M., Tsamardinos, I., & Chatzaki, E. (2024). BRCA1 & BRCA2 methylation as a prognostic and predictive biomarker in cancer: Implementation in liquid biopsy in the era of precision medicine. *Clinical Epigenetics*, *16*(1), 178. https://doi.org/10.1186/s13148-024-01787-8

Paul, A. (2014). The breast cancer susceptibility genes (BRCA) in breast and ovarian cancers. *Frontiers in Bioscience*, *19*(4), 605. https://doi.org/10.2741/4230

Pavelić, K., & Gall-Trošelj, K. (2001). Recent advances in molecular genetics of breast cancer. *Journal of Molecular Medicine*, *79*(10), 566–573. https://doi.org/10.1007/s001090100256

Pelletier, C., Speed, W. C., Paranjape, T., Keane, K., Blitzblau, R., Hollestelle, A., Safavi, K., van den Ouweland, A., Zelterman, D., Slack, F. J., Kidd, K. K., & Weidhaas, J. B. (2011). Rare BRCA1 haplotypes including 3'UTR SNPs associated with breast cancer risk. *Cell Cycle*, *10*(1), 90–99. https://doi.org/10.4161/cc.10.1.14359

Petrovic, N., Davidovic, R., Bajic, V., Obradovic, M., & Isenovic, R. E. (2017). MicroRNA in breast cancer: The association with BRCA1/2. *Cancer Biomarkers*, *19*(2), 119–128. https://doi.org/10.3233/CBM-160319

Polyak, K. (2007). Breast cancer: origins and evolution. *Journal of Clinical Investigation*, *117*(11), 3155–3163. https://doi.org/10.1172/JCI33295

Quann, K., Jing, Y., & Rigoutsos, I. (2015). Post-transcriptional regulation of BRCA1 through its coding sequence by the miR-15/107 group of miRNAs. *Frontiers in Genetics*, *6*. https://doi.org/10.3389/fgene.2015.00242

Radojicic, J., Zaravinos, A., Vrekoussis, T., Kafousi, M., Spandidos, D. A., & Stathopoulos, E. N. (2011). MicroRNA expression analysis in triple-negative (ER, PR and Her2/neu) breast cancer. *Cell Cycle*, *10*(3), 507–517. https://doi.org/10.4161/cc.10.3.14754

Raheem, A. R., Abdul-Rasheed, O. F., Khattab, O. S., Alsammarraie, A. Z., Al-Aubaidy, H., & Abid, H. A. (2024). Circulating miRNA-373 and vascular endothelial growth factor as potential biomarkers for early detection of breast cancer. *Indian Journal of Clinical Biochemistry*. https://doi.org/10.1007/s12291-023-01174-9

Rakha, E. A., El-Sheikh, S. E., Kandil, M. A., El-Sayed, M. E., Green, A. R., & Ellis, I. O. (2008). Expression of BRCA1 protein in breast cancer and its prognostic significance. *Human Pathology*, *39*(6), 857–865. https://doi.org/10.1016/j.humpath.2007.10.011

Rashmi, R., Prasad, K., & Udupa, C. B. K. (2022). Breast histopathological image analysis using image processing techniques for diagnostic purposes: A methodological review. *Journal of Medical Systems*, *46*(1), 7. https://doi.org/10.1007/s10916-021-01786-9

Razack, N. A., & Prabhuswamimath, S. C. (2024). Exploring high-penetrance genes in breast cancer associated genetic syndromes: Insights into genetic predisposition and clinical implications. *International Journal of Health and Allied Sciences*, *13*(1). https://doi.org/10.55691/2278-344X.1091

Rebbeck, T. R., Mitra, N., Wan, F., Sinilnikova, O. M., Healey, S., McGuffog, L., Mazoyer, S., Chenevix-Trench, G., Easton, D. F., Antoniou, A. C., Nathanson, K. L., CIMBA Consortium, Laitman, Y., Kushnir, A., Paluch-Shimon, S., Berger, R., Zidan, J., Friedman, E., Ehrencrona, H., … Andrulis, I. (2015). Association of type and location of BRCA1 and BRCA2 mutations with risk of breast and ovarian cancer. *JAMA*, *313*(13), 1347–1361. https://doi.org/10.1001/jama.2014.5985

Rice, J. C. (2000). Transcriptional repression of BRCA1 by aberrant cytosine methylation, histone hypoacetylation and chromatin condensation of the BRCA1 promoter. *Nucleic Acids Research*, *28*(17), 3233–3239. https://doi.org/10.1093/nar/28.17.3233

Rice, J. C., Ozcelik, H., Maxeiner, P., Andrulis, I., & Futscher, B. W. (2000). Methylation of the BRCA1 promoter is associated with decreased BRCA1 mRNA levels in clinical breast cancer specimens. *Carcinogenesis*, *21*(9), 1761–1765. https://doi.org/10.1093/carcin/21.9.1761

Ricks-Santi, L. J., Sucheston, L. E., Yang, Y., Freudenheim, J. L., Isaacs, C. J., Schwartz, M. D., Dumitrescu, R. G., Marian, C., Nie, J., Vito, D., Edge, S. B., & Shields, P. G. (2011). Association of Rad51 polymorphism with DNA repair in BRCA1 mutation carriers and sporadic breast cancer risk. *BMC Cancer*, *11*(1), 278.

https://doi.org/10.1186/1471-2407-11-278

Ricks-Santi, L. J., Nie, J., Marian, C., Ochs-Balcom, H. M., Trevisan, M., Edge, S. B., Freudenheim, J. L., & Shields, P. G. (2013). BRCA1 polymorphisms and breast cancer epidemiology in the Western New York Exposures and Breast Cancer (WEB) study. *Genetic Epidemiology*, *37*(5), 504–511. https://doi.org/10.1002/gepi.21730

Rio, P. G., Maurizis, J.-C., Peffault de Latour, M., Bignon, Y.-J., & Bernard-Gallon, D. J. (1999). Quantification of BRCA1 protein in sporadic breast carcinoma with or without loss of heterozygosity of theBRCA1 gene. *International Journal of Cancer*, *80*(6), 823–826.

Rivera-Franco, M. M., & Leon-Rodriguez, E. (2018). Delays in breast cancer detection and treatment in developing countries. *Breast Cancer: Basic and Clinical Research*, *12*. https://doi.org/10.1177/1178223417752677

Robson, M., Im, S.-A., Senkus, E., Xu, B., Domchek, S. M., Masuda, N., Delaloge, S., Li, W., Tung, N., Armstrong, A., Wu, W., Goessl, C., Runswick, S., & Conte, P. (2017). Olaparib for metastatic breast cancer in patients with a germline BRCA mutation. *The New England Journal of Medicine*, *377*(6), 523–533. https://doi.org/10.1056/NEJMoa1706450

Rodriguez, I., Smith, C., Pennil, C., Radke, M., Bernards, S., Manhardt, E., Dobrovic, A., Konnick, E., & Swisher, E. (2022). Development and validation of a novel quantitative BRCA1 promoter methylation assay in ovarian carcinoma. *Gynecologic Oncology*, *164*(1), 4. https://doi.org/10.1016/j.ygyno.2021.10.055

Roman Lay, A. A., do Nascimento, C. F., Horta, B. L., & Dias Porto Chiavegatto Filho, A. (2020). Reproductive factors and age at natural menopause: A systematic review and meta-analysis. *Maturitas*, *131*, 57–64. https://doi.org/10.1016/j.maturitas.2019.10.012

Roy, R., Chun, J., & Powell, S. N. (2012a). BRCA1 and BRCA2: different roles in a common pathway of genome protection. *Nature Reviews Cancer*, *12*(1), 68–78. https://doi.org/10.1038/nrc3181

Roy, R., Chun, J., & Powell, S. N. (2012b). BRCA1 and BRCA2: important differences with common interests. *Nature Reviews Cancer*, *12*(5), 372–372. https://doi.org/10.1038/nrc3181-c3

Ruiz de Garibay, G., Fernandez-Garcia, I., Mazoyer, S., Leme de Calais, F., Ameri, P., Vijayakumar, S., Martinez-Ruiz, H., Damiola, F., Barjhoux, L., Thomassen, M., Andersen, L. v. B., Herranz, C., Mateo, F., Palomero, L., Espín, R., Gómez, A., García, N., Jimenez, D., Bonifaci, N., … Pujana, M. A. (2021). Altered regulation of BRCA1 exon 11 splicing is associated with breast cancer risk in carriers of BRCA1 pathogenic variants. *Human Mutation*, *42*(11), 1488–1502. https://doi.org/10.1002/humu.24276

Russi, M., Marson, D., Fermeglia, A., Aulic, S., Fermeglia, M., Laurini, E., & Pricl, S. (2022). The fellowship of the RING: BRCA1, its partner BARD1 and their liaison in DNA repair and cancer. *Pharmacology & Therapeutics*, *232*, 108009. https://doi.org/10.1016/j.pharmthera.2021.108009

Ryser, S., Dizin, E., Jefford, C. E., Delaval, B., Gagos, S., Christodoulidou, A., Krause, K.-H., Birnbaum, D., & Irminger-Finger, I. (2009). Distinct roles of BARD1 isoforms in mitosis: full length BARD1 mediates Aurora B degradation, cancer-associated BARD1β scaffolds Aurora B and BRCA2. *Cancer Research*, *69*(3), 1125–1134. https://doi.org/10.1158/0008-5472.CAN-08-2134

Saelee, P., Chaiwerawattana, A., Ogawa, K., Cho, Y.-M., Tiwawech, D., & Suktangman, V. (2015). Clinicopathological significance of BRCA1 promoter hypermethylation in Thai breast cancer patients. *Asian Pacific Journal of Cancer Prevention*, *15*(24), 10585–10589. https://doi.org/10.7314/APJCP.2014.15.24.10585

Scheiermann, C., Kunisaki, Y., & Frenette, P. S. (2013). Circadian control of the immune system. *Nature Reviews Immunology*, *13*(3), 190–198. https://doi.org/10.1038/nri3386

Schüchner, S., Tembe, V., Rodriguez, J. A., & Henderson, B. R. (2005). Nuclear targeting and cell cycle regulatory function of human BARD1. *Journal of Biological Chemistry*, *280*(10), 8855–8861. https://doi.org/10.1074/jbc.M413741200

Schuuring, E., Verhoeven, E., van Tinteren, H., Peterse, J. L., Nunnink, B., Thunnissen, F. B., Devilee, P., Cornelisse, C. J., van de Vijver, M. J., & Mooi, W. J. (1992). Amplification of genes within the chromosome 11q13 region is indicative of poor prognosis in patients with operable breast cancer. *Cancer Research*, *52*(19), 5229–5234. http://www.ncbi.nlm.nih.gov/pubmed/1394126

Seymour, I. J., Casadei, S., Zampiga, V., Rosato, S., Danesi, R., Falcini, F., Strada, M., Morini, N., Naldoni, C., Paradiso, A., Tommasi, S., Schittulli, F., Amadori, D., & Calistri, D. (2008). Disease family history and modification of breast cancer risk in common BRCA2 variants. *Oncology Reports*, *19*(3), 783–786. https://doi.org/10.3892/or.19.3.783

Shabbeer, S., Omer, D., Berneman, D., Weitzman, O., Alpaugh, A., Pietraszkiewicz, A., Metsuyanim, S., Shainskaya, A., Papa, M. Z., & Yarden, R. I. (2013). BRCA1 targets G2/M cell cycle proteins for ubiquitination and proteasomal degradation. *Oncogene*, *32*(42), 5005–5016. https://doi.org/10.1038/onc.2012.522

Shanazarov, N., Zhapparov, Y., Kumisbekova, R., Turzhanova, D., & Zulkhash, N. (2023). Association of gene polymorphisms with breast cancer risk in the Kazakh population. *Asian Pacific Journal of Cancer Prevention*, *24*(12), 4195–4207. https://doi.org/10.31557/APJCP.2023.24.12.4195

Sharma, B., Preet Kaur, R., Raut, S., & Munshi, A. (2018). BRCA1 mutation spectrum, functions, and therapeutic strategies: The story so far. *Current Problems in Cancer*, *42*(2), 189–207. https://doi.org/10.1016/j.currproblcancer.2018.01.001

Sharma, P., Stecklein, S. R., Kimler, B. F., Sethi, G., Petroff, B. K., Phillips, T. A., Tawfik, O. W., Godwin, A. K., & Jensen, R. A. (2014). The prognostic value of BRCA1 promoter methylation in early stage triple negative breast cancer. *Journal of Cancer Therapeutics and Research*, *3*(1), 2. https://doi.org/10.7243/2049-7962-3-2

Shestakova, E. A. (2016). Epigenetic regulation of BRCA1 expression and its role inbreast cancer stem cell development. *Turkish Journal of Biology*, *40*, 981–989. https://doi.org/10.3906/biy-1507-145

Shiovitz, S., & Korde, L. A. (2015). Genetics of breast cancer: a topic in evolution. *Annals of Oncology*, *26*(7), 1291–1299. https://doi.org/10.1093/annonc/mdv022

Shiu, K.-K., Wetterskog, D., Mackay, A., Natrajan, R., Lambros, M., Sims, D., Bajrami, I., Brough, R., Frankum, J., Sharpe, R., Marchio, C., Horlings, H., Reyal, F., van der Vijver, M., Turner, N., Reis-Filho, J. S., Lord, C. J., & Ashworth, A. (2014). Integrative molecular and functional profiling of ERBB2-amplified breast cancers identifies new genetic dependencies. *Oncogene*, *33*(5), 619–631. https://doi.org/10.1038/onc.2012.625

Shwana, J. J. R., Metwally Gad, Z., Samy Abd Elhafeez, S., Anwar Hama Ghareeb, K., Shwana, S., Mustafa Al Talabani, B. G., Ali Taha, D., Mohialdeen Gubari, M. I., Rahim Fattah, F. H., Rasul Ali, S., & Dlshad Ali, D. (2020). An epidemiological study of female breast cancer in Sulaymaniyah city, Iraqi Kurdistan. *Tabari Biomedical Student Research Journal*. https://doi.org/10.18502/tbsrj.v2i1.2577

Singh, A. K., Pandey, A., Tewari, M., Shukla, H. S., & Pandey, H. P. (2011). Epigenetic silencing of BRCA1 gene associated with demographic and pathologic factors in sporadic breast cancer. *European Journal of Cancer Prevention*, *20*(6), 478–483.

https://doi.org/10.1097/CEJ.0b013e32834761a6

Snell, C., Krypuy, M., Wong, E. M., Loughrey, M. B., & Dobrovic, A. (2008). BRCA1 promoter methylation in peripheral blood DNA of mutation negative familial breast cancer patients with a BRCA1tumour phenotype. *Breast Cancer Research*, *10*(1), R12. https://doi.org/10.1186/bcr1858

Śniadecki, M., Brzeziński, M., Darecka, K., Klasa-Mazurkiewicz, D., Poniewierza, P., Krzeszowiec, M., Kmieć, N., & Wydra, D. (2020). BARD1 and breast cancer: The possibility of creating screening tests and new preventive and therapeutic pathways for predisposed women. *Genes*, *11*(11), 1251. https://doi.org/10.3390/genes11111251

Søkilde, R., Persson, H., Ehinger, A., Pirona, A. C., Fernö, M., Hegardt, C., Larsson, C., Loman, N., Malmberg, M., Rydén, L., Saal, L., Borg, Å., Vallon-Christerson, J., & Rovira, C. (2019). Refinement of breast cancer molecular classification by miRNA expression profiles. *BMC Genomics*, *20*(1), 503. https://doi.org/10.1186/s12864-019-5887-7

Sokolova, A., Johnstone, K. J., McCart Reed, A. E., Simpson, P. T., & Lakhani, S. R. (2023). Hereditary breast cancer: syndromes, tumour pathology and molecular testing. *Histopathology*, *82*(1), 70–82. https://doi.org/10.1111/his.14808

Song, X., Beck, C. R., Du, R., Campbell, I. M., Coban-Akdemir, Z., Gu, S., Breman, A. M., Stankiewicz, P., Ira, G., Shaw, C. A., & Lupski, J. R. (2018). Predicting human genes susceptible to genomic instability associated with Alu / Alu -mediated rearrangements. *Genome Research*, *28*(8), 1228–1242. https://doi.org/10.1101/gr.229401.117

Sotiriou, C., & Pusztai, L. (2003). Gene-expression signatures in breast cancer. *New England Journal of Medicine*, *348*(17), 1715–1717. https://doi.org/10.1056/NEJM200304243481716

Sporn, J. C., Hothorn, T., & Jung, B. (2011). BARD1 expression predicts outcome in colon cancer. *Clinical Cancer Research*, *17*(16), 5451–5462. https://doi.org/10.1158/1078-0432.CCR-11-0263

Ståhlberg, A., Håkansson, J., Xian, X., Semb, H., & Kubista, M. (2004). Properties of the reverse transcription reaction in mRNA quantification. *Clinical Chemistry*, *50*(3), 509–515. https://doi.org/10.1373/clinchem.2003.026161

Starita, L. (2003). The multiple nuclear functions of BRCA1: transcription, ubiquitination and DNA repair. *Current Opinion in Cell Biology*, *15*(3), 345–350. https://doi.org/10.1016/S0955-0674(03)00042-5

Starita, L. M., Horwitz, A. A., Keogh, M.-C., Ishioka, C., Parvin, J. D., & Chiba, N. (2005). BRCA1/BARD1 ubiquitinate phosphorylated RNA polymerase II. *Journal of Biological Chemistry*, *280*(26), 24498–24505. https://doi.org/10.1074/jbc.M414020200

Stefansson, O. A., Hilmarsdottir, H., Olafsdottir, K., Tryggvadottir, L., Sverrisdottir, A., Johannsson, O. T., Jonasson, J. G., Eyfjord, J. E., & Sigurdsson, S. (2020). Brca1 promoter methylation status in 1031 primary breast cancers predicts favorable outcomes following chemotherapy. *JNCI Cancer Spectrum*, *4*(2). https://doi.org/10.1093/jncics/pkz100

Stefansson, O. A., Jonasson, J. G., Olafsdottir, K., Hilmarsdottir, H., Olafsdottir, G., Esteller, M., Johannsson, O. T., & Eyfjord, J. E. (2011). CpG island hypermethylation of BRCA1 and loss of pRb as co-occurring events in basal/triple-negative breast cancer. *Epigenetics*, *6*(5), 638–649. https://doi.org/10.4161/epi.6.5.15667

Stefansson, O. A., Villanueva, A., Vidal, A., Martí, L., & Esteller, M. (2012). BRCA1 epigenetic inactivation predicts sensitivity to platinum-based chemotherapy in breast and ovarian cancer. *Epigenetics*, *7*(11), 1225–1229. https://doi.org/10.4161/epi.22561

Stratton, M. R., & Rahman, N. (2008). The emerging landscape of breast cancer susceptibility. *Nature Genetics*, *40*(1), 17–22. https://doi.org/10.1038/ng.2007.53

Subramanian, K., & Sinha, R. (2024). Functions of differentially regulated miRNAs in breast cancer progression: Potential markers for early detection and candidates for therapy. *Biomedicines*, *12*(3), 691. https://doi.org/10.3390/biomedicines12030691

Sun, X., Wang, X., Zhang, J., Zhao, Z., Feng, X., Liu, L., & Ma, Z. (2021). Efficacy and safety of PARP inhibitors in patients with BRCA-mutated advanced breast cancer: A meta-analysis and systematic review. *The Breast*, *60*, 26–34. https://doi.org/10.1016/j.breast.2021.08.009

Suszynska, M., Kluzniak, W., Wokolorczyk, D., Jakubowska, A., Huzarski, T., Gronwald, J., Debniak, T., Szwiec, M., Ratajska, M., Klonowska, K., Narod, S., Bogdanova, N., Dörk, T., Lubinski, J., Cybulski, C., & Kozlowski, P. (2019). Bard1 is a low/moderate breast cancer risk gene: Evidence based on an association study of the central european p.q564x recurrent mutation. *Cancers*, *11*(6), 740. https://doi.org/10.3390/cancers11060740

Suszynska, M., & Kozlowski, P. (2020). Summary of bard1 mutations and precise estimation of breast and ovarian cancer risks associated with the mutations. *Genes*, *11*(7), 798. https://doi.org/10.3390/genes11070798

Svyatova, G., Berezina, G., Urazbayeva, G., & Murtazaliyeva, A. (2023). Frequencies of dagnostically significant polymorphisms of hereditary breast cancer forms in BRCA1 and BRCA2 genes in the Kazakh population. *Asian Pacific Journal of Cancer Prevention : APJCP*, *24*(11), 3899–3907. https://doi.org/10.31557/APJCP.2023.24.11.3899

Szyf, M. (2000). The DNA methylation machinery as a therapeutic target. *Current Drug Targets*, *1*(1), 101–118. https://doi.org/10.2174/1389450003349362

Tarapara, B., & Shah, F. (2025). BRCA1/2 methylation and expression dynamics in hereditary breast and ovarian cancer: insights from gene, protein, and TCGA analysis. *Clinical and Translational Oncology*, 741–749. https://doi.org/10.1007/s12094-025-03934-w

Tarsounas, M., & Sung, P. (2020). The antitumorigenic roles of BRCA1–BARD1 in DNA repair and replication. *Nature Reviews Molecular Cell Biology*, *21*(5), 284–299. https://doi.org/10.1038/s41580-020-0218-z

Thai, T. (1998). Mutations in the BRCA1-associated RING domain (BARD1) gene in primary breast, ovarian and uterine cancers. *Human Molecular Genetics*, *7*(2), 195–202. https://doi.org/10.1093/hmg/7.2.195

Thakur, C., Qiu, Y., Fu, Y., Bi, Z., Zhang, W., Ji, H., & Chen, F. (2022). Epigenetics and environment in breast cancer: New paradigms for anti-cancer therapies. *Frontiers in Oncology*, *12*. https://doi.org/10.3389/fonc.2022.971288

Torabi Dalivandan, S., Plummer, J., & Gayther, S. A. (2021). Risks and function of breast cancer susceptibility alleles. *Cancers*, *13*(16), 3953. https://doi.org/10.3390/cancers13163953

Tsukamoto, K., Ito, N., Yoshimoto, M., Iwase, T., Tada, T., Kasumi, F., Nakamura, Y., & Emi, M. (1996). Two distinct commonly deleted regions on chromosome 13q suggest involvement of BRCA2 and retinoblastoma genes in sporadic breast carcinomas. *Cancer*, *78*(9), 1929–1934.

Tudini, E., Moghadasi, S., Parsons, M. T., van der Kolk, L., van den Ouweland, A. M. W., Niederacher, D., Feliubadaló, L., Wappenschmidt, B., Spurdle, A. B., & Lazaro, C. (2018). Substantial evidence for the clinical significance of missense variant BRCA1 c.5309G>T p.(Gly1770Val). *Breast Cancer Research and Treatment*, *172*(2), 497–503.

https://doi.org/10.1007/s10549-018-4903-y

Tung, N. M., & Garber, J. E. (2018). BRCA1/2 testing: therapeutic implications for breast cancer management. *British Journal of Cancer*, *119*(2), 141–152. https://doi.org/10.1038/s41416-018-0127-5

Turkoz, F. P., Solak, M., Petekkaya, I., Keskin, O., Kertmen, N., Sarici, F., Arik, Z., Babacan, T., Ozisik, Y., & Altundag, K. (2013). Association between common risk factors and molecular subtypes in breast cancer patients. *The Breast*, *22*(3), 344–350. https://doi.org/10.1016/j.breast.2012.08.005

Turner, N. C., & Reis-Filho, J. S. (2006). Basal-like breast cancer and the BRCA1 phenotype. *Oncogene*, *25*(43), 5846–5853. https://doi.org/10.1038/sj.onc.1209876

Urban, T., Pokorná, P., & Slabý, O. (2024). Significance of aberrant DNA methylation for cancer diagnostics and therapy. *Klinicka Onkologie*, *37*(2), 899–905. https://doi.org/10.48095/ccko202488

van Duijnhoven, F. J. B., Bueno-De-Mesquita, H. B., Ferrari, P., Jenab, M., Boshuizen, H. C., Ros, M. M., Casagrande, C., Tjønneland, A., Olsen, A., Overvad, K., Thorlacius-Ussing, O., Clavel-Chapelon, F., Boutron-Ruault, M.-C., Morois, S., Kaaks, R., Linseisen, J., Boeing, H., Nöthlings, U., Trichopoulou, A., … Riboli, E. (2009). Fruit, vegetables, and colorectal cancer risk: the European Prospective Investigation into Cancer and Nutrition. *The American Journal of Clinical Nutrition*, *89*(5), 1441–1452. https://doi.org/10.3945/ajcn.2008.27120

Vázquez-Arreguín, K., Maddox, J., Kang, J., Park, D., Cano, R. R., Factor, R. E., Ludwig, T., & Tantin, D. (2018). BRCA1 through its E3 ligase activity regulates the transcription factor Oct1 and carbohydrate metabolism. *Molecular Cancer Research*, *16*(3), 439–452. https://doi.org/10.1158/1541-7786.MCR-17-0364

Vega, A. (2013). Breast cancer genes: beyond BRCA1 and BRCA2. *Frontiers in Bioscience*, *18*(4), 1358. https://doi.org/10.2741/4185

Walker, L. C., Whiley, P. J., Houdayer, C., Hansen, T. V. O., Vega, A., Santamarina, M., Blanco, A., Fachal, L., Southey, M. C., Lafferty, A., Colombo, M., De Vecchi, G., Radice, P., & Spurdle, A. B. (2013). Evaluation of a 5-Tier scheme proposed for classification of sequence variants using bioinformatic and splicing assay data: Inter-reviewer variability and promotion of minimum reporting guidelines. *Human Mutation*, *34*(10), 1424–1431. https://doi.org/10.1002/humu.22388

Walsh, T., Casadei, S., Coats, K. H., Swisher, E., Stray, S. M., Higgins, J., Roach, K. C., Mandell, J., Lee, M. K., Ciernikova, S., Foretova, L., Soucek, P., & King, M.-C. (2006). Spectrum of mutations in BRCA1, BRCA2, CHEK2, and TP53 in families at high risk of breast cancer. *JAMA*, *295*(12), 1379. https://doi.org/10.1001/jama.295.12.1379

Wang, B., Matsuoka, S., Ballif, B. A., Zhang, D., Smogorzewska, A., Gygi, S. P., & Elledge, S. J. (2007). Abraxas and RAP80 form a BRCA1 protein complex required for the DNA damage response. *Science*, *316*(5828), 1194–1198. https://doi.org/10.1126/science.1139476

Wang, M., Li, W., Tomimatsu, N., Yu, C. H., Ji, J.-H., Alejo, S., Witus, S. R., Alimbetov, D., Fitzgerald, O., Wu, B., Wang, Q., Huang, Y., Gan, Y., Dong, F., Kwon, Y., Sareddy, G. R., Curiel, T. J., Habib, A. A., Hromas, R., … Zhao, W. (2023). Crucial roles of the BRCA1-BARD1 E3 ubiquitin ligase activity in homology-directed DNA repair. *Molecular Cell*, *83*(20), 3679-3691.e8. https://doi.org/10.1016/j.molcel.2023.09.015

Wei, M., Grushko, T. A., Dignam, J., Hagos, F., Nanda, R., Sveen, L., Xu, J., Fackenthal, J., Tretiakova, M., Das, S., & Olopade, O. I. (2005). BRCA1 promoter methylation in sporadic breast cancer is associated with reduced BRCA1 copy number and chromosome 17 aneusomy. *Cancer Research*, *65*(23), 10692–10699.

https://doi.org/10.1158/0008-5472.CAN-05-1277

Wickham, H. (2011). ggplot2. *WIREs Computational Statistics*, *3*(2), 180–185. https://doi.org/10.1002/wics.147

Widodo, I., Dwianingsih, E. K., Triningsih, E., Utoro, T., & Soeripto, S. (2014). Clinicopathological features of indonesian breast cancers with different molecular subtypes. *Asian Pacific Journal of Cancer Prevention*, *15*(15), 6109–6113. https://doi.org/10.7314/APJCP.2014.15.15.6109

Wielsøe, M., Eiberg, H., Ghisari, M., Kern, P., Lind, O., & Bonefeld-Jørgensen, E. C. (2018). Genetic variations, exposure to persistent organic pollutants and breast cancer risk - A Greenlandic case-control study. *Basic & Clinical Pharmacology & Toxicology*, *123*(3), 335–346. https://doi.org/10.1111/bcpt.13002

Wigginton, J. E., Cutler, D. J., & Abecasis, G. R. (2005). A note on exact tests of Hardy-Weinberg Equilibrium. *The American Journal of Human Genetics*, *76*(5), 887–893. https://doi.org/10.1086/429864

Wittersheim, M., Büttner, R., & Markiefka, B. (2015). Genotype/phenotype correlations in patients with hereditary breast cancer. *Breast Care*, *10*(1), 22–26. https://doi.org/10.1159/000380900

Witus, S. R., Stewart, M. D., & Klevit, R. E. (2021). The BRCA1/BARD1 ubiquitin ligase and its substrates. *Biochemical Journal*, *478*(18), 3467–3483. https://doi.org/10.1042/BCJ20200864

Wong, E. M., Southey, M. C., Fox, S. B., Brown, M. A., Dowty, J. G., Jenkins, M. A., Giles, G. G., Hopper, J. L., & Dobrovic, A. (2011). Constitutional methylation of the BRCA1 promoter is specifically associated with BRCA1 mutation-associated pathology in early-onset breast cancer. *Cancer Prevention Research*, *4*(1), 23–33. https://doi.org/10.1158/1940-6207.CAPR-10-0212

Wu, J., Aini, A., & Ma, B. (2022). Mutations in exon region of BRCA1-related RING domain 1 gene and risk of breast cancer. *Molecular Genetics & Genomic Medicine*, *10*(3), e1847. https://doi.org/10.1002/mgg3.1847

Wu, J., Lu, L.-Y., & Yu, X. (2010). The role of BRCA1 in DNA damage response. *Protein & Cell*, *1*(2), 117–123. https://doi.org/10.1007/s13238-010-0010-5

Wu, J., Vlastos, A., Pelte, M., Caligo, M., Bianco, A., Krause, K., Laurent, G. J., & Irminger-Finger, I. (2006). Aberrant expression of BARD1 in breast and ovarian cancers with poor prognosis. *International Journal of Cancer*, *118*(5), 1215–1226. https://doi.org/10.1002/ijc.21428

Wu, L. C., Wang, Z. W., Tsan, J. T., Spillman, M. A., Phung, A., Xu, X. L., Yang, M.-C. W., Hwang, L.-Y., Bowcock, A. M., & Baer, R. (1996). Identification of a RING protein that can interact in vivo with the BRCA1 gene product. *Nature Genetics*, *14*(4), 430–440. https://doi.org/10.1038/ng1296-430

Xia, Y., Pao, G. M., Chen, H.-W., Verma, I. M., & Hunter, T. (2003). Enhancement of BRCA1 E3 ubiquitin ligase activity through direct interaction with the BARD1 protein. *Journal of Biological Chemistry*, *278*(7), 5255–5263. https://doi.org/10.1074/jbc.M204591200

Xie, S., Wang, Y., Liu, H., Wang, M., Yu, H., Qiao, Y., Li, F., Xie, X., & Zhang, J. (2016). Diagnostic significance of circulating multiple miRNAs in breast cancer: a systematic review and meta-analysis. *Biomarkers in Medicine*, *10*(6), 661–674. https://doi.org/10.2217/bmm-2015-0017

Xu, X., Gammon, M. D., Zhang, Y., Bestor, T. H., Zeisel, S. H., Wetmur, J. G., Wallenstein, S., Bradshaw, P. T., Garbowski, G., Teitelbaum, S. L., Neugut, A. I., Santella, R. M., & Chen, J. (2009). BRCA1 promoter methylation is associated with increased mortality

among women with breast cancer. *Breast Cancer Research and Treatment*, *115*(2), 397–404. https://doi.org/10.1007/s10549-008-0075-5

Xu, Y., Diao, L., Chen, Y., Liu, Y., Wang, C., Ouyang, T., Li, J., Wang, T., Fan, Z., Fan, T., Lin, B., Deng, D., Narod, S. A., & Xie, Y. (2013). Promoter methylation of BRCA1 in triple-negative breast cancer predicts sensitivity to adjuvant chemotherapy. *Annals of Oncology*, *24*(6), 1498–1505. https://doi.org/10.1093/annonc/mdt011

Yamashita, N., Tokunaga, E., Kitao, H., Hitchins, M., Inoue, Y., Tanaka, K., Hisamatsu, Y., Taketani, K., Akiyoshi, S., Okada, S., Oda, Y., Saeki, H., Oki, E., & Maehara, Y. (2015). Epigenetic inactivation of BRCA1 through promoter hypermethylation and its clinical importance in triple-negative breast cancer. *Clinical Breast Cancer*, *15*(6), 498–504. https://doi.org/10.1016/j.clbc.2015.06.009

Yang, Z., & Liu, Z. (2020). The emerging role of microRNAs in breast cancer. *Journal of Oncology*, *2020*, 1–7. https://doi.org/10.1155/2020/9160905

Ye, Q., Hu, Y.-F., Zhong, H., Nye, A. C., Belmont, A. S., & Li, R. (2001). BRCA1-induced large-scale chromatin unfolding and allele-specific effects of cancer-predisposing mutations. *The Journal of Cell Biology*, *155*(6), 911–922. https://doi.org/10.1083/jcb.200108049

Yoshikawa, K., Honda, K., Inamoto, T., Shinohara, H., Yamauchi, A., Suga, K., Okuyama, T., Shimada, T., Kodama, H., Noguchi, S., Gazdar, A. F., Yamaoka, Y., & Takahashi, R. (1999). Reduction of BRCA1 protein expression in Japanese sporadic breast carcinomas and its frequent loss in BRCA1-associated cases. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *5*(6), 1249–1261. http://www.ncbi.nlm.nih.gov/pubmed/10389907

Yoshikawa, K., Ogawa, T., Baer, R., Hemmi, H., Honda, K., Yamauchi, A., Inamoto, T., Ko, K., Yazumi, S., Motoda, H., Kodama, H., Noguchi, S., Gazdar, A. F., Yamaoka, Y., & Takahashi, R. (2000). Abnormal expression of BRCA1 and BRCA1-interactive DNA-repair proteins in breast carcinomas. *International Journal of Cancer*, *88*(1), 28–36. https://doi.org/10.1002/1097-0215(20001001)88:1<28::AID-IJC5>3.0.CO;2-4

Youlden, D. R., Cramb, S. M., Dunn, N. A. M., Muller, J. M., Pyke, C. M., & Baade, P. D. (2012). The descriptive epidemiology of female breast cancer: An international comparison of screening, incidence, survival and mortality. *Cancer Epidemiology*, *36*(3), 237–248. https://doi.org/10.1016/j.canep.2012.02.007

Youlden, D. R., Cramb, S. M., Yip, C. H., & Baade, P. D. (2014). Incidence and mortality of female breast cancer in the Asia-Pacific region. *Cancer Biology & Medicine*, *11*(2), 101–115. https://doi.org/10.7497/j.issn.2095-3941.2014.02.005

Yu, K., Ganesan, K., Miller, L. D., & Tan, P. (2006). A modular analysis of breast cancer reveals a novel low-grade molecular signature in estrogen receptor-positive tumors. *Clinical Cancer Research*, *12*(11), 3288–3296. https://doi.org/10.1158/1078-0432.CCR-05-1530

Zaidi, Z., & Dib, H. A. (2019). The worldwide female breast cancer incidence and survival, 2018. *Cancer Research*, *79*(13_Supplement), 4191–4191. https://doi.org/10.1158/1538-7445.AM2019-4191

Zaleski, M., Kobilay, M., Schroeder, L., Debald, M., Semaan, A., Hettwer, K., Uhlig, S., Kuhn, W., Hartmann, G., & Holdenrieder, S. (2018). Improved sensitivity for detection of breast cancer by combination of miR-34a and tumor markers CA 15-3 or CEA. *Oncotarget*, *9*(32), 22523–22536. https://doi.org/10.18632/oncotarget.25077

Zangana, A., & Garota, S. (2012). Risk factors of breast cancer in a sample of Kurdish women of Kurdistan region - Iraq: A comparative study between pre-menopausal and post-menopausal women. *Zanco Journal of Medical Sciences*, *16*(3), 262–267.

https://doi.org/10.15218/zjms.2012.0044

Zhang, J., & Powell, S. N. (2005). The role of the BRCA1 tumor suppressor in DNA double-strand break repair. *Molecular Cancer Research*, *3*(10), 531–539. https://doi.org/10.1158/1541-7786.MCR-05-0192

Zhang, L., & Long, X. (2015). Association of BRCA1 promoter methylation with sporadic breast cancers: Evidence from 40 studies. *Scientific Reports*, *5*(1), 17869. https://doi.org/10.1038/srep17869

Zhang, X., Xu, X., Ge, G., Zang, X., Shao, M., Zou, S., Zhang, Y., Mao, Z., Zhang, J., Mao, F., Qian, H., & Xu, W. (2018). miR-498 inhibits the growth and metastasis of liver cancer by targeting ZEB2. *Oncology Reports*. https://doi.org/10.3892/or.2018.6948

Zhang, Y., Bianco, A., Malkinson, A. M., Leoni, V. P., Frau, G., De Rosa, N., André, P., Versace, R., Boulvain, M., Laurent, G. J., Atzori, L., & Irminger-Finger, I. (2012). BARD1: An independent predictor of survival in non-small cell lung cancer. *International Journal of Cancer*, *131*(1), 83–94. https://doi.org/10.1002/ijc.26346

Zhang, Z., Bast, R. C., Yu, Y., Li, J., Sokoll, L. J., Rai, A. J., Rosenzweig, J. M., Cameron, B., Wang, Y. Y., Meng, X.-Y., Berchuck, A., van Haaften-Day, C., Hacker, N. F., de Bruijn, H. W. A., van der Zee, A. G. J., Jacobs, I. J., Fung, E. T., & Chan, D. W. (2004). Three biomarkers identified from serum proteomic analysis for the detection of early stage ovarian cancer. *Cancer Research*, *64*(16), 5882–5890. https://doi.org/10.1158/0008-5472.CAN-04-0746

APPENDICES

Appendices

وزارة التعليم العالي والبحث العلمي
جامعة كربلاء
كلية الطب
معاون العميد للشؤون العلمية
شعبة شؤون الدراسات العليا

جمهورية العراق

العدد: د/6/ 6658

التاريخ: 1/ 12/ 2024

أمر اداري

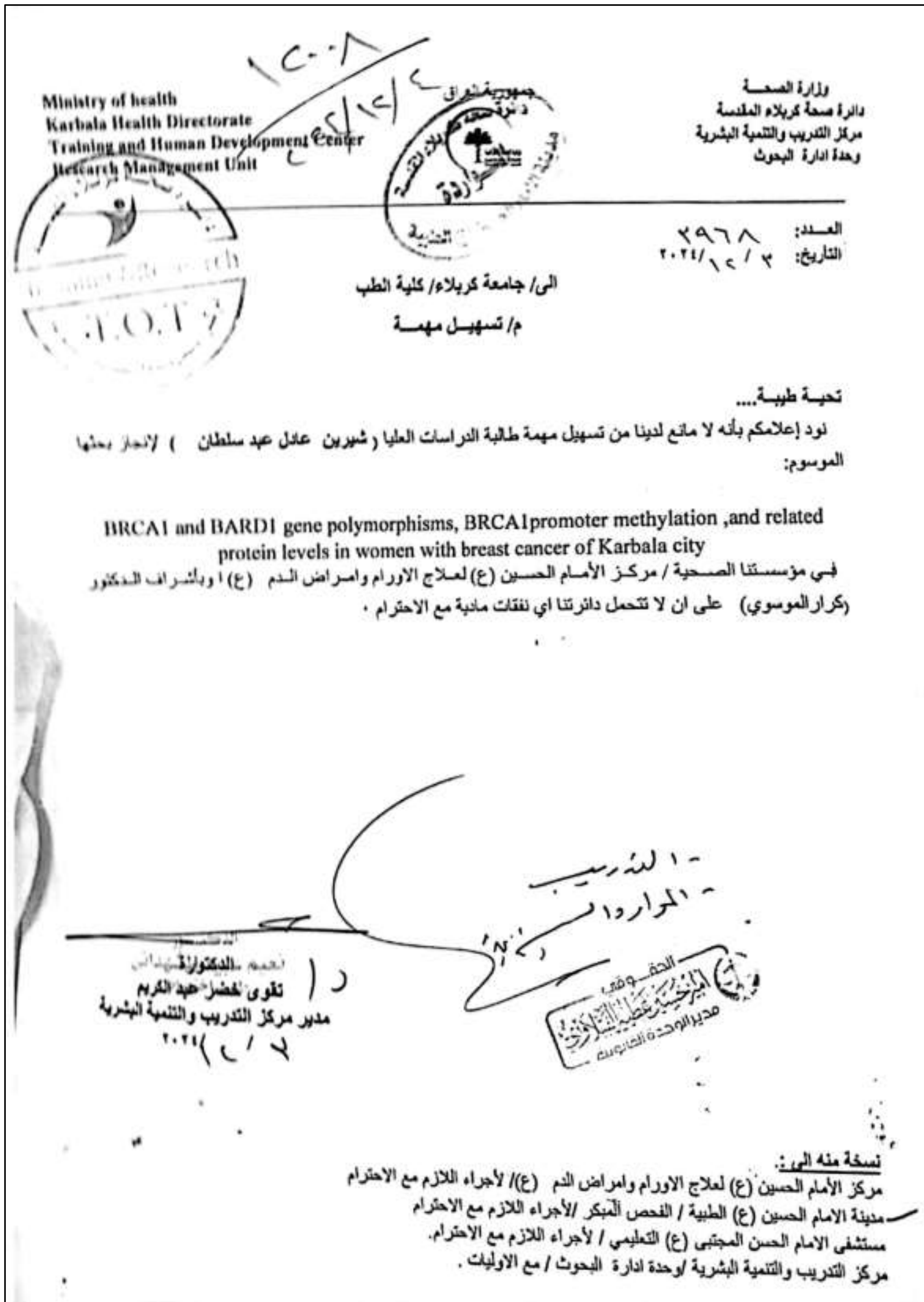
إشارة الى ما جاء في محضر مجلس الكلية بالجلسة الرابعة المنعقدة بتاريخ (2024/11/11) والمصادق عليها من قبل رئاسة جامعة كربلاء /أمانة مجلس الجامعة بكتابهم المرقم (ج/2093 في 2024/11/18) ,واستناداً للصلاحيات المخولة لنا تقرر:

- اعتماد خطط ومشاريع بحوث طلبة الدراسات العليا/ماجستير/كيمياء سريرية وأسماء السادة التدريسيين المشرفين على خطط مشاريع البحوث حسب الجدول ادناه واعتباراً من تاريخ كتاب مصادقة أمانة مجلس الجامعة على محضر مجلس الكلية.

أ.د.علي عبدالرضا ابوطحين
معاون العميد للشؤون العلمية والدراسات العليا
2024/12/1

نسخة منه:
مكتب السيد العميد المحترم للتفضل بالاطلاع مع التقدير.
مكتب معاون العميد للشؤون العلمية المحترم للتفضل بالاطلاع مع التقدير.
فرع الكيمياء والكيمياء الحياتية ... للتفضل بالاطلاع لتبليغ السادة المعنيين.
الحسابات ..للتفضل بالاطلاع ...واتخاذ ما يلزم.
شعبة الدراسات العليا/اضابير الطلبة.
الصادرة.

Supplementary figure 1. Formal approval of the study proposal and its protocols by the College of Medicine, University of Kerbala.



Ministry of health
Karbala Health Directorate
Training and Human Development Center
Research Management Unit

وزارة الصحة
دائرة صحة كربلاء المقدسة
مركز التدريب والتنمية البشرية
وحدة ادارة البحوث

العدد: ٢٩٦٨
التاريخ: ٣ / ١٢ /٢٠٢١

الى/ جامعة كربلاء/ كلية الطب

م/ تسهيل مهمة

تحية طيبة....

نود إعلامكم بأنه لا مانع لدينا من تسهيل مهمة طالبة الدراسات العليا (شيرين عادل عبد سلطان) لانجاز بحثها الموسوم:

BRCA1 and BARD1 gene polymorphisms, BRCA1promoter methylation ,and related protein levels in women with breast cancer of Karbala city

في مؤسستنا الصحية / مركز الأمام الحسين (ع) لعلاج الاورام وامراض الدم (ع) ا وباشراف الدكتور (كرار الموسوي) على ان لا تتحمل دائرتنا اي نفقات مادية مع الاحترام .

د
تقوى خضر عبد الكريم
مدير مركز التدريب والتنمية البشرية
٢٠٢١/١٢/٢

نسخة منه الى:
مركز الأمام الحسين (ع) لعلاج الاورام وامراض الدم (ع)/ لأجراء اللازم مع الاحترام
مدينة الامام الحسين (ع) الطبية / الفحص المبكر /لأجراء اللازم مع الاحترام
مستشفى الامام الحسن المجتبى (ع) التعليمي / لأجراء اللازم مع الاحترام.
مركز التدريب والتنمية البشرية /وحدة ادارة البحوث / مع الاوليات .

Supplementary figure 2. Formal approval of the study proposal and its protocols by the Health Directorate of Karbala.

استمارة الموافقة المسبقة على المشاركة في بحث

عنوان الدراسة:
تعدد الأشكال الجيني لجينات *BRCA1* و *BARD1*، ومثيلة محفز *BRCA1*، ومستويات البروتينات ذات العلاقة لدى النساء المصابات بسرطان الثدي في مدينة كربلاء

الباحث الرئيسي: شيرين عادل عبد سلطان – طالبة ماجستير/ كيمياء سريرية (كلية الطب – جامعة كربلاء)

الدعوة للمشاركة
أنت مدعوة للمشاركة في دراسة بحثية حول سرطان الثدي. توفر هذه الاستمارة معلومات عن الدراسة. يرجى قراءتها بعناية وطرح أي أسئلة قبل اتخاذ قرار المشاركة.

الغرض: تهدف هذه الدراسة إلى دراسة العوامل الجينية والجزيئية المرتبطة بخطر الإصابة بسرطان الثدي لدى النساء في كربلاء، العراق، وإذا وافقت على المشاركة، سوف يطلب منك:
1) اكمال استبيان عن تاريخك الصحي ونمط حياتك 2) السماح لنا بأخذ عينة دم (10 مل)
3) الوصول إلى سجلاتك الطبية او الطبلة (لمريضات سرطان الثدي)

المدة: ستستغرق مشاركتك المباشرة حوالي 30-45 دقيقة. علماً أن المخاطر المرتبطة بهذه الدراسة ضئيلة وتشمل عدم راحة بسيطة من سحب الدم.

الفوائد: قد لا تحصلين على فوائد مباشرة من المشاركة، لكنها بالتأكيد ستساعد في تطوير فهمنا لسرطان الثدي وقد تفيد النتائج مرضى سرطان الثدي في المستقبل.

السرية: سيتم الحفاظ على سرية جميع المعلومات بشكل صارم، وسيتم ترميز بياناتك وتخزينها بشكل آمن، وسيمكن فقط لأعضاء فريق البحث المصرح الوصول إلى البيانات ولن يتم الكشف عن هويتك في أي بحوث او مكان آخر وستُستخدم مُجمعة لغرض البحث العلمي.

طبيعة المشاركة: إن المشاركة طوعية تماماً، ويمكنك الانسحاب في أي وقت تبعات، وانّ قرارك لن يؤثر على رعايتك الطبية مطلقاً. ولا توجد تكاليف للمشاركة في هذه الدراسة.

معلومات الاتصال: للأسئلة حول الدراسة، اتصلي بالباحثة عبر الايميل (shireen.a@s.uokerbala.edu.iq)

إقرار الموافقة:

لقد قرأت وفهمت المعلومات أعلاه. تمت الإجابة على أسئلتي بشكل مُرضٍ. وأوافق على المشاركة في هذه الدراسة.

اسم المشارك	التوقيع	التاريخ
______________	______________	______________

شيرين عادل عبد التميمي	______________	______________
الباحث	التوقيع	التاريخ

Supplementary figure 3. Written informed consent form.

استمارة استبيان (لأغراض البحث العلمي حصراً)

رقم المشارك: __________ التاريخ : __________

أ. المعلومات الديموغرافية:

1 .العمر: ______ سنة 2 .الطول: ______ سم 3 .الوزن: ______ كغم

4 . الحالة الاجتماعية: ☐ عزباء ☐ متزوجة ☐ مطلقة ☐ أرملة

6 .المستوى التعليمي: ☐ ابتدائي ☐ ثانوي ☐ جامعي ☐ دراسات عليا 7 .المهنة: __________

ب. التاريخ الانجابي:

8 .العمر عند أول دورة شهرية: ______ سنة

9 .حالة الطمث (الدورة الشهرية): ☐ قبل انقطاع الطمث ☐ بعد انقطاع الطمث (والعمر عند انقطاع الطمث: ______ سنة)

10 .عدد مرات الحمل: __________ 11 .عدد الولادات الناجحة: __________

12 .العمر عند أول ولادة ناجحة: ______ سنة 13 .هل قمت بالرضاعة الطبيعية؟ ☐ نعم ☐ لا

إذا كانت الإجابة نعم، المدة الإجمالية للرضاعة الطبيعية (لجميع الأطفال): ______ شهر

ج. العوامل الهرمونية:

14 .هل سبق لك استخدام حبوب منع الحمل؟ ☐ نعم ☐ لا (إذا كانت الإجابة نعم، مدة الاستخدام: ______ سنة)

15 .هل سبق لك استخدام العلاج الهرموني البديل؟ ☐ نعم ☐ لا (إذا كانت الإجابة نعم، مدة الاستخدام: ______ سنة)

د. التاريخ العائلي:

16 .هل لديك تاريخ عائلي لسرطان الثدي؟ ☐ نعم ☐ لا إذا كانت الإجابة نعم، يرجى التحديد مما يلي:

☐ الأم ☐ الأخت ☐ الابنة ☐ الجدة ☐ العمة/الخالة ☐ أخرى: __________

17 .عمر التشخيص لأفراد العائلة المصابين: __________

هـ. التاريخ الطبي الشخصي:

18 .هل سبق تشخيصك بأي مرض حميد في الثدي؟ ☐ نعم ☐ لا (إذا كانت الإجابة نعم، يرجى التحديد: __________)

19 .هل سبق لك إجراء تصوير الثدي الشعاعي؟ ☐ نعم ☐ لا (إذا كانت الإجابة نعم، تاريخ آخر تصوير: __________)

20 .هل سبق لك إجراء خزعة للثدي؟ ☐ نعم ☐ لا (إذا كانت نعم، النتيجة: ☐ حميد ☐ خبيث ☐ غير معروف)

و. عوامل نمط الحياة:

21 .**حالة التدخين:** ☐ لم أدخن أبداً ☐ مدخنة سابقة ☐ مدخنة حالية

إذا كنت مدخنة حالية أو سابقة، **عدد السكائر يومياً:** __________ **ومدة التدخين:** ______ سنة

22 .**استهلاك الكحول:** ☐ أبداً ☐ أحياناً ☐ بانتظام (______ مشروب في الأسبوع)

23 .**النشاط البدني:** ☐ خامل ☐ تمارين خفيفة ☐ تمارين معتدلة ☐ تمارين كثيفة (______ ساعة/ في الأسبوع)

24 .**النظام الغذائي:** **كم مرة تتناولين الأطعمة التالية (يومياً/أسبوعياً/شهرياً):**

الفواكه: __________ الخضروات: __________ اللحوم الحمراء: __________

اللحوم المعلبة: __________ اللحوم المصنعة: __________

صفحة 1 من 2

Supplementary figure 4. Questionnaire used for data collection from the participants.

استمارة استبيان (لأغراض البحث العلمي حصراً)

25. **هل تستخدمين أي مكملات غذائية؟** ☐ نعم ☐ لا (إذا كانت الإجابة نعم، يرجى التحديد: ____________)

ز. العوامل البيئية:

26. هل عملك يتضمن التعرض للمواد الكيميائية أو الإشعاع؟ ☐ نعم ☐ لا
إذا كانت الإجابة نعم، يرجى التحديد: ____________
27. عدد السنوات التي عشتها في كربلاء: ______ سنة

ح. للمريضات المصابات بسرطان الثدي فقط:

28. تاريخ تشخيص سرطان الثدي: ____________
29. مرحلة سرطان الثدي عند التشخيص: ☐ الأولى ☐ الثانية ☐ الثالثة ☐ الرابعة ☐ غير معروف
30. **نوع سرطان الثدي:** ☐ قنوي (ductal) ☐ فصيصي (lobular) ☐ أخرى: ____________
31. **حالة مستقبلات الهرمونات:**
ER ☐ إيجابي ☐ سلبي ☐ غير معروف
PR ☐ إيجابي ☐ سلبي ☐ غير معروف
HER2 ☐ إيجابي ☐ سلبي ☐ غير معروف

32. **العلاج الحالي:** ☐ جراحة ☐ علاج كيميائي ☐ علاج إشعاعي ☐ علاج هرموني ☐ علاج بايولوجي (مناعي)

ط. الموافقة على الاختبار الجيني:

33. هل توافقين على إجراء الاختبار الجيني لهذه الدراسة؟ ☐ نعم ☐ لا

ي. اذا كانت لديك أي معلومات أخرى او أسئلة للمشاركة بها (يرجى كتابتها او طرحها شفهيا على الباحثة):

____
____
____
____
____
____
____________.

شكراً لك على إكمال هذا الاستبيان. ستبقى إجاباتك سرية وتستخدم لأغراض البحث فقط.

صفحة 2 من 2

ab214025 – Human IL-1 beta ELISA Kit

For the quantitative measurement of IL-1 beta in human serum, plasma, and cell culture supernatant samples.
For research use only - not intended for diagnostic use.

For overview, typical data and additional information please visit: www.abcam.com/ab214025

Storage and Stability: Store kit at 2-8°C immediately upon receipt. Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Standard Preparation and Reagent preparation sections.

Materials Supplied

Item	Quantity 1 x 96 wells	Quantity 10 x 96 wells	Storage Condition
Human IL-1 beta Capture Antibody 10X	600 µL	10 x 600 µL	+4°C
Human IL-1 beta Detector Antibody 10X	600 µL	10 x 600 µL	+4°C
Human IL-1 beta Lyophilized Recombinant Protein	2 Vials	10 x 2 vials	+4°C
Antibody Diluent 4BI	6 mL	10 x 6 mL	+4°C
Sample Diluent NS	50 mL	2 x 250 mL	+4°C
Wash Buffer PT 10X	20 mL	200 mL	+4°C
TMB Development Solution	12 mL	120 mL	+4°C
Stop Solution	12 mL	120 mL	+4°C
Pre-Coated 96-Well Microplate	96 Wells	10 x 96 Wells	+4°C
Plate Seal	1	10	+4°C

Materials Required, Not Supplied
These materials are not included in the kit, but will be required to successfully utilize this assay:
- Microplate reader capable of measuring absorbance at 450 or 600 nm.
- Method for determining protein concentration (BCA assay recommended).
- Deionized water.
- Multi- and single-channel pipettes.
- Tubes for standard dilution.
- Plate shaker for all incubation steps.
- Optional: Phenylmethylsulfonyl Fluoride (PMSF) (or other protease inhibitors).

Reagent Preparation
- Equilibrate all reagents to room temperature (18-25°C) prior to use. The kit contains enough reagents for 96 wells. The sample volumes below are sufficient for 48 wells (6 x 8-well strips); adjust volumes as needed for the number of strips in your experiment.
- Prepare only as much reagent as is needed on the day of the experiment. Capture and Detector Antibodies have only been tested for stability in the provided 10X formulations.

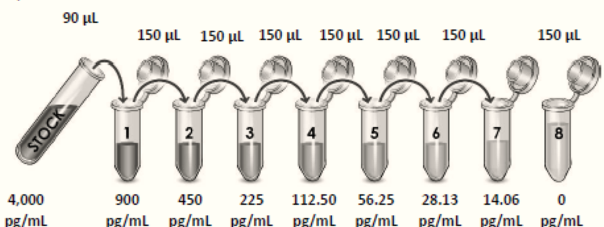
1X Wash Buffer PT: Prepare 1X Wash Buffer PT by diluting Wash Buffer PT 10X with deionized water. To make 50 mL 1X Wash Buffer PT combine 5 mL Wash Buffer PT 10X with 45 mL deionized water. Mix thoroughly and gently.

Version 5a, Last updated 13 September 2024

Antibody Cocktail: Prepare Antibody Cocktail by diluting the capture and detector antibodies in Antibody Diluent 4BI. To make 3 mL of the Antibody Cocktail combine 300 µL 10X Capture Antibody and 300 µL 10X Detector Antibody with 2.4 mL Antibody Diluent 4BI. Mix thoroughly and gently.

Standard Preparation
- Always prepare a fresh set of standards for every use. Discard working standard dilutions after use as they do not store well. The following section describes the preparation of a standard curve for duplicate measurements (recommended).
- **IMPORTANT:** If the protein standard vial has a volume identified on the label, reconstitute the IL-1 beta standard by adding that volume of Sample Diluent NS indicated on the label. Alternatively, if the vial has a mass identified, reconstitute the IL-1 beta standard by adding 500 µL Sample Diluent NS. Hold at room temperature for 10 minutes and mix gently. This is the 4,000 pg/mL **Stock Standard** Solution.

1) Label eight tubes, Standards 1– 8.
2) Add 310 µL Sample Diluent NS into tube number 1 and 150 µL of Sample Diluent NS into numbers 2-8.
3) Use the Stock Standard to prepare the following dilution series. Standard #8 contains no protein and is the Blank control:



Sample Preparation

TYPICAL SAMPLE DYNAMIC RANGE	
Sample Type	Range
Serum*	≤ 50%
Plasma – Citrate*	≤ 50%
Plasma – Heparin*	≤ 50%
Plasma – EDTA*	≤ 50%
Cell culture supernatant	Varies by type

*Based on spiked sample.

Serum: Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 2,000 x g for 10 minutes and collect serum. Dilute samples at least 1: 2 into Sample Diluent NS and assay. Store un-diluted serum at -20°C or below. Avoid repeated freeze-thaw cycles.
Plasma: Collect plasma using citrate, EDTA or heparin. Centrifuge samples at 2,000 x g for 10 minutes. Dilute samples at least 1: 2 into Sample Diluent NS and assay. Store un-diluted plasma samples at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

Supplementary figure 5. ELISA kit's manual for the assessment of IL-1β.

ab46027 Human IL-6 ELISA Kit

For the quantitative measurement of IL-6 in Human serum, plasma, buffered solutions and cell culture media. This product is for research use only and is not intended for diagnostic use.

For overview, typical data and additional information please visit: www.abcam.com/ab46027 (use abcam.cn/ab46027 for China, or abcam.co.jp/ab46027 for Japan)

Materials Supplied and Storage

Store kit at +4°C immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted. Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components.

Item	Quantity		Storage Condition
	1 x 96 tests	2 x 96 tests	
IL-6 Microplate (12 x 8 well strips)	96 wells	2 x 96 wells	+2-8ºC
IL-6 Standard (Lyophilized)	2 vials	4 vials	+2-8ºC
10X Standard Diluent Buffer	15 mL	25 mL	+2-8ºC
Standard Diluent (Serum)	7 mL	2 x 7 mL	+2-8ºC
Control	2 vials	4 vials	+2-8ºC
Biotinylated anti-IL-6	400 µL	2 x 400 µL	+2-8ºC
Biotinylated Antibody Diluent	7.5 mL	13 mL	+2-8ºC
Streptavidin-HRP	2 x 5 µL	4 x 5 µL	+2-8ºC
HRP Diluent	12 mL	23 mL	+2-8ºC
200X Wash Buffer	10 mL	2 x 10 mL	+2-8ºC
Chromogen TMB Substrate Solution	11 mL	24 mL	+2-8ºC
Stop Reagent	11 mL	2 x 11 mL	+2-8ºC
Adhesive Plate Seal	2 units	4 units	+2-8ºC

Note: This ELISA kit will soon contain the "Easy View" colored reagents. The Standard diluent buffer will now be red, and the Streptavidin-HRP Diluent will be green. Please note that while stock lasts you may still receive colorless diluents. This change does not impact the results provided by the kit or the assay procedure.

Materials Required, Not Supplied

- Microplate reader capable of measuring absorbance at 450 nm.
- Precision pipettes to deliver 2 µL to 1 mL volumes.
- Adjustable 1-25 mL pipettes for reagent preparation.
- 100 mL and 1 liter graduated cylinders.
- Absorbent paper.
- Distilled or deionized water.
- Tubes to prepare standard or sample dilutions.
- Log-log graph paper or computer and software for ELISA data analysis.

1. Reagent Preparation

Equilibrate all reagents to room temperature (18-25ºC) prior to use. The kit contains enough reagents for 96 wells. Prepare only as much reagent as is needed on the day of experiment.

1.1 1X Standard Diluent Buffer: Dilute the 10X Standard Diluent Buffer 10-fold in distilled water before use.

Version 7a, Last updated 10 April 2024

1.2 1X Wash Buffer: Dilute the 200X Wash Buffer Concentrate 200-fold in distilled water before use. Mix gently to avoid foaming. Prepare as needed according to the following table:

Number of well strips used	Volume of 200X Wash Buffer Concentrate (mL)	Volume of distilled water (mL)
1-6	5	995
1-12	10	1,990

1.3 1X Control Solution: Lyophilized Control vials must be reconstituted with the most appropriate diluent for your test samples:
For serum and plasma samples: Use Standard Diluent (Serum).
For cell culture supernatants: Use 1X Standard Diluent Buffer.
Control vials must be reconstituted with the volume of appropriate diluent buffer that is indicated on the vial. Reconstitution of the lyophilized material with the indicated volume will yield a solution for which the IL-6 concentration is stated on the vial. Allow the reconstituted 1X Control Solution to stand for 5 minutes with gentle swirling prior to use in the assay procedure. **Do not store the 1X Control Solution after reconstitution.**

1.4 1X Biotinylated anti-IL-6: Prepare the 1X Biotinylated anti-IL-6 immediately prior to use. According to the table below, dilute the Biotinylated anti-IL-6 with the Biotinylated Antibody Diluent based on the number of wells being used in the assay procedure:

Number of well strips used	Volume of Biotinylated anti-IL-6 (µL)	Volume of Biotinylated Antibody Diluent (µL)
2	40	1,060
3	60	1,590
4	80	2,120
6	120	3,180
12	240	6,360

1.5 1X Streptavidin-HRP Solution: Add 500 µL of HRP-Diluent to the Streptavidin-HRP vial prior to use to create a Streptavidin-HRP Concentrate. Do not keep this solution for further experiments. Subsequently, prior to use in the assay procedure, prepare the 1X Streptavidin-HRP Solution by further diluting the Streptavidin-HRP Concentrate with HRP-Diluent. Use the table below to determine the volumes of each solution required to prepare the final 1X Streptavidin-HRP Solution:

Number of well strips used	Volume of Streptavidin HRP (µL)	Volume of HRP Diluent (mL)
2	30	2
3	45	3
4	60	4
6	75	5
12	150	10

2. Standard Preparation

Always prepare a fresh set of standards for every use. Discard working standard dilutions after use as they do not store well. The following section describes the preparation of a standard curve for duplicate measurements (recommended).

2.1 Standard vials must be reconstituted with the appropriate diluent for your samples.
For serum and plasma samples: Use Standard Diluent (Serum).

Supplementary figure 6. ELISA kit's manual for the assessment of IL-6.

ab181421 – Human TNF alpha SimpleStep ELISA® Kit

For the quantitative measurement of TNF alpha in human serum, plasma (heparin), plasma (EDTA), plasma (citrate), cell culture supernatant, and CSF.

For research use only - not intended for diagnostic use.

For overview, typical data and additional information please visit: www.abcam.com/ab181421

This kit is available in a 384-well plate format. This plate utilises smaller volumes of standards and samples per well. Directions for using this format can be found on pg 9.

Storage and Stability: Store kit at 2-8ºC immediately upon receipt. Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Standard Preparation and Reagent preparation sections.

Materials Supplied

Item	Quantity 1 x 96 wells	Quantity 10 x 96 wells	Storage Condition
Human TNF alpha Capture Antibody 10X	600 µL	10 x 600 µL	+4ºC
Human TNF alpha Detector Antibody 10X	600 µL	10 x 600 µL	+4ºC
Human TNF alpha Lyophilized Recombinant Protein	2 Vials	10 x 2 vials	+4ºC
Antibody Diluent 4BR	6 mL	10 x 6 mL	+4ºC
Sample Diluent NS	50 mL	2 x 250 mL	+4ºC
Wash Buffer PT 10X	20 mL	200 mL	+4ºC
TMB Development Solution	12 mL	120 mL	+4ºC
Stop Solution	12 mL	120 mL	+4ºC
SimpleStep Pre-Coated 96-Well Microplate	96 wells	10 x 96 wells	+4ºC
Plate Seal	1	10	+4ºC

Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully utilize this assay:

Microplate reader capable of measuring absorbance at 450 or 600 nm.
Deionized water.
Multi- and single-channel pipettes.
Tubes for standard dilution.
Plate shaker for all incubation steps.
Optional: Phenylmethylsulfonyl Fluoride (PMSF) (or other protease inhibitors).

Reagent Preparation

Equilibrate all reagents to room temperature (18-25ºC) prior to use. The kit contains enough reagents for 96 wells. The sample volumes below are sufficient for 48 wells (6 x 8-well strips); adjust volumes as needed for the number of strips in your experiment.

Prepare only as much reagent as is needed on the day of the experiment. Capture and detector antibodies have only been tested for stability in the provided 10X formulations.

1X Wash Buffer PT: Prepare 1X Wash Buffer PT by diluting Wash Buffer PT 10X with deionized water. To make 50 mL 1X Wash Buffer PT combine 5 mL Wash Buffer PT 10X with 45 mL deionized water. Mix thoroughly and gently.

Antibody Cocktail: Prepare Antibody Cocktail by diluting the capture and detector antibodies in Antibody Diluent 4BR . To make 3 mL of the Antibody Cocktail combine 300 µL 10X Capture Antibody and 300 µL 10X Detector Antibody with 2.4 mL Antibody Diluent 4BR . Mix thoroughly and gently.

Standard Preparation

Always prepare a fresh set of standards for every use. Discard working standard dilutions after use as they do not store well. The following section describes the preparation of a standard curve for duplicate measurements (recommended).

1. Reconstitute the TNF alpha standard sample by adding the volume of Sample Diluent NS indicated on the protein vial label. Hold at room temperature for 10 minutes. Mix thoroughly and gently. This is the 10,000 pg/mL **Stock Standard** Solution.
2. Label eight tubes, Standards 1– 8.
3. Add 360 µL of Sample Diluent NS into tube number 1 and 150 µL of Sample Diluent NS into numbers 2-8.
4. Use the **Stock Standard** to prepare the following dilution series. Standard #8 contains no protein and is the Blank control:

Standard #	Dilution Sample	Volume to Dilute (µL)	Volume of Diluent (µL)	Starting Conc. (pg/mL)	Final Conc. (pg/mL)
1	**Stock Standard**	40	360	10,000	1,000
2	Standard#1	150	150	1,000	500
3	Standard#2	150	150	500	250
4	Standard#3	150	150	250	125
5	Standard#4	150	150	125	62.5
6	Standard#5	150	150	62.5	31.25
7	Standard#6	150	150	31.25	15.63
8	Blank Control	0	150	0	0

Supplementary figure 7. ELISA kit's manual for the assessment of TNF-α.

ab285254 – 8-hydroxy-2'-deoxyguanosine (8-OHdG) ELISA Kit

For *in vitro* quantitative determination of 8-OHdG in serum, plasma, cell culture supernatant, urine and other biological fluids.
For research use only - not intended for diagnostic use.

For overview, typical data and additional information please visit:
http://www.abcam.com/ab285254

Storage and Stability

On receipt entire assay kit should be stored at 4°C. Upon opening, use kit within 6 months.

Materials Supplied

Item	Quantity	Storage Condition
Micro ELISA Plate	8 x 12 strips	4°C
Lyophilized Standard	2 vials	4°C
Sample / Standard dilution buffer	20 ml	4°C
Biotin-detection antibody (Lyophilized)	1 vial	4°C
Antibody dilution buffer	10 ml	4°C
HRP-Streptavidin Conjugate (SABC)	120 µl	4°C
SABC dilution buffer	10 ml	4°C
TMB substrate (avoid light)	10 ml	4°C
Stop Solution	10 ml	4°C
Wash buffer (25X)	30 ml	4°C
Purified water	200 µl	4°C
Plate Sealers	5 units	4°C

Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully utilize this assay:
- Microplate reader capable of measuring absorbance at 450 nm
- 37°C incubator
- Precision pipettes with disposable tips.
- Distilled or deionized water
- Clean Eppendorf tubes for preparing standards or sample dilutions
- Absorbent paper

Reagent Preparation

- Prepare reagents within 30 minutes before the experiment.

Biotin-detection antibody working solution: Centrifuge for 1min at 2000xg and bring down the concentrated biotin-labeled antibody to the bottom of tube. Add 70µl purified water into tube and mix them thoroughly, after the biotin-labeled antibody is dissolved, please store it at 2-8°C. Calculate required total volume of the working solution: 50µl/well x quantity of wells. (It's better to prepare additional 100-200µl.) Dilute the biotinylated detection antibody with antibody dilution buffer at 1/100 and mix them thoroughly. (e.g. Add 10ul concentrated biotin-labeled antibody into 990µl antibody dilution buffer.)

HRP-Streptavidin Conjugate (SABC): Calculate the total volume of the working solution: 0.1 ml/well × quantity of wells with additional 0.1 - 0.2 ml of the total volume. Dilute the SABC with SABC dilution buffer at 1:100 and mix thoroughly.

Version 3b, Last updated 12 February 2025

Wash Buffer: Dilute 30mL of Concentrated Wash Buffer into 750 mL of Wash Buffer with deionized or distilled water. Put unused solution back at 4°C. Crystals formed in the concentrated wash buffer can be heated by water bath at 40°C till complete dissolution. (Heating temperature should be below 50°C.)

Standard Preparation:

1. Reconstitute the lyophilized Human 8-OHdG standard by adding 1 ml of Standard/Sample Dilution Buffer to make the 100 ng/ml standard stock solution.
2. Allow solution to sit at room temperature for 10 minutes, then gently vortex to mix completely. Use within 2 hours of reconstituting.
3. Prepare 0.6 ml of 50 ng/ml top standard by adding 0.3 ml of the above stock solution in 0.3 ml of Standard/Sample Dilution Buffer.
4. Perform 2-fold serial dilutions of the top standards to make the standard curve within the range of this assay. Suggested standard points are: 50, 25, 12.5, 6.25, 3.13, 1.56 ng/ml.

Sample Preparation:
- Samples to be used within 5 days may be stored at 4°C, otherwise samples must be stored at -20°C (≤1 month) or -80°C (≤2 months) to avoid loss of bioactivity and contamination. Homogenized samples are not suitable for use in this assay.
- End user should estimate the concentration of the target protein in the test sample first and select a proper dilution factor to make the diluted target protein concentration falls the optimal detection range of the kit.

Serum: Place whole blood sample at room temperature for 2 hours or put it at 2-8°C overnight and centrifugation for 20 minutes at approximately 1000×g, Collect the supernatant and carry out the assay immediately. Blood collection tubes should be disposable, non-pyrogenic, and non-endotoxin.

Plasma: The concentration of free 8-OHdG in plasma is very low relative to the level of DNA-incorporated 8-OHdG. Glomerular filtration results in excretion of 8-OHdG into the urine, while the DNA incorporated 8-OHdG remains in the blood. The differing fates of free versus DNA-incorporated 8-OHdG should be considered in experimental design. If you choose to measure DNA-incorporated 8-OHdG in plasma, it may be preferable to purify DNA using a commercially available kit and treat the DNA with a combination of nuclease and alkaline phosphatase to liberate the individual bases. Due to the complexities of measuring 8-OHdG in plasma, urine is often a more appropriate matrix.

Urine: Collect urine according to standard procedure into a sterile container. To clarify, centrifuge 2,000 x g for 15 minutes, or filter using a 0.45µm filter to remove precipitate. Assay immediately or store at ≤-20°C in aliquots for later use. Avoid repeated freeze-thaw cycles.

Cell culture supernatant: Collect the supernatant: Centrifuge at 2500 rpm at 2-8°C for 5 minutes, then collect clarified cell culture supernatant to detect immediately. Or you can aliquot the supernatant and store it at -80°C for future assays.

Cultured suspension cells: - Grow 1-5 x 10^6 cells in suspension using complete medium in a suitable tissue culture plate or flask. Count the cells. Harvest cells by centrifugation and remove growth medium. Wash one time with 1X PBS. Suspend cell pellets at 1x10^6 cells/ml in ice-cold 1X PBS. For example, add 5 mL 1X PBS to 5 x 10^6 cells. Aliquot 1 ml into 1.5 ml microcentrifuge tubes. Centrifuge at 10,000 x g for 10 seconds at 2-8 °C. Discard supernatant. Proceed to DNA Extraction. (Cell pellets can be flash frozen in liquid nitrogen and stored at ≤-70°C for later use.)

Supplementary figure 8. ELISA kit's manual for the assessment of 8-OHdG.

MBS2020774 96 Tests
Enzyme-linked Immunosorbent Assay Kit
For Breast Cancer Susceptibility Protein 1 (BRCA1)
Organism Species: Homo sapiens (Human)
Instruction manual

FOR IN VITRO AND RESEARCH USE ONLY
NOT FOR USE IN CLINICAL DIAGNOSTIC PROCEDURES

11th Edition (Revised in July, 2013)

[INTENDED USE]

The kit is a sandwich enzyme immunoassay for in vitro quantitative measurement of BRCA1 in human tissue homogenates, cell lysates and other biological fluids.

[REAGENTS AND MATERIALS PROVIDED]

Reagents	Quantity	Reagents	Quantity
Pre-coated, ready to use 96-well strip plate	1	Plate sealer for 96 wells	4
Standard	2	Standard Diluent	1×20mL
Detection Reagent A	1×120μL	Assay Diluent A	1×12mL
Detection Reagent B	1×120μL	Assay Diluent B	1×12mL
TMB Substrate	1×9mL	Stop Solution	1×6mL
Wash Buffer (30 × concentrate)	1×20mL	Instruction manual	1

[MATERIALS REQUIRED BUT NOT SUPPLIED]

1. Microplate reader with 450 ± 10nm filter.
2. Precision single or multi-channel pipettes and disposable tips.
3. Eppendorf Tubes for diluting samples.
4. Deionized or distilled water.
5. Absorbent paper for blotting the microtiter plate.
6. Container for Wash Solution

[STORAGE OF THE KITS]

1. **For unopened kit:** All the reagents should be kept according to the labels on vials. The **Standard, Detection Reagent A, Detection Reagent B** and the **96-well strip plate** should be stored at -20°C upon receipt while the others should be at 4 °C.
2. **For opened kit:** When the kit is opened, the remaining reagents still need to be stored according to the above storage condition. Besides, please return the unused wells to the foil pouch containing the desiccant pack, and reseal along entire edge of zip-seal.

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC OR THERAPEUTIC PROCEDURES.

Note:

It is highly recommended to use the remaining reagents within 1 month provided this is within the expiration date of the kit. For the expiration date of the kit, please refer to the label on the kit box. All components are stable until this expiration date.

[SAMPLE COLLECTION AND STORAGE]

Tissue homogenates - The preparation of tissue homogenates will vary depending upon tissue type. For this assay, tissues were rinsed in ice-cold PBS(0.01mol/L,pH 7.0-7.2) to remove excess blood thoroughly and weighed before homogenization. Minced the tissues to small pieces and homogenized them in 5-10mL of PBS with a glass homogenizer on ice(Micro Tissue Grinders woks, too). The resulting suspension was sonicated with an ultrasonic cell disrupter or subjected to two freeze-thaw cycles to further break the cell membranes. After that, the homogenates were centrifugated for 5 minutes at 5000×g. Remove the supernate and assay immediately or aliquot and store at ≤ -20°C.

Cell Lysates - Cells must be lysed before assaying according to the following directions.

1. Adherent cells should be detached with trypsin and then collected by centrifugation (suspension cells can be collected by centrifugation directly).
2. Wash cells three times in cold PBS.
3. Resuspend cells in PBS (1×) and the cells was subject to ultrasonication for 4 times (or Freeze cells at ≤ -20°C. Thaw cells with gentle mixing. Repeat the freeze/thaw cycle for 3 times.)
4. Centrifuge at 1500×g for 10 minutes at 2 - 8°C to remove cellular debris.

Other biological fluids - Centrifuge samples for 20 minutes at 1000×g. Remove particulates and assay immediately or store samples in aliquot at -20°C or -80°C. Avoid repeated freeze/thaw cycles.

Note:

1. Samples to be used within 5 days may be stored at 4°C, otherwise samples must be stored at -20°C (≤1 month) or -80°C (≤2 months) to avoid loss of bioactivity and contamination.
2. Sample hemolysis will influence the result, so hemolytic specimen should not be detected.
3. When performing the assay, bring samples to room temperature.

[REAGENT PREPARATION]

1. Bring all kit components and samples to room temperature (18-25°C) before use.
2. **Standard** - Reconstitute the **Standard** with 1.0mL of **Standard Diluent**, kept for 10 minutes at room temperature, shake gently(not to foam). The concentration of the standard in the stock solution is 100ng/mL. Please firstly dilute the stock solution to 10ng/mL and the diluted standard serves as the highest standard (10ng/mL). Then prepare 7 tubes containing 0.5mL Standard Diluent and use the diluted standard to produce a double dilution series according to the picture shown below. Mix each tube thoroughly before the next transfer. Set up 7 points of diluted standard such as 10ng/mL, 5ng/mL, 2.5ng/mL, 1.25ng/mL, 0.625ng/mL, 0.312ng/mL, 0.156ng/mL, and the last EP tubes with **Standard Diluent** is the blank as 0ng/mL.

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC OR THERAPEUTIC PROCEDURES.

Supplementary figure 10. ELISA kit's manual for the assessment of BRCA1.

Human BRCA1 Associated RING Domain Protein 1 (BARD1) Elisa kit

(Competitive ELISA)

96 Tests

Catalog Number: MBS7236583

Store all reagents at 2-8°C

Valid Period: six months

For samples:

Serum, plasma, cell culture supernatants, body fluid and tissue homogenate

Important Note!

Sample Preparation:

With respect to 6.2, we suggest pre-experimenting with neat (undiluted) samples, 1:2 or 1:4 dilutions. Please avoid diluting your samples more than 1:10 as it would exceed the dilution limit set for this kit. If the expected concentration of the target is beyond the detection range of the kit, please contact technical support.

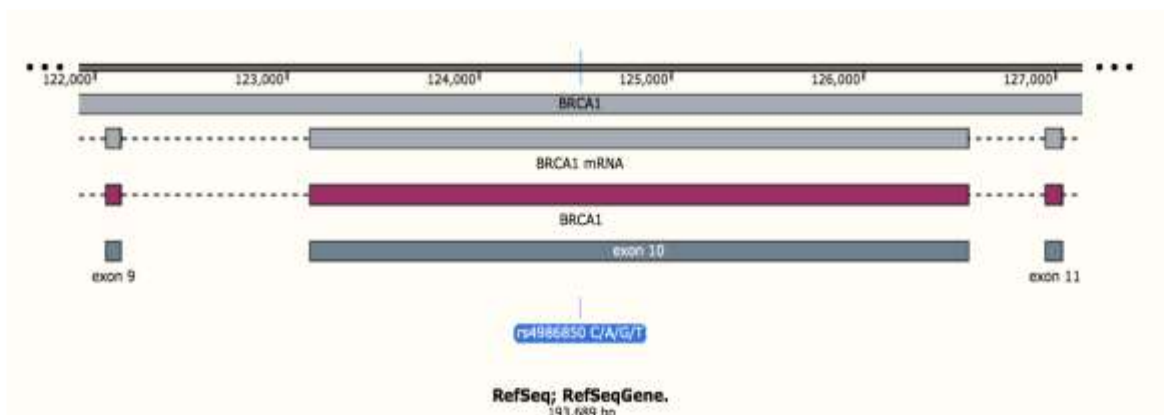
16

1

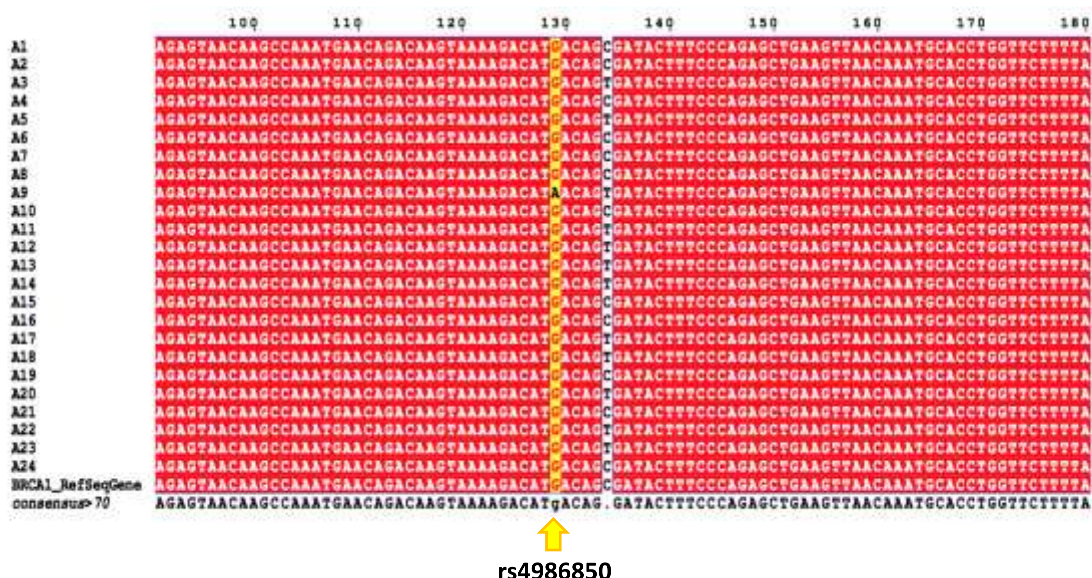
FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

MyBioSource

Supplementary figure 11. ELISA kit's manual for the assessment of BARD1.



Supplementary figure 12. Genomic position of SNP rs4986850 in the exon 10 gene *BRCA1* of *Homo sapiens.*



Supplementary figure 13. Alignment of DNA sequences of all tested samples (patients and healthy) against a deposited RefSeqGene sequence of gene *BRCA1* in the NCBI (Accession number: NG_005905). It shows that identical nucleotides would have white letters inside vertical red boxes. Mismatched nucleotides or SNPs would have either red/black letter inside yellow/white vertical boxes. SNP rs4986850 is highlighted in a vertical line of yellow vertical box. This image is generated using multiple alignment servers in the NCBI database.

Homo sapiens BRCA1 DNA repair associated (BRCA1), RefSeqGene (LRG_292) on chromosome 17

Sequence ID: NG_005905.2 Length: 193689 Number of Matches: 1

Range 1: 124402 to 124781 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
680 bits(368)	0.0	376/380(99%)	0/380(0%)	Plus/Plus

```
Query  1       TAAAGaaaaaaaaGTACAACCAAATGCCAGTCAGGCACAGCAGAAACCTACAACTCATGG  60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  124402  TAAAGAAAAAAAAGTACAACCAAATGCCAGTCAGGCACAGCAGAAACCTACAACTCATGG  124461

Query  61      AAGGTAAAGAACCTGCAACTGGAGCCAAGAAGAGTAACAAGCCAAATGAACAGACAAGTA  120
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  124462  AAGGTAAAGAACCTGCAACTGGAGCCAAGAAGAGTAACAAGCCAAATGAACAGACAAGTA  124521

Query  121     AAAGACATGACAGTGATACTTTCCCAGAGCTGAAGTTAACAAATGCACCTGGTTCTTTTA  180
               |||||||||||| |||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  124522  AAAGACATGACAGCGATACTTTCCCAGAGCTGAAGTTAACAAATGCACCTGGTTCTTTTA  124581

Query  181     CTAAGTGTTCAAATACCAGTGAACTTAAAGAATTTGTCAATCCTAGCCTTCCAAGAGAAG  240
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  124582  CTAAGTGTTCAAATACCAGTGAACTTAAAGAATTTGTCAATCCTAGCCTTCCAAGAGAAG  124641

Query  241     AAAAAGAAGAGAAACTAGAAACAGTTAAAGTGTCTAATAATGCTGAAGACCCCTAAGATC  300
               ||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||
Sbjct  124642  AAAAAGAAGAGAAACTAGAAACAGTTAAAGTGTCTAATAATGCTGAAGACCCCAAAGATC  124701

Query  301     TCAGGTTAAGTGGAGAAAGGGTTTTGCAAACTGAAAGATCTGTAGAGAGTAGCAGTATTT  360
               ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  124702  TCATGTTAAGTGGAGAAAGGGTTTTGCAAACTGAAAGATCTGTAGAGAGTAGCAGTATTT  124761

Query  361     CACTGGTACCTGGTACTGAT  380
               || |||||||||||||||||
Sbjct  124762  CATTGGTACCTGGTACTGAT  124781
```

Supplementary figure 14: Blast of DNA sequence of a PCR product of sample against a deposited sequence of gene *BRCA1* in the NCBI (Accession number: NG_008731.1). It shows that this sample has four variations compared to the corresponding region in the sequence of gene *BRCA1*. Query: SA14 sequence; Sbjct: Registered *BRCA1* sequence at NCBI. This image is generated using the NCBI BLASTn server (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Supplementary figure 15: Location of SNP rs4986850 in the exon 10 gene BRCA1 (Accession number: NG_008731.1) deposited in the NCBI database. Source: https://www.ncbi.nlm.nih.gov/

إضافة الى ذلك، كشف تحليل الارتباط الجزيئي عن اضطرابات حرجة في مريضات السرطان . فُقد الارتباط الإيجابي الطبيعي بين بروتيني BRCA1 وBARD1 الذي تم توثيقه في مجتمع الدراسة الكامل عند قصر تحليل الارتباط في مريضات السرطان فقط، الأمر الذي يُشير بوضوح إلى تضرر آليات إصلاح الحامض النووي. بالمقابل، ظهر ارتباط جديد بين miR-498 (الذي ارتفع 3 أضعاف في المريضات) وبروتين BRCA1 حصرياً في حالات السرطان (ρ=0.475). أكدت التحليلات الكيميائية الحيوية التأثيرات الجهازية، بما في ذلك ارتفاع واسمات الالتهاب (hs-CRP) لغاية 8.18 مقابل 2.00 ملغ/لتر. وقد تؤسس هذه النتائج لملفات جزيئية خاصة بالسكان في مجتمع كربلاء وتختلف عن غيرها من المواقع، مبرزة أهمية البحث المحلي للإدارة الفعالة لسرطان الثدي.

بناءً على هذه النتائج، تظهر عدة توصيات مبنية على النتائج لتحسين نتائج سرطان الثدي في كربلاء. في المقام الأول، يوصى بضرورة التحقق من أهمية وكفاءة اختبار بروتين BARD1 في التفريق بين مريضات سرطان الثدي والنساء السليمات في مجتمعات أخرى. فضلاً عن ذلك، يجب أن تعالج حملات الصحة العامة ومبادرات دائرة الصحة في كربلاء بشكل عاجل الاستخدام المرتفع بشكل استثنائي للعلاج بالهرمونات البديلة. قد تسهم هذه الدراسة في فهم سرطان الثدي لدى النساء العراقيات في مدينة كربلاء، موفرة بيانات جزيئية مهمة يمكنها توجيه التداخلات الموجهة، وتحسين الكشف المبكر، وتقليل العبء المرضي الكبير في هذه المنطقة عالية الإصابة في النهاية.

الكلمات المفتاحية: بروتين BRCA1، أورام الثدي، مثيلة الحمض النووي، الأحماض النووية الرايبية الدقيقة، تعدد أشكال النوكليوتيدة المفردة، الواسمات الحيوية للأورام.

المُلخص

تقدم هذه الأطروحة أول توصيف جزيئي شامل لسرطان الثدي لدى النساء في محافظة كربلاء، العراق، مُعالجةً فجوة معرفية حرجة في مجتمع ذي معدل إصابة مرتفع (80 لكل 100,000). يبقى سرطان الثدي أكثر الأورام الخبيثة انتشاراً بين النساء عالمياً، حيث يصيب 1.4-2.3 مليون امرأة سنوياً، وقد سجّل العراق 8,708 حالة جديدة في عام 2023 وحده. أثبتت البحوث الدولية أهمية المتغيرات الجينية *BRCA1*/*BARD1* والتعديلات اللاجينية والواسمات الجزيئية في سرطان الثدي، ولكن، لحد الآن لم يُجرَ أي تحقيق منهجي في المجتمع العراقي. شملت هذه الدراسة 188 امرأة (90 مريضة بسرطان الثدي و98 امرأة بحالة صحية جيدة كمجموعة ضابطة) من كربلاء بين تشرين الثاني 2024 وحزيران 2025، محللةً تعدد الأشكال الجيني في كل من جين *BRCA1* (rs4986850) وجين *BARD1* (rs1048108). الى جانب ذلك، تمت دراسة مثيلة محفز جين *BRCA1*، ومستويات البروتينات (BRCA1 و BARD1)، وتعبير الحامض النووي الرايبي الدقيق 498 (miRNA-498) عن طريق تقنيات تفاعل البلمرة المتسلسل (PCR) وتحليل التتابع الجيني وتقنية الاليزا (ELISA) وتفاعل PCR في الوقت الفعلي (RT-qPCR).

أظهر التحليل الديموغرافي بعض عوامل الخطر المميزة لمجتمع الدراسة، شملت مستويات التعليم المنخفضة (47.8% من المريضات لديهن تعليم ابتدائي فقط)، والاستخدام الواسع للعلاج بالهرمونات البديلة (65.6% مقابل 8.2% في غير المريضات). سريرياً، فيما يتعلق بالتشخيص، كان هنالك تأخر واضح في طلب المشورة الطبية لدى المريضات فقد كُنّ يحضرن مع حالة متقدمة من المرض وفي ما يقارب 66% من الحالات كُن يحصلن على التشخيص ولديهن إصابة في العقد الليمفاوية، رغم أن 86.7% من الحالات المدروسة كانت إيجابية لمستقبلات الهرمونات. كشف التحليل الجيني عن نمط وقائي: حيث ارتبط الطراز الجيني AA للتغاير rs1048108 في جين *BARD1* بانخفاض في خطر الإصابة بسرطان الثدي نسبته تقارب 66% وكان الأليل البديل (أو الطافر) A يرتبط بوقاية من خطر الإصابة بسرطان الثدي بنسبة 37%. كانت مثيّلة محفز *BRCA1* مرتفعة بشكل ملحوظ في المريضات (17.8%) مقابل (13.3%) وقيمة p كانت 0.043 (أي ذات دلالة إحصائية معنوية عند مستوى الدلالة 0.05)، كما لوحظت انخفاضات كبيرة في مستويات بروتين BRCA1 (4.194 مقابل 7.887 نانوغرام/مل) و BARD1 (3.991 مقابل 10.053 نانوغرام/مل). الملفت للأنتباه، أن بروتين BARD1 أظهر دقة تشخيصية استثنائية مع 93.6% مساحة تحت المنحنى، و88.5% حساسية، و95.8% نوعية.

جامعـــــة كربـــــلاء

كليـــــة الطـــــب

فرع الكيمياء والكيمياء الحياتية

تعدد الأشكال الجيني لـ *BRCA1* و*BARD1*، ومثيلة محفز *BRCA1*، ومستويات البروتينات ذات العلاقة لدى النساء المصابات بسرطان الثدي في مدينة كربلاء

رسالة مقدمة الى

مجلس فرع الكيمياء والكيمياء الحياتية، كلية الطب، جامعة كربلاء

كجزء من متطلبات نيل درجة ماجستير العلوم في تخصص

الكيمياء السريرية

من قبل

شيرين عادل عبد

بكالوريوس علوم الكيمياء (جامعة ديالى، 2022)

بإشراف

الأستاذ الدكتورة

رنا مجيد حميد

دكتوراه كيمياء حياتية

كلية الطب، جامعة كربلاء

الأستاذ المساعد الدكتور

محمد فوزي ال قنبر

بورد (دكتوراه) علم الأمراض

كلية الطب، جامعة السبطين

ذو الحجة، 1446 حزيران، 2025