Vitamin D3 Mediated Regulation of Hormone Receptors in the Pathogenesis of Triple- Negative Breast Cancer

A Thesis Submitted for the Award of the Degree of Doctoral of Philosophy (Ph. D)

By

SHANKARAMURTHY K N

Registration No: 20Ph.D021

Department of Biochemistry

Under the guidance of

Dr. Indira A. Hundekari

BLDE

(Deemed to be University)

Smt. Bangaramma Sajjan Campus, B M Patil Road (Solapur Road), Vijayapura – 586103, Karnataka, India.

June 2025

BLDE

(DEEMED TO BE UNIVERSITY)

Declared as Deemed to be University u/s 3 of UGC Act, 1956

Shri B M Patil Medical College, Hospital and Research Centre

DECLARATION BY THE CANDIDATE

I declare that the thesis entitled **"Vitamin D3 Mediated Regulation of Hormone Receptors in the Pathogenesis of Triple-Negative Breast Cancer"** submitted by me for the degree of Doctor of Philosophy (Ph.D.) is the record of work carried out by me under the guidance of **Dr. Indira A. Hundekari** and has not formed the basis for the award of any Degree, Diploma, Associateship, Fellowship, Titles in this University or any other University or other similar institution of Higher Learning.

Signature of the Candidate
(SHANKARAMURTHY K N)
Department of Biochemistry
Ph.D Scholar. 20Ph.D021

Date: 24/6/25

Place: Vijayapura

BLDE

(DEEMED TO BE UNIVERSITY)

Declared as Deemed to be University u/s 3 of UGC Act, 1956

Shri B M Patil Medical College, Hospital and Research Centre

CERTIFICATE FROM THE GUIDE and (CO-GUIDE, IF ANY)

I/We certify that the thesis entitled **"Vitamin D3 Mediated Regulation of Hormone Receptors in the Pathogenesis of Triple-Negative Breast Cancer"**, submitted for the degree of Doctor of Philosophy (Ph.D.) by **Mr. Shankaramurthy K N**, is the record of research work carried out by him under my/our guidance and supervision, and that this work has not formed the basis for the award of any Degree, Diploma, Associateship, Fellowship or other Titles in this University or any other University or institution of Higher Learning.

23.06.2025

Signature of the Co-Guide

Dr. Praveenkumar Shetty

R&D Director, Nitte University

Mangaluru

Prof. (Dr.) Praveen Kumar Shetty, PhD
Director (Research & Development)
Nitte (Deemed to be University)
Deralakatte, Mangaluru

21/06/2025

Signature of the Guide

Dr. Indira A. Hundekari

Professor, Department of Biochemistry

Shri B M Patil Medical College,

Vijayapura

Professor
Dept. of Biochemistry
BLDE (Deemed to be University)
Shri B.M.Patil Medical College,
Vijayapur-586103.

Date: 24/6/25

Place: Vijayapura

BLDE

(DEEMED TO BE UNIVERSITY)

Declared as Deemed to be University u/s 3 of UGC Act, 1956

Shri B M Patil Medical College, Hospital and Research Centre

CERTIFICATE FROM THE HEAD OF THE INSTITUTION AND DEPARTMENT

I certify that the thesis entitled **"Vitamin D3 Mediated Regulation of Hormone Receptors in the Pathogenesis of Triple-Negative Breast Cancer"** submitted for the degree of Doctor of Philosophy (Ph.D.) is the record of research work carried out by **Mr. Shankaramurthy K N** under the guidance and supervision of **Dr. Indira A. Hundekari** in partial fulfillment for the award of Doctor of Philosophy in the **Faculty of Medicine**, and that this work was carried out by him in the department of **Biochemistry**.

24/6/25

Signature of the HOD

Dr. Deepa Sajjanar

Professor and Head,

Department of Biochemistry

Professor & HOD
Dept. of Biochemistry
BLDE (Deemed to be University)
Shri B.M.Patil Medical College,
Vijayapur-586103.

Date: 24/6/25

Place: Vijayapura

Signature of the Principal

Dr. Aravind V. Patil

Principal, Shri B M Patil Medical College, Hospital and Research Centre

PRINCIPAL
BLDE (Deemed to be University)
Shri B. M. Patil Medical College
Hospital & Research Centre,
VIJAYAPUR-586103

ACKNOWLEDGEMENTS

With deep gratitude and respect, I extend my sincere thanks to all those who have supported and guided me throughout the course of my doctoral journey. This thesis is the outcome of not just my efforts, but the collective encouragement, mentorship, and assistance I have received from many individuals and institutions.

I am profoundly thankful to Dr. M. B. Patil, Hon'ble Chancellor; Dr. Y. M. Jayaraj, Pro-Chancellor; Dr. R. S. Mudhol, Vice-Chancellor; Dr. Arun C. Inamdar, Pro-Vice-Chancellor; and Dr. Raghavendra V. Kulkarni, Registrar, along with Mr. Satish Patil, Deputy Registrar, for their constant encouragement and unwavering institutional support. Their leadership and vision have created a nurturing environment that has enabled the successful completion of my doctoral research.

I sincerely express my gratitude to Dr. Aravind V. Patil, Principal, and Dr. Tejaswini Vallabha, Dean, Faculty of Medicine, for their academic leadership, guidance, and support throughout my research work. Their timely advice and motivation have been instrumental in shaping this study.

I extend my heartfelt thanks to Dr. Kusal Das, Chairperson of my Doctoral Committee, and Dr. Nilima Dongre, Doctoral Committee Member, for their valuable evaluations, insightful suggestions, and continuous academic support throughout my research period.

I am also thankful to Dr. Basavaraj B. Devaranavadagi, former Head of the Department of Biochemistry, for his encouragement, and to Dr. Deepa Sajjannar, Head of Department, for her ongoing support and guidance throughout the research journey.

My deepest gratitude is reserved for my guide, Dr. Indira A. Hundekari, Additional Professor, Department of Biochemistry, for her invaluable mentorship, constant encouragement, and steadfast guidance at every stage of this work. Her expertise and dedication have played a central role in the successful completion of this thesis.

I am equally indebted to my co-guide, Dr. Praveenkumar Shetty, Professor and Director (R&D), Department of Biochemistry, NITTE (Deemed to be University), Mangaluru, for his insightful scientific input, encouragement, and expert guidance, which greatly enriched my research.

I gratefully acknowledge the support of the Research and Development Cell, especially Dr. M. M. Patil, Dr. Chandrika D and Dr. Nirmala, for their facilitation and guidance throughout this research. I also extend my sincere thanks to all the faculty members of the Centre for Advanced Medical Research (CAMR) for their valuable cooperation and motivation.

Finally, I express my appreciation to all the teaching and non-teaching staff of the Department of Biochemistry for their support and assistance during the course of my doctoral research. Their contributions, though often behind the scenes, have been vital to the smooth execution of my work.

A very special thanks to my beloved parents, whose unconditional love, blessings, and sacrifices have been the foundation of my academic journey. Their unwavering support has given me the strength and determination to reach this important milestone.

BLDE

(DEEMED TO BE UNIVERSITY)

Declared as Deemed to be University u/s 3 of UGC Act, 1956
Shri B M Patil Medical College, Hospital and Research Centre

PLAGIARISM VERIFICATION CERTIFICATE

1. Name of Student: Mr. Shankaramurthy K N **Reg. No.:** 20Ph.D021
2. Title of the Thesis: Vitamin D3 Mediated Regulation of Hormone Receptors in the Pathogenesis of Triple-Negative Breast Cancer.
3. Department: Biochemistry
4. Name of Guide & Designation: Dr. Indira A. Hundekari, Additional Professor
5. Name of Co-Guide & Designation: Dr. Praveenkumar Shetty, Professor, Director – R&D, Nitte University

The above thesis was verified for similarity detection. The report is as follows:
Software used: iThenticate **Date:** 19.06.2025
Similarity Index (%): 3% **Total word count:** 46,160
The report is attached for the review by the Student and Guide.
The plagiarism report of the above thesis has been reviewed by the undersigned. The similarity index is below accepted norms.

The similarity index is above accepted norms, because of the following reasons:
- The similarity index is above accepted norms due to standard scientific terminology.
- Common methodological descriptions contribute to the observed overlap.
- Such similarities are typical in research and do not indicate duplication.
- The document has been thoroughly reviewed and adheres to academic integrity standards. The thesis may be considered for submission to the university. The software report is attached.

21/06/2025
Signature of Guide
Dr. Indira Hundekari
Professor
Additional Professor
Dept. of Biochemistry
BLDE (Deemed to be University)
Shri B.M.Patil Medical College,
Vijayapur-586103.

23.06.2025
Signature of Co-Guide
Dr. Praveenkumar Shetty
Professor, Director – R&D
Prof. (Dr.) Praveen Kumar Shetty, PhD
Director (Research & Development)
Nitte (Deemed to be University)
Deralakatte, Mangaluru

Signature of Student
Mr. Shankaramurthy K N

Librarian
Verified by (Signature)
Name & Designation
B.L.D.E. Deemed to be University
Shri B. M. Patil Medical College,
Vijayapur.

INDEX

LIST OF FIGURES

Figure No. **Page No.**

LIST OF TABLES

ABBREVIATION LIST

17β-estradiol	17β-Estradiol
ABAM	Antibiotic-Antimycotic
AI	Artificial Intelligence
AJCC	American Joint Committee on Cancer
ALAN	Artificial Light At Night
BC	Breast Cancer
BCA	Bicinchoninic Acid
BCS	Breast-Conserving Surgery
BMI	Body Mass Index
BRCA	Breast Cancer gene (BRCA1/2)
BRCAs	Breast Cancer Susceptibility Genes
BSA	Bovine Serum Albumin
BSE	Breast Self-Examination
Bak	Bcl-2 Homologous Antagonist/Killer
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma-extra large
BrdU	Bromodeoxyuridine
CA	Cancer Antigen
CDH1	Cadherin 1
CDK4/6	Cyclin-Dependent Kinases 4 and 6
CDK6	Cyclin-Dependent Kinase 6
CDKIs	Cyclin-Dependent Kinase Inhibitors
CDKN1A	Cyclin-Dependent Kinase Inhibitor 1A
COX2	Cyclooxygenase-2
CO_2	Carbon Dioxide
CPOX	Coproporphyrinogen Oxidase
CSCs	Cancer Stem Cells
CXCR4	C-X-C Motif Chemokine Receptor 4
CYP27B1	Cytochrome P450 Family 27 Subfamily B Member 1
CpG island	Cytosine–phosphate–Guanine Island
$CuSO_4$	Copper Sulfate

DAB	3,3′-Diaminobenzidine
DDH_2O	Double Distilled Water
DDT	Dichlorodiphenyltrichloroethane
DES	Diethylstilbestrol
DFS	Disease-Free Survival
DMSO	Dimethyl Sulfoxide
DPX	Distrene Plasticiser Xylene
DSF	Disease-Free Survival
E2	17β-Estradiol
E2F3/E2F7	E2F Transcription Factor 3/7
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic Acid
EGFR	Epidermal Growth Factor Receptor
ER	Estrogen Receptor
ERBB2 gene)	Human Epidermal Growth Factor Receptor 2 (HER2
ERE	Estrogen Response Element
EREs	Estrogen Response Elements
ERα	Estrogen Receptor Alpha
ERβ	Estrogen Receptor Beta
ERβ1	Estrogen Receptor Beta 1
ESR1	Estrogen Receptor 1 (gene encoding ERα)
ESR2	Estrogen Receptor 2 (gene encoding ERβ)
EZH2	Enhancer of Zeste Homolog 2
EpCAM	Epithelial Cell Adhesion Molecule
FFPE	Formalin-Fixed, Paraffin-Embedded
FISH	Fluorescence In Situ Hybridization
FNAC	Fine Needle Aspiration Cytology
GADD45	Growth Arrest and DNA Damage-Inducible 45
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GPER1	G-Protein-Coupled Estrogen Receptor 1
GRB7	Growth Factor Receptor-Bound Protein 7
GWAS	Genome-Wide Association Study

H&E	Hematoxylin and Eosin
HER2	Human Epidermal Growth Factor Receptor 2
HPD	Hematoporphyrin Derivative
HR	Hormonal Receptor
HRT	Hormonal Replacement Therapy
IBC	Invasive Breast Cancer
IDC	Invasive Ductal Carcinoma
IHC	Immunohistochemical
IMP3	IGF2 mRNA-binding protein 3
IMRT	Intensity-Modulated Radiotherapy
IRS	Immunoreactive Score
IU	International Unit
KO	Knockout
LN_2	Liquid Nitrogen
MAPK	Mitogen-Activated Protein Kinase
MCL-1	Myeloid Cell Leukemia 1
MDA-MB	MD Anderson – Metastatic Breast
MEK	Mitogen-Activated Protein Kinase Kinase
MMP-9	Matrix Metalloproteinase-9
MR	Mendelian Randomization
MRI	Magnetic Resonance Imaging
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium
Bromide	
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
NST	No Special Type
OD	Optical Density
OS	Overall Survival
PAHs	Polycyclic Aromatic Hydrocarbons
PAM50	Prediction Analysis of Microarray 50-gene signature
PBS	Phosphate-Buffered Saline
PCBs	Polychlorinated Biphenyls
PD-L1	Programmed Death Ligand-1
PDB	Protein Data Bank

PDCD4	Programmed Cell Death 4
PEM	Positron Emission Mammography
PET/CT	Positron Emission Tomography/Computed Tomography
PGE2	Prostaglandin E2
PI3K/AKT/mTOR	Phosphoinositide 3-Kinase/Protein Kinase B/Mammalian Target of Rapamycin
PR	Progesterone Receptor
PTEN	Phosphatase and Tensin Homolog
PTX	Paclitaxel
PUFAs	Polyunsaturated Fatty Acids
Pcr	Pathological Complete Response
PpIX	Protoporphyrin IX
RAS/MEK/ERK	Rat Sarcoma/Mitogen-Activated Protein Kinase Kinase/Extracellular Signal-Regulated Kinase
RNA	Ribonucleic Acid
RXR	Retinoid X Receptor
RXRα	Retinoid X Receptor Alpha
Rb	Retinoblastoma Protein
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEER	Surveillance, Epidemiology, and End Results Program
SLUG	SNAI2 Transcription Factor
SNPs	Single Nucleotide Polymorphisms
SODs	Superoxide Dismutases
SPECT	Single-Photon Emission Computed Tomography
STATs	Signal Transducer and Activator of Transcription
STK11	Serine/Threonine Kinase 11
TBS	Tris-Buffered Saline
TCGA	The Cancer Genome Atlas
TEMED	Tetramethylethylenediamine
TGFβ/TGFβR	Transforming Growth Factor Beta / Receptor
TNBC	Triple-Negative Breast Cancer
TP53	Tumor Protein p53

P53	Protein p53
TXNRD1	Thioredoxin Reductase 1
UTR	Untranslated Region
VDR	Vitamin D Receptor
VDRE	Vitamin D Response Element
VDREs	Vitamin D Response Elements
VEGF	Vascular Endothelial Growth Factor
VITAL	Vitamin D and Omega-3 Trial
WHO	World Health Organization
WT	Wild-Type
XRCC2	X-Ray Repair Cross Complementing 2
ZEB1	Zinc Finger E-box Binding Homeobox 1
circRNA	Circular RNA
ctDNA	Circulating Tumor DNA
hTERT	Human Telomerase Reverse Transcriptase
kDa	Kilodalton
lncRNA	Long Non-Coding RNA
mRNA	Messenger Ribonucleic Acid
miRNA	MicroRNA
mitoERβ	Mitochondrial-Localized Estrogen Receptor Beta
mutp53	Mutant p53
nM	Nanomolar
pri-miRNA	Primary MicroRNA Transcript
µM	Micromolar
µg/µL	Microgram per Microliter

ABSTRACT

ABSTRACT

Introduction

Breast cancer (BC) is the most prevalent malignancy among women globally. Triple-negative breast cancer (TNBC), characterized by the absence of ER, PR, and HER2 expression, remains a particularly aggressive subtype with limited treatment options and poor prognosis. Recent evidence suggests that the vitamin D receptor (VDR) and estrogen receptor beta 1 (ERβ1) may serve as tumor suppressors in TNBC. This study investigates the therapeutic potential of modulating VDR and ERβ1 pathways using calcitriol and 17β-estradiol, respectively.

Methods

This study was conducted in three phases. In Phase I, immunohistochemical analysis of VDR and ERβ1 expression was performed on 30 formalin-fixed, paraffin-embedded invasive ductal carcinoma samples, spanning 4 molecular BC subtypes. Phase II involved molecular docking simulations to evaluate the binding affinities of calcitriol and 17β-estradiol to VDR, ERβ, EGFR, VEGF, and caspase-3 using Cresset Flare software. In Phase III, *in vitro* assays using MDA-MB-468 TNBC cells were conducted to assess the effects of individual and combined treatments on cell viability (MTT assay) and expression of ERβ1, VDR, EGFR, VEGF, and caspase-3 (Immunoblotting).

Results

The results of this study were obtained across three integrated experimental phases: immunohistochemistry, molecular docking, and *in vitro* functional assays, each contributing to a comprehensive understanding of the therapeutic relevance of VDR and ERβ1 in TNBC.

Immunohistochemical (IHC) findings from Phase I revealed that VDR and ERβ1 are variably expressed across molecular subtypes of BC. Notably, in TNBC cases, VDR was localized to both the cytoplasm and nucleus, whereas ERβ1 showed cytoplasmic expression only. This pattern was distinct from other subtypes such as Luminal A and B, which showed relatively higher nuclear staining, particularly for ERβ1. The exclusive cytoplasmic localization in TNBC suggests altered receptor signaling, possibly indicative of non-genomic pathways or receptor dysfunction.

These VDR and ERβ1 expression may play subtype-specific roles in tumor suppression and provide a basis for evaluating these receptors as therapeutic targets.
In Phase II, molecular docking simulations provided computational insights into the binding interactions between ligands (calcitriol and 17β-estradiol) and their target proteins. Calcitriol showed high binding affinity for VDR, confirming a strong ligand-receptor interaction at the predicted active site. Additionally, it exhibited moderate binding with EGFR and caspase-3, suggesting possible indirect regulatory effects on proliferative and apoptotic signaling. Similarly, 17β-estradiol demonstrated strong affinity for ERβ and VEGF, implicating its potential role in modulating estrogen-responsive and angiogenic pathways. These *in silico* findings support the therapeutic plausibility of targeting multiple signaling axes through ligand-mediated receptor activation.

Phase III involved functional validation through *in vitro* assays using MDA-MB-468 TNBC cells. Treatment with calcitriol (1–5 μM), 17β-estradiol (100–500 nM), and their combination significantly reduced cell viability in a dose- and time-dependent manner. Notably, the combination treatment produced greater reduction in viability compared to either agent alone, indicating potential additive or combination effects.

Immunoblot analysis further validated the molecular impact of treatment. Calcitriol exposure led to decreased ERβ1 expression and downregulation of EGFR and VEGF over time, while increasing caspase-3 levels, suggesting an induction of apoptosis. Treatment with 17β-estradiol similarly modulated ERβ1 expression, with limited effect on EGFR but a notable increase in VEGF modulation and caspase-3 downregulation. Most importantly, combined treatment resulted in the most robust molecular changes, showing simultaneous downregulation of proliferative and angiogenic markers and strong upregulation of caspase-3.

Together, these results demonstrate that dual modulation of VDR and ERβ1 using calcitriol and 17β-estradiol disrupts oncogenic signaling, promotes apoptosis, and may serve as an effective therapeutic strategy for ERβ1-positive TNBC.

Conclusion

This study demonstrates that dual targeting of VDR and ERβ1 using calcitriol and 17β-estradiol elicits favorable antitumor responses in TNBC cells. The combination strategy regulates key oncogenic pathways involved in proliferation, angiogenesis, and apoptosis, highlighting the therapeutic promise of receptor-based induced in ERβ1-positive TNBC. These findings warrant further validation in animal models and could contribute to the development of novel combination treatments for this aggressive BC subtype.

Keywords: Breast cancer, TNBC, VDR, ERβ1, IHC, Vitamin D/D_3, Calcitriol, 17β-Estradiol, Molecular Docking

CHAPTER-1
INTRODUCTION

1. INTRODUCTION

Breast cancer is a malignant neoplasm characterized by the uncontrolled proliferation and invasive behavior of epithelial cells within the breast tissue.[1] Over the past three decades, its incidence and mortality rates have risen globally, posing a significant public health burden.[2] Although considerable progress has been made in characterizing its molecular subtypes, the precise mechanisms underlying BC pathogenesis remain incompletely understood. This gap in knowledge, combined with the persistent issue of delayed diagnosis, continues to impede timely and effective therapeutic intervention. While early detection is widely recognized as a critical factor in improving treatment outcomes, existing diagnostic modalities still face important limitations.[3]

The majority of breast cancers (BCs) arise from the epithelial lining of the lactiferous ducts and are classified as ductal carcinomas. A smaller subset originates in the lobular epithelium, referred to as lobular carcinomas.[4] In contrast, non-epithelial malignancies such as sarcomas and lymphomas though rare develop from the stromal, vascular, or lymphoid components of the breast tissue.[5] The complex histological architecture and diverse cellular composition of the breast contribute to the varied morphological and molecular subtypes of BC; each associated with distinct prognostic and therapeutic implications.[6] The association between BC subtypes and the risk of developing distant metastasis has been well established. Newly diagnosed BCs have been reported to present with bone (3.28%), lung (1.52%), liver (1.2%), and brain (0.35%) metastases at the time of diagnosis.[7] Among the molecular subtypes, TNBC exhibits a higher incidence of brain, liver, and lung metastases, but a significantly lower rate of bone metastases compared to the luminal A subtype. Additionally, patterns of recurrence vary by subtype; HER2-positive and TNBC subtypes demonstrate the highest rates of local and regional recurrence, with recurrence rates of 7.5% and 3.4% for HER2, and 7.6% and 3.3% for TNBC, respectively.[8]

1.1 TRIPLE NEGATIVE BREAST CANCER

TNBC is regarded as the most aggressive and therapeutically challenging subtype of BC.[9] It is defined by the absence of ER, PR, and human epidermal growth HER2 expression. Clinically, TNBC is associated with high invasiveness, elevated

metastatic potential, early disease recurrence, and poor overall prognosis.[10] The lack of hormone receptors and HER2 renders TNBC unresponsive to both endocrine therapies and HER2-targeted treatments, thereby limiting current therapeutic options.[9] As a result, the development of novel, targeted treatment strategies is an urgent priority in the management of TNBC.

1.1.1 Therapeutic Potential of Vitamin D and VDR Signaling Axis in TNBC

Cancer cells, including those in TNBC, often exploit shared regulatory pathways that govern proliferation, differentiation, and apoptosis. Vitamin D signaling has been shown to modulate these critical cellular processes, indicating its potential role in tumor suppression alongside immune regulation.[11] The anticancer effects of vitamin D are largely attributed to its ability to regulate cellular growth and differentiation.[12]

Among the key mediators of vitamin D signaling is the VDR, a nuclear receptor that becomes activated upon binding to the hormonally active form of vitamin D, calcitriol.[13] The ligand-bound VDR forms a heterodimer with retinoid X receptor (RXR) and interacts with vitamin D response elements (VDREs) in the promoter regions of target genes, thereby modulating transcriptional networks that influence cell cycle regulation, apoptosis, angiogenesis, and metastatic potential.[14] In experimental TNBC models, calcitriol has been shown to downregulate genes involved in invasion and metastasis, indicating a tumor-suppressive role.[15] These effects were absent in VDR-knockout mice but were restored upon reintroduction of human VDR, confirming the receptor's essential role in mediating the antitumor activity of calcitriol in TNBC cells.[16]

Clinical evidence further reinforces the functional relevance of VDR signaling in TNBC.16 VDR expression has been identified in a substantial proportion of breast tumors and is consistently associated with favorable clinical outcomes. Patients with VDR-positive tumors exhibit significantly longer disease-free survival compared to those with VDR-negative tumors.[17] Furthermore, high VDR expression is frequently observed in BCs with lower mortality risk and improved prognosis.[18] Conversely, the absence of VDR expression has been correlated with an increased incidence of ER-negative and PR-negative subtypes, which are characteristic features of TNBC.[19] Given the limited treatment options in TNBC and the observed inverse

relationship between VDR expression and tumor aggressiveness, therapeutic strategies aimed at restoring or enhancing VDR signaling are of considerable interest. Notably, emerging data suggest that combining calcitriol with ERβ agonists may effectively reduce tumor invasiveness and offer synergistic benefits in TNBC models, highlighting a potential avenue for targeted intervention in this challenging BC subtype.[20]

1.1.3 Estrogen Receptor Beta and Its Role in TNBC

ERβ, identified in 1996 as a homolog of estrogen receptor alpha (ERα), is encoded by the *ESR2* gene and functions as a ligand-activated transcription factor involved in modulating gene expression, cell proliferation, and differentiation.[21] Unlike ERα, which promotes proliferation in hormone-responsive tissues, ERβ has been recognized for its tumor-suppressive functions in various cancers, including BC.[22] In the context of TNBC, which lacks expression of ERα, PR, and HER2, ERβ has attracted attention as an alternative therapeutic target.[23] Although TNBC is typically considered hormone receptor–negative, subsets of TNBC tumors have been found to express ERβ, and its presence correlates with a less aggressive phenotype and better clinical outcomes.[24] Experimental studies demonstrate that ERβ expression in TNBC cell lines leads to inhibition of proliferation, reduced invasiveness, and suppression of tumorigenicity both *in vitro* and *in vivo*.[25]

Among the various isoforms of ERβ, ERβ1 is the only full-length, transcriptionally active form capable of binding DNA and regulating gene expression. ERβ1 has been identified as a functionally distinct tumor suppressor in TNBC. *In vivo* xenograft models using human TNBC cells overexpressing ERβ1 showed significant reductions in primary tumor growth and metastatic dissemination.[26] Mechanistically, elevated ERβ1 expression in TNBC is associated with downregulation of epithelial-to-mesenchymal transition (EMT) markers and BC stem cell markers, alongside increased expression of genes involved in suppressing cell invasiveness and metastasis.[27] Furthermore, genomic and transcriptomic analyses have identified both ligand-dependent and ligand-independent ERβ1 target genes, many of which are relevant to tumor progression control.[25]

The natural ligand of ERβ1 is 17β-estradiol (E2), which upon binding activates ERβ1-mediated transcriptional regulation. In TNBC models, treatment with 17β-estradiol has been shown to enhance the antitumor effects of ERβ1, including induction of G1 cell cycle arrest, reduction in proliferation, and promotion of apoptosis.[26,27] Notably, these effects are dependent on an intact DNA-binding domain of ERβ1, underscoring the importance of its nuclear function.[28]

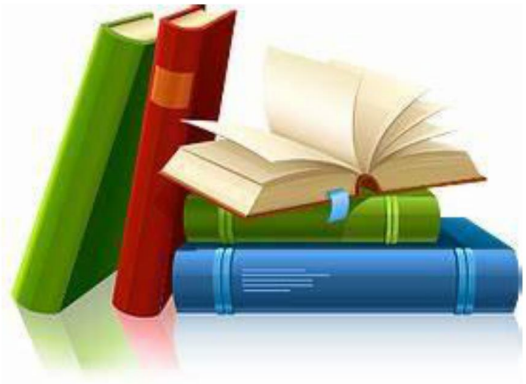
Beyond the classical genomic pathway where the 17β estradiol-ER complex translocates to the nucleus to modulate gene expression, recent studies have highlighted the importance of rapid, non-genomic estrogen signaling. These non-genomic actions are mediated by membrane-bound estrogen receptors and are capable of triggering rapid cellular responses independent of direct gene transcription. Such mechanisms have garnered growing interest for its role in cancer progression and their potential as novel endocrine-associated therapeutic targets.[29] Targeting ERβ1 using 17β-estradiol or selective ERβ agonists represents a promising therapeutic approach for ERβ1-positive TNBC. IHC studies using the isoform-specific PPG5/10 monoclonal antibody have reported ERβ1 positivity in approximately 18% of TNBC tumors.[30] Based on this potential, an ongoing phase II clinical trial (NCT03941730) is currently evaluating the efficacy of ERβ1 stimulation using estradiol in patients with advanced or metastatic ERβ1-positive TNBC.[31] Although emerging evidence highlights the individual tumor-suppressive functions of ERβ1 and the VDR in TNBC, their combined therapeutic targeting remains underexplored. Specifically, there is a lack of integrated data on the co-expression and subcellular localization of VDR and ERβ1 across BC subtypes, particularly in ERβ1 positive TNBC. Moreover, while calcitriol and 17β-estradiol have been investigated independently, the mechanistic interplay between VDR and ERβ1 signaling pathways, including their influence on downstream targets such as EGFR, VEGF, and caspase-3, has not been comprehensively characterized. To date, no study has systematically combined immunohistochemistry, molecular docking, and *in vitro* functional assays to elucidate the biological relevance of dual receptor modulation in ERβ1-positive TNBC.

Therefore, our study is designed to fill this critical gap by systematically evaluating the expression and biological significance of VDR and ERβ1 signaling in TNBC. Collectively, these insights provide a mechanistic rationale for targeting both

VDR and ERβ1 using calcitriol and 17β-estradiol, respectively, as a dual-targeted therapeutic approach.

To investigate this, in the present study, we planned to evaluate the expression patterns and functional significance of VDR and ERβ1 in TNBC.

Through this integrative approach, the study aims to elucidate the molecular crosstalk between VDR and ERβ1 signaling pathways activated by calcitriol and 17β-estradiol, and to provide a mechanistic basis for dual receptor-targeted strategies in the management of ERβ1-positive TNBC.



CHAPTER-2
REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 ANATOMY OF THE BREAST

The female breast is composed of multiple integrated structures. Externally, it is covered by skin and includes the areola and nipple. Internally, the breast comprises adipose tissue and glandular components such as lactiferous glands and lactiferous ducts, which are responsible for the production and transport of milk. These ducts converge at the nipple. The glandular tissue is embedded within supportive connective and fatty tissues.[32] Posteriorly, the breast is situated over the pectoralis major muscle and the ribs, with the intercostal muscles forming the deeper thoracic wall.[33] Structurally, the gland consists of 15 to 20 lobes arranged radially around the nipple, each drained by an individual lactiferous duct, as shown in Figure 1. Although these lobes are separated by fibrous connective tissue septa, they are not distinctly identifiable during surgical procedures due to their close integration within the glandular stroma.[34] This anatomical organization underpins both the physiological functions and structural support of the breast.

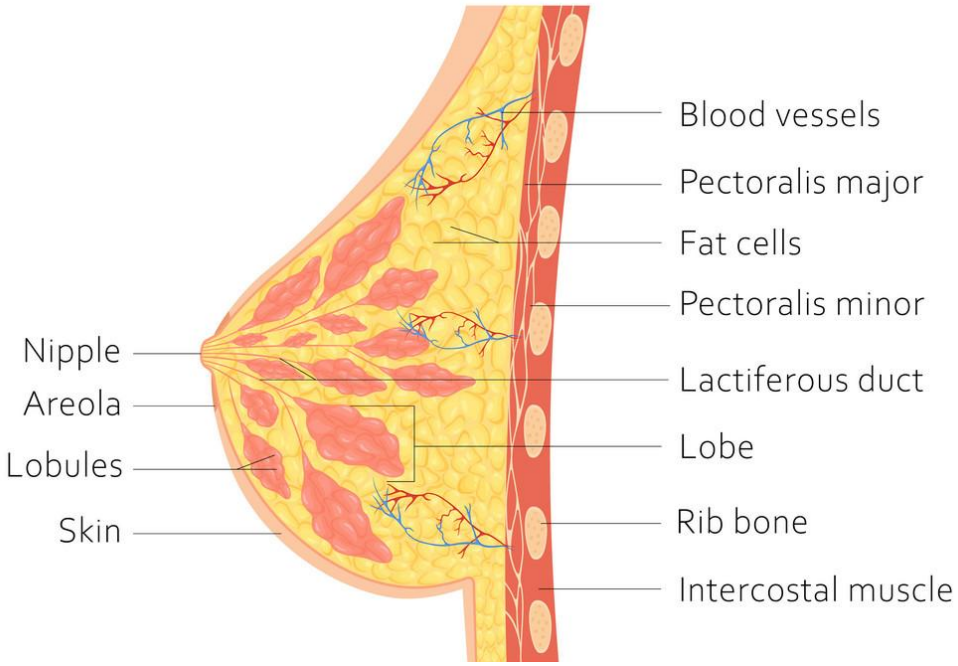


Figure 1. Anatomical structure of the female breast.

A sagittal section of the female breast illustrating key components, including the skin, adipose tissue, areola, nipple, lactiferous ducts and glands, as well as the underlying pectoralis major and intercostal muscles. The breast lies over the ribs and consists of glandular tissue supported by connective and fatty tissue, with ducts converging toward the nipple for milk secretion. **Source:** **https://www.vectorstock.com/royalty-free-vector/female-breast-cross-section-anatomy-vector-41766610** Accepted on 16.06.2024.

2.1.2 Lymphatic System of the Breast

The lymphatic system plays a critical role in the metastatic spread of BC. It consists of a network of lymphatic vessels and lymph nodes that facilitate immune surveillance and drainage of interstitial fluid.[35] Lymph nodes are small, bean-shaped structures containing immune cells that help fight infections. In the context of BC, malignant cells can infiltrate the lymphatic vessels and disseminate to regional lymph nodes, particularly those in the axillary, and internal mammary regions. The presence of cancer cells in lymph nodes is a key prognostic indicator, as it significantly increases the likelihood of systemic spread to distant organs.[36]

2.1.3 Benign Breast Lumps

The majority of palpable breast lumps are benign and do not indicate cancer.[37] Benign breast tumors typically arise from non-cancerous proliferations such as fibrosis (formation of fibrous connective tissue) and cysts (fluid-filled sacs).[38] These lesions can cause the breast to feel nodular or lumpy, and in some cases, may be associated with a slightly cloudy nipple discharge. Although benign, such findings warrant thorough clinical and radiological evaluation to distinguish them from malignant lesions and guide appropriate management.[38]

2.1 OVERVIEW OF BREAST CANCER

BC is a heterogeneous group of malignant neoplasms originating primarily from the epithelial components of the breast tissue.[39] Anatomically, as illustrated in Figure 2, the female breast comprises;

i. Lobules (milk-producing glands)
ii. Ducts (channels that transport milk to the nipple)
iii. Stromal elements including adipose and fibrous connective tissue
iv. Blood vessels, and an extensive lymphatic network.[32]

The majority of BCs arise from the epithelial lining of the lactiferous ducts and are classified as ductal carcinomas. A smaller subset originates in the lobular epithelium, referred to as lobular carcinomas.[4] In contrast, non-epithelial malignancies such as sarcomas and lymphomas though rare develop from the stromal, vascular, or lymphoid components of the breast tissue.[5] The complex histological architecture and diverse cellular composition of the breast contribute to the varied morphological and

molecular subtypes of BC; each associated with distinct prognostic and therapeutic implications.[6] The association between BC subtypes and the risk of developing distant metastasis has been well established. Newly diagnosed BCs have been reported to present with bone (3.28%), lung (1.52%), liver (1.2%), and brain (0.35%) metastases at the time of diagnosis.[7]

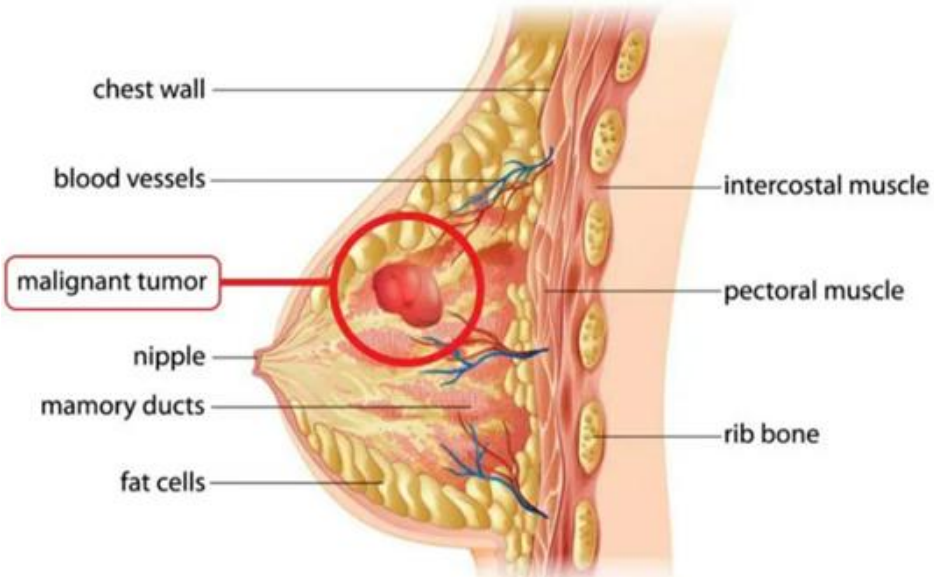


Figure 2. Schematic representation of a malignant tumor breast.

The image depicts the anatomical structures of the female breast, including the nipple, mammary ducts, fat cells, blood vessels, and underlying muscular layers such as the pectoral and intercostal muscles. A malignant tumor is localized within the glandular tissue, demonstrating its potential proximity to the ductal system, vasculature, and chest wall structures, which can influence tumor progression and metastasis.

Source: https://alaskasurgicaloncology.com/treatment/breast-cancer-treatment-in-anchorage/

Accepted on 10.06.2025.

Among the molecular subtypes, TNBC exhibits a higher incidence of brain, liver, and lung metastases, but a significantly lower rate of bone metastases compared to the luminal A subtype. Additionally, patterns of recurrence vary by subtype; HER2-positive and TNBC subtypes demonstrate the highest rates of local and regional recurrence, with recurrence rates of 7.5% and 3.4% for HER2 and 7.6% and 3.3% for TNBC, respectively.[8]

2.3 BREAST CANCER SYMPTOMS

BC presents with a range of symptoms that can vary in severity and visibility. One of the most common signs is a painless lump in the breast, often detected during self-examination or routine screening. Some women experience pain in the breast or armpit, sometimes accompanied by changes in breast size or shape. A noticeable lump in the breast or underarm area may also be present. Additional symptoms include nipple rash, redness or swelling of the breast skin, and discharge or bleeding from the nipple. In certain cases, swelling of lymph nodes in the armpit can occur, indicating possible early metastasis. Heaviness, breast deformity, or retraction of the nipple may appear in more advanced stages. These signs may develop gradually, and early symptoms are often subtle or absent, emphasizing the importance of regular breast self-examinations and adherence to screening guidelines for timely detection and intervention.[40, 41]

2.4 BREAST CANCER CAUSES

The exact cause of BC remains unknown, but researchers have identified several factors that increase its risk. These include hormonal influences, lifestyle choices such as diet and alcohol consumption, and environmental exposures. However, not all individuals with risk factors develop BC, and some without any known risks may still be affected. This suggests that BC likely results from a complex interaction between genetic predispositions and external influences.

BC begins when there is a mutation or alteration in the DNA of cells within breast tissue. Normally, DNA directs healthy cells to grow, divide, and die in a controlled manner. In cancerous cells, however, these instructions are altered, leading the cells to multiply rapidly and evade normal cell death. This uncontrolled growth may result in a mass or tumor, which can invade surrounding tissues and, over time, spread to other parts of the body. This process is known as metastasis.

Most BC originate in the cells lining the milk ducts, termed invasive ductal carcinoma. Others may begin in the milk-producing lobules, known as invasive lobular carcinoma. Though rare, cancer can also arise from other cell types within the breast, highlighting the complexity of BC development.[42]

2.5 RISK FACTORS OF BREAST CANCER

BC is a multifactorial disease influenced by both non-modifiable and modifiable risk factors, illustrated in Table 1. Understanding these determinants is essential for individualized risk assessment, early detection, and the development of targeted prevention strategies. The following sections present an overview of established and emerging risk factors supported by epidemiological and molecular evidence.

2.5.1 NON-MODIFIABLE RISK FACTORS

2.5.1.1 Sex

Female is a major determinant of BC risk due to prolonged exposure to estrogen and progesterone. Unlike men, women possess hormonally sensitive breast tissue, and elevated or imbalanced hormone levels increase the risk of carcinogenesis.[43] Although BC in men accounts for less than 1% of cases, factors such as older age, BRCA mutations, Klinefelter syndrome, and elevated estrogen levels can raise their risk.[44]

Table 1. Modifiable and non-modifiable risk factors of BC.

Non-Modifiable Factors	Modifiable Factors
Sex	Hormonal replacement therapy
Older age	Diethylstilbestrol
Family history (of breast or ovarian cancer)	Physical activity
Genetic mutations	Overweight/obesity
Race/ethnicity	Alcohol intake
Pregnancy and breastfeeding	Smoking
Menstrual period and menopause	Insufficient vitamin supplementation
Density of breast tissue	Excessive exposure to artificial light
Previous history of BC	Intake of processed food
Non-cancerous breast diseases	Exposure to chemicals
Previous radiation therapy	Other drugs

2.5.1.2 Advancing Age

Age is a strong predictor of BC risk, with approximately 80% of cases occurring in women over 50 years and more than 40% in women over 65 years. The lifetime risk increases with age 1.5% at 40, 3% at 50, and over 4% by 70. [45] Younger women (<40 years) are more likely to develop aggressive triple-negative subtypes, while older women (>70 years) often present with luminal A types.[46]

2.5.1.3 Family History

Having a first-degree relative with BC cancer increases risk significantly. Between 13–19% of BC patients report such familial history [47], with the risk amplified if the relative was diagnosed before age 50. Epigenetic factors and shared environmental exposures contribute to this association.[48]

2.5.1.4 Genetic Mutations

Mutations in BRCA1 (chromosome 17) and BRCA2 (chromosome 13) confer a high risk for BC.[49] Inherited mutations in p53, PTEN, CDH1, and STK11 also increase susceptibility.[50- 53] Recent studies have linked XRCC2 mutations with increased risk as well.[54]

2.5.1.5 Race and Ethnicity

Race and ethnicity impact BC outcomes. While white non-Hispanic women have the highest incidence, Black women have higher mortality and poorer survival, partly due to later-stage diagnoses and higher prevalence of aggressive subtypes.[55]

2.5.1.6 Reproductive History

Hormonal fluctuations during menarche, pregnancy, breastfeeding, and menopause influence BC risk. Early menarche and late menopause increase lifetime estrogen exposure, raising risk.[56] Early first full-term pregnancy and extended breastfeeding offer protective effects.[57] No association has been established between abortion and increased BC risk.[58]

2.5.1.7 Breast Tissue Density

High breast density, commonly observed in younger women and those on hormone therapy, is associated with a greater risk of BC.[59] Breast density can obscure tumor detection on mammograms and is being explored as a screening parameter.

2.5.1.8 Personal or Benign Breast Disease History

A history of BC or benign lesions like atypical hyperplasia or carcinoma in situ increases the risk of recurrence or new tumors.[60] Histological subtype and familial predisposition further modulate this risk.[61]

2.5.1.9 Previous Radiation Therapy

Patients who received radiation therapy, especially before the age of 30, are at increased risk for BC.[62] Techniques like tangential field intensity-modulated radiotherapy (IMRT) are associated with lower secondary cancer risk compared to multi-field methods. Additional radiation boosts may reduce local recurrence risk.[63]

2.5.2. MODIFIABLE RISK FACTORS

2.5.2.1 Pharmacologic Agents

Use of diethylstilbestrol (DES) during pregnancy has been associated with BC in both mothers and offspring.[64] Prolonged hormonal replacement therapy (HRT), especially over 5–7 years, significantly increases risk.[65] The role of antidepressants, antibiotics, and other drugs (e.g., NSAIDs, statins, antihypertensives) remains inconclusive but is under investigation.[66]

2.5.2.2 Physical Inactivity

Regular physical activity reduces BC risk across menopausal stages. Mechanisms may involve lowering sex hormones, improving insulin sensitivity, and modulating immune response.[67] Both pre- and postmenopausal women benefit, though studies differ on which group shows stronger associations.[68]

2.5.2.3 Obesity and Body Mass Index

Obesity, especially in postmenopausal women, elevates estrogen production via adipose tissue and is linked to estrogen-receptor-positive BC.[69] Obesity also correlates with worse prognosis, higher-grade tumors, and increased lymph node involvement.[70]

2.5.2.4 Alcohol Consumption

Alcohol increases estrogen levels and promotes DNA damage, contributing to breast carcinogenesis.[71] Risk is particularly elevated with consumption prior to first pregnancy[72] and is more pronounced in estrogen receptor-positive tumors.[73]

2.5.2.5 Tobacco Smoking

Active and passive smoking increases the risk of mutations in oncogenes and tumor suppressor genes (e.g., p53).[74] Risk is further elevated when smoking begins before the first full-term pregnancy, especially in individuals with a family history.[75]

2.5.2.6 Vitamin Deficiency

Deficiencies in vitamins particularly vitamin D have been associated with increased BC risk.[76] Higher serum 25(OH)D levels are inversely correlated with BC incidence and mortality[77], though evidence remains inconsistent.[78]

2.5.2.7 Artificial Light Exposure

Exposure to artificial light at night (ALAN) disrupts melatonin secretion and circadian rhythms, potentially increasing BC risk via epigenetic changes.[79] Evidence on the effects of electronic devices remains mixed.

2.5.2.8 Diet and Processed Food Intake

A diet high in ultra-processed foods, saturated fats, and sugars is linked to increased BC risk, likely through its impact on obesity and inflammation.[80] Conversely, diets rich in vegetables, fiber, phytonutrients, and omega-3 polyunsaturated fatty acids (PUFAs) are considered protective.[81]

2.5.2.9 Environmental Chemical Exposure

Long-term exposure to chemicals like DDT, PCBs, and PAHs has been associated with increased BC risk, especially with early-life exposure.[82] Disruption of mammary gland development and hormonal signaling are key mechanisms.

2.5.2.10 Other Medications

Various drugs including NSAIDs, statins, calcium channel blockers, and antihypertensives have been studied for possible associations with BC, but current evidence remains inconclusive. [45]

2.6 BREAST CANCER CLASSIFICATION

2.6.1 Histological Classification

Invasive BCs (IBCs) comprise a heterogeneous group of tumors with substantial variation in morphology, clinical behavior, and biological characteristics. The World Health Organization (WHO) recognizes at least 18 different histological types of BC.[83] Among these, invasive breast carcinoma of no special type (NST) formerly referred to as invasive ductal carcinoma, is the most prevalent, accounting for approximately 40–80% of all cases.[84] This category is defined by exclusion, as it includes tumors that do not fit into any of the specific histological subtypes.[83] Approximately 25% of IBCs, however, demonstrate distinct cytological and architectural patterns and are classified into special subtypes, including invasive lobular carcinoma, tubular carcinoma, mucinous types A and B, neuroendocrine carcinoma, among others, illustrated in Figure 1. [85]

2.6.2 Molecular Subtypes of Breast Cancer

Independent of histology, BCs are also classified into molecular subtypes based on gene expression profiles.

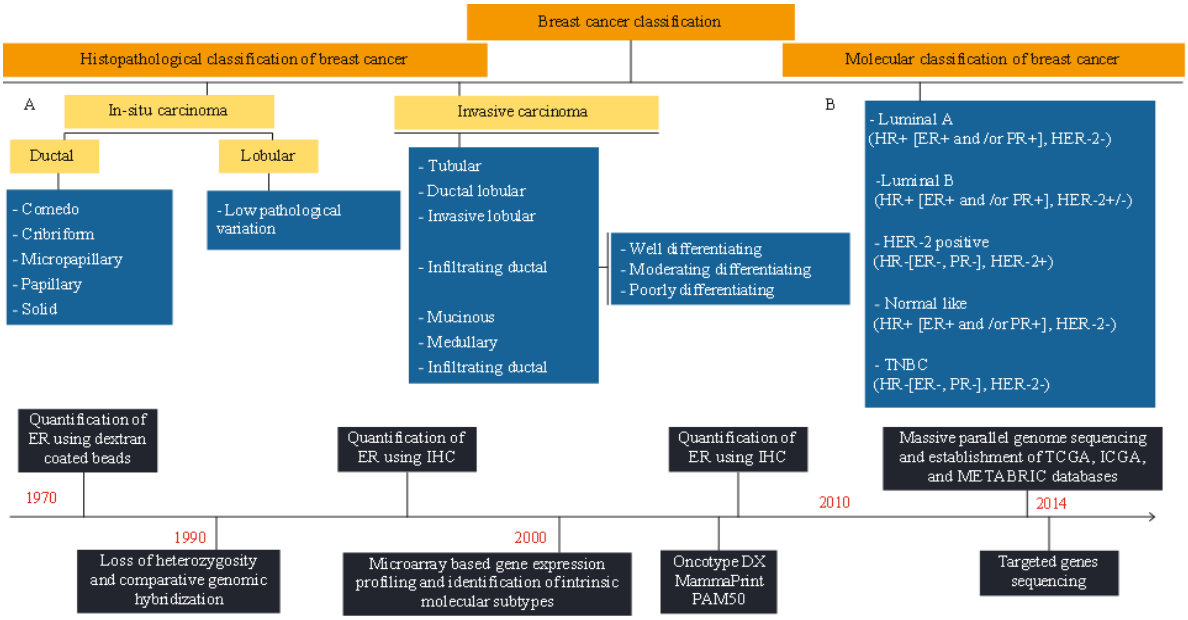


Figure 3: Schematic overview of BC classification. Illustrating (a) histopathological subtypes of in-situ and invasive carcinomas, (b) molecular subtypes based on hormone receptor and HER2 status, and (c) a timeline of major milestones in BC, including advancements in ER quantification, molecular profiling, and genomic sequencing technologies. Source: DOI: 10.1155/2022/9605439
Abbreviations: BC: Breast cancer, HRE2: Human epithelial growth factor receptor 2, ER: Estrogen receptor

In a landmark study in 2000, Perou et al. identified four intrinsic molecular subtypes, Luminal, HER2-enriched, Basal-like, and Normal Breast-like through microarray analysis of mRNA expression data in 38 breast tumors.[86] Later studies refined this classification by dividing the Luminal group into Luminal A and Luminal B subtypes.[87, 88]

The "Normal Breast-like" group was eventually excluded as it is now believed to represent contamination with normal tissue. The Cancer Genome Atlas (TCGA) project further validated this classification by analyzing over 300 primary tumors at the DNA, RNA, and protein levels, reaffirming the existence of four molecular subtypes, Luminal A, Luminal B, HER2-enriched, and Basal-like based on transcriptomic data, as depicted in Figure 3.[89] In 2007, an additional subtype Claudin-low was identified through integrated analysis of human and mouse mammary tumors.[90]

Parker et al. introduced the PAM50 classifier, a 50-gene signature capable of assigning tumors to intrinsic subtypes with a reported accuracy of 93%.[91] This

classifier has since been implemented in clinical practice using the NanoStringna Counter® platform, forming the basis of the Prosigna® test. Prosigna® integrates the PAM50 gene expression profile with clinical parameters to estimate the risk of distant recurrence in postmenopausal women with hormone receptor-positive, node-negative or node-positive early-stage BC, and assists in the decision-making process regarding adjuvant chemotherapy.[92-94]

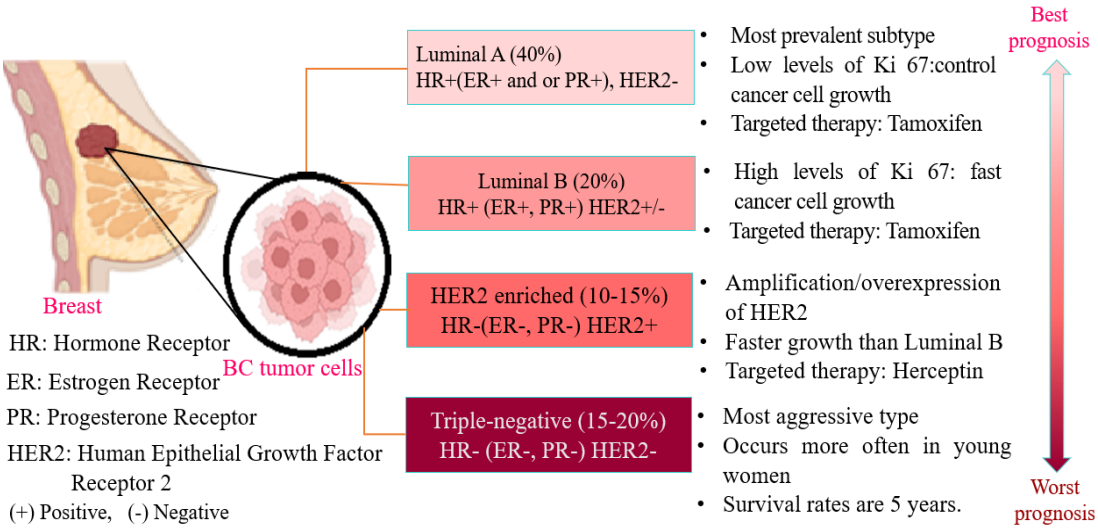


Figure 4: Molecular subtypes of breast cancer.

Subtypes vary based on hormone receptor and HER2 status, showing differences in prognosis, proliferative index (Ki-67), and responsiveness to targeted therapies.

Source: DOI:10.3390/biomedicines9080876

2.6.2.1 Luminal Breast Cancer

Luminal BCs are characterized by ER positivity and constitute nearly 70% of all BC cases in Western populations.[95] These tumors frequently present as invasive carcinomas of no special type but may occasionally exhibit features of other histological subtypes, including invasive lobular, tubular, cribriform, mucinous, and micropapillary carcinomas.[4,96] Luminal subtypes are distinguished primarily by differential expression of proliferation-associated and luminal-associated genes.

2.6.2.2 Luminal A tumors exhibit ER and/or PR positivity with HER2 negativity, as presented in the Figure 4. In this group, ER transcriptional activity drives the expression of genes associated with luminal epithelial differentiation.[97,98] These

tumors have low proliferative indices and demonstrate favorable clinical features, including low grade, slow growth, and excellent prognosis.[99]

2.6.2.3 Luminal B tumors are more proliferative, exhibit higher histological grade, and have worse prognosis. While also ER positive, they may be PR negative and/or HER2 positive and are characterized by elevated expression of proliferation-related genes such as MKI67.[100-102] Luminal B tumors typically show reduced expression of luminal differentiation markers, including PR, [98] as illustrated in Figure 4.

2.6.2.4 HER2-Enriched Breast Cancer

HER2-enriched BCs represent approximately 10 - 15% of all cases and are defined by high expression of HER2 (ERBB2) and absence of both ER and PR, as shown in Figure 4. This subtype is dominated by the expression of proliferation-related genes such as ERBB2/HER2 and GRB7, rather than luminal or basal gene signatures.[102, 103] Recent research suggests that mutagenesis in this group may be driven by APOBEC3B, a member of the cytidine deaminase family responsible for inducing cytosine-to-uracil transitions and generating clustered mutations.[104]

HER2-enriched cancers are typically high-grade and rapidly growing. Before the introduction of targeted HER2 therapies, they were associated with poor outcomes. Importantly, HER2-enriched is a molecular subtype and not synonymous with clinically HER2-positive disease; some ER-positive/HER2-positive tumors are actually luminal B, while approximately 30% of HER2-enriched tumors may be HER2-negative by IHC or FISH.[105]

2.6.2.5 Basal-Like/TNBC

TNBC is a heterogeneous group of ER-negative, PR-negative, and HER2-negative tumors (see Figure 4**)**, accounting for about 20% of all BCs. TNBC is more common in women under 40 and among African-American populations.[105] Up to 80% of tumors in patients with BRCA1 germline mutations are TNBC, and 11–16% of TNBC cases harbour BRCA1 or BRCA2 mutations. These tumors are aggressive and often have a poor prognosis.[106] Histologically, TNBC typically presents as invasive ductal carcinoma but may also manifest as medullary-like, metaplastic, or adenoid cystic carcinomas.[107]

While the terms "basal-like" and "TNBC" are often used interchangeably, gene expression profiling reveals distinct TNBC subtypes: Basal-like 1 and 2 (BL1, BL2), mesenchymal (M), mesenchymal stem-like (MSL), immunomodulatory (IM), luminal androgen receptor (LAR), and an unspecified group (UNS).[108, 109] The clinical utility of these subtypes remains under investigation, and their application to treatment decision-making is still evolving.[110]

2.6.2.6 Claudin-Low Breast Cancer

Claudin-low (CL) BCs are typically ER-, PR-, and HER2-negative and represent 7–14% of all BCs.[96] They do not differ significantly in prognosis from other poor-outcome subtypes like Luminal B, HER2-enriched, or Basal-like. CL tumors are defined by low expression of tight junction proteins such as claudins 3, 4, and 7, occludin, and E-cadherin, along with high expression of epithelial-to-mesenchymal transition (EMT) and stem cell-associated genes.[111,112] They are also characterized by prominent immune and stromal infiltration [113] and their relatively undifferentiated state contributes to genomic stability, potentially mediated by EMT-regulating transcription factors like ZEB1.[114]

2.6.2.7 Surrogate Marker-Based Classification

Due to the high cost and limited availability of genomic assays, clinical practice often relies on surrogate classification using immunohistochemical markers. St. Gallen's 2013 guidelines advocate for using IHC-based markers such as ER, PR, HER2, Ki-67, and others for treatment decisions.[115] These markers guide subtype identification and predict therapeutic response.[116] Additional markers like cytokeratin 5/6 and EGFR help identify basal-like tumors within the TNBC group.[117] However, IHC-based subtyping is an approximation; discordance with gene expression-based subtypes can reach up to 30%.[118]

2.6.2.8 American Joint Committee on Cancer (AJCC) Classification

The AJCC staging system, first established in 1977, traditionally relied on anatomical criteria: tumor size (T), lymph node involvement (N), and metastasis (M). The 8th edition (2018) incorporates biologic markers such as ER, PR, HER2, grade, and multigene assay results into prognostic staging.[119] The Elston - Ellis modification of

the Scarff -Bloom - Richardson grading system, or Nottingham grade, is commonly used and assesses tubule formation, nuclear pleomorphism, and mitotic count.[120, 121] Multigene assays like the 21-gene Oncotype DX® test provide additional prognostic value and are integrated into staging for patients with hormone receptor-positive, HER2-negative, node-negative tumors smaller than 5 cm. Patients with a recurrence score below 11 achieve high survival rates (98.6% at 6.9 years) with endocrine therapy alone, enabling omission of chemotherapy.[122, 123] The AJCC system distinguishes between clinical and pathological prognostic staging, incorporating molecular and pathological findings before and after surgical resection. Validation of the updated staging system using large datasets (e.g., SEER, MD Anderson Cancer Center) has confirmed its superior prognostic accuracy compared to purely anatomical staging.[124, 125]

2.7 DIAGNOSIS OF BREAST CANCER

2.7.1 History and Physical Examination

Diagnosis begins with a detailed medical history to assess BC risk. Key factors include age at menarche, menopausal status, use of hormone therapy, prior pregnancies, and personal or family history of breast or ovarian cancer. Patients are evaluated for symptoms such as breast pain, bone pain, fatigue, nipple discharge, and weight loss. A thorough physical examination involves palpation of the breasts, axillary, and supraclavicular lymph nodes to detect lumps or abnormalities.[126,127]

2.7.2 Self-Examination

Though its impact on mortality reduction is debated, breast self-examination (BSE) remains a recommended method for women to familiarize themselves with their breast structure. Awareness campaigns, including SMS-based education, have improved BSE practice and knowledge among women and healthcare students.[128]

2.7.3 Imaging Techniques

- *Digital Mammography*: Gold standard for early detection, though less sensitive in dense breasts.[129]
- *MRI*: More accurate in detecting tumors in high-risk women and those with dense breasts or BRCA mutations.[130]

- *PEM and MRI*: Both offer high sensitivity for invasive and in situ cancers; PEM is a good alternative when MRI is not preferred.[131]
- *Ultrasound***:** Especially useful in evaluating palpable lumps and distinguishing cystic from solid lesions, particularly in dense breasts.[132]

2.7.4 Nuclear Medicine Techniques

- *SPECT*: Utilizes gamma-emitting radionuclides (e.g., technetium-99m) for accurate detection of primary and metastatic lesions.[133]
- *PET/CT***:** Uses [18F]-FDG to detect hypermetabolic cancer cells. It is highly sensitive for staging, detecting occult metastases, and monitoring treatment response.[134]

2.7.5 Tumor Markers

- *CA 15-3*: Elevated in around 70% of advanced-stage cases; useful in monitoring treatment efficacy.[135]
- *CA 27.29***:** Comparable to CA 15-3; not superior in early detection and can be elevated in other conditions.[136]

2.7.6 Receptor and Molecular Testing

- *Estrogen and Progesterone Receptors*: Detection of ER, PR, and HER2 helps classify tumor subtypes and guides hormonal and targeted therapy decisions.[137]
- *IHC*: Crucial for confirming diagnosis and subtype classification. IHC enhances diagnostic accuracy when combined with H&E staining.[138]

2.7.7 Biopsy Techniques

*Fine Needle Aspiration cytology (FNAC)**:*** Extracts individual cells for cytological evaluation; often guided by ultrasound.[139]

- *Core Biopsy*: Provides tissue samples with preserved architecture; performed under local anesthesia.[139]
- *Vacuum-Assisted Stereotactic Core Biopsy**:*** Offers multiple samples through a small incision; guided by mammogram, MRI, or ultrasound.[140]

- *Surgical Biopsy***:** Performed when non-invasive methods are inconclusive; involves excising tissue under general anesthesia.[141]

2.7.8 Artificial Intelligence (AI)

- Asif Hassan Syed studied the importance of AI in oncology. He reported that AI has emerged as a transformative tool in precision oncology, offering promising applications in early BC diagnosis and prognosis assessment.
- Each modality offers unique advantages in identifying tumors at an early, asymptomatic stage, thus enabling timely medical or surgical intervention. However, variations in sensitivity, specificity, and access to these technologies across different healthcare systems have led to inconsistent outcomes, particularly in low- and middle-income regions.[142]

2.8 PROGNOSTIC BIOMARKERS

2.8.1 Estrogen Receptor

The ER serves as a critical diagnostic and therapeutic marker, with approximately 70–75% of invasive breast carcinomas showing high ER expression.[143] Evaluation of ER status is mandatory for both primary tumors and recurrences to guide endocrine therapy, which includes selective estrogen receptor modulators, estrogen receptor downregulators, and aromatase inhibitors.[144] While ER assessment is pivotal for selecting appropriate treatment, it also holds prognostic relevance, as high ER levels are linked to favorable outcomes.[145] A positive family history correlates with ER expression, enhancing its role as a diagnostic marker in hereditary BC.[146] Furthermore, Konan et al. found that ERα-36 could serve both as a target and prognostic marker in PR-positive BCs.[147]

2.8.2 Progesterone Receptor

PR expression is prevalent (>50%) in ER-positive patients but uncommon in ER-negative tumors. As a downstream target of ER, PR expression reflects an intact ER signaling axis.[148] Both ER and PR are co-expressed in many BCs and jointly act as prognostic and diagnostic markers, especially in hormone-responsive subtypes.[149] Higher PR levels are associated with improved survival, delayed recurrence, and

reduced treatment failure, whereas lower levels suggest aggressive disease and poorer outcomes.[150] Despite its clinical value, PR predictive utility remains debated.[151]

2.8.3 Human Epidermal Growth Factor Receptor 2

HER2 is overexpressed in 15–25% of BC cases and has substantial implications for targeted therapy selection; its overexpression often occurs early during oncogenesis.[152] HER2 detection enhances the identification of metastatic or relapsed disease, raising detection rates from 50% to over 80%.[153] Serum HER2 serves as a promising real-time indicator of disease burden or recurrence.[154] HER2 amplification drives activation of oncogenic pathways, leading to unchecked proliferation and poor outcomes.[155] Additionally, HER2 overexpression correlates with reduced disease-free survival (DSF) and is linked to tumor histology, pathological stage, and nodal metastasis.[156]

2.8.4 Antigen Ki-67

Ki-67 is a nuclear protein indicative of cell proliferation, and its index is a validated marker for estimating tumor aggressiveness and treatment response in BC.[157] The Ki-67 index is integral in determining appropriate therapeutic strategies and monitoring for recurrence. Nevertheless, limitations in assay reproducibility necessitate cautious interpretation in clinical decisions. A meta-analysis involving 12,155 cases from 68 studies confirmed that elevated Ki-67 is linked to worse outcomes.[158] High Ki-67 expression is also indicative of reduced survival rates in BC patients.[159]

2.8.5 Circulating Circular RNA

Circular RNAs (circRNAs), a class of non-coding RNAs, are increasingly recognized for their role in oncogenic processes such as proliferation, apoptosis evasion, and metastasis.[160] Notable circRNAs in BC include circFBXW7, a potential diagnostic and therapeutic candidate in TNBC, and hsa_circ_0072309, linked with poor survival.[161]

2.8.6 p53

p53 gene mutations particularly loss-of-function alterations are common in many cancers, including breast, leukemia, osteosarcoma, and brain tumors.[162] The p53

protein governs essential responses like cell cycle arrest, apoptosis, DNA repair, and senescence.[163] p53 mutations frequently arise in early tumorigenesis, especially in TNBC (up to 80%) and less commonly in Luminal A cancers (10%).[164] Numerous studies underline the prognostic importance of p53 loss in BC.[165] Missense mutations may confer oncogenic gain-of-function activity in addition to loss of native function.[166] p53 IHC status divides TNBC into two subgroups: p53-negative, resembling normal breast tissue, and p53-positive, basal-like tumors with poorer survival.[167] Yet, neither mutational analysis nor IHC has been universally accepted for prognosis in routine practice.[168]

2.8.7 MicroRNA

MicroRNAs (miRNAs) are short, non-coding RNAs (19–25 nt) that regulate gene expression in various biological pathways.[169] Many miRNAs contribute to tumor initiation, progression, and therapy response.[170] A meta-analysis by Adhami et al. identified consistent upregulation of miR-21 and miR-210 and downregulation of six miRNAs including miR-145, miR-139-5p, miR-195, miR-99a, miR-497, and miR-205 across BC studies.[171] Still, more robust studies are necessary to validate their utility as specific and sensitive diagnostic tools.

2.9 TREATMENT STRATEGIES

2.9.1 Surgery

Surgery remains the primary intervention for early-stage BC and typically involves mastectomy or breast-conserving surgery (BCS).[172] Mastectomy is preferred in multicentric tumors and in patients unable to receive radiotherapy.[173] BCS followed by radiotherapy offers survival rates equivalent to mastectomy in suitable cases.[174] Axillary lymph node dissection (ALND) and sentinel lymph node biopsy (SLNB) are crucial for nodal staging and therapeutic planning.[175] SLNB has replaced ALND in clinically node-negative cases due to lower morbidity.[176]

2.9.2 Radiotherapy

Radiotherapy is integral to BC management, particularly after BCS, to reduce local recurrence.[177] It is also indicated following mastectomy in patients with large tumors or nodal involvement.[178] Hypo fractionated radiotherapy has shown comparable

efficacy to conventional regimens with fewer side effects.[179] Additionally, advanced techniques like IMRT improve dose distribution and minimize toxicity.[180] Radiation also enhances locoregional control and overall survival (OS) in high-risk patients.[181]

2.9.3 Chemotherapy

Chemotherapy is administered in neoadjuvant, adjuvant, and metastatic settings. It is crucial for triple-negative and HER2-positive subtypes.[182] Common regimens include anthracyclines (e.g., doxorubicin), taxanes (e.g., paclitaxel), and alkylating agents (e.g., cyclophosphamide).[183] Neoadjuvant chemotherapy improves operability and enables breast conservation.[184] The achievement of pathological complete response (pCR) correlates with better outcomes, especially in TNBC and HER2-positive cases.[185] However, chemotherapy has limited benefit in low-risk Luminal A tumors.[186]

2.9.4 Hormone Therapy

Hormone therapy targets ER and/or PR-positive tumors and significantly improves survival.[187] Tamoxifen, a selective estrogen receptor modulator, is widely used in premenopausal women.[188] Aromatase inhibitors (e.g., anastrozole, letrozole) are preferred in postmenopausal women to suppress peripheral estrogen synthesis.[189] Ovarian suppression with gonadotropin-releasing hormone (GnRH) analogs enhances outcomes in premenopausal women when combined with other endocrine therapies.[190] Endocrine resistance remains a clinical challenge, prompting combination approaches with targeted therapies.[191]

2.9.5 Targeted Therapy

Targeted therapies have revolutionized the treatment of HER2-positive BCs. Trastuzumab, a monoclonal antibody against HER2, significantly improves disease-free and OS.[192] It is often combined with chemotherapy or pertuzumab in dual-targeted regimens.[193] Small molecule tyrosine kinase inhibitors like lapatinib and neratinib also show efficacy in HER2-positive disease, especially in the metastatic setting.[194] CDK4/6 inhibitors (palbociclib, ribociclib, abemaciclib) are used in combination with endocrine therapy in ER-positive, HER2-negative metastatic BC.[195] Additionally, PI3K/AKT/mTOR pathway inhibitors and PARP inhibitors (e.g.,

olaparib) have shown promise in selected molecular subtypes, including BRCA-mutated TNBC.[196]

2.9.6 Immunotherapy

Immunotherapy is gaining traction in the treatment of triple-negative BC. Immune checkpoint inhibitors, such as anti-PD-1 and anti-PD-L1 antibodies, enhance T-cell-mediated antitumor responses.[25] Atezolizumab, in combination with nab-paclitaxel, has shown improved progression-free survival in PD-L1-positive TNBC.[197] However, benefits are limited to subsets with high immune infiltration or biomarker positivity. Immunotherapy remains under investigation for broader application in other BC subtypes.[198]

2.9.7 Challenges in TNBC Management

TNBC presents significant clinical challenges due to its aggressive nature, heterogeneity, and lack of well-defined molecular targets.[199] Unlike hormone receptor-positive or HER2-overexpressing subtypes, TNBC does not benefit from endocrine or HER2-targeted therapies, limiting treatment options to surgery, chemotherapy, and radiotherapy.[200] Although many TNBCs initially respond to chemotherapy, the risk of relapse remains high, and long-term survival is comparatively lower.[201]

The lack of specific biomarkers further hinders the development of targeted therapies. While PARP inhibitors show efficacy in BRCA1/2-mutated TNBC, their benefit is confined to a small subset of patients.[201] Similarly, immune checkpoint inhibitors have shown promise only in PD-L1-positive tumors, with limited success in unselected populations.[202] Moreover, immune-related adverse effects and the cost of immunotherapy limit widespread application.[203] Another major concern in TNBC is the early onset and higher prevalence among younger women and those of African ancestry, often associated with more aggressive disease and poorer outcomes. Socioeconomic disparities, delayed diagnosis, and limited access to specialized care further exacerbate survival differences in these populations.[204] Resistance to chemotherapy, both intrinsic and acquired, remains a pressing issue. Mechanisms such as EMT, overexpression of drug efflux pumps, and activation of compensatory signaling pathways contribute to treatment failure.[201] Furthermore, the absence of

validated predictive and prognostic biomarkers restricts precision medicine approaches in TNBC. Liquid biopsies, circulating tumor DNA (ctDNA), and multi-omics profiling hold potential for early detection and real-time monitoring but are not yet routinely used in clinical settings.[205]

2.10 EFFECTS OF 1,25(OH)$_2$D$_3$ ON CANCER STEM CELLS (CSCs)

CSCs, first identified in acute myeloid leukemia, represent a subpopulation with the ability to initiate, sustain, and propagate tumors, contributing to recurrence and therapeutic resistance.[206] Saeg and Anbalagan et al. stated that several critical signaling cascades, including Notch, Wnt/Frizzled/β-catenin, Hippo, and Hedgehog, govern the maintenance and self-renewal of CSCs, and their dysregulation is intricately linked to BC development. CSCs in BC, particularly those with a basal-like phenotype, are phenotypically defined by a $CD44^+/CD24^-$ surface marker profile. CD44, a transmembrane adhesion receptor involved in cell - cell and cell - matrix interactions, facilitates tumor cell dissemination and is widely accepted as a CSC marker. [206, 207] CD24, a sialo glycoprotein acting as a ligand for P-selectin on endothelial cells, enables intravascular migration of cancer cells, thus promoting metastasis.[208, 209] Al-Hajj et al. reported that the co-expression and plasticity of CD44 and CD24 in various malignancies prompted their establishment as core CSC markers. They demonstrated that $CD44^+/CD24^-$/low cells possess greater tumor-initiating capacity compared to $CD44^+/CD24^+$ cells, reinforcing their role as functional markers of breast CSCs.[210]

Calcitriol and its analogues modulate CSC characteristics through several mechanisms. Treatment of basal-like MCF10DCIS cells with the Gemini vitamin D$_3$ analogue BXL0124 led to significant reductions in CD44 mRNA and protein expression.[211, 212] Transcriptomic profiling of mammospheres derived from these cells revealed that 1,25(OH)$_2$D$_3$ and BXL0124 downregulated genes essential for CSC maintenance (e.g., GDF15), EMT, invasion, and metastasis (e.g., LCN2, S100A4), and chemoresistance (e.g., NGFR, PPP1R1B, AGR2), while upregulating basal-like differentiation markers (e.g., KRT6A, KRT5) and tumor suppressor genes (e.g., EMP1).[213]

2.11 BIOCHEMICAL BASIS OF CANCER

Cancer cells exhibit distinct characteristics that enable uncontrolled proliferation, evasion of programmed cell death, and reliance on lactate as the principal metabolic fuel.[214] Hanahan and Weinberg identified ten hallmark capabilities that drive the neoplastic transformation of normal cells. These hallmarks include genome instability, evasion of immune surveillance, resistance to growth suppressors, enabling replicative immortality, evasion of apoptosis, tumor-promoting inflammation, sustained proliferative signaling, induction of angiogenesis, activation of invasion and metastasis, and deregulation of cellular energetics, as detailed in Figure 5.[215] Biochemically, the key metabolic alteration in tumor cells is the Warburg effect, characterized by an increased rate of aerobic glycolysis. This phenomenon, observed nearly a century ago, involves reduced mitochondrial function despite adequate oxygen availability. As a result, glucose is predominantly converted into lactate, which supports rapid cell division by contributing to the production of biomolecules (lipids, proteins, and nucleotides).[216, 217]

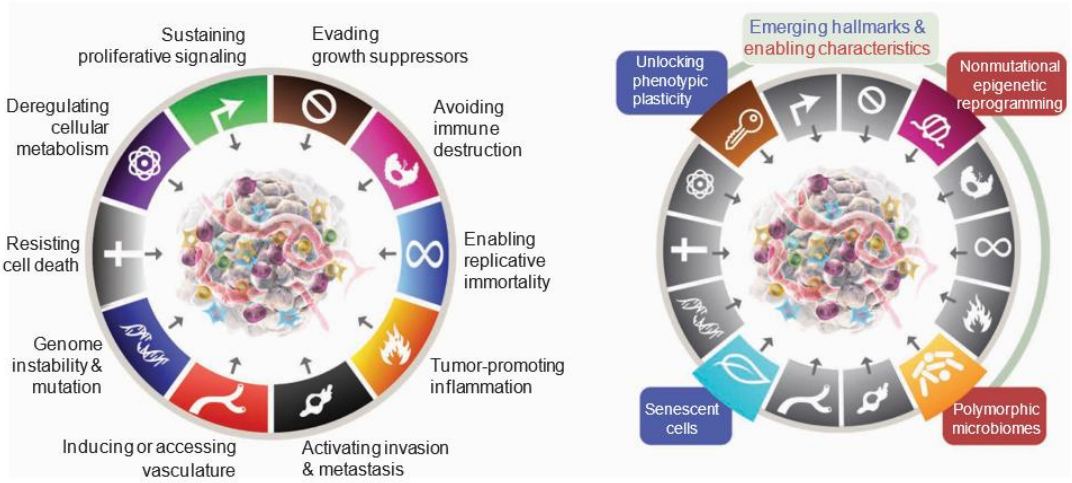


Figure 5: Hallmarks of cancer - updated framework (circa 2022).

The figure summarizes the evolving understanding of cancer biology. The left panel illustrates the core hallmarks and enabling characteristics, including the original six hallmarks (e.g., sustaining proliferative signaling, evading growth suppressors) along with validated additions such as deregulating cellular metabolism and avoiding immune destruction. The enabling characteristics, genome instability and tumor-promoting inflammation—facilitate acquisition of these traits. The right panel highlights newly proposed emerging hallmarks and enabling features, including unlocking phenotypic plasticity, nonmutational epigenetic reprogramming, senescent cells, and polymorphic microbiomes. Adapted from Hanahan and Weinberg.

Source: DOI: 10.1158/2159-8290.CD-21-1059

2.12 DEFICIENCY OF VITAMIN D IN BREAST CANCER

Díaz et al. highlighted that in both *in vitro* and *in vivo* models, calcitriol has demonstrated anti-proliferative, pro-differentiative, and pro-apoptotic effects on cancer cells, suggesting its potential in limiting cancer progression or even preventing it. A wealth of observational studies correlates low circulating levels of cholecalciferol with an increased risk of cancer and poorer prognoses. However, the role of vitamin D in cancer risk, incidence, and mortality remains controversial. In cancer metastasis, a cascade of events, including extravasation and the subsequent outgrowth of disseminated cells, leads to epithelial-mesenchymal transition at secondary sites. Additionally, acquired resistance to chemotherapy remains a significant challenge in treating BC. Despite the presence of the VDR and the enzymatic machinery for vitamin D metabolism in breast epithelial cells, the association between serum 25OHD levels and BC risk remains debated. While some studies find no significant relationship, others report an inverse correlation between serum 25OHD levels and BC risk.[218, 219] Villaseñor et al. reported that limited evidence suggests vitamin D status may influence outcomes in BC survivors, with higher 25OHD levels being associated with improved OS, though not necessarily with cancer-specific survival.[220] Prentice, R.L. et al reported reduced BC risk in certain cohort after supplementation with calcium and vitamin D.[221] Nevertheless, whether hypovitaminosis D is a cause or a consequence of BC remains unclear, as does the optimal 25OHD level for cancer prevention. However, serum 25OHD levels exceeding 52 ng/mL are linked to a 50% reduced risk of BC compared to women with levels below 13 ng/mL, highlighting the importance of preventing vitamin D deficiency.[222, 223] Consequently, hypovitaminosis D emerges as a modifiable risk factor for BC, which can be mitigated through supplementation or adequate sun exposure.

2.12.1 Effects of Calcitriol in Cancer Prevention and Treatment

Calcitriol non-calcemic effects have gained considerable attention, with evidence showing that its role in cancer prevention extends beyond regulating calcium and phosphate levels. It influences various organ systems in a paracrine and autocrine manner, offering a promising approach to cancer treatment. It is estimated that by raising serum calcidiol levels to 40 - 60 ng/mL, over 220,000 cases of breast and colorectal cancer could be prevented annually worldwide.[224] The mechanisms by

which calcitriol exerts its anti-cancer effects are diverse, and its actions vary depending on tissue type. The VDR is essential for these effects, and its loss in malignant cells can lead to resistance to calcitriol.[225] Calcitriol supplements can activate the VDR, thereby restoring balance by recruiting Sirt1, which reduces pro-inflammatory factors such as NF-κB and enhances anti-inflammatory factors like interleukin 10 (IL-10).[225] Jeon and Shin noted that vitamin D_3 is primarily obtained through dietary sources such as fatty fish or synthesized in the skin following sunlight exposure (UVB radiation). Sun exposure is a significant factor influencing vitamin D synthesis, and geographical location, season, and lifestyle can impact vitamin D levels, as illustrated in Figure 6. [226]

Christakos et al. explained that the biologically active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), is produced through two hydroxylation steps. Initially, vitamin D_3 is converted to 25-hydroxyvitamin D_3 ($25(OH)D_3$) in the liver, which serves as a marker of vitamin D status. Subsequently, in the kidney, CYP27B1 hydroxylates $25(OH)D_3$ to form the active $1,25(OH)_2D_3$. The enzyme CYP24A1 regulates the levels of $1,25(OH)_2D_3$ by degrading both $1,25(OH)_2D_3$ and $25(OH)D_3$, thus maintaining homeostasis.[227]

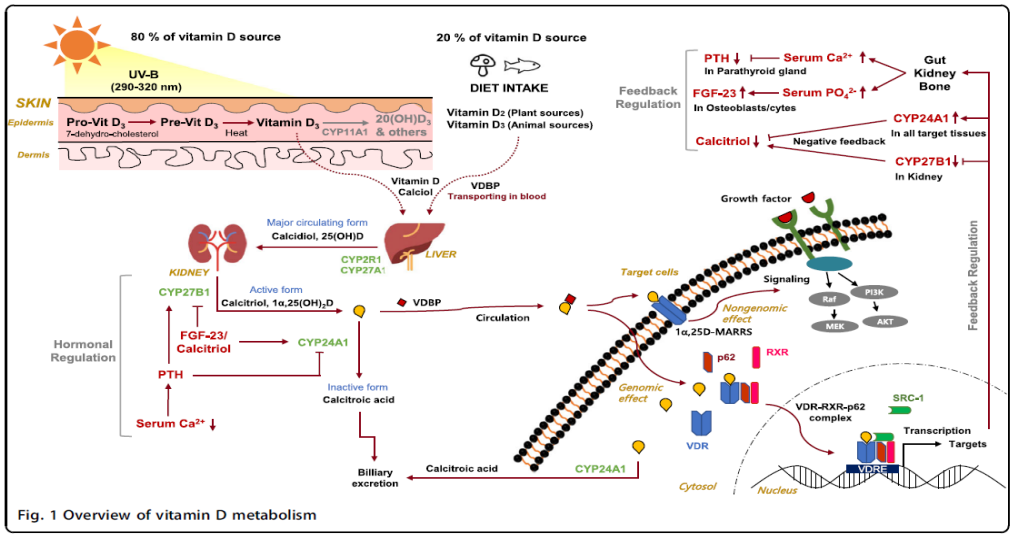


Figure 6: Schematic overview of vitamin D metabolism.

Vitamin D is synthesized via UV-B exposure and obtained from dietary sources, followed by hepatic and renal hydroxylation. Its metabolism is regulated by PTH and FGF-23, with feedback mechanisms. The active form, calcitriol, exerts genomic and nongenomic actions through VDR-mediated signaling in target cells. Source: DOI:10.1038/s12276-018-0038-9

Abbreviations: VDR: Vitamin D receptor, UV-B: Ultraviolet B, PTH: Parathyroid hormone, FGF-23: Fibroblast growth factor 23.

Once formed, 1,25(OH)2D3 binds to the VDR, which forms a heterodimer with the retinoid X receptor (RXR), a member of the nuclear receptor superfamily. The VDR-RXR complex then binds to vitamin D response elements (VDREs) in the DNA of target genes, modulating transcriptional activity by recruiting co-activators and releasing co-repressors.[227] While the primary function of 1,25(OH)2D3 is to regulate calcium and phosphate homeostasis, the VDR is expressed not only in tissues involved in mineral metabolism but also in various cancerous tissues. Both *in vitro* and *in vivo* studies have shown that 1,25(OH)2D3 modulates signaling pathways involved in cell proliferation, apoptosis, differentiation, inflammation, invasion, and angiogenesis, as shown in Figure 7.[227, 228]

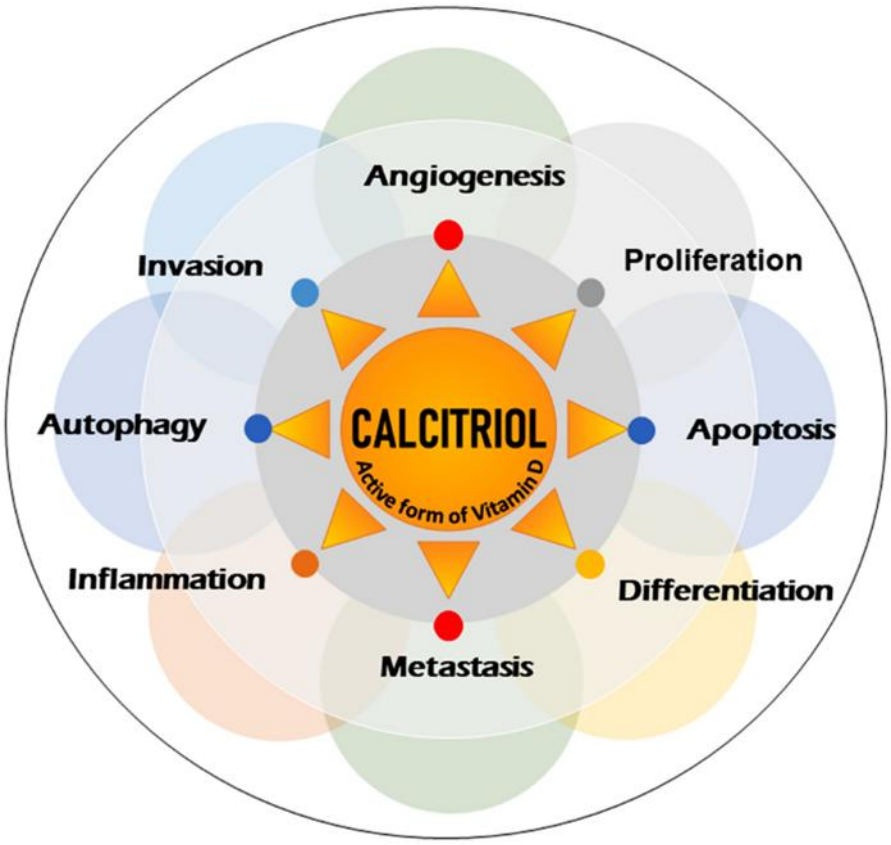


Figure 7: Pleiotropic effects of calcitriol on cancer-associated cellular processes.
Calcitriol exerts pleiotropic regulatory functions by modulating a range of cancer-relevant pathways, including proliferation, apoptosis, differentiation, metastasis, angiogenesis, inflammation, invasion, and autophagy. Source: DOI: 10.1007/s12032-022-01855-0

2.13 BREAST CANCER AND VITAMIN D: MECHANISTIC INSIGHTS

Breast epithelial cells are capable of locally converting vitamin D precursors into their active forms due to the presence of the enzymatic machinery for vitamin D metabolism, including 1α-hydroxylase and 24-hydroxylase, along with the expression of the VDR. Calcitriol plays an essential role in normal mammary gland development. This was evidenced in VDR knockout mouse models, where mammary morphogenesis was disrupted, and an exaggerated proliferative response to estrogen and progesterone was observed relative to wild-type controls.[229-231] Several studies have demonstrated higher serum calcitriol levels in early-stage BC relative to advanced and metastatic disease, suggesting progressive dysregulation of vitamin D signaling during cancer evolution.

Notably, aberrant overexpression of the CYP24A1 gene, encoding 24-hydroxylase the enzyme responsible for calcitriol degradation has been identified in BC tissues, which may result in attenuation of calcitriol-mediated growth control.[232, 233] Eisman et al. reported that the presence of functional VDR in both normal and malignant breast cells suggests that neoplastic cells remain partially responsive to vitamin D metabolites.[234] Experimental data in multiple *in vitro* and *in vivo* BC models have confirmed the antiproliferative and pro-apoptotic actions of calcitriol and its analogs. However, the magnitude of these effects is modulated by tumor subtype, disease stage, VDR expression status, and combination with other therapies.[235-237] Comparative studies show that malignant breast tissues exhibit higher VDR expression than benign counterparts, suggesting a compensatory or regulatory role of VDR signaling in cancerous contexts.[238] Mechanistically, calcitriol exerts tumor-suppressive functions by modulating multiple cellular pathways, including inhibition of cell proliferation, induction of differentiation, G1 phase cell cycle arrest, suppression of oncogene expression, and attenuation of pro-inflammatory cytokine production.[214,239] Chen et al. noted that these effects provide the rationale for exploring calcitriol and its analogs as therapeutic agents in oncology.[240]

The clinical relevance of VDR expression extends across multiple BC subtypes, including ER-positive, PR-positive, and HER2-positive tumors where it has been associated with favorable prognostic features and less aggressive phenotypes.[17] Koshizuka et al. demonstrated that calcitriol and its analogs, EB1089 and 1,25$(OH)_2$-16-ene-23-yne-19-nor-26,27-F6-D_3, synergistically enhanced the antitumor efficacy

of paclitaxel *in vivo*. Among these, EB1089, a non-calcemic analog, produced the most potent additive antineoplastic response. Given the high prevalence of VDR expression in breast tumors, exploiting VDR activation in conjunction with conventional chemotherapeutics emerges as a promising strategy.[241] Further validation using MCF-7 xenograft models showed that the vitamin D analog CB1093 also potentiated paclitaxel cytotoxicity more effectively than cisplatin, highlighting subtype-specific chemo potentiation.[242] Wang et al. later expanded this observation to ER-positive (MCF-7, T-47D) and TNBC (MDA-MB-231) cell lines. Pretreatment with calcitriol led to a significant reduction in the IC_{50} of paclitaxel (up to 100-fold) and doxorubicin (up to 10-fold), primarily through enhanced apoptosis and Bcl-2 phosphorylation a key marker of chemotherapeutic response.[243]

2.14 VITAMIN D AND BREAST CANCER

2.14.1 Observational and Meta-Analytical Evidence

Initial epidemiological observations in the 1980s linked higher cancer incidence to regions with reduced sunlight exposure, particularly at higher latitudes. This geographic variation was hypothesized to reflect differences in cutaneous vitamin D synthesis due to lower ultraviolet B (UVB) radiation (Figure. 6), implicating vitamin D deficiency as a potential cancer risk factor.[244, 245] Subsequent studies assessing baseline 25-hydroxyvitamin D [25(OH)D] concentrations at diagnosis confirmed that BC patients often present with vitamin D deficiency.[245] Karthikayan et al. observed that this deficiency appears to be particularly pronounced in patients with higher-grade tumors, non-luminal molecular subtypes, and ER-negative status.[246] Observational studies have aimed to delineate the relationship between vitamin D status including 25(OH)D serum levels and dietary intake and BC risk, prognosis, and survival. However, limitations in sample size, study design, and confounders often preclude definitive conclusions. To address this, several meta-analyses have pooled results from independent cohorts to enhance statistical power. These analyses have demonstrated a modest protective association between higher serum 25(OH)D concentrations and decreased BC risk, with some reporting a 6% risk reduction per 5 nmol/L increment in vitamin D levels.[247-250] Notably, the protective effect was more evident in premenopausal women.[248]

Despite these findings, no consistent link has been observed between dietary vitamin D intake BC risk.[248] While some meta-analyses support a reduction in BC progression or mortality with higher serum 25(OH)D levels.[251]

2.14.2 Mendelian Randomization (MR) Studies

To overcome confounding inherent in observational designs, MR studies have been employed. These studies utilize single nucleotide polymorphisms (SNPs) as instrumental variables to assess causality between genetically determined vitamin D status and BC risk. Initial MR studies identified SNPs in key vitamin D metabolism genes GC (encoding vitamin D binding protein), DHCR7, CYP2R1, and CYP24A1 as significant determinants of serum 25(OH)D levels. A large-scale genome-wide association study (GWAS) involving over 79,000 individuals later identified two additional loci: SEC23A and AMDHD1.[252]

Jiang et al. analyzed six SNPs in a large dataset of 122,977 BC cases; however, no association was found between these genetic determinants of vitamin D status and BC risk.[253] Expanding this approach, an MR study incorporating 138 SNPs across 69 vitamin D-associated loci also failed to establish a causal role for serum 25(OH)D in BC development.[254] Bouillon et al. found that these findings were consistent across both hormone receptor - positive and - negative subtypes. Thus, despite the increased statistical power and rigor of MR analyses, there remains no genetic evidence to support a causal role for vitamin D insufficiency in BC etiology. [255]

2.14.3 Randomized Controlled Trials (RCTs)

RCTs are the gold standard for evaluating causal relationships between interventions and outcomes. The VITamin D and OmegA-3 TriaL (VITAL) is one of the largest RCTs to assess vitamin D effect on cancer prevention. It enrolled 25,871 individuals in a randomized, double-blind, placebo-controlled design to test daily supplementation with 2000 IU of vitamin D_3, alone or combined with omega-3 fatty acids. The mean baseline 25(OH)D level was 30 ± 10 ng/mL, increasing to 41.8 ng/mL in the intervention group after one year.[256] Although no significant difference in overall BC incidence was found, secondary analyses revealed a reduction in metastatic and fatal cancers, particularly in normal-weight participants (BMI <25).[257]

Lappe et al. conducted a 4-year randomized controlled trial involving 2,303 postmenopausal women, in which daily supplementation with 2000 IU of vitamin D_3 and 1500 mg of calcium did not significantly reduce cancer incidence. Other RCTs have similarly explored the relationship between vitamin D_3 supplementation and cancer outcomes.[258] The ViDa study, utilizing high-dose monthly supplementation (100,000 IU), also failed to show an effect on overall cancer incidence.[259] Arnaout et al. conducted a targeted trial assessing the impact of vitamin D on tumor and found inconclusive results. In their study, preoperative high-dose vitamin D_3 supplementation (40,000 IU/day for 2–6 weeks) did not affect markers of proliferation (Ki67) or apoptosis (cleaved caspase-3) in breast tumor tissue, despite increased serum 25(OH)D levels.[260] Likewise, a study in high-risk premenopausal women receiving 20,000 IU/week for 12 months did not reduce mammographic density, a known surrogate marker of BC risk.[261]

Recent meta-analyses of pooled RCTs also failed to confirm any significant reduction in BC risk with vitamin D supplementation.[78, 262] Furthermore, no major RCTs in the last 5 years have addressed the effect of vitamin D on survival or therapeutic response in BC patients. However, two ongoing trials are exploring this domain: one evaluating the impact of neoadjuvant vitamin D_3 (50,000 IU/week) on pathological complete response (NCT03986268), and another assessing 5-year DFS following neoadjuvant vitamin D_3 therapy (NCT01608451). In sum, RCTs to date do not support a clear preventive or therapeutic role for vitamin D supplementation in BC. The heterogeneity in trial design, dosing regimens (daily vs. monthly), baseline vitamin D status, and uncontrolled self-supplementation during study periods pose challenges in interpreting outcomes. Thus, the optimal dosing strategy and target serum 25(OH)D level for BC prevention remain unresolved.[263]

2.15 VDR EXPRESSION IN HUMAN BREAST CANCER CELLS

The VDR is expressed in various cell types of the mammary gland, including lobular and ductal epithelial cells, where it contributes significantly to mammary gland development during key physiological stages such as puberty, lactation, and pregnancy periods marked by intense tissue growth and remodeling.[17, 264] Zinser, Packman, and Welsh observed that in murine models, VDR expression peaks in the differentiated cells of terminal end buds during puberty, with relatively lower levels in

proliferative zones.[265] Knockout (KO) models lacking VDR exhibit accelerated ductal morphogenesis and branching compared to wild-type mice, highlighting VDR regulatory role during pubertal development. Additionally, in MMTV-neu transgenic mice, VDR deletion was associated with mammary fat pad atrophy, estrogen deficiency, weight loss, and reduced survival by 12 months of age.[266] Adipocytes also influence mammary gland development, and VDR ablation in adipose tissue increases epithelial density during hormone-driven glandular expansion.[267] Welsh et al. highlighted that adipose tissue serves as a reservoir for vitamin D metabolites, and VDR-mediated signaling between adipocytes and epithelial cells is implicated not only in physiological development but also in breast carcinogenesis.[268] Furthermore, VDR is expressed in cancer-associated fibroblasts (CAFs), where its activation by $1,25(OH)_2D_3$ downregulates proliferative genes such as Neuregulin-1 (NRG1), suggesting a potential anti-tumorigenic function.[269]

In human BC tissues, VDR expression is inversely associated with tumor aggressiveness. Studies have shown significantly higher VDR levels in benign lesions compared to both *in situ* and invasive carcinomas.[270] Multiple reports indicate a progressive decline in VDR expression during tumor advancement, correlating with reduced responsiveness to vitamin D3.[268] Indeed, BC cell lines lacking or exhibiting minimal VDR expression show poor sensitivity to $1,25(OH)_2D_3$ or its analogs.[271] Several studies have explored VDR as a prognostic biomarker for cancer progression and survival.[272] Elevated total VDR expression, both nuclear and cytoplasmic, has been linked with favorable tumor characteristics such as lower histological grade, smaller tumor size, ER/PR positivity, reduced Ki-67 proliferation index, and improved BC specific survival.[16, 264] Heublein et al. observed that in BRCA1-mutated BC cases, significantly elevated VDR expression is correlated with extended OS.273 Xu et al. conducted a meta-analysis of seven studies and found no overall correlation between VDR expression and BC OS or DFS. However, subgroup analyses revealed that high total VDR expression in both nuclear and cytoplasmic compartments was positively associated with OS. Moreover, when immunoreactive score (IRS) thresholds other than IRS >5 or IRS >25 were used, a strong association emerged between higher VDR expression and improved survival outcomes.[272] IRS is a semi-quantitative immunohistochemical scoring method based on the product of the percentage of positive cells (scale 0–4) and staining intensity (scale 0-3), yielding a

composite score ranging from 0 to 12.[274] Although Murray et al. did not find a significant correlation between VDR expression and DFS overall, they did observe a positive association within luminal A BC subtypes, with no corresponding association in basal-like, HER2-positive, or luminal B subtypes. Collectively, these findings suggest the potential utility of VDR expression levels as a prognostic indicator for tumor progression. [271]

Further studies have examined the regulation of vitamin D metabolism in BC via CYP27B1 and CYP24A1. These enzymes modulate VDR signaling and are frequently deregulated during tumor dedifferentiation and progression.[268] Most analyses indicate a reduction in CYP27B1 (responsible for converting $25(OH)D_3$ to the active $1,25(OH)_2D_3$) and an upregulation of CYP24A1 (which inactivates $1,25(OH)_2D_3$) in invasive carcinomas, a pattern suggestive of tumor cells evading vitamin D mediated antitumor effects.[270, 233] Albertson et al. consequently recognized CYP24A1 as an emerging oncogene in BC.[232] Beyond epithelial cells, CYP27B1 is also expressed in mammary adipose tissue, facilitating the local activation of $25(OH)D_3$ and paracrine modulation of neighboring cells.[268] Notably, transcriptional induction of CYP24A1 by $1,25(OH)_2D_3$ is stronger in CAFs than in normal fibroblasts, potentially accelerating vitamin D catabolism in the tumor microenvironment.[269] However, a more recent investigation found reduced CYP24A1 mRNA expression in breast tumor tissues, with lower levels correlating with improved OS.[275]

2.16 PRECLINICAL ANTI-NEOPLASTIC EFFECTS OF $1,25(OH)_2D_3$ ON BREAST CANCER

2.16.1 Effects of $1,25(OH)_2D_3$ on cell proliferation

1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), the active hormonal form of vitamin D, exerts its antiproliferative effects on BC cells primarily through the activation of the VDR. Binding of $1,25(OH)_2D_3$ to VDR initiates a cascade of transcriptional events that suppress cell cycle progression. Notably, in VDR-knockout cells, these growth-inhibitory effects are absent, confirming the VDR-dependent mechanism of action.[14, 276] Upon activation, $1,25(OH)_2D_3$ upregulates the expression of cyclin-dependent kinase inhibitors (CDKIs), including CDKN2D (p19), CDKN1A (p21), and CDKN1B (p27), while simultaneously downregulating the expression of cyclins (D1, D3, A1,

E1) and cyclin-dependent kinases CDK2 and CDK4.[277-279] This results in the suppression of CDK4/6 activity, reducing phosphorylation of the retinoblastoma (Rb) protein, a key cell cycle checkpoint regulator. The hypophosphorylated form of Rb retains its ability to bind and sequester E2F transcription factors, thereby blocking the transcription of E2F-regulated genes essential for G1/S transition, such as CDK2.[227] Dhawan, Weider, and Christakos reported that inhibition of E2F-driven gene expression leads to cell cycle arrest at the G0/G1 phase. Additionally, in ER-positive MCF7 cells, $1,25(OH)_2D_3$ upregulates the transcription factor C/EBPα, which enhances the expression of VDR itself, establishing a positive feedback loop that amplifies antiproliferative signaling.[280] Beyond direct gene regulation, $1,25(OH)_2D_3$ influences non-coding RNA pathways, particularly microRNAs (miRNAs), to exert growth control. miR-1204, for instance, has been shown to promote BC cell proliferation, EMT, and invasiveness both *in vitro* and *in vivo*. Mechanistic studies revealed that miR-1204 directly targets the 3′ untranslated region (UTR) of VDR mRNA, leading to translational suppression of VDR. Silencing miR-1204 results in increased VDR expression and a corresponding reduction in proliferation and invasion, indicating a crucial role of the miR-1204 VDR axis in tumorigenesis.[281]

The antiproliferative effects of $1,25(OH)_2D_3$ are further supported by *in vivo* findings. In the MMTV-PyMT transgenic mouse model, continuous subcutaneous administration of either $1,25(OH)_2D_3$ or its precursor $25(OH)D_3$ via osmotic minipumps significantly reduced the expression of proliferative markers such as Ki67, ErbB2, and cyclin D1, and suppressed overall tumor growth.[282] Importantly, while $25(OH)D_3$ enhanced local intratumoral conversion to $1,25(OH)_2D_3$ without inducing hypercalcemia, direct $1,25(OH)_2D_3$ infusion resulted in elevated serum calcium levels. However, these effects are not universal. In a xenograft model derived from highly proliferative BC tissues, intratumoral injection of $1,25(OH)_2D_3$ failed to reduce proliferation (measured by BrdU incorporation, Ki67, CDKN1A, CDKN1B) or induce apoptosis (assessed by Bcl-2 levels), suggesting that tumor type and local microenvironment influence the efficacy of vitamin D treatment.[283]

In addition to $1,25(OH)_2D_3$, its metabolite 24R,25-dihydroxyvitamin D_3 ($24R,25(OH)_2D_3$) also modulates BC cell behavior. *In vitro*, $24R,25(OH)_2D_3$ stimulates DNA synthesis in ER-positive MCF7 and T47D cells through a caveolae-associated phospholipase D-dependent pathway involving crosstalk with estrogen

receptors. Interestingly, *in vivo* administration of 24R,25$(OH)_2D_3$ in MCF7 xenograft models reduced tumor burden and enhanced survival. These effects were accompanied by the downregulation of pro-metastatic markers such as Snail1 and the CXCR4/CXCL12 chemokine axis, indicating an inhibitory role in invasion and metastasis.[284]

2.16.2 Effects of 1,25$(OH)_2D_3$ on apoptosis

1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) induces apoptosis in BC cells by modulating multiple apoptotic signaling pathways in a cell type specific manner. The hormone promotes a shift in the apoptotic balance by downregulating anti-apoptotic proteins such as Bcl-2 and Bcl-xL, while concurrently upregulating pro-apoptotic mediators like Bax and Bak. This altered Bcl-2/Bax ratio favors mitochondrial outer membrane permeabilization and subsequent cell death over survival.[285] Christakos et al. and Zheng et al. demonstrated that one of the key molecular pathways targeted by 1,25$(OH)_2D_3$ is the RAS/MEK/ERK axis, which plays a critical role in both proliferation and anti-apoptotic signaling. Treatment of both ER-positive MCF7 and ER-negative MDA-MB-453 BC cells with 1,25$(OH)_2D_3$ significantly reduced RAS expression and inhibited phosphorylation of downstream kinases MEK and ERK1/2. This signaling blockade led to suppression of cell survival, and reactivation of RAS signaling reversed the antiproliferative effects of 1,25$(OH)_2D_3$, indicating that inhibition of this pathway is essential for its pro-apoptotic function.[227, 286] Weitsman et al. provided further mechanistic insight into mitochondrial apoptosis by showing that pre-treatment of MCF7 cells with 1,25$(OH)_2D_3$ sensitized them to reactive oxygen species (ROS)-induced cytotoxicity. This occurred through a loss of mitochondrial inner membrane potential, triggering cytochrome c release into the cytosol, a hallmark of the intrinsic apoptotic pathway. The culmination of this cascade is caspase activation and cell death.[287] Thus, 1,25$(OH)_2D_3$ acts as a mitochondrial stress sensitizer in hormone receptor positive BC cells.

Duffy, Synnott, and Crown reported that the tumor suppressor gene p*53*, which encodes p53, is mutated in a high proportion of BCs, particularly in TNBC (80%) and HER2-positive subtypes (70%), while it is less commonly altered in luminal A (10%) and luminal B (30%) tumors.[168] Notably, the VDR gene has been identified as a direct transcriptional target of p53 and its family members.[288] However, in cells harbouring

mutant p53 (mutp53), a paradoxical interaction occurs between mutp53 and VDR. These mutant forms of p53 not only bind VDR but also functionally modify its activity, converting vitamin D from a pro-apoptotic to an anti-apoptotic agent. This phenomenon has been observed in TNBC cell lines such as MDA-MB-231 and MDA-MB-468, which endogenously express mutp53R280K and mutp53R273H, respectively. Although the exact mechanism underlying this functional conversion remains unclear. This suggests that additional molecular alterations may cooperate with mutp53 to mediate an anti-apoptotic response to vitamin D3 in certain tumor contexts.[289]

2.17 MicroRNA AND REGULATION OF VITAMIN D SIGNALING

MicroRNAs (miRNAs) are a class of small, non-coding RNAs approximately 18 - 22 nucleotides in length that regulate gene expression post-transcriptionally by binding to complementary sequences in the 3′ untranslated region (3′-UTR) of target mRNAs, resulting in mRNA degradation or translational repression.[290] The biogenesis of miRNAs is a tightly regulated, as outlined in Figure 8, multistep process, and these molecules play key roles in various physiological and pathological processes including development, differentiation, proliferation, and apoptosis.[290] Iorio and Croce and Croce highlighted that aberrant miRNA expression has been implicated in numerous malignancies, where miRNAs function either as tumor suppressors or oncogenes depending on the cellular context. [170, 291]

Mohri et al. Zhang et al. Chen et al. and Li et al**.** demonstrated that microRNAs (miRNAs) modulate key components of the vitamin D signaling pathway, including the VDR, the activating enzyme CYP27B1, the deactivating enzyme CYP24A1, and the nuclear co-receptor retinoid X receptor alpha (RXRα). Specifically, four miRNAs, miR-125b, miR-27b, miR-298, and miR-346 have been shown to directly target the VDR transcript, as represented in Figure 9. [292-295] The first such interaction was identified by Mohri et al. who demonstrated that miR-125b suppresses VDR expression in MCF-7 BC cells, abolishing the antiproliferative effects of $1,25(OH)_2D_3$.[292]

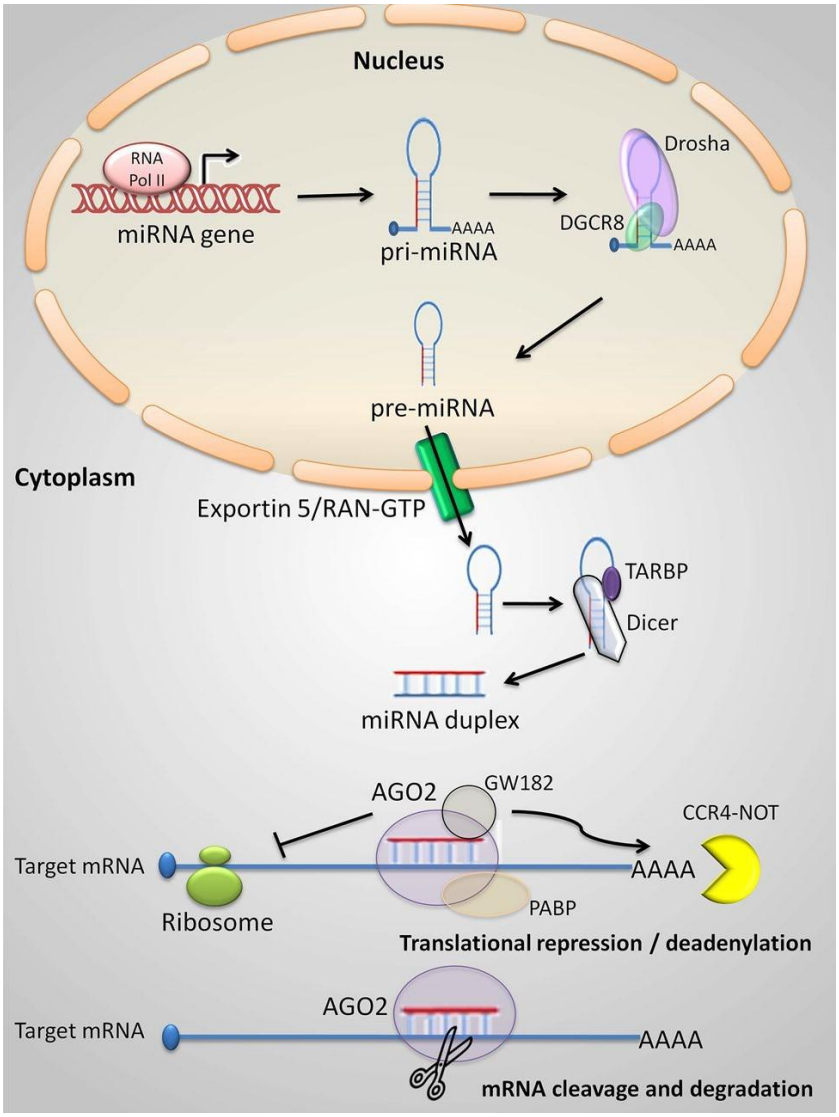


Figure 8: Canonical pathway of miRNA biogenesis.

In the nucleus, RNA polymerase II transcribes the miRNA gene, producing the primary transcript (pri-miRNA). This is processed by the Drosha-DGCR8 complex to generate precursor miRNA (pre-miRNA), which is then exported to the cytoplasm via Exportin 5/RAN-GTP. In the cytoplasm, Dicer and TAR RNA-binding protein (TARBP) further process pre-miRNA into a mature miRNA duplex. One strand of the duplex is incorporated into the RNA-induced silencing complex (RISC), composed of AGO2, GW182, and PABP. Imperfect base pairing between miRNA and target mRNA leads to translational repression or deadenylation mediated by CCR4-NOT, while perfect complementarity results in mRNA cleavage and degradation.

Source: DOI: 10.1007/s00438-017-1301-9.

Similarly, miR-27b regulates VDR expression in melanoma, colon (LS-180), pancreatic (PANC1), and lung fibroblast (MRC5) cell lines. Li et al. confirmed, using a luciferase reporter assay, that miR-27b directly binds the VDR 3′UTR, reducing VDR protein expression without affecting mRNA levels.[295] Pan, Gao, and Yu found that the binding site for miR-298 in the VDR 3′UTR is evolutionarily conserved across humans, rats, and mice, and verified its interaction with VDR through luciferase-based assays.[296]

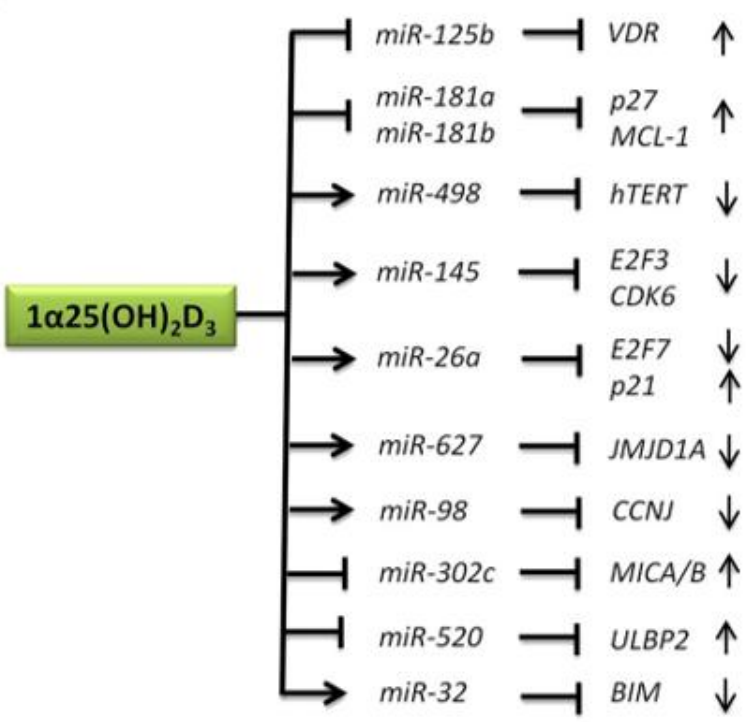


Figure 9: MicroRNAs regulated by 1α,25-dihydroxyvitamin D_3 and their associated target genes in various cancer types. This figure illustrates specific miRNAs modulated by $1\alpha,25(OH)_2D_3$, along with their respective target genes. The direction of regulation (upregulation or downregulation) of both miRNAs and their targets is indicated, highlighting the potential role of vitamin D in influencing oncogenic or tumor-suppressive pathways.

Abbravations: VDR, vitamin D receptor; p27, CDKN1B—cyclin-dependent kinase inhibitor 1B; MCL-1, myeloid cell leukemia 1; hTERT, human telomerase reverse transcriptase; E2F3, E2F transcription factor 3; CDK6, cyclin-dependent kinase 6; p21, CDKN1A (WAF1/CIP1)—cyclin-dependent kinase inhibitor 1A; E2F7, E2F transcription factor 7; JMJD1A, Jumonji domain containing 1A; MICA/B, MHC class I polypeptide-related sequence A/B; ULBP2, UL16-binding

In the context of inflammation, miR-346 was reported to downregulate VDR expression in intestinal epithelial cells by direct binding to the VDR 3′UTR.[294] Other components of the vitamin D pathway are also regulated by specific miRNAs. CYP24A1, the enzyme responsible for catabolizing $1,25(OH)_2D_3$, is regulated by miR-125b and the oncogenic miR-17~92 cluster. Functional studies in KGN and MCF-7 cell lines validated that miR-125b directly targets CYP24A1 mRNA.[297] On the other hand, the activating enzyme CYP27B1 was shown to be targeted by miR-21 in Mycobacterium leprae-infected monocytes, where luciferase reporter assays confirmed the direct interaction between miR-21 and CYP27B1.[298]

Ji et al. demonstrated that miR-27a and miR-27b downregulate RXRα expression in hepatic stellate cells, while Adlakha et al. confirmed that miR-128-2 suppresses RXRα expression in HEK293T cells via luciferase assay. RXRα, which forms a heterodimer with VDR to mediate vitamin D transcriptional responses, is regulated by several miRNAs, including miR-27a, miR-27b, miR-128-2, and miR-574-3p.[299, 300] Furthermore, Guérit et al. reported that miR-574-3p negatively regulates RXRα during chondrogenic differentiation of mesenchymal stem cells.[301] Collectively, these findings illustrate that miRNAs are critical post-transcriptional regulators of the vitamin D signaling pathway, influencing VDR activity, ligand metabolism, and nuclear co-receptor interactions. Their dysregulation can significantly alter vitamin D responsiveness in both physiological and pathological contexts, including cancer.

2.18 VITAMIN D MODULATES miRNA EXPRESSION IN BREAST CANCER

Vitamin D influences miRNA expression through multiple mechanisms that span both transcriptional and post-transcriptional regulation. Upon activation, the VDR binds to vitamin VDREs located in the promoter regions of specific miRNA genes, thereby regulating their transcription. Additionally, vitamin D can modulate miRNA biogenesis by altering the expression of key enzymes involved in miRNA processing, such as Drosha and Dicer, or by influencing the stability of mature miRNAs.[302] Kasiappan et al. demonstrated that vitamin D alters miRNA expression profiles that contribute to tumor suppression. A dose-dependent transcriptional induction of miR-498 by vitamin D was observed in both MCF-7 breast and Ishikawa endometrial cancer cell lines, indicating a conserved mechanism across hormone-responsive

cancers. This upregulation of miR-498 has been implicated in mediating part of vitamin D anticancer activity.[303] Additionally, in MCF-7 and MDA-MB-231 TNBC cells, vitamin D treatment led to reduced expression of miR-302c and miR-520, two miRNAs known to suppress the immune surveillance pathway. As a result, vitamin D enhanced the susceptibility of cancer cells to natural killer (NK) cell-mediated cytotoxicity by upregulating NKG2D pathway ligands such as MICA/B and ULBP2, which are known targets of these miRNAs.[304]

Further supporting a regulatory role of VDR in miRNA expression, Alimirah et al. demonstrated that the miR-199a/miR-214 cluster (including miR-199a-3p, miR-199a-5p, and miR-214) is negatively regulated by VDR through modulation of the Dnm3os gene in BC cell lines MCF-7 and T47D, as well as in murine VDR wild-type (WT-145) and knockout (VDRKO) mammary tumor models. Treatment of T47D cells with 50 nM of vitamin D for 24 hours led to a marked induction of VDR and p21 at both mRNA and protein levels.[305] Interestingly, overexpression of miR-214 was found to attenuate vitamin D signaling in both MCF-7 and T47D cells, suggesting a feedback loop where certain miRNAs can dampen VDR-mediated transcriptional activity and partially reverse the anticancer effects of vitamin D.[305]

2.19 MicroRNA GENE REGULATION IN TNBC

miRNAs play a critical role in the post-transcriptional regulation of gene expression and have emerged as key regulators in the molecular pathology of TNBC. Several miRNAs have been identified as either tumor suppressors or oncogenes in TNBC, and their dysregulation contributes to cancer progression, cell cycle disruption, apoptosis resistance, and metastasis. One such tumor suppressor miRNA is miR-205, which is frequently downregulated in TNBC. miR-205 expression is positively regulated by p53, a well-known tumor suppressor that enforces cell cycle checkpoints and prevents the propagation of damaged DNA. Loss of p53, common in TNBC, leads to reduced miR-205 levels and impaired control over proliferative and invasive pathways.[306, 307] Functionally, p53 ensures that cells do not transition through the G1/S checkpoint with unrepaired DNA damage, a safeguard lost in many cancers.[308]

Similarly, the Rb, another key tumor suppressor responsible for enforcing G1 checkpoint fidelity, is often inactivated in TNBC. Loss of Rb function has been mechanistically linked to the post-transcriptional downregulation of Smurf2, an E3

ubiquitin ligase involved in the regulation of cell polarity, migration, and apoptosis, as presented in Figure 10. This downregulation is mediated by miRNA interference, highlighting the role of miRNAs as key regulators of tumor suppressive networks in TNBC.[309, 310] Filipowicz et al. explained that microRNAs typically act through mechanisms such as mRNA cleavage, chromatin remodeling, and translational repression, ultimately leading to decreased protein synthesis from target transcripts.[311] Additional miRNAs with tumor suppressor functions in TNBC include miR-203, which inhibits BIRC5 and LASP1, leading to reduced cell proliferation and migration and miR-200c, which suppresses the anti-apoptotic protein XIAP, thereby enhancing apoptosis and limiting tumor progression.[312] Park et al. reported that miR-200c, a member of the miR-200 family, is essential for maintaining the epithelial phenotype of cancer cells by inhibiting EMT.[313] Jang et al. found that the loss of other miR-200 family members, such as miR-200a, has similarly been associated with aggressive tumor behavior and metastatic potential in BC.[314] Conversely, certain miRNAs exhibit oncogenic properties in TNBC. For example, miR-221 promotes proliferation and survival; its knockdown results in cell cycle arrest and apoptosis induction, indicating its pro-tumorigenic role.[315] Dong et al. reported that miR-21, a well-characterized oncomiR, is significantly overexpressed in TNBC tissues and is associated with poor prognosis. It modulates multiple oncogenic pathways, including *PTEN* and *PDCD4*, thereby contributing to tumor cell proliferation and immune evasion.[316]

Similarly, miR-182 is overexpressed in TNBC and has been linked to enhanced cell migration and invasion, thereby facilitating metastatic spread.[317] Kong et al. demonstrated that miR-155 exemplifies oncogenic activity by targeting the tumor suppressor gene *von Hippel–Lindau* (VHL), which plays a key role in inhibiting angiogenesis. Downregulation of VHL by miR-155 promotes vascularization and tumor growth.[318] Collectively, these findings emphasize that miRNAs are central modulators of TNBC. Tumor-suppressive miRNAs are frequently downregulated, allowing oncogenic pathways to dominate, while oncomiRs are upregulated to support unchecked proliferation, resistance to apoptosis, and metastasis. Understanding these regulatory circuits provides opportunities for developing miRNA-based diagnostics and targeted therapies. Additional findings on miRNAs in TNBC are summarized in Table 2.

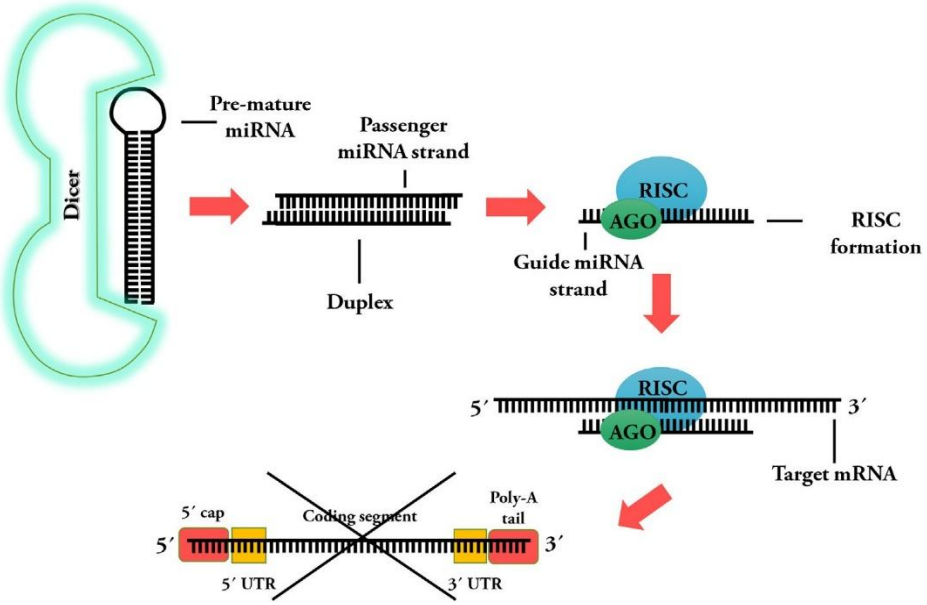


Figure 10: Post-transcriptional mechanism of microRNA action.

This schematic illustrates the processing of pre-microRNA by Dicer into a miRNA duplex, consisting of a guide strand and a passenger strand. The guide strand is incorporated into the Argonaute-containing RNA-induced silencing complex (RISC), enabling recognition of complementary target mRNA. Binding of RISC to the 3′ untranslated region (UTR) of target mRNAs leads to translational repression or degradation. Abbreviations: miRNA – microRNA; AGO – Argonaute; UTR – untranslated region.

Table 2. Various microRNAs and their regulatory role in TNBC.

microRNA	**Significance in TNBC**
miR-106b, miR-17/92, miR-200 (a,b,c), miR-21, miR-155	Upregulated in TNBC
miR-126, miR-145, miR-205	Downregulated in TNBC
miR-424, miR-125a, miR-627, miR-579, miR-101	Metastasis
miR-520g, miR-149, miR-342, miR-107, miR-520g-h, miR-155, miR-30c, miR-382	Markers for ER status
miR-520g, miR-520d, miR-328, miR-373, miR-217, miR-504, miR-485-3p	Markers for PR status
miR-520d, miR-30b, miR-217, miR-363, miR-383, miR-377, miR-130a, miR-422a	Markers for HER2/neu status
miR-342, miR-27b, miR-150	Prognostic markers

Abbreviations: miR, microRNA; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

2.20 MicroRNAs AND METASTASIS IN TNBC

Several miRNAs have been implicated in the regulation of metastatic processes in TNBC, functioning either as metastasis suppressors or promoters. Among these, miR-31 has been identified as a key anti-metastatic miRNA. Augoff et al. demonstrated that downregulation of miR-31 enhances metastatic potential in TNBC. The reduced expression of miR-31 was attributed to epigenetic silencing, specifically through hypermethylation of its promoter CpG island. Treatment with demethylating agents restored miR-31 expression, confirming that promoter methylation directly regulates its transcriptional silencing. These findings suggest that restoring miR-31 expression could be explored as a therapeutic strategy to inhibit metastasis in TNBC.[319]

In a related mechanism, miR-200b has also been shown to function as a metastasis-suppressive miRNA in TNBC. Humphries et al. reported that miR-200b is significantly downregulated in metastatic TNBC tissues, and this downregulation correlates with increased invasive behavior. Mechanistically, miR-200b targets protein kinase C alpha (PKCα), a key modulator of cytoskeletal dynamics and cell motility. Loss of miR-200b leads to unchecked PKCα expression, thereby promoting cell migration and metastatic dissemination. These findings highlight miR-200b as a potential therapeutic target for preventing or reversing metastasis in aggressive TNBC subtypes.[320]

2.21 VITAMIN D SIGNALING IN TNBC

Den Hollander, Savage, and Brown noted that unlike hormone receptor - positive or HER2-positive tumors, TNBC lacks effective targeted therapies and is currently managed with conventional chemotherapy, with or without radiotherapy. To date, no prophylactic agents have been approved for TNBC.[321] Studies have reported significantly lower serum vitamin D levels in patients with TNBC, particularly in those with poor prognoses, compared to other BC subtypes.[322] Thakkar et al. demonstrated that a majority of TNBC tumors express VDR, and that VDR agonists exert antiproliferative effects by inducing apoptosis and cell cycle arrest in TNBC cell lines. Growing interest has therefore emerged around the therapeutic potential of targeting the VDR pathway in TNBC.[323] These findings suggest that VDR ligands could serve as adjuncts to standard chemotherapy. Supporting this, Chiang et al. reported that the calcitriol analog MART-10, when combined with calcitriol,

significantly suppressed metastatic potential in TNBC cell lines, with MART-10 demonstrating greater potency than calcitriol alone.[324] Additional evidence from LaPorta and Welsh and Flanagan et al. also confirmed the growth-suppressive effects of calcitriol and its analogs in TNBC cell lines such as SUM-159PT and WT145.[14, 325] Ferronato et al. further advanced this line of inquiry by evaluating two vitamin D analogues, EM1 and UVB1, in both HER2-positive and TNBC patient-derived xenograft (PDX) models.[326] Among African-American women, who often present with lower baseline 25(OH)D levels, inverse associations were observed between vitamin D supplementation and TNBC risk, particularly with increased sun exposure.[327] These population-level observations underscore a plausible association between vitamin D deficiency and BC. However, the inability to eliminate confounding variables in observational studies renders the causal inference difficult to establish.[328, 329] VDR expression levels are notably higher in luminal A subtypes relative to TNBC, the most aggressive BC variant.[17, 268] These analogs significantly reduced tumor viability, with UVB1 additionally showing antiproliferative effects in trastuzumab-emtansine resistant cell lines and modulating VDR expression in PDXs. A mechanistic review by Blasiak et al. suggested that vitamin D may exert protective effects in BRCA1-mutated TNBC by stabilizing p53BP1 (tumor protein p53 binding protein 1) and preventing its degradation by cathepsin L. Vitamin D was also proposed to interact with proteins from the growth arrest and DNA damage-inducible 45 (GADD45) family, further contributing to DNA repair and tumor suppression.[15] Despite these promising findings, some TNBC cell lines, such as MDA-MB-157, MDA-MB-231, and MDA-MB-468, have shown resistance to vitamin D treatment.[330] Stambolsky et al. and Hirshfield and Ganesan observed that the ineffectiveness of vitamin D in these models was attributed to the absence or dysfunction of the p53 gene, resulting in a paradoxical anti-apoptotic rather than pro-apoptotic effect. They further emphasized that the presence of mutp53 may confer resistance to vitamin D signaling.[289, 331]

Santos-Martínez et al. found that calcitriol, through VDR activation, induced functional expression of ERα in ER-negative BC cells. This re-expression of ERα restored responsiveness to anti-estrogen therapy and inhibited cell proliferation.[332] Zheng et al. demonstrated the therapeutic synergy between calcitriol and paclitaxel (PTX) in TNBC. Calcitriol enhanced the antitumor efficacy of paclitaxel by

downregulating matrix metalloproteinase-9 (MMP-9) and Bcl-2, while upregulating E-cadherin expression.[333] Moreover, it counteracted the elevation of C-C motif chemokine ligand 2 (CCL2) and $Ly6C^+$ monocytes induced by paclitaxel, both of which are associated with metastatic spread. Supporting this, the combination of paclitaxel and calcitriol delivered via pH-sensitive micelles significantly suppressed primary tumor growth and lung metastasis in 4T1 tumor-bearing mice, a model of stage IV BC.[333] These findings suggest a clinically translatable strategy to overcome the pro-metastatic side effects of chemotherapy.

In addition, treatment of TNBC cells with ERβ agonists was shown to reduce invasiveness, while ERβ knockdown resulted in enhanced cell migration and invasion. These data indicate that combining calcitriol with ERβ agonists may offer an effective therapeutic strategy in ERβ-expressing TNBC cells.[334] Due to the absence of hormone receptors and HER2 expression, TNBC is typically managed using broad-spectrum chemotherapeutic agents such as platinum compounds (e.g., cisplatin, carboplatin), taxanes (e.g., paclitaxel, docetaxel), anthracyclines (e.g., doxorubicin, epirubicin), antimetabolites (e.g., 5-fluorouracil, methotrexate), and alkylating agents (e.g., cyclophosphamide).[335,336] Interestingly, approximately one-third of TNBC tumors express VDR, and this expression has been inversely correlated with mitotic score, histological grade, proliferation index, and recurrence rate.[337] Patients with VDR-positive TNBC tumors also exhibit prolonged OS (26 months) compared to VDR-negative cases. More recently, RNA-sequencing data from basal-like PDX models confirmed that VDR is among the most highly expressed genes in TNBC, further highlighting its potential as a therapeutic target.[338]

2.22 MECHANISTIC INSIGHTS INTO CALCITRIOL AND VDR SIGNALING IN BREAST CANCER AND TNBC

Blasiak et al. illustrated that vitamin D_3 acts through VDR-mediated genomic and non-genomic mechanisms. Recent literature increasingly highlights VDR signaling as a context-dependent, subtype-specific, and therapeutically exploitable pathway, particularly in TNBC, as shown in Figure 11.[15] Huss et al. provided compelling evidence that nuclear membrane-localized vitamin D receptor (VDR^num) expression in breast tumors is strongly associated with favorable prognosis.[339] In a cohort of 878 BC patients, immunohistochemical analysis revealed

that VDR^num positivity correlated with better tumor differentiation, ER and PR positivity, and significantly prolonged BC free interval and OS. This effect was particularly pronounced in large, clinically detected tumors, suggesting that the transcriptionally active nuclear-bound form of VDR may represent a functional biomarker for endocrine-responsive BC subtypes.[339] Veeresh et al. supported these findings through *in vitro* and *in vivo* studies, demonstrating that cholecalciferol reduced viability of BC cells by inducing G0/G1 or G2/M arrest and triggering apoptosis via upregulation of p53 and Bax and downregulation of Bcl-2 and cyclin D1. These antiproliferative effects were corroborated in mouse models bearing Ehrlich ascites carcinoma (EAC), where intraperitoneal administration of vitamin D3 significantly decreased ascitic volume and tumor burden. This dual-level evidence emphasized the applicability of vitamin D3 across diverse subtypes, particularly in HER2-negative and TNBC, where conventional hormone therapies remain ineffective.[340]

Wong et al. evaluated the combination of talazoparib, a PARP inhibitor, with calcitriol in BRCA1-wild-type and BRCA1-deficient TNBC cells (BT-20 and MDA-MB-468). This combination therapy markedly increased S and G2/M phase arrest, triggered apoptosis and necrosis, and more effectively inhibited cell migration and viability than monotherapies. Notably, calcitriol potentiated the action of talazoparib by activating VDR-mediated transcription of cell cycle and apoptosis-related genes, while talazoparib impaired DNA repair by inhibiting PARP activity, offering a strategic pairing for BRCA-associated or triple-negative tumors.[341] Similarly, Schneider et al. demonstrated the synergistic efficacy of calcitriol and ruxolitinib, a JAK2 inhibitor, in HER2-enriched and TNBC subtypes. Their combined treatment in MDA-MB-468 and SKBR3 cells significantly inhibited proliferation and induced G0/G1 and G2/M arrest through downregulation of c-Myc, cyclin D1, CDK1/4, and phosphorylated JAK2, while simultaneously upregulating p21, p27, p53, and cleaved PARP. These mechanistic findings were further validated *in vivo* using *xenograft* models, confirming reduced tumor growth and minimal toxicity.[342]

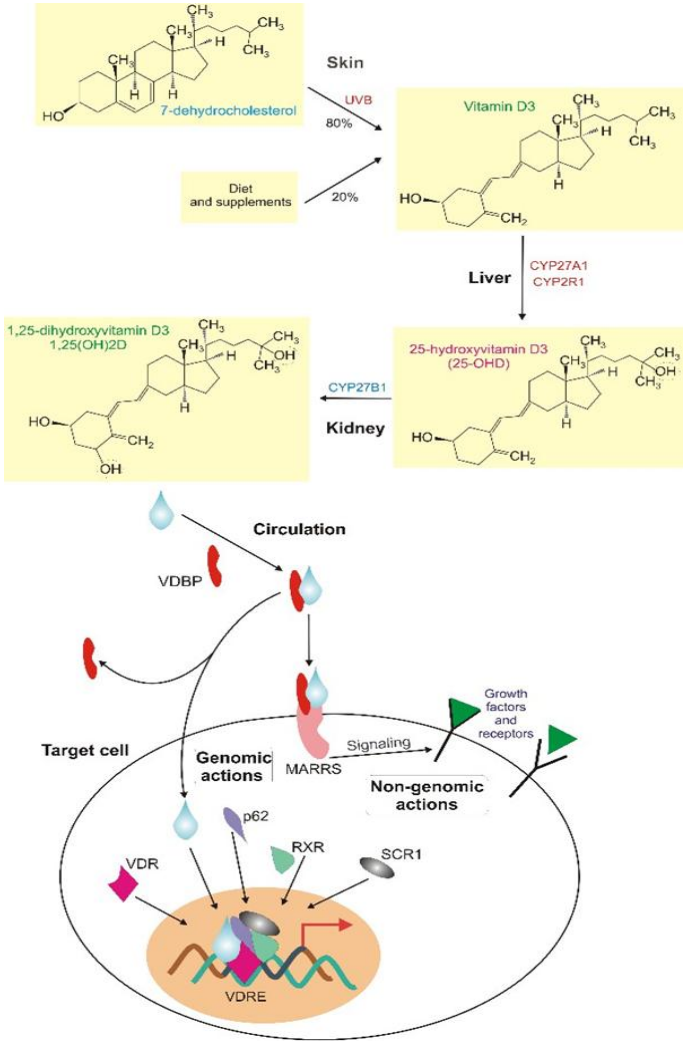


Figure 11. Synthesis, activation, and cellular actions of vitamin D_3 in cancer regulation.

The figure illustrates the biosynthesis and molecular mechanisms by which vitamin D_3 exerts anticancer effects. Vitamin D_3 is synthesized in the skin from 7-dehydrocholesterol upon UVB exposure or obtained through dietary and supplemental sources. Following absorption, it undergoes hepatic hydroxylation by CYP27A1 and CYP2R1 enzymes to form 25-hydroxyvitamin D_3 \[$25(OH)D_3$], the primary circulating form. Renal conversion by CYP27B1 then produces the hormonally active form, 1,25-dihydroxyvitamin D_3 \[$1,25(OH)_2D_3$].

In the context of cancer, $1,25(OH)_2D_3$ binds to the vitamin D receptor (VDR), which forms a heterodimer with retinoid X receptor (RXR) and translocates to the nucleus. This complex binds to vitamin D response elements (VDREs) in DNA, modulating the transcription of genes involved in tumor suppression, including those regulating proliferation, differentiation, apoptosis, angiogenesis, and metastasis. Additionally, $1,25(OH)_2D_3$ can initiate non-genomic actions through membrane-associated rapid response steroid-binding (MARRS) proteins and intracellular signaling intermediates such as p62 and SCR1. These pathways influence cancer cell behavior by interacting with growth factor receptors and downstream signaling cascades. Together, the genomic and non-genomic actions of vitamin D_3 contribute to its anticancer potential, particularly in aggressive subtypes such as TNBC. Source: DOI: 10.3390/ijms21103670

Mechanistic studies have further elaborated the diverse genomic and non-genomic functions of vitamin D in BC, revealing its interference with signaling cascades, metabolic circuits, immune modulation, and epigenetic regulation. Vanhevel et al. offered a detailed systems-level description of calcitriol-mediated tumor suppression, highlighting its capacity to halt the cell cycle via upregulation of CDK inhibitors (p21, p27), repress cyclins and CDKs, and prevent Rb phosphorylation. Apoptosis was induced through BAX and BAK activation, while EMT was reversed via SLUG suppression and E-cadherin restoration. In addition to its effects on tumor cell dynamics, calcitriol impaired CSC maintenance by suppressing CD44 and OCT4 and reprogrammed energy metabolism by downregulating LDH, PC, and SLC1A5. Furthermore, it enhanced tumor immunogenicity by increasing CD8+ T cell infiltration and sensitized cells to oxidative stress.[343] Complementarily, Gkotinakou et al. demonstrated that calcitriol inhibits hypoxia-driven oncogenesis in breast tumors by suppressing HIF-1α and HIF-2α expression through blockade of PI3K/AKT/mTOR and ERK pathways. This repression reduced VEGF and EGFR levels, limiting angiogenesis and proliferation. However, they also noted that hypoxic conditions promote calcitriol inactivation via CYP24A1 overexpression, highlighting the need for dual-targeting approaches to sustain VDR pathway integrity under unfavorable tumor microenvironments.[345]

The regulatory interplay between calcitriol and epigenetic elements has emerged as another promising frontier in BC research. Blasiak et al. examined the vitamin D and VDR axis in relation to long noncoding RNAs (lncRNAs), including MALAT1, LINC00511, MEG3, and HOTAIR, and showed that calcitriol modulates these lncRNAs to suppress proliferation, EMT, and CSC phenotypes. Through both genomic and non-genomic routes, VDR influences epigenetic remodeling, transcription factor availability, and miRNA interactions. These findings suggest that restoring tumor-suppressive lncRNAs while repressing oncogenic ones via VDR activation may offer a precision-medicine strategy in BC therapy.[346] Segovia-Mendoza et al. further strengthened this notion by highlighting that calcitriol sensitizes TNBC cells to paclitaxel and cisplatin, and exhibits synergistic effects when combined with agents like curcumin, genistein, and melatonin. These combinations target multiple pathways, MAPK, β-catenin, and RelB, enhancing radiosensitivity, reversing drug resistance, and eliminating $ALDH1^+$ CSC populations. Such evidence

consolidates the rationale for using calcitriol as a central adjuvant in multidrug regimens.[229] Notably, calcitriol and BXL0124 suppress ductal carcinoma progression *in vivo* and inhibit the expansion of CSCs in mammosphere cultures, largely by inducing cellular differentiation and reducing expression of pluripotency-associated markers in TNBC models. [212, 347]

From a prognostic standpoint, variable VDR expression patterns in breast tumors have shown significant correlations with clinical outcomes, with studies suggesting a focality-specific dimension to VDR function. Zehni et al. found that in unifocal tumors, VDR positivity correlated with low grade, smaller size, and nodal negativity, indicating a tumor-suppressive role. In contrast, multifocal tumors with high VDR expression paradoxically showed higher metastatic staging and worse DSF, suggesting that spatial tumor distribution influences VDR functionality.[348] Martínez-Reza et al. introduced a new layer by showing that calcitriol induces autocrine production of IL-1β and TNF-α in TNBC cells, contributing to its antiproliferative effect. This mechanism was reversed upon blocking IL-1R1 and TNFR1, underscoring the relevance of immune-modulating cytokine signaling downstream of VDR activation.[349] Huss et al. observed that both nuclear and cytoplasmic VDR staining were associated with lower tumor grade, ER/PR positivity, and longer survival, with cytoplasmic localization indicating possible non-genomic effects. These studies collectively point toward VDR as both a prognostic biomarker and therapeutic target, the utility of which may depend on localization, receptor status, and tumor heterogeneity.[17]

Further advancing the molecular understanding, earlier studies revealed crucial resistance mechanisms and metabolic interactions limiting vitamin D efficacy in BC. Fleet et al. (2012) outlined that calcitriol exerts its anti-tumor actions by upregulating apoptosis (Bax, caspase-3), cell cycle arrest (p21, p27), DNA repair (GADD45α), autophagy (Beclin-1), and antioxidant defense (SODs, TXNRD1). However, the overexpression of CYP24A1, silencing of VDR, and downregulation of CYP27B1 were identified as key factors that reduce calcitriol bioavailability and signaling in aggressive tumors.[350] García-Quiroz et al. demonstrated that astemizole, an antihistamine drug, enhances calcitriol efficacy by inhibiting Eag1 potassium channels, repressing CYP24A1, and amplifying VDR-RXR signaling. The combined treatment more effectively reduced Ki-67 and Eag1 expression and increased VDR

accumulation.[351] Narvaez et al. emphasized that while VDR expression is largely retained in TNBC, tumor progression can disrupt its signaling due to ligand depletion and overactive metabolic degradation.[352] LaPorta and Welsh (2014) further showed that $1,25(OH)_2D_3$ regulates transcription of invasion-related and tumor suppressor genes in basal-like TNBC. VDR knockout abolished these effects, whereas restoration of human VDR reinstated responsiveness, confirming receptor dependency. These foundational insights reveal not only the mechanistic versatility of VDR signaling but also the potential for reversing resistance through metabolic or epigenetic intervention.[14]

Earlier studies have laid the groundwork for the current understanding of vitamin D and VDR signaling as a multifaceted regulatory axis in BC. Ditsch et al. provided one of the earliest comprehensive analyses of VDR expression in breast tumors using immunohistochemistry, reporting that 92% of tumors exhibited detectable levels of VDR protein. Importantly, high VDR immunoreactivity scores correlated with smaller tumor size, absence of lymph node metastasis, and improved progression-free and OS. Both nuclear and cytoplasmic VDR staining patterns were observed, suggesting that VDR mediates its tumor suppressive effects via both genomic (nuclear) and non-genomic (cytoplasmic) mechanisms, as indicated in Figure 11. These findings confirmed the prognostic significance of VDR in BC and established a benchmark for evaluating receptor localization in future clinical studies.[353] Around the same time, García-Quiroz et al. investigated the use of calcitriol in combination with astemizole, a known potassium channel blocker and CYP24A1 inhibitor. They demonstrated that this combination enhanced VDR expression and potentiated the antiproliferative effects of calcitriol by blocking the catabolism of $1,25(OH)_2D_3$, effectively increasing its half-life and transcriptional activity. Downregulation of Ki-67 and Eag1 protein levels confirmed reduced proliferation, even at sub-cytotoxic doses, suggesting the feasibility of using calcitriol in combination therapies for hormone receptor-independent BC subtypes.[351]

Adding further mechanistic depth, Banwell et al. uncovered that resistance to $1,25(OH)_2D_3$ in aggressive BC cell lines such as MDA-MB-231 could be attributed not to mutations in the VDR gene, but rather to epigenetic silencing of its downstream signaling. Despite having a wild-type VDR, these cells exhibited high expression of nuclear co-repressors such as SMRT and NCoR1, which recruited histone

deacetylases (HDACs) to VDR-bound chromatin. This led to transcriptional silencing of vitamin D-responsive genes, ultimately attenuating its antiproliferative effects. Remarkably, treatment with the HDAC inhibitor Trichostatin A restored VDR signaling and re-sensitized cells to vitamin D analogs such as Ro26-2198, highlighting a new therapeutic direction based on overcoming transcriptional repression. This early demonstration of VDR-related epigenetic resistance was pivotal in shaping subsequent strategies that combined calcitriol with HDAC inhibitors or CYP24A1 antagonists to enhance its anti-tumor efficacy, particularly in TNBC.[352]

The systemic relevance of vitamin D levels in BC incidence and prognosis was further supported by Wu et al. who showed through epidemiological analysis that higher serum 25(OH)D levels were associated with reduced incidence of BC and improved survival, especially in premenopausal women. This observation complemented mechanistic data by demonstrating the physiological importance of maintaining adequate vitamin D levels not only for bone health but also as a potential preventive strategy against BC development.[353] Friedrich et al. also identified a potent anti-inflammatory and anti-proliferative synergy between calcitriol and the COX2 inhibitor celecoxib in both hormone receptor-positive and TNBC cell lines. Calcitriol suppressed COX2 mRNA and protein expression, thereby reducing the pro-inflammatory prostaglandin E2 (PGE2) levels, and this effect was amplified in combination with celecoxib. In addition, this dual therapy downregulated aromatase activity, implicating a crosstalk between vitamin D and estrogen biosynthesis regulation, especially relevant in hormone-responsive tumors.[355]

Peng et al. introduced a novel application of vitamin D in photodynamic therapy (PDT) for BC by demonstrating that calcitriol pretreatment enhances the efficacy of hematoporphyrin derivative (HPD)-mediated PDT. Calcitriol upregulated the expression of coproporphyrinogen oxidase (CPOX), a key enzyme in the heme biosynthesis pathway, leading to increased accumulation of protoporphyrin IX (PpIX), the active photosensitizer in PDT. Upon light activation, this enhanced PpIX level produced significantly more reactive oxygen species (ROS), leading to increased apoptosis and tumor cell death. These findings revealed that calcitriol can be effectively employed as a sensitizing agent to improve PDT outcomes, especially in TNBC cells where limited therapeutic options exist. This strategy reflects a shift in

how vitamin D could be integrated into multimodal therapies that extend beyond its classical role as a transcriptional regulator.[356]

These foundational studies laid the critical groundwork for contemporary translational research on vitamin D and VDR in BC. They highlighted the receptor role in tumor suppression via regulation of proliferation, apoptosis, differentiation, inflammation, and angiogenesis. The discovery of resistance mechanisms such as CYP24A1 overexpression, histone deacetylation, and hypoxia-mediated calcitriol degradation has prompted the development of new therapeutic strategies combining VDR agonists with metabolic inhibitors, epigenetic modulators, or targeted therapies. Collectively, this body of evidence supports the hypothesis that vitamin D signaling when intact and pharmacologically reinforced can offer a significant advantage in managing aggressive and receptor-negative BC subtypes such as TNBC. This evolving field continues to uncover deeper layers of VDR function and therapeutic application, reinforcing its potential as a cornerstone in future oncologic interventions.

2.23 ROLE OF ERα AND ERβ IN BREAST CANCER

Estrogens regulate key cellular processes including proliferation, differentiation, and survival through receptor-mediated mechanisms that function via both genomic and non-genomic pathways. Initially, estrogenic activity was believed to be mediated solely by a single estrogen receptor, now termed ERα.[357] However, the discovery of a second isoform, ERβ, introduced a paradigm shift, as this receptor exhibits a distinct tissue distribution and regulatory profile compared to ERα.[358] Jefferson et al. reported that ERα and ERβ are widely expressed across mammalian tissues, and their transcriptional activity is influenced by promoter specificity and receptor-binding preferences. These factors may account for the tissue- and isoform-specific effects of estrogens.[359]

ERβ, encoded by the ESR2 gene, belongs to the nuclear transcription factor superfamily. Structurally, the DNA-binding domain of ERβ shares 96% homology with ERα, while the ligand-binding domain shares 60%, indicating both functional overlap and divergence.[360] Although ERβ is primarily detected in normal mammary epithelial cells, it is also present in 20–30% of BCs [Hawse et al., 2020]. Later studies

suggest that the positivity rate of ERβ in breast tumors exceeds 60%, although its biological role remains incompletely defined.[360]

Oueslati et al. demonstrated that ERβ acts as a negative regulator of ERα, opposing its oncogenic effects and correlating with better prognosis and prolonged DSF.[361] Functional assays *in vitro* have confirmed that ERβ inhibits angiogenesis, tumor growth, and metastatic potential through repression of proliferation, migration, and invasion of BC cells.[362]

2.24 ERβ SIGNALING AND THERAPEUTIC RELEVANCE IN BREAST CANCER

Chen et al. observed an association of ERβ expression with aggressive features such as high proliferation rates and distant metastases.[363] However, its co-expression with ERα has also been linked to high-grade tumors and metastatic behavior, indicating a complex interaction between the isoforms.[364] Grober et al. demonstrated that ERβ can inhibit ERα transcriptional activity by competing for estrogen response elements (EREs) or by recruiting distinct co-repressors.[365] This competitive inhibition highlights the importance of the ERα:ERβ ratio in determining cellular outcomes.

Although ERα and ERβ bind EREs similarly, their ability to recruit different transcription factors and cofactors can result in divergent gene expression profiles. This duality leads to both overlapping and isoform-specific transcriptional programs.[366] Notably, ERα functions predominantly as an oncogene by upregulating genes like cyclin D1 and Bcl-2, whereas ERβ functions as a tumor suppressor by promoting apoptosis and maintaining cellular differentiation.[366] Miziak et al. reported a role of estrogen–estrogen receptor in mammary gland development and is a major determinant of BC.[367]

Oueslati et al. noted that resistance to endocrine therapies such as tamoxifen often arises due to crosstalk between ER and EGFR or their downstream effectors.[361] In this context, ERβ-selective agonists have been proposed as potential therapeutic agents, particularly in early-stage ERα-positive ductal carcinomas where they may delay or prevent disease progression. For advanced lobular carcinomas, ERα antagonists may be more beneficial.[368]

Clinical observations support these mechanistic insights. Chang et al. reported a significant reduction in ERβ gene expression in breast tissue samples from 120 patients following chemotherapy in phase II–IIIA stages, suggesting therapy-induced ERβ suppression.[369] In contrast, another study in 78 postmenopausal women with invasive stage II–III disease reported no significant change in ERβ levels before and after endocrine therapy, pointing to variable responses depending on treatment type and tumor.[362]

2.24.1 Prognostic and Predictive Implications of ERβ in Breast Cancer

The precise prognostic value of ERβ remains under investigation. Recent studies have focused on epigenetic mechanisms, such as hypermethylation of the ERβ promoter, and on the predictive relevance of ERβ:ERα ratios in response to therapy.[360, 370] Despite inconsistent findings across cohorts, cumulative evidence supports the role of ERβ as a potential independent prognostic and predictive biomarker, particularly in patients treated with chemotherapy and hormone therapy. ERβ isoform-specific analyses have shown that high expression of ERβ2 and ERβ5 correlates with poor OS, particularly in ERα-negative subtypes and TNBC. Elevated Ki-67 and poor prognostic indicators are frequently associated with these isoforms, suggesting an isoform-dependent divergence in ERβ function.[371] Studies also support the role of ERβ as a predictive and prognostic biomarker in benign and malignant breast tissues.[372]

2.24.2 Structure and Functional Domains of ERβ

The discovery of ERβ in rat prostate and ovary by Kuiper et al. in 1996 expanded the classical understanding of estrogen signaling pathways.[373] ERβ belongs to the nuclear receptor superfamily (type I), functioning as a ligand-activated transcription factor. In its inactive state, ERβ resides in the cytoplasm and translocates to the nucleus upon ligand binding, forming homodimers that interact with palindromic EREs on DNA.[374] The ERβ protein is structurally composed of five domains arranged from the N-terminal to the C-terminal: A/B (NTD), C (DBD), D (hinge), and E/F (LBD), as illustrated in Figure 12. The ESR2 gene, located on chromosome 14q23.2, encodes ERβ and spans approximately 61.2 kb, whereas ERα is encoded by the ESR1 gene on chromosome 6q25.1.[363]

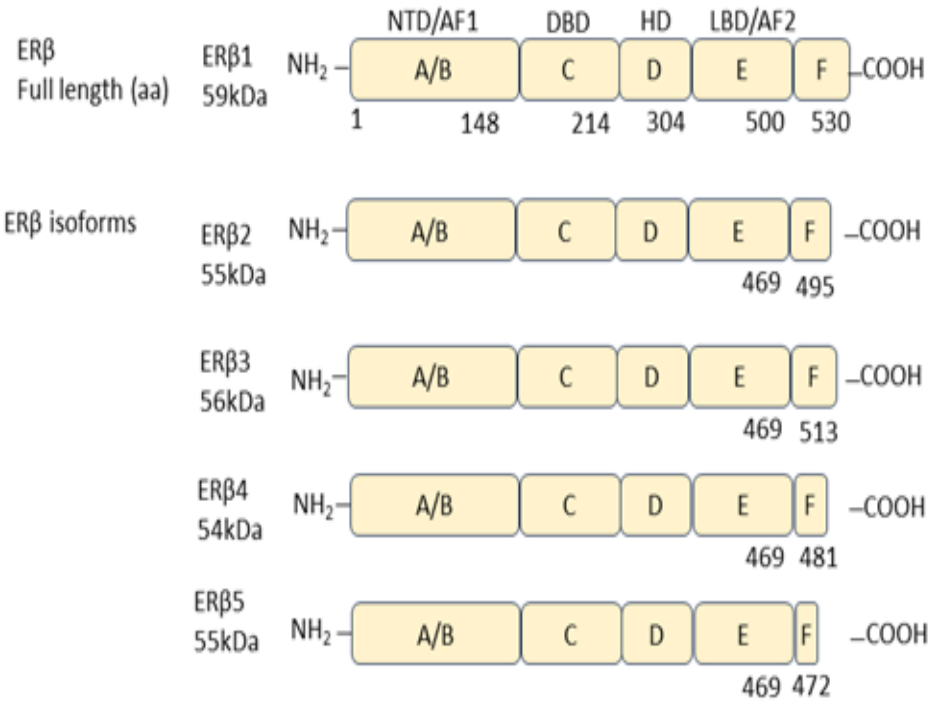


Figure 12. The structure of human ERβ1 and other human ERβ1 isoforms.

Aa, Amino acids; NTD, NH2-terminal domain; AF1, activation function 1; HD, hing domine; DBD, DNA-binding domain; LBD, ligand-binding domine, AF-2, activation function 2; COOH, carboxyl terminal.

Full-length ERα and ERβ consist of 595 and 530 amino acids, corresponding to molecular weights of approximately 66 kDa and 59 kDa, respectively. The A/B domain (NTD) confers receptor specificity and contains activation function 1 (AF1), which mediates ligand-independent transcriptional activity. The highly conserved C domain (DBD) enables specific DNA binding. The hinge region (D domain) contains nuclear localization signals and connects to chaperone proteins like heat shock proteins, stabilizing DNA binding. The E/F domain (LBD) includes activation function 2 (AF2), responsible for ligand-dependent transcriptional regulation.[375] Comparative analysis reveals that ERα and ERβ share 97% homology in their DBDs, 59% in LBDs, and only 16% in NTDs, indicating significant divergence in transcriptional regulation and coregulator interaction.[363]

Swedenborg et al. reported that alternative splicing generates ERβ isoforms (ERβ2–5), which differ in the ligand-binding domain (LBD) sequence, resulting in truncated activation function 2 (AF2) domains and compromised ligand-binding

capacity. Among these, only ERβ1 retains full-length functionality, enabling it to bind estrogens and mediate downstream gene regulation, while the other isoforms exhibit limited or no estrogen responsiveness.[376]

2.24.3 Estrogen Receptor Signaling Mechanisms: Genomic and Non-Genomic Pathways

Estrogen signaling in cells is mediated via two principal mechanisms: genomic and non-genomic. In the genomic pathway, estrogen (primarily 17β-estradiol, E2) binds to intracellular ERα or ERβ, forming receptor dimers that translocate to the nucleus. These dimers interact with EREs and other cis-regulatory elements such as AP-1 and Sp1 on promoters of target genes (Figure 13), regulating transcription.[377] Chen et al. explained that the resulting transcriptional program influences various cellular processes, including proliferation, apoptosis, differentiation, and vascular function. This genomic response is relatively slow, typically occurring over hours due to the need for chromatin remodeling and mRNA synthesis.[363]

In contrast, the non-genomic mechanism involves rapid signal transduction initiated by membrane-localized receptors. E2 can activate G-protein-coupled estrogen receptor 1 (GPER1) or membrane-associated ERα/ERβ, triggering downstream cascades such as MAPK, STATs, and Src-family tyrosine kinases.[376] These rapid responses occur within seconds to minutes and do not require direct transcriptional engagement, although they can influence gene expression indirectly via kinase-mediated phosphorylation of transcription factors.

2.24.4 Functions of ERβ in TNBC

Multiple studies have elucidated the non-genomic actions of estrogen signaling in TNBC, which operate independently of classical ERE. Wang et al. and De Francesco et al. demonstrated that 17β-estradiol suppresses VEGF expression and angiogenesis through activation of GPER-1.[378, 379] Tao et al. identified the E2/GPER/miR-148a/HOTAIR axis as a mediator of metastatic behavior in TNBC, complementing previous findings that estrogen-mediated vascular regulation in ER-negative tumors involves ERK1/2 activation and HIF1α induction. This signaling cascade provides a mechanistic basis for estrogen-driven effects in the absence of classical estrogen receptors.[380] Fuentes and Silveyra reviewed the interplay between genomic (ERE-

dependent) and non-genomic (kinase-activated) pathways, highlighting the involvement of co-regulators such as PELP1, SRC-1, and CBP/p300 in modulating estrogen receptor function.[357] These studies collectively underscore the significance of cytoplasmic signaling events and long non-coding RNAs in shaping ERβ's functions within TNBC cells.

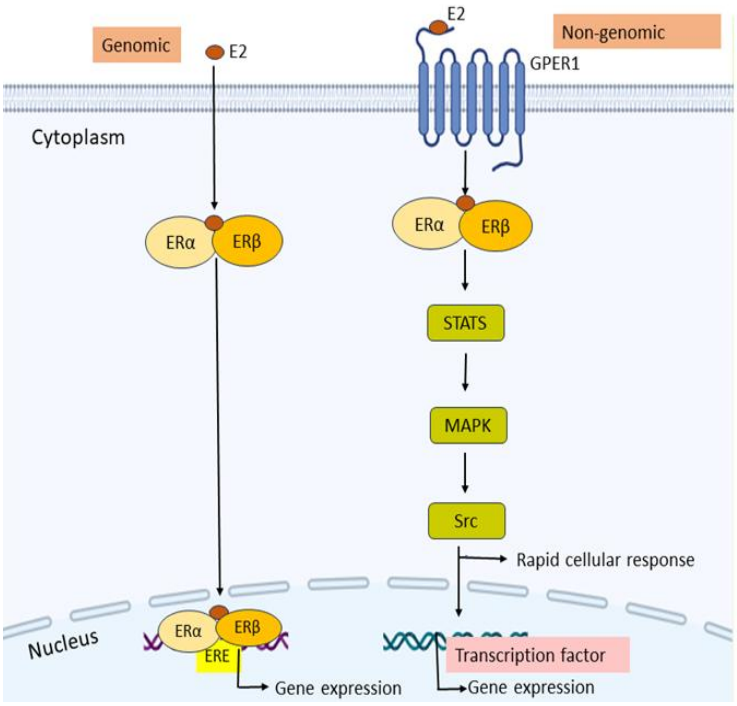


Figure 13: Mechanisms of estrogen action.

E2 participates in the genomic route by attaching to intracellular ERα and ERβ receptors to produce a complex that reaches the nucleus and binds to DNA to control gene transcription. In the non-genomic route, E2 binds to a GPER1 and initiates fast signal transduction that involves STATS, and MAPK and Src. The non-genomic response happens in seconds to minutes, while the genomic process takes hours.

Abbreviations: GPER1, G protein-coupled estrogen receptor 1; ERα, estrogen receptor alpha; ERβ, Estrogen receptor beta; STATS, signal transducers and activators of transcription; MAPK, mitogen-activated protein kinase; Src, proto-oncogene tyrosine-protein kinase.

Further insights into ERβ1 transcriptional regulation and prognostic implications in TNBC were provided by Shanle et al., who used inducible ERβ expression models in MDA-MB-468 cells to show suppression of Wnt/β-catenin signaling alongside upregulation of CDKN1A and CDH1. Despite ERβ1 being expressed in a subset of TNBC tumors and its correlation with Ki-67 in one cohort, its prognostic utility remained inconsistent, likely due to inter-study differences in

detection techniques and tumor heterogeneity.[24] Expanding the functional landscape, Song et al. reported that mitochondrial-localized ERβ (mitoERβ) regulates energy metabolism through interaction with mtDNA, enhancing both ATP and ROS generation.[381] Reese et al. further validated the therapeutic relevance of ERβ1 by demonstrating its capacity to sensitize ERα positive BC cells to anti-estrogen therapies and to suppress proliferation in TNBC models.[382] In addition, Bado et al. revealed that ERβ1 counteracts mutp53-driven oncogenic signaling and upregulates epithelial markers such as E-cadherin and SHARP1, thereby mitigating metastatic progression.[383]

Clinical investigations have offered translational relevance to these preclinical findings. In a phase II trial, Wisinski et al. administered high-dose oral estradiol to metastatic TNBC patients and reported modest efficacy restricted to ERβ-positive cases, supporting the selective utility of estrogen therapy in this subgroup.[384] Rajah et al. demonstrated that genistein and 17β-estradiol synergistically reduced cell proliferation and induced apoptosis in MDA-MB-231 cells by modulating Bax/Bcl-2 ratios.[385] In parallel, Yang et al. reported that heteronemin triggered apoptosis in TNBC by inhibiting ERK1/2 and STAT3 pathways, revealing another potential avenue for therapeutic modulation.[3] Zhao et al., through Mendelian randomization, provided epidemiological evidence that genetically elevated estradiol levels are associated with reduced systemic inflammation, suggesting estrogen broader immunomodulatory potential beyond its roles in tumor.[386]

Foundational research established the early framework for understanding ERβ role in BC subtypes. Mann et al. first identified ERβ expression in approximately 47% of ERα-negative BCs, with a positive correlation to improved clinical outcomes among patients receiving adjuvant hormone therapy.[387] These findings positioned ERβ as an independent prognostic factor and challenged the prevailing ERα-centric diagnostic paradigm. Harvey et al. contributed methodological advancements by demonstrating the superiority of IHC over ligand-binding assays in assessing ER status and predicting tamoxifen responsiveness.[388] Ross et al. added a genetic dimension by reporting that ESR1 mutations can drive ligand-independent estrogen signaling, even when ER protein remains IHC - positive, potentially explaining some therapy-resistant phenotypes.[389] Thomsen et al. advocated for combining ESR1

mRNA in situ hybridization with IHC to improve diagnostic accuracy, particularly in tumors with borderline or heterogeneous ER expression.[390] Earlier, Mur et al. had demonstrated that prolonged estradiol exposure suppressed proliferation in ER-negative cells, likely via non-genomic pathways, offering one of the first insights into alternative estrogen receptor signaling mechanisms.[391] Collectively, these foundational and contemporary studies provide a comprehensive framework for advancing ERβ-targeted strategies in TNBC management.

2.24.5 ERβ in TNBC: Suppressor Role and Clinical Correlation

ERβ plays a tumor-suppressive role in TNBC, a BC subtype characterized by the absence of ERα, PR, and HER2. Approximately 20–30% of TNBC tumors express ERβ, and this expression is linked to improved prognosis and survival outcomes.[30, 392] Aspros et al. demonstrated that ERβ forms a transcriptional co-repressor complex with enhancer of zeste homolog 2 (EZH2), which suppresses NFκB/p65 signaling, a known driver of oncogenic inflammation and tumor progression in TNBC. This complex formation is dependent on the presence of p65, suggesting that ERβ functions dually as a transcriptional repressor and a modulator of oncogenic signaling.[30] The suppressive activity of ERβ has been visualized in molecular pathway studies (Figure 14), which illustrate its ability to reprogram EZH2 and block tumor-promoting transcription. Mukhopadhyay et al. reported that the biological effects of ERβ in TNBC are influenced by p53 mutation status, which alters its functional consequences. In TNBC cells harboring wild-type p53, ERβ overexpression promotes cell proliferation, whereas ERβ knockdown induces apoptosis. Conversely, in p53-mutant TNBC cells, ERβ acts as a tumor suppressor by promoting apoptosis and inhibiting proliferation, suggesting that p53 status is a key determinant of ERβ functionality.[393]

2.24.6 Influence of p53 Mutations on ERβ-Mediated Therapeutic Responses

Mut-p53 alters the transcriptional landscape of TNBC and significantly affects ERβ-mediated signaling. When p53 is mutated, ERβ expression correlates with anti-proliferative effects, while in wild-type p53 backgrounds, ERβ may paradoxically promote proliferation.[394] Scarpetti et al. documented a clinical case of a TNBC patient with brain metastasis who responded to tamoxifen therapy. The patient exhibited

elevated ERβ expression and a reduced metastatic burden following treatment withdrawal, highlighting a potential anti-proliferative synergy between mut-p53 and ERβ.[395]

Further, ERβ upregulation has been associated with enhanced doxorubicin sensitivity in ERβ-positive TNBC cell lines (MDA-MB-231 and BT-549) through inhibition of the PI3K/AKT/mTOR pathway. Combination therapy using doxorubicin and liquiritigenin (a selective ERβ agonist) has shown synergistic anti-proliferative effects *in vitro*, reinforcing the therapeutic promise of ERβ modulation in chemoresistant TNBC.[396]

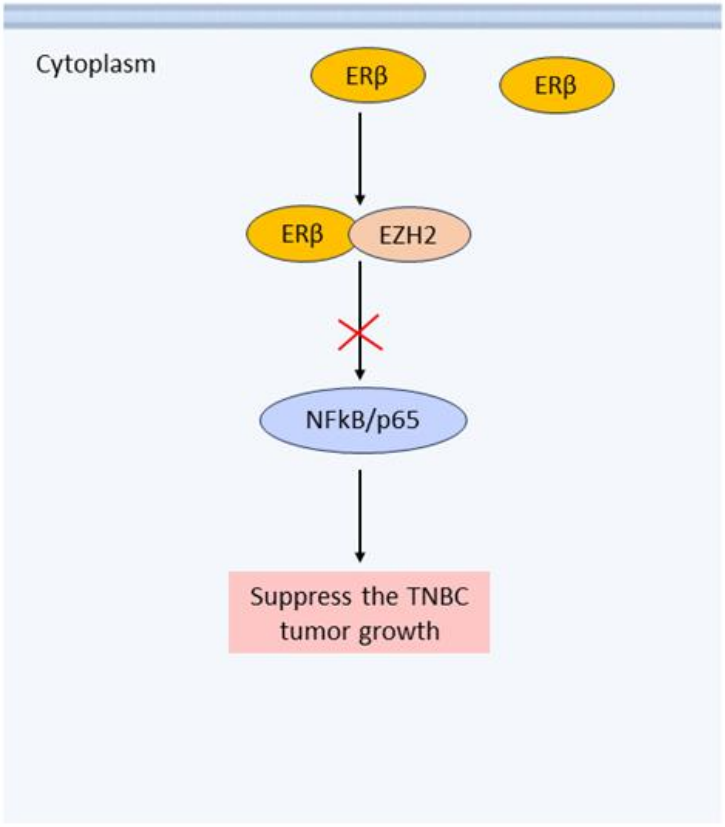


Figure 14. ERβ-mediated tumor suppression via EZH2 and NF-κB pathway blockade in TNBC. ERβ acts as a tumor suppressor in TNBC by forming a complex with EZH2, blocking the NFκB/p65 oncogenic signaling pathway.

Abbreviations: ERβ, Estrogen receptor beta; EZH2, enhancer of zeste homolog 2; NF-κB, nuclear factor kappa B.

2.24.7 Potential Benefits of Targeting ERβ in TNBC: Mechanistic and Translational Perspectives

Basal expression of ERβ is generally low in TNBC cell lines; however, its inducible upregulation has demonstrated promising translational relevance. Activation of ERβ via specific ligands reduces survivin expression, induces tumor suppressors, and inhibits cell proliferation, migration, and invasion through both ligand-independent and ligand-dependent mechanisms.[3] These findings support the feasibility of ERβ-targeted therapies to counteract metastatic progression in TNBC. A clinical study found reduced ERβ levels in breast tumor tissue compared to adjacent normal tissue, with mitoERβ levels inversely correlating with recurrence risk. In animal models, enforced mitoERβ expression hindered both TNBC cell growth and tumor volume.[381] Furthermore, data from African-American TNBC patients revealed high ERβ expression in tumor tissue, with IGF2 shown to transcriptionally activate ERβ expression in TNBC cell lines. The IGF2 ERβ axis may thus represent a novel targetable mechanism for managing aggressive TNBC phenotypes.[397]

At the molecular level, ERβ2/cx and ERβ4 - 5 isoforms promote tumor aggressiveness by enhancing hypoxia signaling, correlating with early relapse in TNBC patients. While ERβ1, ERβ2/cx, and ERβ5 share sequence identity except at the C-terminus, truncation in ERβ2 and ERβ5 compromises ligand-binding capacity, affecting dimerization and signaling. Despite their truncated domains, these isoforms may still heterodimerize with ERα, thereby modifying estrogen responsiveness in TNBC.[398]

2.25 ERβ1 EXPRESSION IN TNBC: PROGNOSTIC ROLE AND CLINICAL UTILITY

ERβ1, the full-length isoform with an intact ligand-binding domain, has been studied extensively for its potential tumor-suppressive functions in TNBC. Zambo et al. proposed that ERβ1 expression correlates with reduced tumor aggressiveness, but its prognostic value remains subject to cohort-specific variability.[392] Yan et al. reported that IHC analysis of familial BC subtypes (BRCA1, BRCA2, and BRCAX) revealed that BRCA1-mutated tumors were significantly more likely to be ERα-negative and ERβ1 - positive. Notably, nuclear ERβ1 expression predicted a favorable response to endocrine therapy at a 15 years follow-up.[399]

Nagandla and Thomas and Honma et al. reported that approximately 18 - 27% of TNBC tumors express ERβ1, and several studies support its independent prognostic value, particularly in postmenopausal women. In this subgroup, ERβ1 positivity was associated with significantly higher survival rates, challenging the traditional view of TNBC as a hormone-insensitive disease.[400] Shanle et al. evaluated two TNBC cohorts using the VECTRA™ automated IHC platform and found that cytoplasmic and nuclear ERβ1 expression levels were correlated. However, a significant association with Ki-67 was observed in only one of the cohorts.[401] Takano et al. similarly reported that ERβ1 expression, whether assessed using the Allred score or percentage positivity, was not predictive of clinical outcomes in TNBC.[402]

Furthermore, the functional role of ERβ1 is isoform-dependent, with ERβ1 exerting tumor-suppressive effects, while ERβ2 and ERβ5 have been implicated in promoting tumorigenesis. Yan et al. demonstrated that these isoforms modulate distinct signaling axes, reinforcing the importance of isoform-specific evaluation in prognostic stratification.[25]

2.26 MOLECULAR MECHANISMS AND THERAPEUTIC IMPLICATIONS OF ERβ1 IN TNBC

The tumor-suppressive functions of ERβ1 in TNBC involve its interaction with several oncogenic signaling pathways. Assunta Sellitto et al. demonstrated that ERβ1 interacts with the PTEN/PI3K/pAKT axis, where the ERβ1(+)/pAKT(−) status correlates with the most favorable prognosis in TNBC patients.[21] Loss or downregulation of ERβ1 activates the transforming growth factor-beta receptor (TGFβR) pathway, leading to the upregulation of matrix metalloproteinase-13 (MMP-13) and CXCL8, which are critical mediators of tumor invasion and metastasis. This activation is antagonized by cystatin secretion, which inhibits TGFβR signaling a response enhanced by ERβ1 activation, presented in Figure 15.[25]

Gene expression profiling revealed that ERβ1 activation via chloroindazole (CLI), an ERβ-selective agonist, results in downregulation of pro-invasive genes and suppression of tumor migration and invasion, highlighting its therapeutic potential.[26] Furthermore, ERβ1 directly inhibits the PI3K/AKT/mTOR signaling pathway, sensitizing TNBC cells to doxorubicin, a key chemotherapeutic agent.[396]

Samanta et al. demonstrated that ERβ1 modulates EGFR-dependent signaling and indirectly suppresses IGF2 mRNA-binding protein 3 (IMP3), a known pro-metastatic factor in TNBC.[403] In addition, Salahuddin et al. reported that ERβ1 activation downregulates VEGF expression, thereby impairing neovascularization within the tumor microenvironment. This inhibition of angiogenesis represents another crucial mechanism through which ERβ1 exerts its tumor-suppressive effects in TNBC.[404]

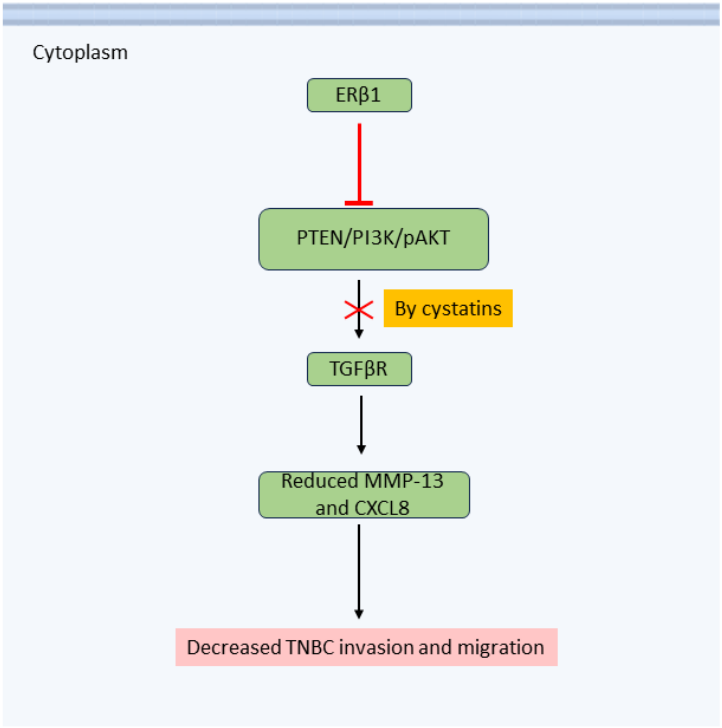


Figure 15. ERβ1 inhibits PTEN/PI3K/pAKT and TGFβR signaling. ERβ1 mediates inhibition of the PTEN/PI3K/pAKT pathway and blocks the TGFβR, leading to subsequent cystatin production, reduction in MMP-13 and CXCL8 production, and decreased TNBC invasion and migration.
Abbreviations: ERβ1, Estrogen receptor beta 1; PTEN, phosphatase and tensin homolog; PI3K, phosphatidylinositol-3 kinase; AKT, protein kinase B; TGFβR, transforming growth factor beta receptor; MMP-13, matrix metalloproteinase 13; CXCL8, C-X-C motif chemokine ligand 8.

Clinically, ERβ1 expression has been positively associated with both OS and DSF in patients treated with tamoxifen, suggesting that ERβ1 may serve as a predictive biomarker and therapeutic target, especially in TNBC subtypes that were traditionally considered hormone-unresponsive.[405]

2.26.1 Tumor-Suppressive Effects of ERβ1 and 17β-Estradiol in TNBC Cell Lines

In vitro studies have established that ERβ1 expression suppresses tumor growth and metastatic potential in TNBC cell lines such as MDA-MB-231, MDA-MB-468, and Hs578T. ERβ1 inhibits cell proliferation, induces G1 cell cycle arrest, suppresses colony formation, and significantly reduces xenograft tumor volume in animal models, as represented in Figure 16.[401, 406]

Reese et al. observed that approximately 30% of TNBC cases express ERβ, and treatment with 17β-estradiol (E2) significantly reduces proliferation in ERβ-positive TNBC cell lines. E2 further enhances these anti-proliferative effects through ERβ1 activation.[27] These findings affirm that E2-ERβ signaling exerts a tumor-suppressive effect, contrasting with its traditionally perceived oncogenic role in ERα-positive BC.

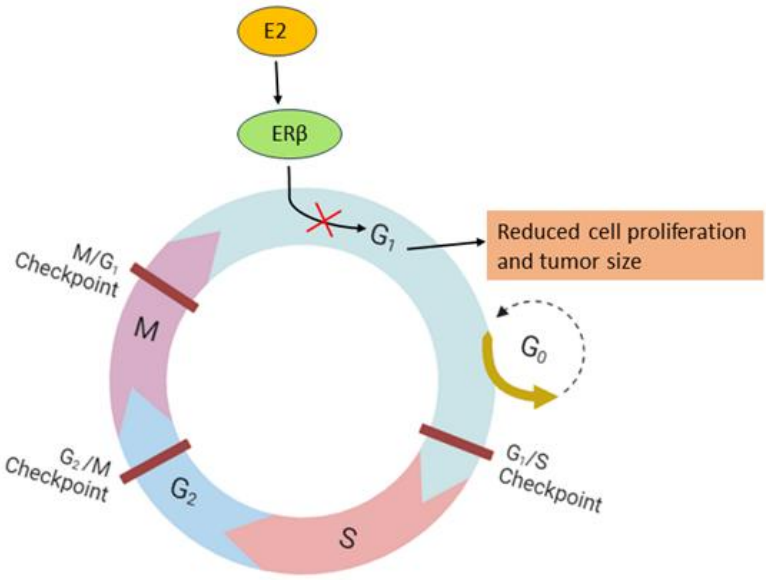


Figure 16. E2 activation of ERβ blocks G1 phase. E2 stimulates the ERβ and blocks the G1 phase, inhibiting colony formation and reducing tumor growth**.**

Abbravations: E2, Estradiol; ERβ, Estrogen receptor beta; G1; Gap 1 phase; G0, resting phase; S, DNA synthesis; G2, Gap phase 2; M, mitosis.

Treeck et al. reported that the functional effects of 17β-estradiol (E2) in TNBC are context-dependent. E2 can exert anti-tumoral activity through activation of ERβ and GPER-1, while also interacting with estrogen-related receptors (ERRs), which modulate estrogen signaling in both ligand-dependent and ligand-independent

manners.[401] The dual capacity of ERβ to influence gene transcription through direct ligand binding or through coactivator-independent pathways underscores its complex role in hormonal regulation.[407,408]

Importantly, E2-mediated ERβ1 activation has been linked to reduced proliferation in TNBC lines, as demonstrated by Reese et al., who showed that both E2 and ERβ-selective agonists inhibit TNBC cell growth via ERβ1-dependent mechanisms.[21] Cittelly et al. suggested that these findings provide a rationale for combining E2-depletion strategies, such as aromatase inhibitors, with immune checkpoint blockade (e.g., PD-1 inhibitors) and radiotherapy, particularly in metastatic or recurrent TNBC.[409]

Collectively, these data position ERβ1 as a central regulator of tumor suppression in TNBC, acting through multiple interconnected pathways, including inhibition of PI3K/AKT, EGFR, TGFβ, and angiogenic signaling. Its ability to mediate E2 - driven anti-tumor responses in ERα negative contexts broadens the therapeutic landscape for TNBC and supports its utility as a dual prognostic and therapeutic target.

2.26.2 Clinical Translation and its limitation

Ongoing clinical trials are exploring the utility of ERβ activation in TNBC management. A Phase II trial titled "Therapeutic Targeting of ER β in TNBC" (ClinicalTrials.gov Identifier: NCT03941730) aims to evaluate the clinical efficacy of E2-based interventions in ERβ-positive TNBC cases.

2.26.3 Limitations

Despite the mechanistic support for E2-induced ERβ activation, a Phase II clinical study involving an unselected but largely ERβ-positive metastatic TNBC cohort failed to show a significant survival advantage with E2 therapy.[410] Wisinski et al. demonstrated in xenograft models expressing inducible ERβ that administration of E2 led to G1 cell cycle arrest and tumor regression. E2–ERβ interactions were associated with reduced tumor formation and growth, thereby validating ERβ as a potential therapeutic target for tumor suppression *in vivo*. These results underscore the importance of patient selection, isoform-specific expression, and functional receptor status in determining therapeutic outcomes.[384]

The reviewed literature underscores the distinct, context-specific roles of both VDR and ERβ1 signaling in BC, especially in triple-negative subtypes. Calcitriol and VDR signaling demonstrates broad anti-tumor mechanisms through genomic, non-genomic, metabolic, and immunologic pathways. Simultaneously, ERβ1 emerges as a tumor suppressor with isoform-specific and immune-modulatory functions in TNBC. However, gaps remain in translating these mechanistic insights into clinical applications, particularly in understanding the combinatorial effects of VDR and ERβ1 modulation. These findings provide the rationale for the current investigation into the cooperative effects of calcitriol and 17β-estradiol in ERβ1-positive TNBC models.

CHAPTER-3
AIM and OBJECTIVES

AIM

- To demonstrate the role of vitamin D_3 and 17β-estradiol in TNBC progression and its therapeutic implications.

OBJECTIVES

- To demonstrate the association between the expression of vitamin D receptor and ERβ1 in triple negative breast cancer.
- To analyze and compare the *in silico* binding affinities of vitamin D_3 and 17β-estradiol with key target proteins (ERβ, EGFR, VEGF and Caspase 3) in triple-negative breast cancer.
- To elucidate the molecular mechanism of vitamin D_3 and 17β-estradiol agonist treatment in triple negative breast cancer progression using *in vitro* model.

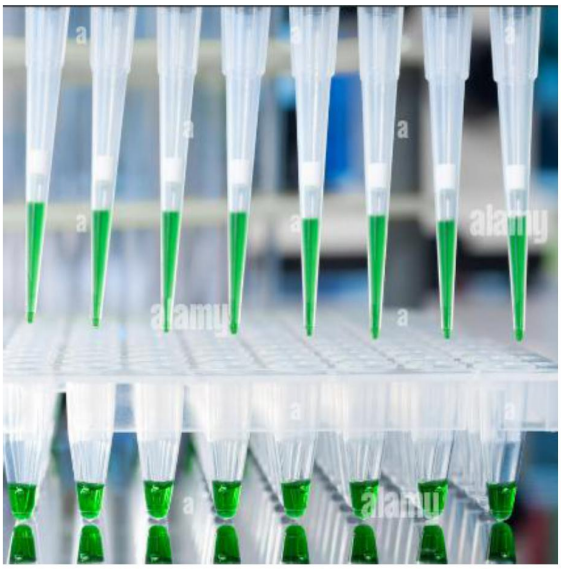
NEED FOR THE STUDY & YPOTHESIS

Need for the study

TNBC is an aggressive subtype with limited targeted treatment options and poor prognosis. Vitamin D_3, through activation of the VDR, and 17β-estradiol, through ERβ1, have shown tumor-suppressive effects. However, the combined role of VDR and ERβ1 activation in TNBC progression has not been adequately investigated. This study is needed to explore whether vitamin D_3 with 17β-estradiol can modulate key oncogenic pathways and offer a novel therapeutic strategy for TNBC management.

Hypothesis

We hypothesize that calcitriol, through activation of the VDR, and 17β-estradiol, via ERβ1, individually and in combination, exert regulatory effects on TNBC progression by modulating key oncogenic pathways including proliferation (EGFR), angiogenesis (VEGF), and apoptosis (caspase-3). Their combined receptor-targeted modulation may offer a promising therapeutic approach for ERβ1-positive TNBC.



CHAPTER-4
MATERIALS & METHODS

4. MATERIALS and REAGENTS

This study was designed to explore the therapeutic potential of calcitriol and 17β-estradiol in ERβ1-positive TNBC through a combination of experimental and computational approaches. This study was conducted from 2020 to 2025 using a structured, multi-phase design.

It was carried out in three sequential phases:

i. Immunohistochemical profiling of tumor tissues.
ii. Molecular docking studies to evaluate receptor–ligand interactions.
iii. *In vitro* assays using TNBC cell lines to assess functional responses to treatment.

4.1 STUDY DESIGN

Type of study: Cross-sectional and experimental.

Study Duration: 2020-2025

STUDY POPULATION

The study focused specifically on invasive ductal carcinoma (IDC), given its high prevalence among BC subtypes and its well-documented clinical relevance. Human breast cancer tissue samples were used in this study, and all procedures were conducted in accordance with institutional ethical guidelines. Ethical clearance was obtained from the Institutional Ethical Committee of BLDE (Deemed to be University), Vijayapura, Karnataka, India (Approval No. BLDE(DU)/IEC/631-C/2022-2023, dated 28/05/2022). FFPE tissue blocks were retrieved from the archives of the Department of Pathology, Shri B. M. Patil Medical College, Hospital and Research Centre, Vijayapura, for the period between August 2020 and May 2023. A total of 30 histologically confirmed cases of IDC in female patients aged 30 to 75 years (mean age: 56 years) were included in the study.

BC molecular subtyping was performed using IHC evaluation of hormone receptor status, ER, PR and HER2 expression. Based on IHC profiles, tumors were classified into the following molecular subtypes:[410]

- Luminal A: ER+, PR+, HER2−
- Luminal B: ER+, PR+/−, HER2+
- HER2-enriched: ER−, PR−, HER2+
- TNBC: ER−, PR−, HER2−

The distribution of cases was as follows: TNBC (n = 15), Luminal A (n = 8), Luminal B (n = 4), and HER2-enriched (n = 3). Clinicopathological data, including patient age, receptor status, histological subtype, and tumor grade (I and II), were obtained from the immunohistochemistry registry.

4.2 General Consideration of IHC

IHC is a technique that utilizes the specific binding between antigens and antibodies to detect protein expression within tissue sections. The core principle of IHC is based on antigen–antibody interactions, where a primary antibody binds selectively to its target antigen. This is followed by the application of a secondary antibody conjugated with an enzyme, typically horseradish peroxidase (HRP), which facilitates signal detection. Visualization is achieved through the enzymatic conversion of a chromogenic substrate, such as 3,3'-diaminobenzidine (DAB), producing a colored precipitate at the site of antigen localization. This enables morphological assessment and semi-quantitative evaluation of protein expression under light microscopy. IHC plays a vital role in cancer diagnosis, classification, and biomarker research due to its high specificity and tissue context preservation.

Requirements

i. Formalin-fixed, paraffin-embedded tissue blocks
ii. Diagnostic Biosystems kit
iii. Pressure cooker
iv. VDR (anti-VDR, sc-13133, Santa Cruz Biotechnology, CA, USA)
v. ERβ1 (ERβ1-sc-390243, Santa Cruz Biotechnology, CA, USA)
vi. Microscopic slides
vii. Coplin staining jars
viii. Mayer’s hematoxylin
ix. Coverslip
x. Distrene Plasticiser Xylene **(**DPX)
xi. Microscope
xii. AR pro software
xiii. Image J software

4.2.1 Optimized IHC Protocol [411]

The tissue sections were cut into 3–4 µm thickness.

↓

Deparaffinized using multiple changes of xylene (a few minutes each).

↓

Treated with isopropyl alcohol, then rinsed in water.

↓

Performed antigen retrieval in EDTA buffer at 120°C for 15 minutes.

↓

Washed with Tris-buffered saline (TBS, pH 7.4) for a few minutes.

↓

Incubated with tissue primer for 5 minutes; follow with TBS wash.

↓

Applied background blocker (5 minutes), then incubate with primary antibodies, VDR and ERβ1 (each 1 hour).

↓

Washed in TBS, apply enhancer reagent (15–20 minutes).

↓

After TBS wash, incubated with Poly HRP secondary antibodies (30 minutes).

↓

Performed another TBS wash, then developed with DAB chromogen (3–5 minutes).

↓

Rinsed with distilled water; counterstained with Mayer's hematoxylin.

↓

Rinsed again, allowed slides to air-dry, and mounted with DPX

Figure 17: Stepwise representation of the optimized IHC protocol.

4.2.2 Patterns of VDR Expression in TNBC Subtypes

VDR expression was assessed across different subcellular compartments of tumor cells within TNBC subtypes. Immunohistochemical evaluation revealed the presence of VDR staining in both the cytoplasm and nucleus of tumor cells (Figure 18). The cytoplasmic expression of VDR was semi-quantitatively assessed based on staining intensity, which was graded into four distinct categories: negative (0), weak (1+), moderate (2+), and strong (3+). A sample was considered VDR-positive in the cytoplasm if at least 20% of the tumor cells demonstrated staining at the highest intensity grade.[339] This threshold was established to ensure consistency in identifying cases with biologically meaningful expression.

Nuclear expression of VDR was also assessed. A tumor was considered positive for nuclear VDR if 10% or more of the tumor cell nuclei showed visible immunoreactivity. This dual localization both cytoplasmic and nuclear suggests possible functional diversity in VDR-mediated signaling pathways within TNBC subtypes.[339]

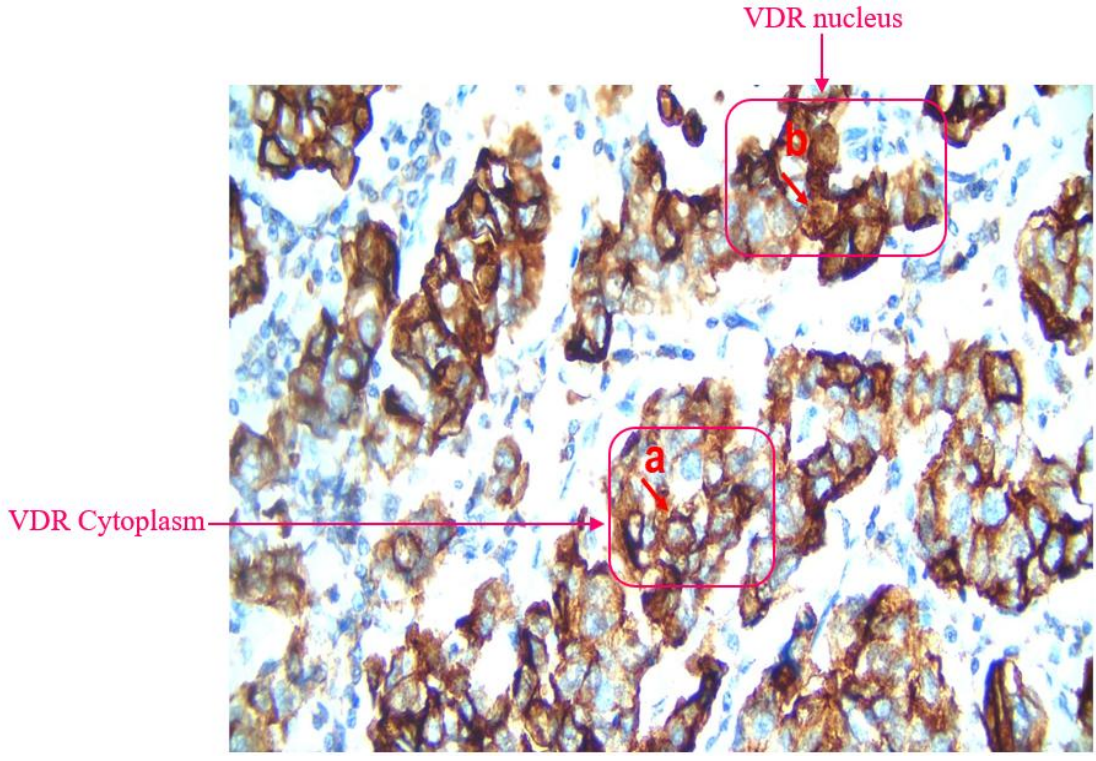


Figure 18. Immunohistochemical staining of VDR expression in ERβ1-positive TNBC tumour cells (40X). The image shows distinct subcellular localization of VDR: (a) cytoplasmic staining, (b) nuclear staining.

4.3 ERβ1 RECEPTOR EXPRESSION

In addition to VDR, the expression of ERβ1 was evaluated in both TNBC and luminal A tumor tissues using a scoring system that accounted for both the extent and intensity of cytoplasmic staining.[382] ERβ1 was considered positive when more than 10% of tumor cells exhibited cytoplasmic immunoreactivity.[412] This criterion was chosen based on previous studies that demonstrated its relevance for categorizing functional ERβ1 expression in breast tumors. The extent of ERβ1 staining was scored as follows: score 0 for <1% of positive cells, score 1 for 1%–25%, score 2 for 26%–50%, score 3 for 51%–75%, and score 4 for 76%–100% positive cells. The intensity of staining was recorded using the four-tier system: negative (0), weak (1+), moderate (2+), and strong (3+).

To provide a comprehensive assessment, a combined score was calculated by summing the extent and intensity scores. Based on this cumulative score, ERβ1 expression was categorized into three levels: low or negative (scores 0–2), moderate (scores 3–5), and high (scores 6–7).[382] This classification allowed a nuanced differentiation between tumors with negligible expression and those with potentially functional ERβ1 signaling, as illustrated in Figure 19**.**

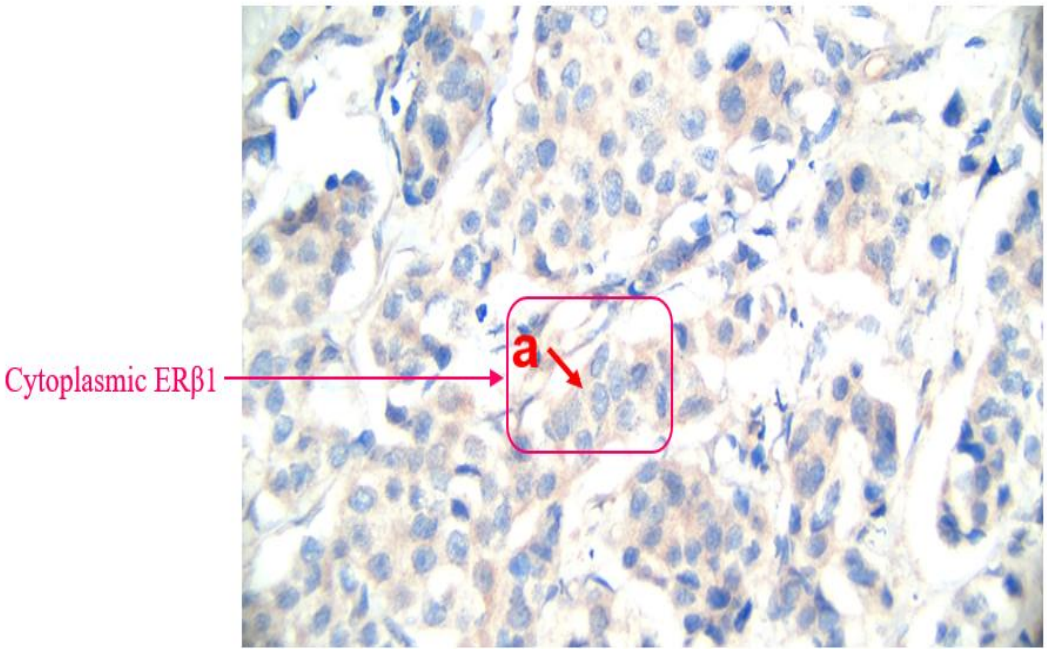


Figure 19. Immunohistochemical staining of ERβ1 receptor showing cytoplasmic localization in ERβ1-positive TNBC tumor cells (40X). (a) ERβ1 receptor expression in cytoplasm.

4.4 PROTEIN and LIGAND PREPARATION

4. 4.1 General consideration

Accurate preparation of protein and ligand structures is essential for reliable molecular docking and interaction studies. The predictive success of computational modeling is largely dependent on the quality of the input structures, particularly the completeness and chemical accuracy of both receptors and ligands.

For protein targets, only high-resolution crystal structures (≤2.5 Å) are considered to ensure fidelity in binding site definition and atomic positioning. These structures, typically obtained from the Protein Data Bank (PDB), often require preprocessing due to missing atoms, unresolved residues, or the presence of non-essential crystallographic components such as water molecules and bound ligands. Standard preparation steps involve hydrogen addition, structural correction, and geometry optimization to ensure readiness for docking.

Ligand preparation similarly focuses on ensuring the molecules reflect their biologically active conformations. This involves obtaining 3D structures from trusted chemical databases like PubChem and optimizing them through appropriate force fields to achieve accurate bond angles, stereochemistry, and energy-minimized geometry. Proper preparation directly influences the docking pose accuracy and binding affinity estimations.

4.4.2 Protocol

High-resolution 3D crystal structures of four key protein targets relevant to TNBC were selected based on their functional roles in proliferation, angiogenesis, and apoptosis. The following PDB IDs were used:

- Estrogen Receptor Beta (ERβ) – PDB ID: 1QKM
- Epidermal Growth Factor Receptor (EGFR) – PDB ID: 1A28
- Vascular Endothelial Growth Factor (VEGF) – PDB ID: 2BCW
- Caspase-3 – PDB ID: 3MZW

Protein refinement was conducted using Cresset Flare v9.0, which facilitates structure optimization and receptor–ligand interaction modeling.

The refinement process included:

- Addition of polar hydrogen atoms.
- Correction of missing residues or atoms based on structural validation reports.

- Removal of non-essential water molecules.
- Energy minimization and geometry optimization to ensure docking compatibility.

For ligand preparation, the bioactive compounds investigated were:

- Calcitriol (PubChem CID: 5280453).
- 17β-Estradiol (PubChem CID: 5757).

These ligands were downloaded from the PubChem database in SDF format. The structures were converted into PDBQT format for compatibility with docking tools. Following preparation, both proteins and ligands were validated and aligned with the predicted active site residues using Flare's binding site analysis tools. These optimized and pre-processed structures were used for downstream molecular docking studies, ensuring a high level of structural accuracy and consistency.

4.5 CELL LINE and REAGENTS

Requirements

- T25 flask
- Serological pipettes
- Hand gloves
- 70% alcohol
- Inverted microscope.
- MDA-MB-468 cell line
- Leibovitz's L-15 medium
- Fetal bovine serum (FBS)
- Antibiotic-antimycotic (ABAM) solution
- Trypsin-ethylenediaminetetraacetic acid (EDTA)
- 5% CO_2 incubator

The MDA-MB-468 TNBC cell line was obtained from the National Centre for Cell Science (NCCS), Pune, India. Cells were cultured in Leibovitz's L-15 medium supplemented with 10% FBS and 1% antibiotic-antimycotic solution. All cell culture media and reagents were procured from HiMedia Laboratories Pvt. Ltd., Mumbai, India. Cultures were maintained at 37°C in a humidified incubator under atmospheric conditions, as L-15 medium does not require CO_2 supplementation. Subculturing was

performed using trypsin-EDTA, and cells were regularly observed for morphology and confluence under an inverted microscope.

The compounds used for treatment were calcitriol and 17β-estradiol. Calcitriol (Cat. No. S1466) was obtained from Selleckchem (Houston, TX, USA) and was dissolved in DMSO to prepare stock solutions. 17β-estradiol (Cas No. 50-28-2), purchased from Sigma-Aldrich (St. Louis, MO, USA), was dissolved in ethanol. Working concentrations were freshly prepared by diluting the stock solutions in culture medium immediately prior to each experiment.

All cell handling procedures were performed under aseptic conditions in a biosafety cabinet. Reagents were freshly prepared, and all experiments were conducted in triplicate to ensure data accuracy and reproducibility.

4.5.1 Cell Culture

4.5.2 Revival of MDA-MB-468 Cell Line

Removed required reagents (e.g., complete medium, Leibovitz's L-15 + 10% FBS + 1% ABAM solution, from the refrigerator and kept them in a 37°C water bath for 15-20 minutes.

↓

Wiped the exterior of all reagent bottles with 70% ethanol before placing them into the biosafety cabinet (BSC)

↓

Retrieved the cryovial containing MDA-MB-468 cells from liquid nitrogen (LN_2) storage.

↓

Thawed the cryovial quickly in a 37°C water bath for 1–2 minutes until just liquified.

↓

Clean the surface with 70% ethanol and transfer the cryovial to the biosafety cabinet (BSC)

↓

Transferred the vial contents into a 15 mL sterile centrifuge tube.

↓

3 mL of complete medium was added to it

↓

Centrifuged at 800–1000 rpm for 4 minutes to pellet the cells.

↓

Carefully discarded the supernatant without disturbing the cell pellet.

↓

Gently resuspended the pellet by tapping the tube.

↓

4 mL of complete medium was added, and mixed gently.

↓

Transferred the cell suspension into a T25 cell culture flask.

↓

Examined the cells under an inverted microscope for morphology

↓

Incubated the flask at 37°C in a humidified CO_2 incubator under standard conditions.

Figure 20: Revival procedure of the MDA-MB-468 cell line

4.5.2 Post-revival monitoring

Changed the complete medium every 3 days.

↓

Observed daily for contamination and color change in the medium (indicative of pH shift).

↓

Once MDA-MB-468 cells reached approximately 80% confluence, proceeded with the subculturing procedure.

Figure 21: Post-revival monitoring procedure of the MDA-MB-468 cell line.

4.5.4 Subculturing of MDA-MB-468 cells

(Performed when cells reach 80% confluence)

Complete medium and trypsin-ethylenediaminetetraacetic acid solution kept in a 37°C water bath for 25-30 minutes.

↓

Transferred the reagents into the BSC

↓

Observed the cells under an inverted microscope to confirm 80% confluence.

↓

Carefully aspirated and discarded the spent medium from the T25 flask.

↓

Gently rinsed the cell monolayer with 1–2 mL of sterile PBS to remove residual serum that may inhibit trypsin activity.

↓

Discarded the PBS and add 0.5–1 mL of trypsin-EDTA solution to the flask.

↓

Tilt the flask to ensure even coverage of the cell surface.

↓

Incubated at 37°C for 3–5 minutes, monitoring periodically under the microscope until cells begin to round and detach for T25 flask.

↓

Once detachment is complete, 3–4 mL of complete medium was added to neutralize the trypsin.

↓

Gently pipette up and down to form a single-cell suspension.

↓

Transfered the suspension into a 15 mL centrifuged tube and centrifuge at 800–1000 rpm for 4 minutes.

↓

Discarded the supernatant and gently resuspended the cell pellet in fresh complete medium.

↓

Seeded the required volume of the cell suspension into a new culture T25 flask or 96 well plate (e.g., split 1:2 or 1:3 depending on experimental needs).

↓

Incubated the flask at 37°C in a humidified CO_2 incubator.

↓

Labeled the flask with passage number, date, and cell line name for record-keeping.

Figure 22. Depicting the subculturing procedure of the MDA-MB-468 cell line.

4.6. MTT ASSAY - Measurement of Viable Cells

4.6.1 General Consideration

The MTT assay is a colorimetric method used to evaluate cell viability by measuring the metabolic activity of viable cells. It relies on the enzymatic reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into insoluble formazan crystals by nicotinamide adenine dinucleotide phosphate [NAD(P)H] dependent oxidoreductase enzymes within the mitochondria. The intensity of the purple color formed is proportional to the number of metabolically active cells.

This assay is widely used for screening the cytotoxic effects of test compounds due to its simplicity, cost-effectiveness, and reproducibility. However, the results may be influenced by factors such as seeding density, incubation time, and uniform solubilization of formazan. Consistent handling of reagents and standardization of assay conditions are essential to generate reliable and interpretable data. Optical density (OD) is typically measured at 570 nm using a microplate reader, and the percentage of viable cells is calculated relative to untreated controls.[413]

Requirements

- Leibovitz's L-15 medium
- MDA-MB-468 cell line
- Hand gloves
- 70% alcohol
- Calcitriol
- 17β-Estradiol
- MTT
- 96-well flat-bottom plates
- 5% CO_2 incubator
- DMSO
- Cytation microplate reader

Aim

To evaluate the cytotoxic effects of calcitriol, 17β-estradiol, and their combination on MDA-MB-468 cells using the MTT assay.

4.6.2 Treatment Groups

- Calcitriol: 1, 2, 3, 4, and 5 µM.
- 17β-Estradiol: '100, 200, 300, 400, and 500 nM.
- Combination: 5 µM calcitriol + 500 nM 17β-estradiol.
- Control: untreated cells.

Treatment Duration

- 8, 16, 24, and 32 hours.

4.6.3 Protocol

MDA-MB-468 cells were seeded in sterile 96-well flat-bottom plates

↓

Cell density: 2×10^4 cells per 96 well

↓

Incubated overnight at 37°C for adherence

↓

After treatment (calcitriol 1-5µM and 17-beta -estradiol 100-500nM), 10% MTT solution (v/v) was added to each well

↓

Plates were incubated for 4 hours at 37°C in a 5% CO_2 humidified incubator

↓

Medium was discarded carefully to retain the formed formazan

↓

100 µL of DMSO was added to each well to dissolve the formazan crystals

↓

Plates were gently shaken to ensure complete dissolution

↓

Absorbance was measured at 570 nm using a Cytation microplate reader (BioTek Instruments Inc., USA)

↓

Cell viability (%) was calculated using the formula: [413]

↓

$$\frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}} \text{X } 100$$

Experiments were performed in triplicate for statistical reliability

Figure 23: MTT assay procedure for the MDA-MB-468 cell line.

4.7. IMMUNOBLOT STUDY

Requirements

- Leibovitz's L-15 plain medium
- Calcitriol
- 17β-estradiol
- Phosphate buffer saline (PBS)
- MDA-MB-468 cells
- 60mm cell culture dishes

4.7.1 Cell Growth

Cell growth was continued from the previous experiment and maintained through regular subculturing.

4.7.2 Treatment Studies/Protocol

MDA-MB-468 cells were seeded at a density of 1×10^6 cells per 60 mm culture plate and allowed to adhere overnight. Once the cells reached approximately 80% confluence, they were treated with calcitriol (5 μM), 17β-estradiol (500 nM), or their combination. Untreated cells served as the control group.

4.7.3 Treatment groups

- MDA-MB-468 cells were treated with calcitriol and observed at multiple time points to study the temporal effects of treatment.

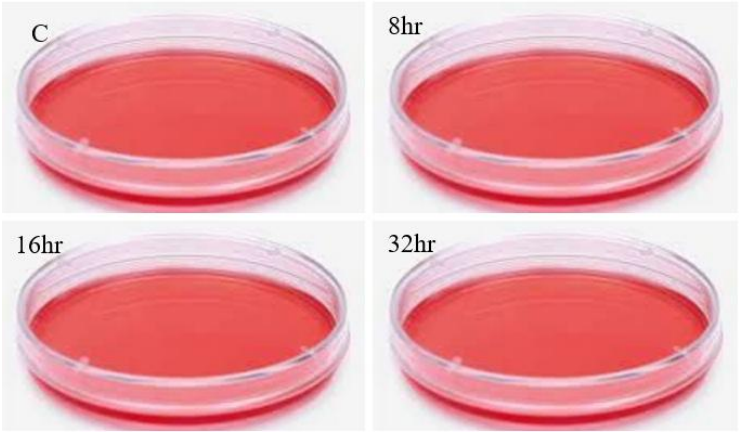


Figure 24. Calcitriol (5 μM) treatment in MDA-MB-468 cells observed at different time intervals (Control, 8 hr, 16 hr, and 32 hr). Cells were treated after reaching 80% confluence to assess time-dependent effects. C; control, hr; hour.

- MDA-MB-468 cells were treated with 17β-estradiol and observed at multiple time points to study the temporal effects of treatment.

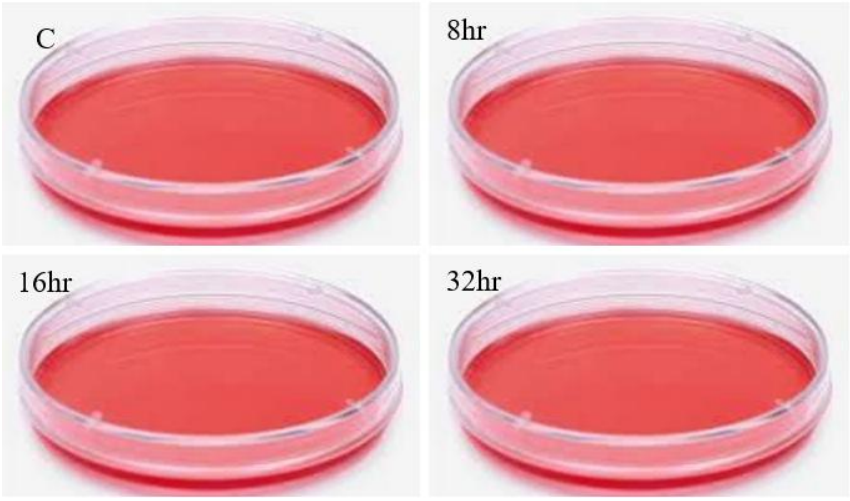


Figure 25. 17β-estradiol (500 nM) treatment in MDA-MB-468 cells observed at different time intervals (Control, 8 hr, 16 hr, and 32 hr). Treatment was initiated after cells reached 80% confluence to evaluate time-dependent cellular responses**.** C; Control, hr; hour.

- MDA-MB-468 cells were treated with a combination of calcitriol and 17β-estradiol and monitored at different time points to assess the time-dependent effects of combined treatment.

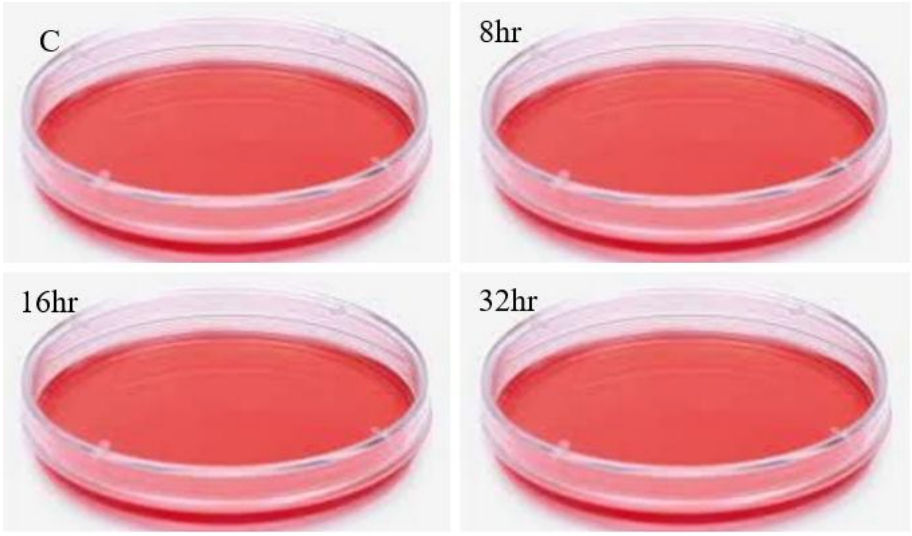


Figure 26. Combination treatment of calcitriol (5 µM) and 17β-estradiol (500 nM) in MDA-MB-468 cells observed at various time intervals (Control, 8 hr, 16 hr, and 32 hr). Cells were treated after reaching 80% confluence to evaluate the effects of dual exposure over

4.8. PREPARATION OF CELL LYSATE

Requirements

- Sterile cell scraper
- Cell lysis buffer (with protease and phosphatase inhibitor cocktail)
- 1.5 mL microcentrifuge tubes
- Sonicator
- −80°C freezer

After treatment, cell lysates were collected at designated time intervals for downstream protein analysis.

4.8.1 Following Steps

Scraped the cells gently from a 60 mm cell culture dish using a sterile cell scraper in the presence of lysis buffer supplemented with protease and phosphatase inhibitor cocktail.

↓

Collected the lysate and store at −80°C until further use.

↓

Thawed the lysate and sonicated on ice for 10 seconds, repeated twice, with cooling between cycles.

↓

Centrifuged the lysate at 12,000–14,000 rpm for 20–30 minutes at 4°C to removed cellular debris.

↓

Carefully transfered the clear supernatant to a fresh 1.5 mL microcentrifuge tube.

↓

The resulting lysate was either stored at −80°C or immediately used for protein quantification using the Bicinchoninic Acid (BCA) assay.

Figure 27: Preparation of cell lysate from treated MDA-MB-468 cells for

4.9 PROTEIN ESTIMATION BY BCA METHOD

4.9.1 General Consideration

The BCA assay is a colorimetric method used for the quantitative estimation of total protein concentration in biological samples. The assay is based on the biuret reaction, in which peptide bonds reduce Cu^{2+} to Cu^{+} under alkaline conditions. The reduced copper ions then form a purple-colored complex with bicinchoninic acid, which absorbs strongly at 562 nm. The absorbance is directly proportional to the protein concentration.

The BCA assay offers several advantages;

- High sensitivity
- Broad linear range
- Compatibility with detergent-containing buffers
- Minimal interference compared to Bradford or Lowry assays

Both standards and unknown samples were incubated with the BCA reagent, and absorbance is measured at 562 nm after incubation at 37°C for 30 minutes.

Reagent Preparation

- Bovine serum albumin - Standard.

Reagent A

- Bicinchoninic Acid – 1 g
- Anhydrous sodium carbonate – 1.72 g
- Sodium potassium tartrate – 160 mg
- Sodium hydroxide – 400 mg
- Sodium bicarbonate – 950 mg

  → Dissolved in 80 mL double-distilled water (DDH_2O), pH was adjusted to 11.25.

  → Made up to 100 mL and stored at 4°C.

  → Adjusted the pH using NaOH (to increase) or sodium bicarbonate (to decrease).

Reagent B

- $CuSO_4$ – 400 mg dissolved in 100 mL DDH_2O.
  → Stored in an amber bottle at room temperature.

Working reagent

• Reagent A and B were mixed in a 50:1 ratio immediately before use.

4.9.2 PROTOCOL

Table 3. Composition of BCA assay reaction mixtures for BSA standards and unknown MDA-MB-468 cell lysate samples used in protein quantification.

Components	Blank	S1	S2	S3	S4	S5	Unknown
BSA (1µg/µL)	-	2µl	4µl	6µl	8µl	10µl	-
DDH_2O	10µl	8µl	6µl	4µl	2µl	-	-
Cell lysate	-	-	-	-	-	-	5µl
BCA [50:1]	200µl	200µl	200µl	200µl	200µl	200µl	200µl

- Incubated at 37°C for 30 minutes.
- Absorbance was measured at 562 nm using a spectrophotometer or microplate reader.

Protein Concentration Calculation

- Protein content per microliter (µg/µL) was calculated using the known absorbance values corresponding to standard BSA concentrations using the formula;

$$\frac{\text{Test - Blank}}{\text{Standard - Blank}} \text{ X } \frac{\text{Conc.}}{\text{Vol.}}$$

Where

Test: Absorbance value of test
Standard: Absorbance value of sample
Conc.: Concentration of standard taken
Vol.: Volume of sample
Blank: Absorbance of blank

4.10 ELECTROPHORESIS [Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE)]

4.10.1 General Consideration

SDS-PAGE operates on a discontinuous buffer system to separate proteins based on molecular weight.

The gel matrix is composed of two distinct layers

i. Stacking gel – A microporous gel of low acrylamide concentration with pH 6.8, designed to concentrate proteins into a sharp band.
ii. Separating (resolving) gel – A microporous gel of higher acrylamide concentration with pH 8.8, used for the effective separation of proteins during electrophoresis.

The running buffer in the electrophoresis tank typically has a pH of 8.3, creating a discontinuous system in combination with the stacking and separating gels. Upon initiation of the electric field, chloride ions (from the gel), glycine ions (from the running buffer), and bromophenol blue migrate towards the anode, forming an ionic front. As glycine in the running buffer (pH 8.3) enters the stacking gel (pH 6.8), its dissociation decreases due to the lower pH being close to glycine's isoelectric point, resulting in a drop in charge and reduced mobility. Meanwhile, protein samples, which are less affected by pH change than glycine, retain greater mobility. Thus, in the stacking gel, the migration order becomes:

Cl^- < proteins < bromophenol blue < glycine.

The large pore size of the stacking gel offers minimal resistance, allowing proteins to concentrate into sharp bands between the leading ion (Cl^-) and the trailing ion (glycine), forming a localized high potential gradient. This gradient rapidly compresses and aligns proteins into narrow bands, enhancing resolution during separation. As the ions and proteins migrate into the resolving gel (pH 8.8), glycine fully dissociates, gains charge, and resumes normal migration. The tighter pore size of the resolving gel causes differential migration based on protein molecular size, as the sieving effect becomes dominant. Proteins coated with SDS (imparting a uniform negative charge) were separated by size, with smaller proteins migrating faster than larger ones. This system enables high-resolution separation and quantitative analysis of protein samples prior to transfer or staining.

4.10.2 SDS-PAGE PROTOCOL

4.10.3 General Consideration

SDS-PAGE is a widely used technique to separate proteins primarily based on their molecular weight. It is typically suitable for resolving proteins ≤ 10 kDa, as smaller proteins bind less efficiently to SDS and are harder to resolve. The system operates on a discontinuous buffer system involving a stacking and separating gel with different pH and acrylamide concentrations.

4.10.4 Gel Preparation

- System used: Bio-Rad Mini-PROTEAN® vertical gel electrophoresis system.
- All glass plates, spacers, and combs should be clean and completely dry.
- Assemble the gel cassette according to the manufacturer's instructions.

Reagents and Buffers

4.10.5 30% Acrylamide Stock Solution

- Acrylamide 29.2 g
- Bisacrylamide 0.8 g
- Dissolved in 100 mL of distilled water (DH_2O)

4.10.6 Tris-HCl Buffer (0.5 M, pH 6.8) – For Stacking Gel

- Tris base 60.5 g in 800 mL DH_2O
- Adjusted 'pH to 6.8 using concentrated HCl
- Made up to 1liter (L)with DH_2O

4.10.7 Tris-HCl Buffer (1.5 M, pH 8.8) – For Separating Gel

- Tris base 182 g in 800 mL DH_2O
- Adjusted pH to 8.8 with concentrated HCl
- Made up to 1L with DH_2O

Table 4. Resolving/separating gel composition (1.5M, Tris-HCl, pH 8.8)

Components	**8%**	**10%**	**15%**
DH2O	4.7mL	4.1mL	2.4mL
Acrylamide -30%	2.7%	3.3%	5.0mL
Tris-HCl- pH 8.8	2.5mL	2.5mL	2.5mL
Ammonium persulfate (APS) – 10%	0.1mL	0.1mL	0.1mL
Tetramethylethylenediamine (TEMED)	10µl	10µl	10µl

- After polymerization, the overlaying liquid was poured off, and the surface was cleaned with tissue paper.

Table 5. Stacking gel composition (0.5M, Tris-HCl, pH 6.8)

Components	**Quantity**
DH2O	3.45mL
Acrylamide - 30%	0.83 mL
Tris-HCl - pH 6.8	0.63 mL
APS - 10%	50µL
TEMED	15µL

- Poured the stacking gel directly over the polymerized separating gel and immediately inserted the comb carefully to avoid air bubbles.
- Allow polymerization and removed the comb before sample loading.

Precaution

After adding TEMED, mixed quickly and poured the gel solution immediately to avoid premature polymerization.

Note

- Covered the separating gel with 0.1% SDS or overlay with isopropanol or water (for gels ≤8% acrylamide) or isopropanol (for gels ≥10%) during polymerization.
- Gels were stored at 4°C for up to 1–2 weeks, wrapped in plastic and kept moist with a wet tissue.

4.10.8 Sample Preparation

- Protein concentration in each sample was determined.
- Transfer the calculated volume of each sample to a 200 µL tube or PCR tube.
- 5 µL of loading dye (Laemmli buffer) was added to it.
- Heated the samples at 90°C for 3–5 minutes.
- Allowed to cool and kept at room temperature until ready to load.

4.10.9 Electrophoresis Tank Buffer/Running Buffer (1X)

Preparation of 5X Stock Buffer

(Stored at 4°C)

- Tris base – 7.5 g
- Glycine – 36 g
- SDS – 2.5 g
- Dissolved in 500 mL of DH_2O
- Mixed well until fully dissolved

Preparation of 1X Working Buffer

(Prepared fresh and stored at room temperature in a brown-colored bottle)

- 5X Stock Buffer – 200 mL
- DH_2O – 800 mL
- Mixed thoroughly to obtain 1 L of 1X running buffer

4.10.10 Steps to Run Electrophoresis

Remove the polymerized gel cassette from the casting stand and inserted it into the electrode assembly.

↓

Secured the assembly inside the electrophoresis tank with the short glass plate facing inward.

↓

If necessary, sealed edges with sealing gel to prevent leakage.

↓

Filled the inner chamber (between the plates) and the outer tank with 1X running buffer.

↓

Carefully loaded the prepared protein samples into the wells.

↓

Connected the tank to the power supply.

↓

Run the gel at 65 V until the samples migrate from the stacking to the separating gel.

↓

Increased the voltage to 90 V and continued the electrophoresis until the tracking dye reaches the bottom of the gel.

Figure 28: Steps involved in running SDS-PAGE electrophoresis for protein separation

4.11 IMMUNOBLOT REAGENTS PREPARATION

4.11.1 General Consideration

In immunoblotting (Western blotting), buffer systems are critical for efficient protein transfer, membrane stabilization, and antibody interactions. Each chemical used in transfer and wash buffers serves a specific purpose:

- **Tris base**: Acts as a buffering agent, maintaining stable pH during electrophoretic transfer and washing.
- **Glycine**: Serves as an ionic carrier in the transfer buffer, allowing current flow during protein migration from the gel to the membrane.
- **Methanol**: Facilitates protein binding to nitrocellulose membrane by removing SDS and stabilizing protein structures; also reduces gel swelling.
- **NaCl**: Maintains ionic strength and isotonicity in TBS, supporting proper antibody-antigen interactions.
- **Tween 20**: A non-ionic detergent added to tris buffer saline T-20 (TBST) to minimize non-specific antibody binding by blocking hydrophobic interactions.

All reagents were prepared using high-purity chemicals and DH_2O. Solutions were stored appropriately to ensure consistency and reliability in experimental outcomes.

4.11.2 TRANSFER BUFFER

5X Transfer Buffer (Store at 4°C)

- Tris base – 15 g *(Buffering agent, pH stabilizer)*
- Glycine – 72.5 g *(Provides ions for current flow during transfer)*
- Methanol – 200 mL *(Facilitates protein binding to nitrocellulose membrane, removes SDS)*
- DH_2O – Added to make up to 1L

1X Transfer Buffer (Working solution; store at room temperature)

- 5X Transfer Buffer – 200 mL
- Methanol – 200 mL
- DH_2O – Added to make up to 1L
  → Mixed thoroughly before use

4.11.3 WASH BUFFERS

1M Tris-HCl Buffer (pH 8.0) (Store at room temperature)

- Tris base – 121 g *(Maintains pH during wash steps)*
- DH_2O – 800 mL
- Adjust pH to 8.0 using concentrated HCl *(pH adjustment)*
- Made up final volume to 1L with DH_2O

5X TBS Buffer (Tris-buffered saline), pH 8.0 (Store at room temperature)

- 1 M Tris-HCl Buffer – 400 mL *(Buffering agent)*
- NaCl – 45 g '*(Provides ionic strength and osmotic balance for optimal antibody binding)*
- DH_2O – Add to make up to 1L
  → Stirred until completely dissolved

1X TBST Buffer (TBS with Tween 20) (Working solution; store at room temperature)

- 5X TBS Buffer – 200 mL
- Tween 20 – 1 mL *(Reduces non-specific binding by blocking hydrophobic sites)*
- DH_2O – Add to make up to 1L
  → Mixed gently before use

4.11.4 IMMUNOBLOTTING PROTOCOL

Following SDS-PAGE, immunoblotting was performed to detect the expression levels of ERβ1, EGFR, VEGF, and caspase-3 in MDA-MB-468 cell lysates using a nitrocellulose membrane.

Requirements

- Nitrocellulose membrane (0.45 µm pore size)
- Primary antibodies: ERβ1, EGFR, VEGF, caspase-3, and GAPDH (internal control) – all monoclonal antibodies obtained from Santa Cruz Biotechnology, CA, USA.
- HRP-conjugated anti-mouse IgG secondary antibody (Santa Cruz Biotechnology)
- Blocking buffer: 2% BSA in TBS
- TBST (TBS + 0.1% Tween 20) wash buffer
- ECL substrate (Bio-Rad, UK)
- Gel documentation system: G:BOX Chemi-XR5 (Syngene, UK)
- Image analysis software: ImageJ

Protein Transfer

- After SDS-PAGE, the nitrocellulose membrane was soaked in distilled water and equilibrated in transfer buffer.
- A standard wet transfer sandwich was assembled as follows:

Sponge → filter paper → gel → nitrocellulose → filter paper → sponge

- It was performed at 300 V for 90 minutes at 4°C.
- Membrane staining with Ponceau S confirmed protein transfer.

Blocking

- The membrane was blocked in 2% BSA in TBS for 45 minutes at room temperature with gentle agitation to prevent non-specific binding.

Primary Antibody Incubation

- The blocked membrane was incubated overnight at 4°C in 1% BSA in TBS, with all primary antibodies diluted 1:1000:
  - ERβ1 (sc-390243)
  - EGFR (sc-53274)
  - VEGF (sc-7269)
  - Caspase-3 (sc-56046)
  - GAPDH (sc-137179) – Housekeeping gene

Washing (post-primary)

- The membrane was washed 10–12 times for 5 minutes each in TBST to remove unbound primary antibodies.

Secondary Antibody Incubation

- The membrane was incubated with HRP-conjugated anti-mouse IgG, diluted 1:3000 in TBS, for 2 hours at room temperature with gentle rocking.

Washing (post-secondary)

- Post-incubation, the membrane was washed again 10–12 times for 5 minutes each in TBST to remove any excess secondary antibody.

Detection and Imaging

- The membrane was treated with ECL substrate for 1–2 minutes.
- Protein bands were visualized using the G:BOX Chemi-XR5 imaging system, as illustrated in Figure 29.
- GAPDH was used as a housekeeping control to normalize protein expression.

Quantification

- Band intensities were quantified using ImageJ software.
- Protein expression was normalized to GAPDH, and background subtraction was performed to ensure accurate comparisons across treatment groups.

All experimental procedures were conducted following standardized protocols to ensure the accuracy, reproducibility, and reliability of the data. The combination of immunohistochemical analysis, molecular docking, and *in vitro* assays provided a comprehensive framework for evaluating the receptor-mediated effects of calcitriol and 17β-estradiol in ERβ1-positive TNBC models. The results derived from these methods are presented and discussed in the following chapter.

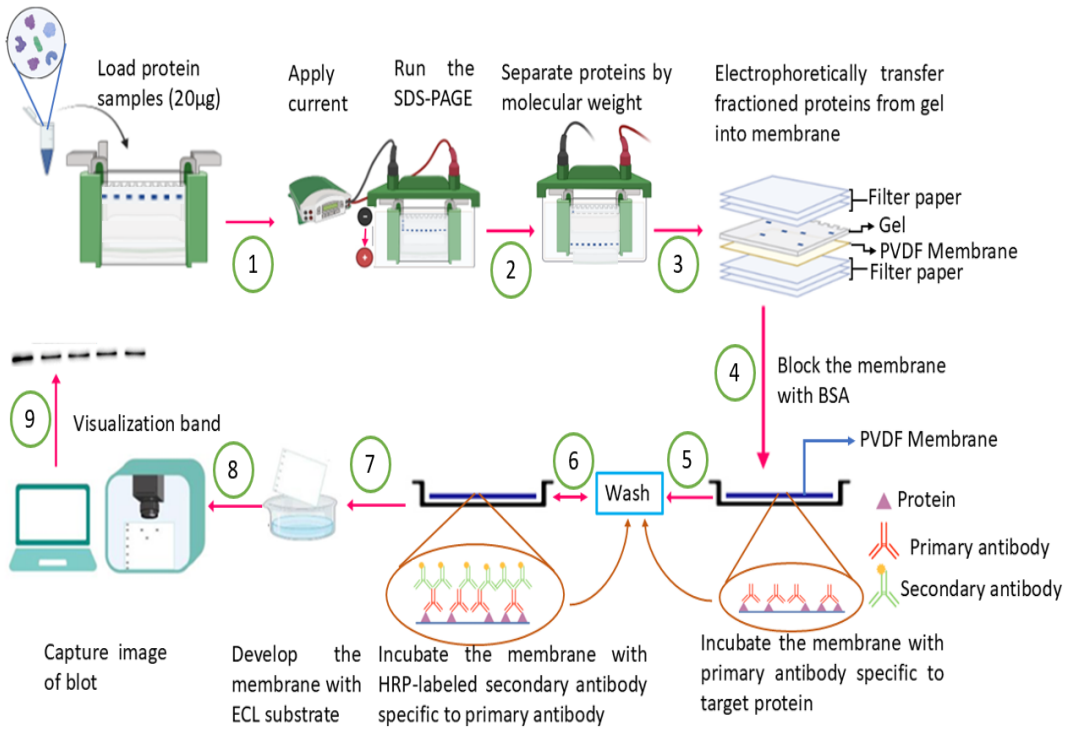


Figure 29: Summary of SDS-PAGE and Immunoblot

STATISTICAL ANALYSIS

STATISTICAL ANALYSIS

For immunohistochemical data, categorical variables such as receptor expression patterns were presented as frequencies and percentages. The Chi-square (χ^2) test was applied to assess associations between VDR and ERβ1 expression and BC molecular subtypes, including luminal A, luminal B, HER2-enriched, and TNBC categories. A *p*-value of <0.05 was considered statistically significant. All analyses were performed using IBM SPSS Statistics for Windows, Version 28.0 (IBM Corp., Armonk, NY, 2021), ensuring rigorous statistical evaluation.

For *in vitro* experiments, including cell viability assays, results were obtained from a minimum of three independent replicates (n = 3) and expressed as mean ± standard error of the mean (SEM)**.** Statistical significance was determined using one-way analysis of variance (ANOVA)**,** followed by Tukey's post-hoc test for multiple group comparisons. A *p*-value ≤0.05 was considered statistically significant. Data visualization and graph generation were carried out using GraphPad Prism version 8 to facilitate accurate and effective presentation of experimental outcomes.

CHAPTER-5
RESULTS

5.1 Patterns of VDR Expression in Breast Cancer Subtypes

The current study aimed to evaluate the differential expression and subcellular localization of VDR across major breast cancer subtypes, with a particular focus on TNBC. Immunohistochemical analysis was performed on FFPE BC tissue blocks, categorized into luminal A, luminal B, HER2-enriched and TNBC subtypes. The subcellular localization of VDR, specifically nuclear versus cytoplasmic was examined, and the expression patterns were statistically compared across subtypes to understand potential subtype-specific VDR signaling roles.

In the TNBC, VDR expression exhibited a distinct pattern characterized by significant cytoplasmic localization. High cytoplasmic VDR staining was observed in (n = 10, 33.33%), while nuclear localization was noted in (n = 5, 16.6%) as shown in Figure 30. Importantly, dual cytoplasmic and nuclear expression was evident in some samples, suggesting that VDR may function through both genomic and non-genomic pathways in TNBC. The cytoplasmic predominance may reflect activation of rapid signaling cascades, while the nuclear presence indicates potential involvement in transcriptional regulation. This dual localization was statistically significant ($p < 0.042$), highlighting a potentially unique regulatory mechanism in TNBC that differs from receptor-positive subtypes.

In luminal A tumors, a comparatively lower level of VDR expression was observed. High cytoplasmic expression was detected in (n = 6, 20%), whereas nuclear expression was limited (n = 2, 6.6%) ($p < 0.042$), as illustrated in Figure 30. The relatively restricted nuclear localization in luminal A tumors suggests that VDR signaling may be less transcriptionally active in this subtype or that its function is more compartmentalized within the cytoplasm. Compared to TNBC, the overall VDR expression, especially the dual localization was markedly reduced, possibly due to differing hormonal environments or receptor-mediated regulatory controls prevalent in luminal A tumors.

The luminal B subtype exhibited a consistent trend with exclusive cytoplasmic localization. VDR expression was confined to the cytoplasm in (n = 4, 13.3%), and no nuclear staining was observed ($p < 0.042$). This pattern reinforces the hypothesis that in luminal B tumors, VDR may exert its effects primarily through non-genomic mechanisms. The absence of nuclear VDR could be attributed to differential co-

regulator expression or altered intracellular trafficking, both of which can impact VDR nuclear translocation. The limited expression may also indicate a diminished role for VDR in the biological behavior of luminal B cancers.

In HER2-enriched tumors, a distinctly different VDR expression pattern was noted. No cytoplasmic VDR expression was observed in any of the samples, whereas nuclear VDR localization was present in (n = 3, 10%) ($p < 0.042$), as depicted Figure 30.

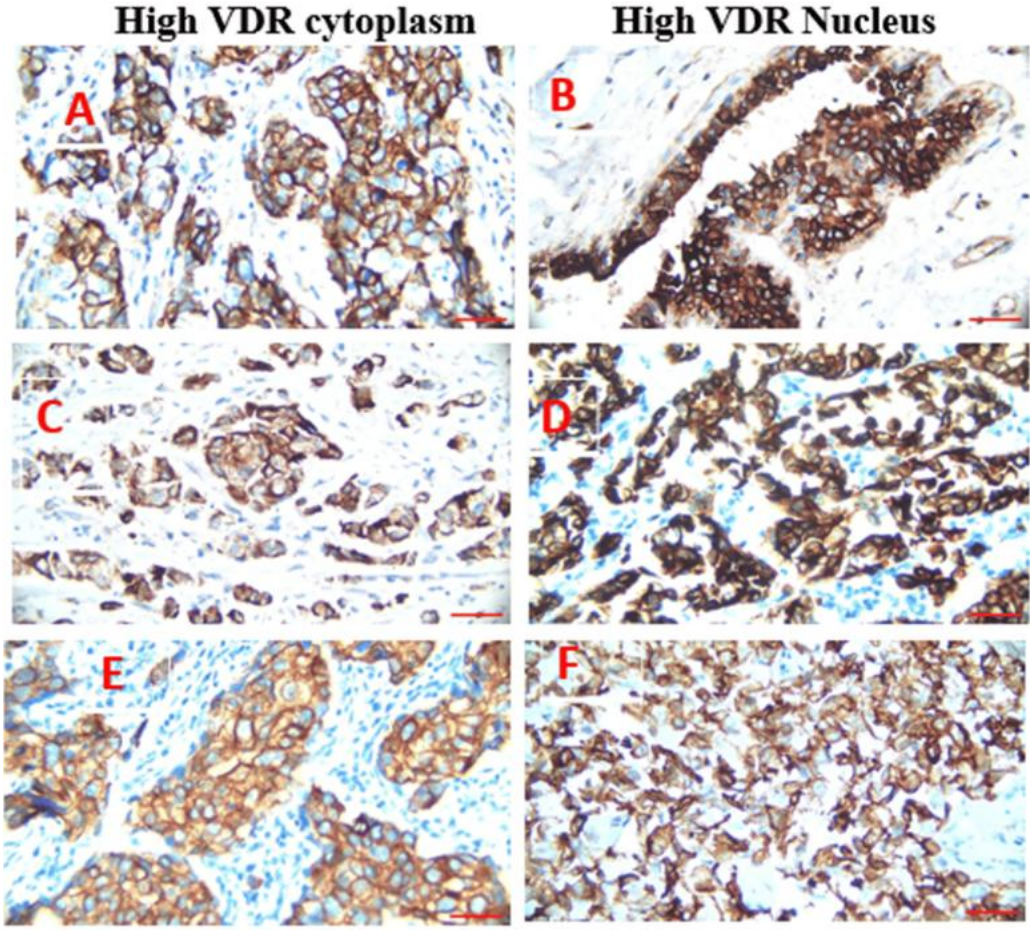


FIGURE 30: VDR localization (40X) in TNBC subtypes showing high expression in the cytoplasm and nucleus. (A-B) TNBC: (A) High VDR expression in the cytoplasm, (B) high VDR expression in the nucleus. (C-D) Luminal A: (C) High VDR expression in the cytoplasm, (D) high VDR expression in the nucleus. (E) Luminal B: High VDR expression in the cytoplasm. (F) HER2-enriched: High VDR expression in the nucleus. Scale bar = 13.75 µm **Abbreviations:** VDR, vitamin D receptor; HER2, human epithelial growth factor receptor 2; TNBC, triple-negative breast cancer.

This subtype-specific nuclear expression suggests that in HER2-driven tumors, VDR may engage in direct transcriptional regulation, potentially interacting with oncogenic pathways unique to this group. The absence of cytoplasmic expression may imply limited involvement in non-genomic signaling or altered receptor stability in the cytoplasmic compartment. When comparing the four subtypes collectively, TNBC demonstrated the most prominent and dual localization of VDR, with both cytoplasmic and nuclear expression detected in a subset of tumors. This pattern contrasts with luminal A and B tumors, where expression was predominantly cytoplasmic, and HER2-enriched tumors, where VDR was confined to the nucleus.

TABLE 6: Expression of VDR in tumor cells in molecular subtypes of breast cancer.

Staining intensity	**VDR expression in cellular location**	**TNBC (n = 15)**	**Luminal A (n = 8)**	**Luminal B (n = 4)**	**HER2-enriched (n = 3)**	***p*-value**
High	VDR cytoplasm, n (%)	10 (33.3)	6 (20)	4 (13.3)	0 (0)	*P* <0.042*
	VDR nucleus, n (%)	5 (16.6)	2 (6.6)	0 (0)	3 (10)	

*Indicates statistical significance ($p < 0.05$).

Abbreviations: VDR, vitamin D receptor; TNBC, triple-negative breast cancer; HER2, human epithelial growth factor receptor 2.

The statistical analysis confirmed that the differences in VDR localization among subtypes were significant ($p < 0.042$), reinforcing the biological relevance of the expression patterns. A summary of VDR localization and frequency across the subtypes is presented in Table 1, and representative immunohistochemical images illustrating the subtype-specific expression patterns are shown in Figure 30.

5.2 Cytoplasmic ERβ1 Immunoreactivity in TNBC and Luminal A Subtypes

In addition to examination of VDR expression, this study also investigated the immunohistochemical expression of ERβ1, with a specific focus on its cytoplasmic localization in two molecular subtypes of BC: TNBC and luminal A. A total of 18 histologically confirmed IDC cases were included in this analysis, comprising TNBC (n = 10) and luminal A (n = 8) subtypes. Other molecular subtypes, including luminal B and HER2-enriched, were excluded from this analysis due to insufficient tissue

sections availability. The assessment was restricted to cytoplasmic expression, as nuclear staining was absent in all analyzed samples. In TNBC cases, cytoplasmic ERβ1 expression was observed in all tumors, albeit with variable intensity. Moderate cytoplasmic expression was the most prominent pattern, present in (n = 9, 50%) and (n = 1, 5.5%), exhibited high cytoplasmic ERβ1 expression, as showed in Figure 31. This distribution pattern indicates that ERβ1 is retained in the cytoplasm in a majority of TNBC tumors.

The moderate to high expression levels observed in TNBC support the hypothesis that cytoplasmic ERβ1 may play a regulatory role, potentially through non-genomic signaling pathways, in the absence of ERα and PR signaling. The frequency distribution in TNBC was statistically significant ($p < 0.025$), reinforcing the biological relevance of cytoplasmic ERβ1 in this aggressive subtype.

In contrast, luminal A tumors demonstrated a distinctly different pattern of ERβ1 expression. Among the 8 cases analyzed, high cytoplasmic expression was observed in (n = 5, 27.7%), while moderate expression was noted in (n = 3, 16.6%), as represented in Figure 31.

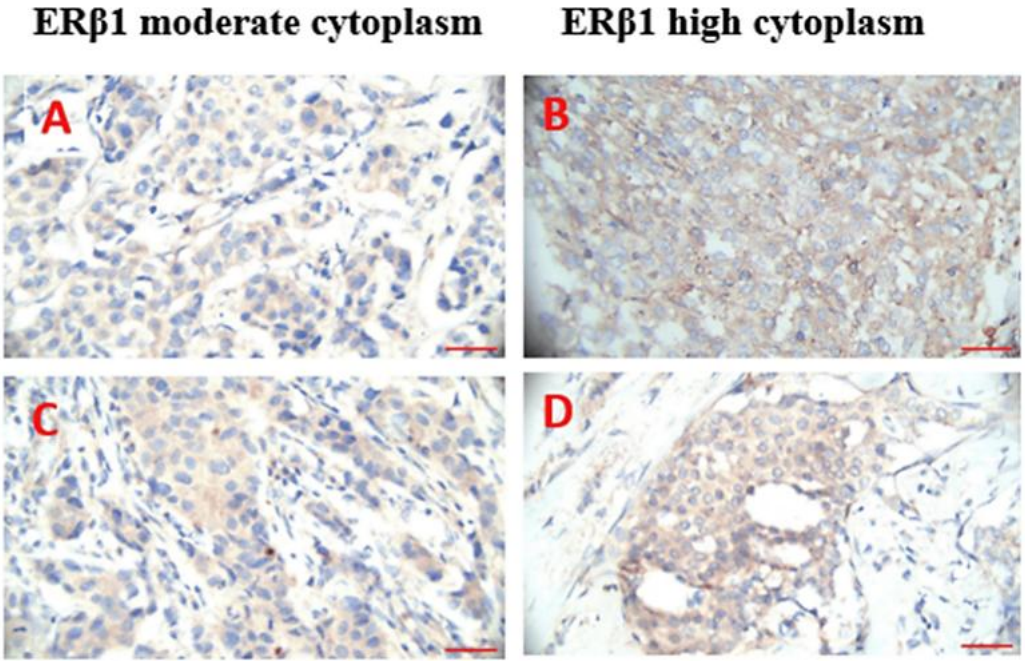


FIGURE 31: ERβ1 moderate to high expression in the cytoplasm (40X) in TNBC and luminal A. (A-B) TNBC: (A) Moderate ERβ1 expression in the cytoplasm, (B) High ERβ1 expression in the cytoplasm. (C-D) Luminal A: (C) Moderate ERβ1 expression in the cytoplasm, (D) High ERβ1 expression in the cytoplasm. Scale bar: 13.75 µm **Abbreviations:** ERβ1, estrogen receptor beta 1; TNBC, triple-negative breast cancer.

Notably, no luminal A tumors showed low or absent ERβ1 expression. Despite the higher proportion of tumors with strong cytoplasmic staining, the total frequency of moderate expression was considerably lower in luminal A compared to TNBC.

TABLE 7: Expression of ERβ1 in TNBC and luminal

Staining intensity	**ERβ1 expression in cellular location**	**TNBC (n = 10)**	**Luminal A (n = 8)**	***p*-value**
ERβ1 cytoplasm	Moderate, n (%)	9 (50)	3 (16.6)	P <0.025*
	High, n (%)	1 (5.5)	5 (27.7)	

*Indicates statistical significance ($p < 0.05$)

Abbreviations: ERβ1, estrogen receptor beta 1; TNBC, triple-negative breast cancer.

These subtype-specific expression trends were summarized in Table 2 and representative immunohistochemical staining images of ERβ1 expression are presented in Figure 31. The data illustrate that while both TNBC and luminal A tumors exhibit cytoplasmic ERβ1 staining, the intensity distribution varies between them. TNBC exhibits a predominance of moderate expression, suggesting a consistent but possibly sub-optimized regulatory role. In contrast, the higher proportion of strongly stained luminal A cases suggests enhanced receptor stabilization or signaling efficiency in this subtype.

The comparative findings also underscore the potential utility of ERβ1 as a molecular biomarker for TNBC characterization. Its consistent cytoplasmic expression in TNBC supports its consideration as a candidate for functional studies and therapeutic targeting, especially in tumors lacking ERα. In luminal A tumors, where ERα-driven genomic signaling predominates, cytoplasmic ERβ1 may serve a modulatory or inhibitory function.

Furthermore, the significant difference in the pattern of ERβ1 expression between TNBC and luminal A ($p < 0.025$) provides a rationale for exploring ERβ1 as a discriminative biomarker for subtype classification and stratification in future studies. These findings contribute to the growing evidence that ERβ1 plays a context-dependent role in BC and may have clinical implications for the design of receptor-targeted therapies in TNBC, particularly in the context of dual-modulation strategies involving VDR and ERβ1.

In our study, cytoplasmic ERβ1 immunoreactivity was consistently detected in TNBC and luminal A BC tissues, with differential intensity patterns suggesting subtype-specific functional implications. The moderate expression in TNBC and high expression in luminal A may reflect underlying biological differences in ERβ1 activity, potentially contributing to distinct tumor phenotypes and therapeutic responses.

II Objective

To analyze and compare the *in silico* binding affinities of vitamin D_3 and 17β-estradiol with key target proteins (ERβ, VDR, EGFR, and VEGF) in triple-negative breast cancer.

5.3 Molecular Docking Analysis of Calcitriol and 17β-Estradiol with Target Proteins in TNBC

To evaluate and compare the binding potential of calcitriol and 17β-estradiol with critical molecular targets implicated in TNBC, a comprehensive molecular docking analysis was conducted. The study specifically assessed the interactions of these two compounds with estrogen receptor beta (ERβ), VDR, EGFR, VEGF, and caspase 3. These targets were selected based on their established relevance to hormone receptor signaling, cellular proliferation, angiogenesis, and apoptosis regulation in TNBC. Docking results were analyzed based on binding energy values and key hydrogen bonding interactions with specific amino acid residues.

5.3.1 Docking with VDR

The interaction of calcitriol and 17β-estradiol with the VDR yielded informative contrasts. Calcitriol, the natural ligand of VDR, showed a strong binding energy of −9.15 kcal/mol, consistent with a high-affinity interaction. It engaged residues such as LEU-A414, GLN-A152, and ASP-A149 (Figure 32C). These findings validate the structural docking reliability and reflect VDR's natural binding conformation for calcitriol. On the other hand, 17β-estradiol displayed a weaker binding affinity of −6.37 kcal/mol, interacting with residues such as SER-A398, ASN-A394, ARG-A391, SER-A265, ARG-A343, and ASP-A342 (Figure 32D).

5.2.2 Docking with ERβ

Calcitriol and 17β-estradiol exhibited favorable binding affinities with ERβ, with 17β-estradiol showing a stronger interaction. The binding energy for calcitriol with ERβ was −7.85 kcal/mol, while 17β-estradiol demonstrated a significantly lower (more favorable) binding energy of −9.08 kcal/mol. As shown in Figure 32A and 32B, calcitriol formed hydrogen bonds with LYS-A315, GLU-A274, PRO-A277, GLU-A276, HIS-A394, and TRP-A345. In contrast, 17β-estradiol interacted with HIS-A475, PHE-A356, ARG-A346, and GLU-A305.

5.3.2 Docking with EGFR

In EGFR docking, calcitriol demonstrated the strongest binding among all evaluated interactions, with a binding energy of −10.04 kcal/mol. This significant affinity was supported by hydrogen bond interactions with LYS-A56, GLU-A78, THR-A57, ASP-51, and NAG-A1032 (Figure 33A). These interactions indicate a potential for calcitriol to modulate EGFR signaling, possibly contributing to growth inhibition in TNBC. Conversely, 17β-estradiol showed a binding energy of −7.10 kcal/mol with EGFR, forming interactions with THR-A406, LYS-A407, ASN-A12, SER-A11, and water molecule HOH-A2438 (Figure 33B).

5.3.3 ‘Docking with VEGF

VEGF, 17β-estradiol displayed a more favorable interaction (−7.06 kcal/mol) compared to calcitriol (−5.72 kcal/mol). Calcitriol bound with GLN-H115 and GLY-H9 (Figure 33C), while 17β-estradiol interacted with ASN-A214 and LEU-H118 (Figure 33D). Despite its weaker binding affinity, calcitriol interactions may indicate indirect modulation of VEGF-related angiogenesis.

Overall, the docking results indicate that calcitriol exhibits the highest binding affinity for EGFR (−10.04 kcal/mol) and VDR (−9.15 kcal/mol), highlighting its potential to regulate both receptor-mediated and growth factor signaling pathways. In contrast, 17β-estradiol shows superior affinity for ERβ (−9.08 kcal/mol) and VEGF (−7.06 kcal/mol), supporting its role in hormonal and angiogenic regulation. These findings are tabulated in Table 3 for clarity and comparison.

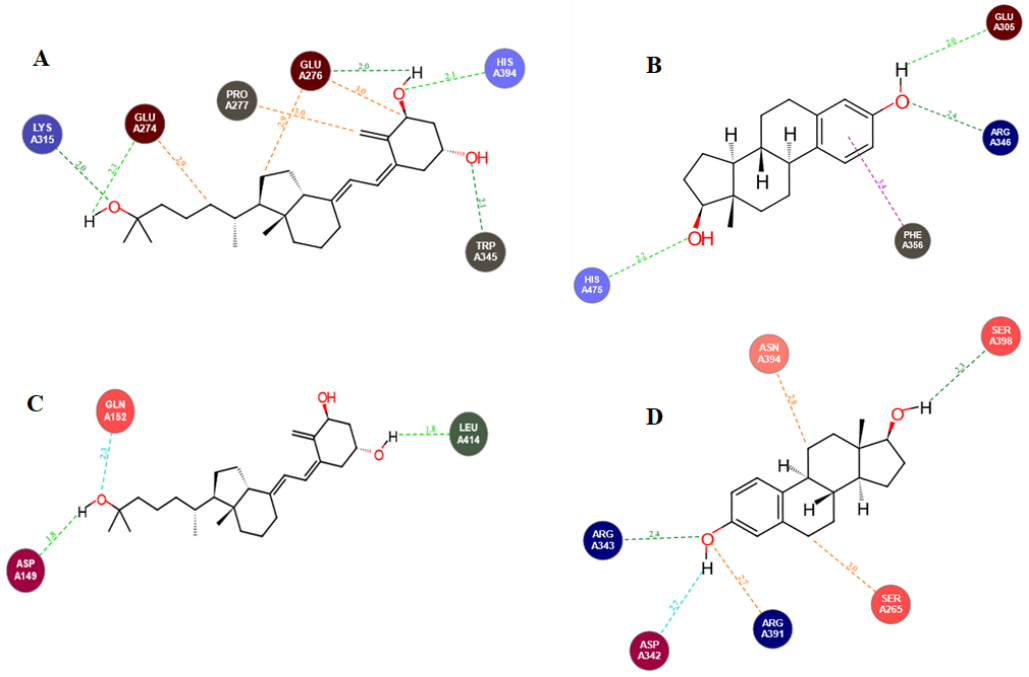


Figure 32: Molecular docking interactions of calcitriol and 17β-estradiol with ERβ and VDR. Calcitriol (A, C) and 17β-estradiol (B, D) were docked with ERβ (A, B) and VDR (C, D), respectively.

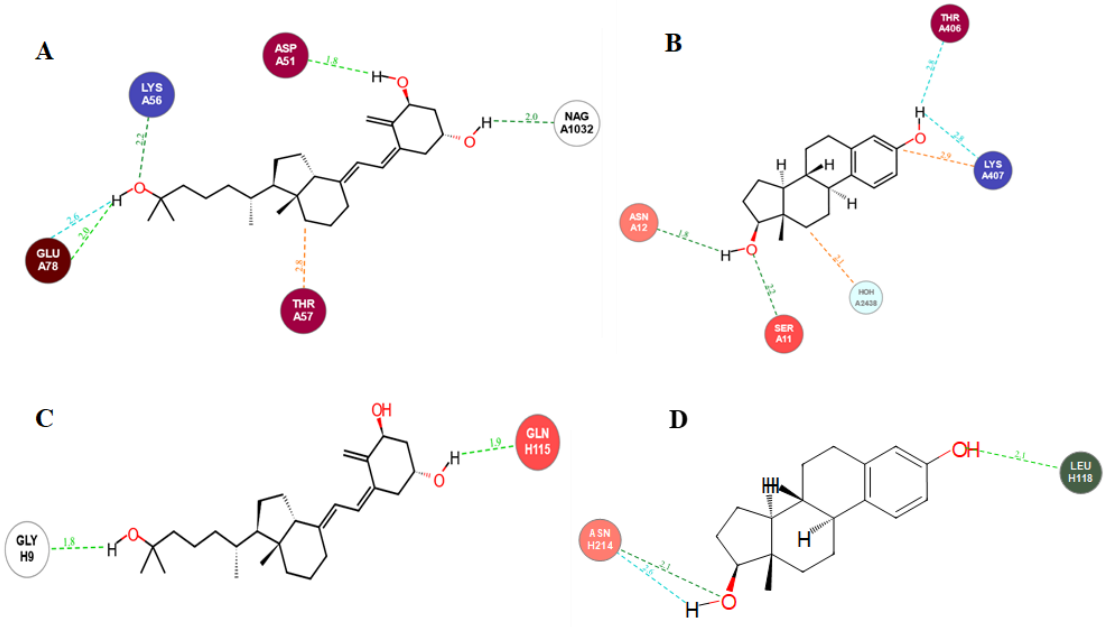


Figure 33: Molecular docking interactions of calcitriol and 17β-estradiol with EGFR and VEGF. Calcitriol (A, C) and 17β-estradiol (B, D) were docked with EGFR (A, B) and VEGF (C, D),

5.3.4 Special Interactions Involving N-acetylglucosamine (NAG) and Structural Water Molecules (HOH – Water Molecule (H_2O))

In the docking interaction of calcitriol with EGFR, one notable hydrogen bond was formed with NAG-A1032, which refers to N-acetylglucosamine, a carbohydrate moiety frequently found as part of glycosylation sites on membrane-bound proteins like EGFR. This interaction suggests that calcitriol may not only engage with amino acid residues but also interact with glycosylated regions of the receptor, potentially influencing receptor stability or ligand accessibility. Similarly, in the docking of 17β-estradiol with EGFR, a hydrogen bond was observed with HOH-A2438, representing a crystallographic water molecule. These water-mediated interactions often play a stabilizing role in ligand binding by bridging between the ligand and the receptor's active site residues. The involvement of HOH-A2438 indicates that water molecules may facilitate or stabilize 17β-estradiol orientation within the EGFR binding pocket.

Table 8. Binding energy and hydrogen bond interactions of calcitriol and 17β-estradiol with ERβ, EGFR, and VEGF.

Target protein	Compound	Binding energies (kcal/mol)	Key interacting residues
VDR	Calcitriol	-9.15	LEU-A414, GLN-A152, ASP-A149
	17β-Estradiol	-6.37	SER-A398, ASN-A394, ARG-A391, SER-A265, ARG-A343, ASP-A342
ERβ	Calcitriol	-7.85	LYS-A315, GLU-A274, PRO-A277, GLU-A276, HIS-A394, TRP-A345
	17β-Estradiol	-9.08	HIS-A475, PHE-A356, ARG-A346, GLU-A305
EGFR	Calcitriol	-10.04	LYS-A56, GLU-A78, THR-A57, ASP-51, NAG-A1032
	17β-Estradiol	-7.10	THR-A406, LYS-A407, ASN-A12, SER-A11, HOH-A2438
VEGF	Calcitriol	-5.72	GLN-H115, GLY-H9
	17β-Estradiol	-7.06	ASN-A214, LEU-H118

III Objective

To elucidate the molecular mechanism of vitamin D_3 and 17β-estradiol agonist treatment in triple negative breast cancer progression using *in vitro* model.

5.4 Effect of Calcitriol, 17β-Estradiol, and Their Combination on MDA-MB-468 Cell Viability

To explore the molecular mechanisms by which calcitriol and 17β-estradiol influence TNBC progression, the cytotoxic potential of these agents, individually and in combination, was evaluated using the MDA-MB-468 cell line, a well-characterized *in vitro* model. Cell viability was measured using the MTT assay across a time course of 8-, 16-, 24-, and 32-hours following treatment with varying concentrations of each compound.

a) Calcitriol monotherapy (1, 2, 3, 4, and 5 μM) demonstrated a clear dose- and time-dependent cytotoxic effect. As shown in Figure 34A, cell viability decreased progressively with both increasing dose and extended exposure time. The most substantial reduction was seen at the highest concentration (5 μM), with viability decreasing to 16 hr (74%), 24 hr (65%), and 32 hr (50%).
b) 17β-estradiol treatment (100, 200, 300, 400, and 500 nM) also resulted in a significant reduction in cell viability, though the response profile differed slightly. As shown in Figure 34B, notable declines in viability were recorded at the 500 nM concentration, 16 hr (76%), 24 hr (68%), and 32 hr (50%). These findings indicate a moderate but consistent cytotoxic response.
c) The combination treatment (calcitriol 5 μM + 17β-estradiol 500 nM) produced the most pronounced effects, as represented in Figure 34C. Cell viability was reduced to 16 hr (70%), 24 hr (61%), and 32 hr (50%), showing an additive cytotoxic effect compared to individual treatments. This enhanced suppression may be attributed to simultaneous activation of VDR and ERβ1, leading to more comprehensive inhibition of oncogenic signaling pathways, including proliferation (EGFR), angiogenesis (VEGF), and apoptosis regulation (caspase-3).

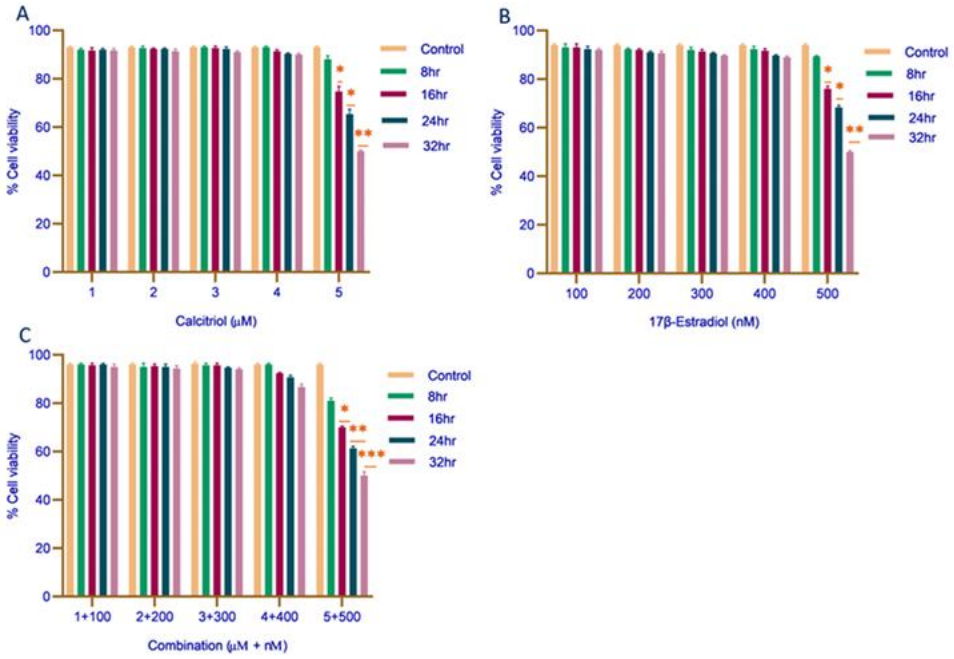


FIGURE 34: Effect of calcitriol, 17β-estradiol, and its combination on MDA-MB-468 cell viability. Cell viability was assessed using the MTT assay following treatment with (A) Calcitriol (1, 2, 3, 4, and 5 μM), (B) 17β-estradiol (100, 200, 300, 400, and 500 nM), and (C) combination treatment (calcitriol, 5 μM + 17β-estradiol, 500 nM) at the indicated concentrations for 8, 16, 24, and 32 hours. Data are expressed as mean ± SEM from three independent experiments. Statistically significant differences in cell viability compared to the untreated control at each time point are indicated (*p < 0.01, **p < 0.001, *p < 0.0001). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. Abbreviations: SEM: standard error mean; μM: micromolar; nM: nanomolar; hr: hours.

While early time points (8 hours) did not exhibit substantial changes in any of the treatment groups, a progressive decline in viability was evident beyond 16 hours, indicating that the onset of cytotoxic effects is time-dependent. Notably, the greatest differences emerged between 24 and 32 hours, a window that appears to correspond to sustained receptor-mediated transcriptional changes and accumulation of apoptotic signals within the TNBC cells.

Statistical analysis confirmed the significance of observed effects using one-way ANOVA followed by Tukey's post hoc test, with p-values indicating highly significant reductions in viability at later time points for all three treatment conditions (*p < 0.01, **p < 0.001, ***p < 0.0001).

These findings confirm that both calcitriol and 17β-estradiol exhibit potent cytotoxic effects on MDA-MB-468 cells in a time- and dose-dependent manner, with combination therapy producing an enhanced inhibitory effect. These data support the therapeutic potential of dual receptor targeting, via VDR and ERβ1, as a viable strategy in the management of ERβ1-positive TNBC.

5.5 IMMUNOBLOT ANALYSIS

5.5.1 Effect of Calcitriol on ERβ1, EGFR, VEGF, and Caspase-3 Expression in MDA-MB-468 Cells

To further elucidate the molecular mechanisms underlying the cytotoxic effects of calcitriol in TNBC, Western blot analysis was conducted to evaluate the expression levels of key regulatory proteins, including ERβ1, EGFR, VEGF, and caspase-3. MDA-MB-468 cells were treated with 5 μM calcitriol and harvested at 8, 16, 24, and 32 hours to assess time-dependent modulation of protein expression.

5.3.3 ERβ1 Expression

As shown in Figure 35A and quantified in Figure 35B, ERβ1 expression exhibited a progressive decline following calcitriol treatment. While the change between 8 and 16 hours was not statistically significant (p = ns), a marked decrease was observed at 24 and 32 hours ($p < 0.0001$), indicating that prolonged calcitriol exposure exerts a sustained suppressive effect on ERβ1 protein levels. The continued reduction from 24 to 32 hours ($p < 0.0001$) suggests that calcitriol not only initiates but maintains repression of ERβ1, a tumor suppressor that modulates survival pathways in TNBC. This downregulation could potentially reflect feedback signaling or altered receptor stability under sustained ligand stimulation.

5.5.2 EGFR Expression

EGFR, a critical mediator of proliferative and survival signaling in TNBC, was significantly suppressed in response to calcitriol (Figure 35C). No significant change was detected between 8 and 16 hours (p = ns), but a significant decrease occurred from 16 to 24 hours ($p < 0.0001$), with an additional reduction between 24 and 32 hours ($p = 0.005$). This time-dependent inhibition suggests that calcitriol

interferes with EGFR-driven signaling, which may contribute to its antiproliferative effects. The delayed but consistent suppression of EGFR supports the idea that calcitriol impairs receptor-mediated mitogenic signaling through sustained VDR activation.

5.5.3 VEGF Expression

VEGF expression, an angiogenic factor essential for tumor vascularization, also declined significantly over time (Figure 35D). A robust reduction was observed from 8 to 32 hours ($p < 0.0001$), with a further decrease from 16 to 32 hours ($p < 0.0001$), and a smaller yet statistically significant change between 24 and 32 hours ($p = 0.01$). These findings suggest that calcitriol may impair angiogenesis in TNBC cells, likely by downregulating VEGF transcription or translation. The consistent decrease over extended exposure highlights the anti-angiogenic potential of calcitriol, supporting its role as a suppressor of tumor progression via vascular inhibition.

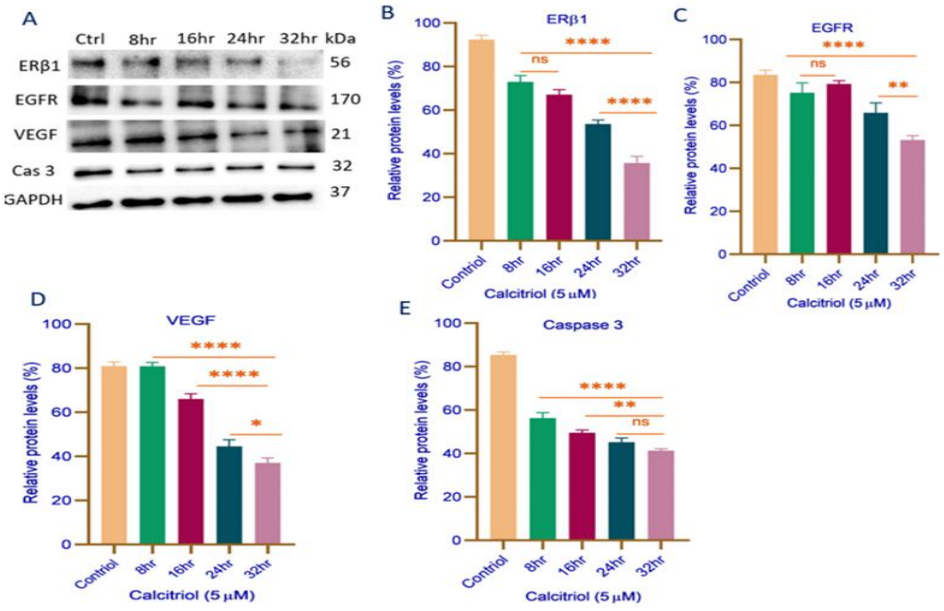


FIGURE 35: Representative immunoblot analysis (A) and quantification of protein expression levels of ERβ1 (B), EGFR (C), VEGF (D), and caspase-3 (E) in MDA-MB-468 cells treated with 5 μM calcitriol at different time intervals (8, 16, 24, and 32 hours). Protein expression levels were normalized to GAPDH and are presented as relative percentages. Data are expressed as mean ± SD. Statistical significance is indicated as *$p < 0.01$, **$p < 0.005$, ***$p < 0.001$, ****$p < 0.0001$. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. **Abbreviations:** ERβ1: estrogen receptor beta 1; EGFR: epidermal growth factor receptor; VEGF: vascular endothelial growth factor; Cas 3: caspase 3; GAPDH: glyceraldehyde phosphate dehydrogenase; Ctrl: control; hr: hours; kDa: kilodalton; μM: micromolar; ns: not significant.

5.5.4 Caspase-3 Expression

Interestingly, caspase-3, an essential effector of the apoptotic cascade, also showed time-dependent downregulation following calcitriol treatment (Figure 35E). A significant reduction was seen from 8 to 32 hours ($p < 0.0001$), and between 16 to 32 hours ($p = 0.001$). However, expression levels remained unchanged between 24 and 32 hours (p = ns), suggesting a plateau phase in apoptotic regulation. These findings could indicate that calcitriol initially activates apoptotic pathways, but sustained exposure leads to compensatory downregulation or exhaustion of caspase-3 expression as the apoptotic program reaches completion. Alternatively, it may reflect a time-dependent switch from pro-apoptotic to survival or clearance phases in treated cells.

Collectively, these results demonstrate that calcitriol exerts multi-faceted regulatory effects on key TNBC-associated proteins. ERβ1, EGFR, and VEGF were all progressively suppressed, reflecting inhibition of receptor-mediated signaling and angiogenesis. The downregulation of caspase-3, while initially counterintuitive, may represent a temporal response linked to apoptotic progression. The most pronounced changes occurred between 24 and 32 hours, a time frame consistent with the observed reduction in cell viability. These findings reinforce the hypothesis that calcitriol anti-cancer activity in TNBC involves coordinated repression of proliferation, angiogenesis, and survival signaling, thereby providing a mechanistic basis for its therapeutic potential.

5.6 Effect of 17β-Estradiol on ERβ1, EGFR, VEGF, and Caspase-3 Expression in MDA-MB-468 Cells

To investigate the temporal regulation of key oncogenic and tumor-suppressive proteins in TNBC, MDA-MB-468 cells were treated with 500 nM 17β-estradiol. Western blot analysis was performed to assess the protein expression levels of ERβ1, EGFR, VEGF, and caspase-3 at multiple time points (8, 16, 24, and 32 hours), providing insights into the hormonal modulation of signaling pathways associated with tumor progression, angiogenesis, and apoptosis.

5.6.1 ERβ1 Expression

ERβ1, a tumor suppressor and nuclear hormone receptor, displayed a complex temporal pattern following 17β-estradiol treatment. As seen in Figure 36A and quantified in Figure 36B, ERβ1 levels declined from 8 to 24 hours, indicating a time-dependent downregulation, which reached statistical significance from 8 to 24 hours ($p < 0.001$). Notably, the reduction between 16 and 24 hours was not statistically significant (p = ns), suggesting a transient stabilization of expression. While, from 24 to 32 hours, a significant increase in ERβ1 expression was observed ($p = 0.0002$), suggesting a potential compensatory feedback mechanism or receptor reactivation after prolonged ligand exposure. This late-stage re-induction of ERβ1 may reflect receptor recycling or cellular adaptation to hormonal signaling, consistent with the known ligand-activated dynamics of ERβ1.

5.6.2 EGFR Expression

EGFR, a transmembrane receptor tyrosine kinase involved in proliferation and survival signaling, showed a consistent decline in expression following 17β-estradiol exposure.

As illustrated in Figure 36C, EGFR levels decreased significantly from 8 to 32 hours ($p < 0.0001$), with an additional significant drop between 16 and 32 hours ($p = 0.003$). However, the change between 24 and 32 hours was not statistically significant (p = ns), suggesting a plateau in suppression, potentially indicating that maximal downregulation was achieved by 24 hours. These results suggest that 17β-estradiol may interfere with EGFR-mediated proliferative signaling in ERβ1-positive TNBC cells through sustained transcriptional or post-translational mechanisms.

5.6.3 VEGF Expression

VEGF, a major pro-angiogenic factor involved in tumor vascularization, also exhibited time-dependent suppression in response to 17β-estradiol. A statistically significant decrease was observed from 8 to 32 hours ($p < 0.0001$), as shown in Figure 36D. VEGF expression declined significantly between 16 and 24 hours ($p < 0.01$), but the subsequent reduction from 24 to 32 hours was not significant (p = ns), again suggesting expression stabilization at late time points. The overall reduction in VEGF

expression highlights the anti-angiogenic potential of 17β-estradiol treatment, possibly mediated through ERβ1 activation, which is known to agonise VEGF expression in BC models.

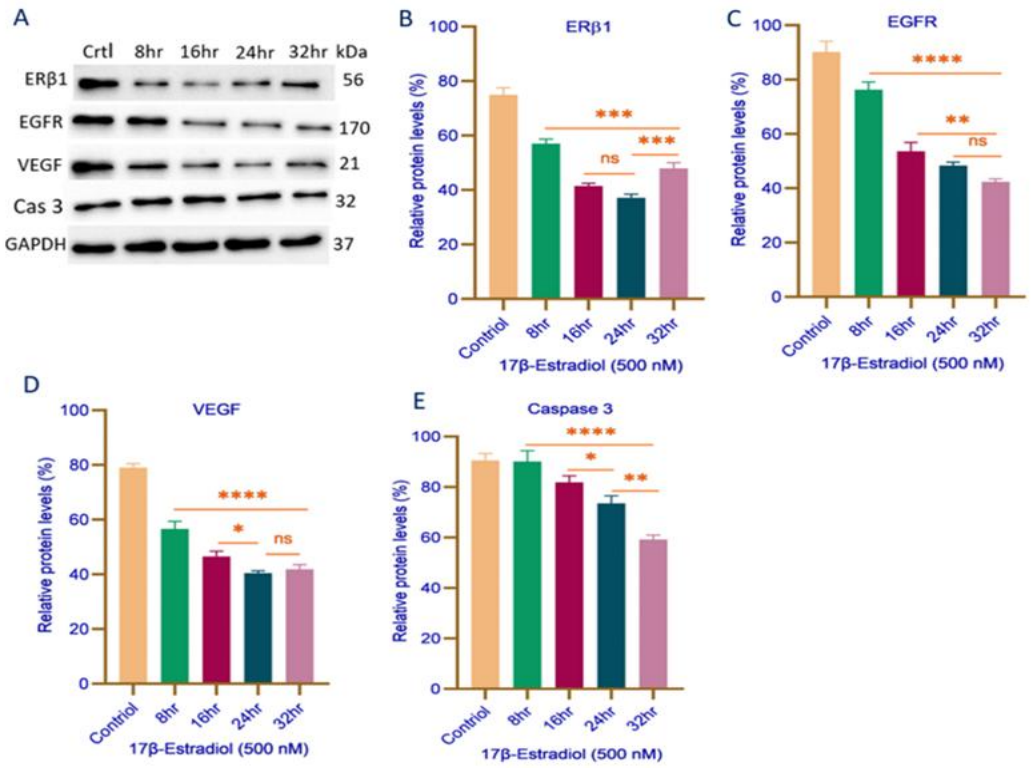


FIGURE 36: Representative immunoblot analysis (A) and quantification of protein expression levels of ERβ1 (B), EGFR (C), VEGF (D), and caspase-3 (E) in MDA-MB-468 cells treated with 500 nM 17β-estradiol at different time intervals (8, 16, 24, and 32 hours). Protein expression levels were normalized to GAPDH and are presented as relative percentages. Data are expressed as mean ± SD. Statistical significance is indicated as *p < 0.01, **p < 0.003, ***p < 0.001, ****p < 0.0001. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. **Abbreviations**: ERβ1: estrogen receptor beta 1; EGFR: epidermal growth factor receptor; VEGF: vascular endothelial growth factor; Cas 3: caspase 3; GAPDH:

5.6.4 Caspase-3 Expression

Caspase-3, a crucial executioner of apoptosis, also showed a gradual and sustained reduction following 17β-estradiol exposure. As shown in Figure 36E, expression was significantly reduced from 8 to 32 hours (p = 0.0001), with further reductions noted between 16 and 32 hours (p = 0.003) and between 24 and 32 hours (p = 0.001). These findings suggest that 17β-estradiol may modulate apoptotic signaling in a delayed

manner, possibly through ERβ1-dependent pathways. The consistent suppression of caspase-3 across later time points could reflect either completion of apoptosis induction or inhibitory feedback mechanisms suppressing its further activation.

These results collectively demonstrate that 17β-estradiol treatment induces time-dependent modulation of key regulatory proteins in TNBC. While ERβ1, EGFR, VEGF, and caspase-3 initially decline in expression, certain proteins, particularly ERβ1, exhibit late-stage reactivation or stabilization, suggesting complex regulatory feedback during hormone-mediated signaling. The suppression of EGFR and VEGF implies reduced proliferative and angiogenic potential, whereas changes in caspase-3 suggest altered apoptotic dynamics. These findings reinforce the role of 17β-estradiol in modulating multiple tumor-regulatory pathways and support its potential therapeutic value when targeted through ERβ1 positive TNBC.

5.7 Combined Effect of Calcitriol and 17β-Estradiol on ERβ1, EGFR, VEGF, and Caspase-3 Expression in MDA-MB-468 Cells

To assess whether the combination of calcitriol (5 μM) and 17β-estradiol (500 nM) produces a more potent regulatory effect than individual treatments, protein expression of key markers ERβ1, EGFR, VEGF, and caspase-3 was analyzed using Western blot in ERβ1-positive TNBC cells (MDA-MB-468). Cells were harvested at 8, 16, 24, and 32 hours after combination treatment, and protein levels were quantified relative to GAPDH.

5.7.1 ERβ1 Expression

As shown in Figure 37A and quantified in Figure 37B, ERβ1 expression declined progressively over time in response to the combination treatment. Although no statistically significant reduction was observed between 8 and 16 hours ($p = ns$), a significant reduction was evident from 8 to 32 hours ($p = 0.0001$), with a further sharp decrease from 24 to 32 hours ($p = 0.0001$). This pattern suggests an initial resistance or delayed response to the combined ligands, followed by a robust and sustained suppression of ERβ1 expression. Compared to individual treatments, the combination induced a more prolonged and consistent downregulation, indicating an effect on ERβ1 signaling in TNBC cells.

5.7.2 EGFR Expression

EGFR, a critical mediator of proliferative and pro-survival signaling in TNBC, exhibited substantial downregulation across all time points (Figure 37C). The expression significantly decreased from 8 to 32 hours, as well as between 16–24 hours and 24–32 hours ($p = 0.0001$ for each interval), demonstrating the combination treatment ability to continuously inhibit EGFR over time. This finding supports the hypothesis that simultaneous activation of VDR and ERβ1 pathways results in enhanced suppression of EGFR expression, thereby weakening proliferative signaling cascades in TNBC.

5.7.3 VEGF Expression

The expression of VEGF, a key pro-angiogenic factor, also showed significant time-dependent suppression. As illustrated in Figure 37D, VEGF levels significantly decreased from 8 to 32 hours ($p < 0.0001$), and notable reductions occurred between 16–24 hours ($p = 0.009$) and 24–32 hours ($p = 0.0004$). The persistent decrease in VEGF expression highlights the anti-angiogenic potential of the combination treatment. While both calcitriol and 17β-estradiol individually reduced VEGF, the combined approach produced a more consistent and profound suppression over the full treatment course, suggesting enhanced inhibition of angiogenesis-related pathways in TNBC cells.

5.7.4 Caspase-3 Expression

Caspase-3, a downstream executor of apoptosis, exhibited a strong and continuous decrease in expression following combination treatment (Figure 37E). Protein levels were significantly downregulated from 8 to 32 hours ($p < 0.0001$), with additional significant reductions between 16–32 hours ($p = 0.0002$) and 24–32 hours ($p = 0.008$). This sustained decline suggests either activation and consumption of caspase-3 during apoptosis or post-translational regulation suppressing its availability. Compared to monotherapies, the combination treatment demonstrated greater efficacy in suppressing caspase-3 expression, indicating a more robust apoptotic response in TNBC cells.

Together, these results demonstrate that the combination of calcitriol and 17β-estradiol exerts a more pronounced inhibitory effect on ERβ1, EGFR, VEGF, and caspase-3 expression in MDA-MB-468 cells compared to individual treatments. The delayed but significant suppression of ERβ1 and sustained inhibition of EGFR and VEGF point toward a combination modulation of tumor-promoting pathways, including proliferation and angiogenesis.

Meanwhile, the gradual and continued downregulation of caspase-3 may reflect enhanced apoptosis induction under dual receptor targeting conditions. The most substantial suppression across all markers was observed between 24 and 32 hours, suggesting that prolonged combination exposure is necessary to achieve maximal therapeutic efficacy.

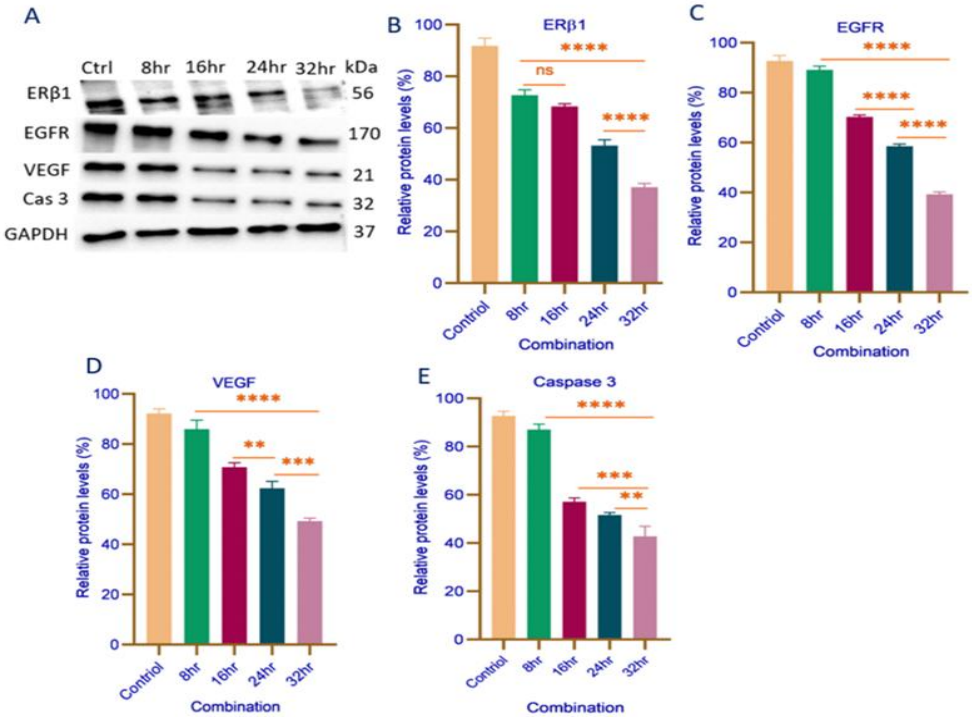


FIGURE 37: Representative immunoblot analysis (A) and quantification of protein expression levels of ERβ1 (B), EGFR (C), VEGF (D), and caspase-3 (E) in MDA-MB-468 cells treated with the combination of calcitriol (5 µM) and 17β-estradiol (500 nM) at different time intervals (8, 16, 24, and 32 hours). Protein expression levels were normalized to GAPDH and are presented as relative percentages. Data are expressed as mean ± SD. Statistical significance is indicated as **p < 0.008, ***p < 0.004, ****p < 0.0001. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. **Abbreviations:** ERβ1: estrogen receptor beta 1; EGFR: epidermal growth factor receptor; VEGF: vascular endothelial growth factor; Cas 3: caspase 3; GAPDH: glyceraldehyde phosphate dehydrogenase; Ctrl: control; hr: hours; kDa: kilodalton; ns: not significant.

These findings underscore the therapeutic potential of dual targeting of VDR and ERβ1 pathways in TNBC and provide a mechanistic foundation for future combinatorial strategies aimed at hormone receptor-positive subsets within the TNBC spectrum.

CHAPTER-6
DISCUSSION

6. DISCUSSION

The current study elucidates the mechanistic implications of VDR and ERβ1 expression across BC subtypes, with particular emphasis on TNBC. The observed differential expression patterns suggest distinct functional roles of VDR and ERβ1 in modulating tumor growth through genomic and non-genomic pathways.

6.1 VDR Expression and Functional Implications in TNBC

VDR, mediates its actions primarily via ligand-activated transcriptional regulation. Upon binding with calcitriol, VDR heterodimerizes with RXR and binds to VDREs in the promoter regions of target genes, initiating transcriptional modulation of proliferation, apoptosis, and immune regulation.

In our cross-sectional analysis, nuclear VDR expression was observed in 16.6% of TNBC cases, indicating that a subset of tumors retains the capacity for VDR-mediated genomic signaling. Cytoplasmic VDR expression was detected in 33.3% of cases, suggesting partial receptor presence within the cellular compartment. Although the overall nuclear localization of VDR was limited, these findings are consistent with previous studies reporting VDR negativity in over half of TNBC tumors (56.6%).[339] The detection of nuclear VDR in a subset of TNBC cases highlights the potential for selective responsiveness to vitamin D-based interventions, particularly in tumors exhibiting retained nuclear receptor expression. This pattern aligns with earlier findings that associate low nuclear VDR expression with adverse clinical outcomes and heightened tumor aggressiveness in TNBC.[16, 339] The absence of moderate to high nuclear VDR expression in TNBC further underscores its disrupted genomic signaling axis. This supports the hypothesis that insufficient nuclear translocation and transcriptional activity of VDR compromise its tumor-suppressive functions.

Interestingly, luminal A subtypes showed relatively higher cytoplasmic VDR expression (20%) and limited nuclear presence (6.6%), suggesting that VDR expression is retained in these tumors, its subcellular distribution may influence functional outcomes. Contrasting with Huss et al., who reported complete nuclear VDR negativity in 6.6% of luminal A cases.[339] our findings hint at subtype-specific regulation of VDR trafficking and activity. Similarly, luminal B subtypes revealed 13.3% cytoplasmic VDR expression without nuclear localization. This is lower than

the 25.6% nuclear expression observed by Huss et al.[339] possibly indicating variability in receptor activation thresholds or post-translational modifications among subtypes.
In HER2-enriched tumors, nuclear VDR expression was limited (10%) and cytoplasmic staining was absent. Huss, L. *et al.* noted that high cytoplasmic VDR in HER2-positive cases.[339] Suggesting a discrepancy possibly linked to sample size or HER2-driven alterations in receptor localization dynamics. Mechanistically, these findings indicate that HER2 signaling may modulate the VDR pathway through PI3K/Akt or MAPK-mediated phosphorylation events that hinder VDR nuclear import.[414] Collectively, these findings support a model wherein impaired VDR signaling, whether due to receptor loss, cytoplasmic sequestration, or reduced nuclear translocation, contributes to aggressive tumor behavior in TNBC. Restoration of VDR signaling via pharmacological ligands like calcitriol may thus reinstate regulatory control over genes involved in cell cycle arrest and apoptosis, offering a potential therapeutic avenue in TNBC.

6.2 ERβ1 Localization and Its Role in Non-Genomic Signaling in TNBC

ERβ1, unlike its alpha counterpart (ERα), functions as a tumor suppressor in certain contexts, including TNBC. ERβ1 may act through classical genomic mechanisms or initiate rapid non-genomic signaling cascades via membrane or cytoplasmic pools. In our study, TNBC samples exhibited high levels of cytoplasmic ERβ1, with 50% of cases demonstrating moderate expression and 5.5% showing high expression. This cytoplasmic predominance suggests a dominant role for ERβ1 in non-genomic signaling pathways in TNBC. Such pathways may include PI3K/Akt, Src kinase, or MAPK cascades that drive rapid cellular responses such as migration, angiogenesis, and proliferation modulation independent of direct gene transcription.[415]

These observations are consistent with Reese et al., who reported high cytoplasmic ERβ1 expression in 70.1% of TNBC samples.[382] The high frequency of cytoplasmic expression, despite the ERα-negative status of TNBC, implies an alternative estrogen-mediated signaling mechanism retained via ERβ1. Further, cytoplasmic ER immunoreactivity has been found in 23% of ER-negative and only 1.4% of ER-positive breast cancer cases.[412] reinforcing the selective importance of ERβ1 TNBC. Notably, luminal A tumors demonstrated lower ERβ1 cytoplasmic expression (moderate in 16.6%, high in 27.7%), and nuclear ERβ1 expression was

absent. This restricted distribution limits ERβ1 potential role in classical estrogen-responsive gene transcription within luminal A tumors. Consistent with our findings, Reese et al. noted that ERβ1 nuclear expression was rare and typically low in non-TNBC subtypes.[382]

These expression patterns suggest a mechanistic divergence wherein ERβ1 exerts predominantly non-genomic influence in TNBC but remains functionally quiescent in luminal A tumors due to limited nuclear and cytoplasmic presence. The functional significance of ERβ1 cytoplasmic presence in TNBC may relate to its interaction with membrane-associated proteins or scaffolding molecules, thereby influencing intracellular kinase cascades, cell motility, and apoptotic resistance.[416]

6.3 Interplay of VDR and ERβ1 in TNBC Progression

The concurrent low expression of nuclear VDR and elevated cytoplasmic ERβ1 in TNBC implies a dual impairment of tumor-suppressive genomic signaling alongside heightened activation of potentially pro-survival non-genomic pathways. This unique molecular profile may underlie TNBC aggressive phenotype and resistance to conventional hormone therapies.

Mechanistically, the lack of VDR-mediated transcription impairs regulation of pro-apoptotic genes such as p21, Bax, and caspase-3, while cytoplasmic ERβ1 may potentiate oncogenic signaling through PI3K/Akt activation.[417] The absence of nuclear ERβ1 further restricts its suppressive functions on EMT and stemness-associated gene transcription.[418]

The interaction between VDR and ERβ1 signaling may also be reciprocal. Evidence suggests that VDR activation can modulate estrogen receptor expression and *vice versa*.[419] Therefore, therapeutic activation of VDR using calcitriol may indirectly influence ERβ1 signaling pathways, potentially restoring apoptotic control and inhibiting tumor growth. These findings provide mechanistic insights into the complex receptor-mediated landscape of TNBC. The impaired genomic activity of VDR and functional diversion of ERβ1 toward non-genomic signaling contribute synergistically to the pathogenesis of TNBC. This supports the rationale for exploring combined therapeutic strategies that restore VDR function and modulate ERβ1 signaling.

Objective II

The molecular docking analysis provides the first mechanistic evidence that calcitriol and 17β-estradiol differentially but complementarily target key oncogenic pathways in TNBC.

6.4 Calcitriol Binding Affinity and Mechanism of Interaction

Calcitriol demonstrated high-affinity binding to VDR (−9.15 kcal/mol), validating the docking model and confirming its strong receptor-ligand compatibility. The ligand engaged core residues such as LEU-A414, GLN-A152, and ASP-A149, which are integral to the VDR ligand-binding domain. This interaction pattern is consistent with established structural data and reinforces VDR suitability as a therapeutic target in TNBC. Importantly, this high-affinity interaction suggests that calcitriol can facilitate VDR nuclear translocation and transcriptional activation of vitamin D-responsive genes, thereby promoting anti-proliferative and pro-apoptotic effects in VDR-expressing TNBC cells.[11]

In addition to its classical nuclear receptor, calcitriol also exhibited notable binding to EGFR, with the strongest docking score across all interactions (−10.04 kcal/mol). Hydrogen bond contacts with LYS-A56, GLU-A78, THR-A57, ASP-51, and NAG-A1032 suggest a potential for calcitriol to engage both the protein backbone and glycosylated regions of EGFR. These interactions may influence EGFR stability, receptor dimerization, or ligand affinity, possibly impairing downstream PI3K/Akt and MAPK signaling.[420] This finding is particularly significant as EGFR overexpression is common in TNBC, and calcitriol interaction with EGFR may contribute to growth suppression via receptor inhibition or internalization mechanisms.[421] Calcitriol also showed a moderately favorable binding affinity to ERβ (−7.85 kcal/mol), engaging key residues such as GLU-A276, LYS-A315, and TRP-A345. Although not the natural ligand for ERβ, this interaction suggests a possible allosteric modulation or partial agonistic behavior, which may influence receptor conformation or its association with co-regulators. Moreover, the engagement of calcitriol with apoptotic effector caspase-3 (−9.15 kcal/mol) indicates potential regulatory activity over apoptosis execution phases, possibly through stabilization of active site residues involved in caspase activation. The binding residues, LEU-A414,

GLN-A152, and ASP-A149 highlight the ligand interaction near catalytically relevant regions, which may promote pro-apoptotic signaling cascades in cancer cells.[418] Interestingly, calcitriol demonstrated a comparatively lower binding affinity for VEGF (−5.72 kcal/mol), interacting with GLN-H115 and GLY-H9. These interactions may not directly block VEGF-mediated angiogenesis but could modulate paracrine or autocrine signaling if calcitriol downregulates VEGF gene expression via upstream nuclear receptor signaling.[422]

Overall, the data support a mechanistic model wherein calcitriol targets multiple oncogenic pathways in TNBC: (1) activation of VDR for transcriptional control of growth and apoptosis-related genes, (2) potential interference with EGFR-mediated proliferative signaling, (3) partial modulation of ERβ, and (4) engagement with caspase-3 to support apoptotic execution.

6.5 17β-Estradiol Binding Affinity and Functional Implications

As expected of a natural estrogen receptor ligand, 17β-estradiol displayed the strongest binding affinity for ERβ (−9.08 kcal/mol), with key hydrogen bonds formed with HIS-A475, PHE-A356, ARG-A346, and GLU-A305. These residues lie within the canonical ligand-binding domain of ERβ, indicating a stable and functionally relevant receptor engagement. This strong interaction is consistent with ERβ known physiological responsiveness to estrogenic ligands and supports the role of 17β-estradiol in modulating ERβ-mediated tumor suppressive functions in TNBC.[23] The interaction is particularly relevant for ERβ1-expressing TNBC subsets, as ERβ1 has been associated with anti-proliferative and anti-invasive signaling.

In contrast to calcitriol, 17β-estradiol showed weaker binding to VDR (−6.37 kcal/mol), with multiple interactions involving SER-A398, ASN-A394, and ARG-A343. While this interaction is likely non-physiological, the binding profile suggests some off-target affinity, potentially influencing VDR behavior under conditions of receptor overexpression or altered conformation. However, given the significantly lower affinity compared to calcitriol, VDR is unlikely to be a major target of estradiol in TNBC.

The binding of 17β-estradiol to EGFR (−7.10 kcal/mol) involved both protein and solvent-mediated interactions, including hydrogen bonds with THR-A406, ASN-A12, and water molecule HOH-A2438. Water-mediated bridging interactions are

known to stabilize ligand positioning and could support partial modulation of EGFR kinase activity or dimer interface. Although not as potent as calcitriol in EGFR engagement, the interaction hints at potential indirect effects on proliferative signaling in TNBC.[423] A notable observation was the relatively stronger binding affinity of 17β-estradiol to VEGF (−7.06 kcal/mol) compared to calcitriol. Residue interactions with ASN-A214 and LEU-H118 suggest that 17β-estradiol may modulate angiogenic signaling, possibly through interference with VEGF receptor activation or ligand dimerization. This is mechanistically relevant as angiogenesis is a key feature of TNBC aggressiveness, and estrogen-mediated VEGF upregulation has been previously reported in BC.[424]

Binding to caspase-3 was less favorable (−6.37 kcal/mol) compared to calcitriol. Nevertheless, interactions with ASP-A342, ARG-A391, and SER-A265 suggest that 17β-estradiol may still exert weak regulatory effects on apoptotic machinery, potentially via indirect pathway modulation rather than direct activation.[425]

6.6 Comparative Mechanistic Insights and Therapeutic Implications

The comparative docking profiles reveal that calcitriol exhibits its highest affinities toward EGFR and VDR, aligning with its dual function in nuclear hormone signaling and growth factor receptor modulation. These interactions support a mechanistic rationale for calcitriol anti-proliferative and pro-apoptotic properties in TNBC.

Conversely, 17β-estradiol showed stronger binding to ERβ and VEGF, reinforcing its role in modulating hormone receptor pathways and angiogenesis. Its favorable interaction with ERβ supports its potential as a targeted agent in ERβ1-positive TNBC. Importantly, these findings have not been reported in prior TNBC docking studies, establishing a novel framework for dual ligand targeting. The complementary receptor engagement VDR/EGFR by calcitriol and ERβ/VEGF by 17β-estradiol suggests the feasibility of a combinatorial therapeutic strategy to address TNBC heterogeneity by simultaneously modulating genomic, non-genomic, proliferative, and angiogenic pathways.

The involvement of additional moieties such as N-acetylglucosamine (NAG) and structural water molecules (HOH) in EGFR docking further supports the realism and depth of ligand-receptor interactions modeled here. These interactions contribute

to binding pocket stability and orientation, which are often overlooked in simplified docking models.

These findings reinforce the hypothesis that a dual-compound therapeutic strategy may offer a multi-axis approach to managing ERβ1-positive TNBC and warrant further *in vitro* validation.

6.7 Mechanistic Interpretation of *In Vitro* Findings

Calcitriol and 17β-estradiol, as endogenous ligands for VDR and ERβ respectively, have garnered significant attention for their potential roles in cancer biology, particularly within hormone receptor-modulated pathways. In this study, their time-dependent effects on MDA-MB-468 TNBC cells were investigated in terms of cytotoxicity and modulation of key protein targets involved in proliferation, angiogenesis, and apoptosis. The observed responses reveal both individual and combined therapeutic potential, underscoring their relevance in ERβ1-positive TNBC subtypes.

5.7.1 Time-Dependent Cytotoxic Effects and Viability Modulation

The cytotoxic effects of calcitriol in MDA-MB-468 cells were shown to increase in a time-dependent manner, with the most prominent reductions in cell viability recorded at 16, 24, and 32 hours. This finding aligns with previous studies that demonstrated the antiproliferative properties of vitamin D in BC cell models. Bajbouj et al. showed a substantial decrease in cell viability in MDA-MB-231 cells following high-dose vitamin D exposure, highlighting vitamin D pro-apoptotic and anti-proliferative actions through both receptor-dependent and receptor-independent mechanisms.[426]

Similarly, 17β-estradiol induced a marked decline in cell viability over time, with statistically significant reductions observed from 16 to 32 hours. These effects correspond with earlier findings where MDA-MB-468 cells transfected with ERβ showed over 60% inhibition of cell proliferation upon exposure to 17β-estradiol.[427] The inhibition was attributed to ERβ-mediated suppression of oncogenic signaling, including c-Myc downregulation and altered cell cycle control. The current study reinforces this pathway, as 17β-estradiol appears to act via ERβ1-mediated repression of proliferative signals.

The combined treatment of calcitriol and 17β-estradiol exhibited an additive cytotoxic effect, resulting in enhanced reduction of cell viability at all examined time points. The observed combination likely reflects the complementary targeting of multiple signaling axes. While calcitriol predominantly modulates nuclear hormone receptor and growth factor pathways, 17β-estradiol strongly influences estrogen receptor-mediated non-genomic processes. This multi-target modulation strategy may provide a promising therapeutic approach in managing ERβ1-expressing TNBC.

6.7.2 ERβ1 Modulation and Hormonal Signaling Dynamics

ERβ1, a tumor suppressor isoform of ERβ, is of particular interest in TNBC. In the present study, calcitriol treatment led to a gradual, time-dependent reduction in ERβ1 expression, with significant downregulation observed between 24 and 32 hours. This downregulation likely represents a ligand-induced regulatory mechanism. Nuclear hormone receptors are well known to undergo ligand-mediated repression via transcriptional silencing, proteasomal degradation, or disruption of coactivator complexes.[362] Swami et al. demonstrated that calcitriol binding to VDR can inhibit ER signaling, either by recruiting transcriptional repressors or by modulating co-regulator recruitment at estrogen-responsive elements.[428] Thus, calcitriol may indirectly repress ERβ1 expression by altering nuclear receptor crosstalk or chromatin remodeling.

Interestingly, treatment with 17β-estradiol produced a biphasic modulation of ERβ1. Expression levels remained stable at 8 hours, followed by mild reduction at 16 hours and a significant decline at 24 and 32 hours. This pattern is characteristic of ligand-induced feedback regulation. Initial receptor stabilization through ligand binding is often followed by downregulation due to receptor internalization, degradation, or repression of receptor gene transcription. Reports by Jia et al. and Lazennec et al. highlight such temporal dynamics of ER regulation, where ligand binding triggers initial receptor activation and downstream signaling, which subsequently leads to self-regulatory feedback suppression.[429, 430] Therefore, the observed biphasic trend may represent a transition from receptor activation to proteolytic or transcriptional inhibition.

The complementary downregulation of ERβ1 by both calcitriol and 17β-estradiol suggests that these compounds converge on the regulation of ER pathways. Calcitriol appears to modulate ERβ1 expression indirectly via VDR-mediated signaling crosstalk, while 17β-estradiol regulates ERβ1 through ligand-dependent receptor turnover and feedback control.

6.7.3 EGFR Downregulation and Growth Signal Interference

The EGFR is frequently overexpressed in TNBC and contributes to tumor proliferation, invasion, and therapeutic resistance. In this study, calcitriol induced a pronounced, time-dependent suppression of EGFR expression, with significant reductions observed at 24 and 32 hours. These findings align with previous research indicating that vitamin D and its analogs downregulate EGFR expression, thereby suppressing PI3K/Akt and MAPK-driven proliferation in BC cells.[431] Mechanistically, calcitriol may achieve this repression by inhibiting EGFR gene transcription through VDR-RXR complex binding at EGFR promoter elements or by enhancing EGFR ubiquitination and lysosomal degradation.

Similarly, 17β-estradiol also induced a consistent reduction in EGFR levels, particularly evident at later time points. Khode et al. reported that 17β-estradiol treatment suppressed EGFR expression in MDA-MB-231 cells, suggesting that estrogen signaling may exert growth-inhibitory effects in ERβ1-positive TNBC by attenuating growth factor receptor signaling.[432] The regulation may be mediated by ERβ1 recruitment to transcriptional silencers at the EGFR promoter or through indirect effects on growth factor signal transduction cascades.

Combined treatment with both agents could enhance EGFR suppression through converging mechanisms: calcitriol acting via transcriptional inhibition and degradation, and 17β-estradiol attenuating transcription via ERβ1. This dual regulation may represent a viable therapeutic strategy to curb EGFR-driven progression in TNBC.

6.7.4 VEGF Suppression and Angiogenesis Inhibition

Angiogenesis, driven by VEGF, plays a crucial role in TNBC metastasis and tumor expansion. Calcitriol treatment significantly reduced VEGF expression in a time-dependent fashion, with marked suppression evident at 16 and 24 hours and sustained

reduction at 32 hours. This supports earlier findings where vitamin D was shown to inhibit hypoxia-inducible factor-1 (HIF-1) and VEGF transcription in BC models.[433] VDR activation likely interferes with HIF-1α binding to the VEGF promoter or promotes expression of anti-angiogenic factors such as thrombospondin.

Similarly, 17β-estradiol exerted a time-dependent suppressive effect on VEGF expression, with the most significant downregulation occurring between 16 and 24 hours. Although a further reduction was not statistically significant between 24 and 32 hours, VEGF levels remained consistently low, indicating a plateau in inhibitory response. This trend suggests that estrogen-mediated angiogenic regulation may occur via a temporally limited activation window, possibly governed by receptor saturation or downstream feedback inhibition. ERβ has been implicated in the transcriptional repression of VEGF in TNBC, acting either through direct promoter interaction or via inhibition of co-activator recruitment.[378]

The ability of both agents to independently downregulate VEGF highlights a significant anti-angiogenic potential. Their concurrent administration may augment VEGF suppression, potentially disrupting the tumor vasculature and impairing nutrient supply, thereby contributing to overall tumor regression.

6.7.5 Caspase-3 Modulation and Apoptotic Response

Caspase-3 is a central executioner of apoptosis, responsible for the cleavage of key structural and regulatory proteins during programmed cell death. In this study, calcitriol treatment resulted in a progressive, statistically significant decrease in caspase-3 expression from 8 to 32 hours, with additional suppression between 16 and 32 hours. This apparent downregulation may initially seem paradoxical; however, it likely reflects dynamic activation-degradation kinetics of caspase-3. Upon activation, caspase-3 undergoes autocatalysis, followed by proteasomal degradation once apoptosis is underway. Hence, the observed reduction in expression may correspond to the terminal stages of caspase-3 activity after pro-apoptotic signals have been executed.[342]

17β-estradiol also induced significant downregulation of caspase-3 between 24 and 32 hours, suggesting a delayed but definitive engagement of apoptotic pathways. Estrogen has been reported to influence both intrinsic and extrinsic apoptotic cascades in TNBC models, often through ERβ1-mediated transcriptional

regulation of pro-apoptotic and anti-apoptotic genes.[434] Estradiol may also indirectly modulate apoptotic machinery by affecting mitochondrial integrity and cytochrome c release, leading to caspase activation followed by downstream repression.[435]

The dual modulation of caspase-3 by calcitriol and 17β-estradiol may reflect their overlapping roles in triggering and regulating apoptotic processes. Early activation followed by degradation could signify effective induction of apoptosis and clearance of apoptotic proteins as cells undergo programmed death.

6.7.6 Integrated Mechanism and Therapeutic Relevance

The collective findings suggest that calcitriol and 17β-estradiol modulate multiple cellular pathways in a time-dependent manner to exert their anti-cancer effects in ERβ1-positive TNBC cells. Calcitriol exerts high-affinity interaction with VDR and strongly modulates EGFR and VEGF expression while regulating apoptosis via caspase-3. In parallel, 17β-estradiol preferentially targets ERβ1, downregulates EGFR and VEGF, and engages apoptotic regulation. Their combined application enhances cytotoxicity and downregulation of key oncogenic proteins, supporting the hypothesis of a complementary and potentially interactive effect.

From a translational perspective, these findings advocate for further exploration of combined vitamin D and ER modulation as a therapeutic avenue for TNBC patients exhibiting ERβ1 expression. The study also establishes temporal benchmarks for the maximal response to each agent, guiding dosing and scheduling strategies for future preclinical or clinical trials.

6.8 PROPOSED MECHANISM OF ACTION OF CALCITRIOL AND 17β-ESTRADIOL IN ERβ1-POSITIVE TNBC CELLS

The schematic illustrates the complementary and multi-targeted mechanism through which calcitriol and 17β-estradiol (E2) modulate cellular pathways involved in TNBC pathogenesis. These pathways influence proliferation, apoptosis, angiogenesis, and metastatic potential via both genomic and non-genomic actions.

6.8.1 Calcitriol Mechanism via VDR Signaling

Calcitriol binds to the VDR, which upon activation forms a heterodimer with the RXR. This VDR-RXR complex translocates to the nucleus and binds to VDREs on

target gene promoters. This transcriptional regulation results in DNA damage response activation and the expression of genes responsible for apoptosis, reduced proliferation, decreased survival, and suppressed metastasis, as shown in Figure 38. Calcitriol also interacts with membrane-bound receptors and intracellular pathways that indirectly modulate other signaling molecules such as EGFR and VEGF. In this context, calcitriol downregulates EGFR (a key driver of proliferation) and VEGF (a central mediator of angiogenesis), thereby suppressing tumor growth and vascular support.

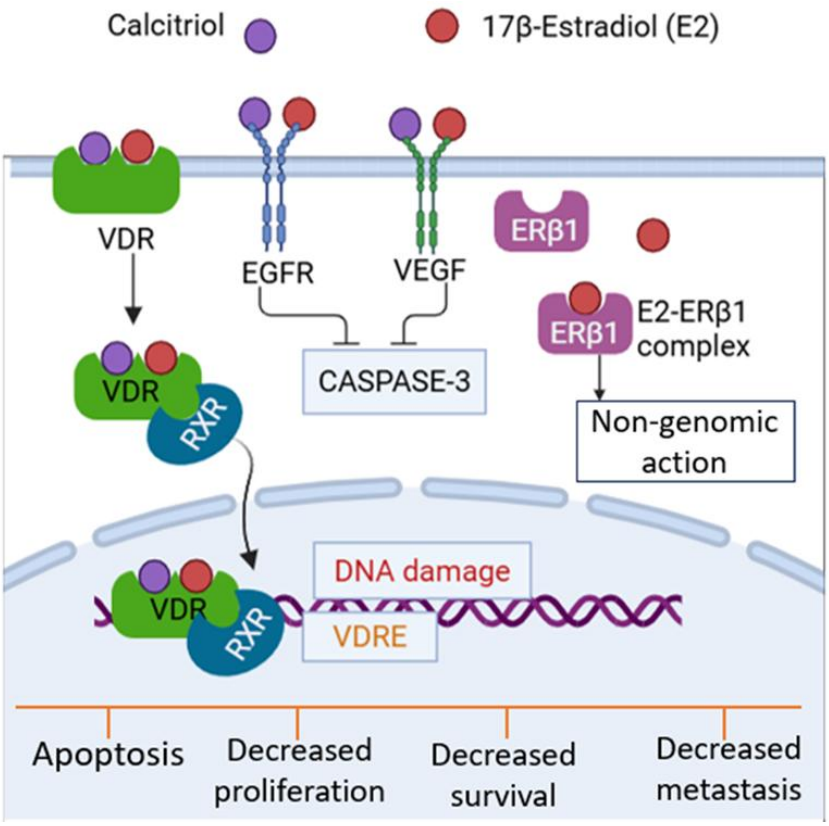


Figure 38. Proposed mechanism of VDR and ERβ1-mediated regulation in TNBC.

Calcitriol binds to the VDR, which forms a heterodimer with the RXR and translocates to the nucleus, where it binds to the VDRE to regulate the expression of genes involved in apoptosis, proliferation, and cell survival. Simultaneously, 17β-estradiol interacts with ERβ1, promoting non-genomic signaling pathways. The combined downregulation of ERβ1, EGFR, VEGF, and caspase-3 contributes to decreased proliferation, metastasis, and survival, while promoting apoptosis in TNBC cells.

Abbreviations: VDR, vitamin D receptor; RXR, retinoid X receptor; VDRE, vitamin D response element; ERβ1, estrogen receptor beta 1.

6.8.2 17β-Estradiol Mechanism via ERβ1 Activation

17β-estradiol binds specifically to ERβ1, forming the E2–ERβ1 complex. This complex, localized in the cytoplasm, primarily mediates non-genomic signaling, as illustrated in Figure 38. It rapidly modulates intracellular signaling cascades without directly altering gene transcription, often affecting kinase activation, apoptosis regulation, and cross-talk with growth factor signaling pathways. The non-genomic effects can suppress cell proliferation and support differentiation or cell death, depending on cellular context.

5.8.3 Convergent Effects on Downstream Targets

Both VDR and ERβ1 pathways converge on critical downstream effectors, particularly caspase-3, which is a central executioner in the apoptotic cascade. The combined treatment results in reduced expression of EGFR, VEGF, and caspase-3, indicating suppression of survival and angiogenic signaling along with potential apoptotic regulation.

6.8.4 Therapeutic Implications

This dual targeting strategy, activating VDR through calcitriol and modulating ERβ1 through 17β-estradiol, leverages both genomic and non-genomic signaling mechanisms. It leads to coordinated suppression of tumor-promoting pathways and enhances anti-tumor responses. These findings support the rationale for using hormone-based combination therapy in ERβ1-positive TNBC to achieve multi-pathway inhibition and durable therapeutic efficacy.

CONCLUSION

This study reveals significant variations in the expression patterns of VDR and ERβ1 across TNBC subtypes. The predominance of cytoplasmic VDR expression in TNBC, accompanied by limited nuclear localization, suggests a potentially distinct functional role for VDR in this aggressive phenotype. Similarly, the exclusive cytoplasmic localization of ERβ1 in both TNBC and luminal A tumors indicates subtype-specific differences in receptor signaling and regulation. These findings underscore the importance of investigating the spatial dynamics of receptor expression as a potential determinant of tumor behavior and therapeutic responsiveness.

In parallel, the *in vitro* analysis demonstrated that calcitriol and 17β-estradiol modulate the expression of ERβ1, EGFR, VEGF, and caspase-3 in a time-dependent manner. This regulation points to their involvement in key oncogenic pathways governing proliferation, angiogenesis, and apoptosis in ERβ1-positive TNBC. Notably, the combination treatment exhibited potential additive effects, supporting its relevance as a therapeutic strategy for dual-target modulation.

Taken together, these findings provide preliminary evidence for the therapeutic relevance of co-targeting VDR and ERβ1 in TNBC.

LIMITATIONS

The present study did not include *in vivo* experiments, which limits the translational validation of the observed in vitro effects of calcitriol and 17β-estradiol in ERβ1-positive TNBC.

FUTURE PERSPECTIVES

Future studies should aim to further elucidate the molecular pathways underlying these receptor interactions, particularly those involved in hormone receptor signaling and downstream effector cascades. Additionally, to validate and extend these observations, larger multicenter clinical trials are warranted to evaluate VDR and ERβ1 expression across diverse demographic groups and clinical settings.

Preclinical validation using appropriate TNBC xenograft models will also be essential to assess the translational potential of hormone-based combination therapies in ERβ1-positive TNBC.

SUMMARY

SUMMARY

TNBC, characterized by the absence of ER, PR, and HER2, remains one of the most aggressive and therapeutically challenging breast cancer subtypes. Due to its poor prognosis and lack of targeted therapies, identifying novel molecular targets is a clinical imperative. This thesis explores the biological significance and therapeutic potential of VDR and ERβ1 in TNBC, using an integrative approach combining IHC, molecular docking, and *in vitro* experiments.

IHC analysis was conducted to investigate the differential expression and subcellular localization of VDR and ERβ1 across various BC subtypes. Notably, TNBC tissues showed predominant cytoplasmic VDR expression with partial nuclear localization, whereas luminal A tumors exhibited lower expression with limited nuclear involvement. Luminal B tumors demonstrated exclusive cytoplasmic staining, and HER2-enriched tumors displayed only nuclear localization. These subtype-specific patterns suggest that VDR may function through both genomic and non-genomic mechanisms in TNBC, in contrast to the more compartmentalized signaling in other subtypes. The statistical analysis confirmed significant variation in VDR localization across subtypes ($p < 0.042$), highlighting its potential role in tumor-specific signaling dynamics.

Similarly, ERβ1 expression in TNBC and luminal A tumors revealed exclusive cytoplasmic localization, with TNBC cases predominantly exhibiting moderate expression and luminal A tumors showing higher intensity. Nuclear ERβ1 staining was absent in all cases. This differential cytoplasmic expression pattern, significant between TNBC and luminal A tumors ($p < 0.025$), underscores ERβ1 potential non-genomic role and its suitability as a subtype-specific molecular marker. The findings suggest that ERβ1, though traditionally regarded as a tumor suppressor, may participate in alternative signaling pathways when localized in the cytoplasm, particularly in ERβ1 positive TNBC.

To explore the binding interactions of calcitriol and 17β-estradiol with key molecular targets, molecular docking studies were performed. Calcitriol exhibited strong binding affinity to VDR (−9.15 kcal/mol) and EGFR (−10.04 kcal/mol), indicating potential regulation of receptor-mediated signaling and cell proliferation pathways. In contrast, 17β-estradiol showed high affinity for ERβ (−9.08 kcal/mol) and VEGF (−7.06 kcal/mol), suggesting a role in modulating hormonal and angiogenic signaling. These *in silico* interactions support the hypothesis that calcitriol

and 17β-estradiol act on complementary pathways, with possible synergy when used in combination. These findings provide a molecular rationale for co-targeting VDR and ERβ1 in TNBC management.

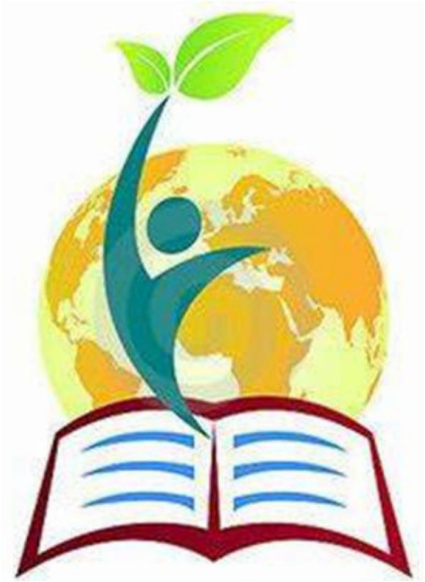
Further, *in vitro* assays using MDA-MB-468 cells assessed the cytotoxic effects of calcitriol and 17β-estradiol, both individually and in combination. MTT assays demonstrated time- and dose-dependent reductions in cell viability, with the combination treatment producing the most pronounced inhibitory effect, especially between 24 and 32 hours. This additive cytotoxicity suggests enhanced suppression of oncogenic pathways when both ligands are applied together, likely due to dual activation of VDR and ERβ1 signaling axes.

To delineate the downstream molecular mechanisms, immunoblot analysis was conducted to measure the expression of ERβ1, EGFR, VEGF, and caspase-3 following treatment. Calcitriol significantly suppressed ERβ1, EGFR, and VEGF in a time-dependent manner, reinforcing its role in repressing tumor-promoting pathways. Notably, caspase-3 levels also declined over time, possibly reflecting activation-consumption during apoptosis or regulatory feedback mechanisms.

Treatment with 17β-estradiol showed a complex expression profile, with early downregulation of ERβ1 followed by reactivation at 32 hours, indicative of receptor recycling or compensatory feedback. EGFR and VEGF levels consistently declined, while caspase-3 expression was progressively suppressed. These results suggest that 17β-estradiol may modulate apoptosis and angiogenesis via ERβ1-mediated pathways.

The combination treatment produced the most robust and sustained suppression of all four markers. ERβ1 expression declined consistently, EGFR and VEGF were markedly downregulated, and caspase-3 showed sustained inhibition. The enhanced effect of the combination treatment confirms the advantage of dual-targeting VDR and ERβ1 for simultaneously disrupting proliferation, angiogenesis, and apoptotic regulation in ERβ1-positive TNBC.

Overall, this study identifies distinct patterns of VDR and ERβ1 expression in BC subtypes, particularly TNBC, and establishes the molecular basis for their functional involvement in tumor biology. The findings provide compelling evidence that co-targeting these receptors using calcitriol and 17β-estradiol holds therapeutic promise.



BIBLIOGRAPHY

BIBLIOGRAPHY

1. Tong Y, Ning H, Zhang Z, Zhang X, Tu H, Yang M, et al. Global burden of disease changes related to high red meat diets and breast cancer from 1990 to 2021 and its prediction up to 2030. Front Nutr. 2025;12. Available from: http://dx.doi.org/10.3389/fnut.2025.1586299

2. Deng T, Zi H, Guo X-P, Luo L-S, Yang Y-L, Hou J-X, et al. Global, regional, and national burden of breast cancer, 1990-2021, and projections to 2050: A systematic analysis of the Global Burden of disease study 2021. Thorac Cancer. 2025;16(9):e70052. Available from: http://dx.doi.org/10.1111/1759-7714.70052

3. Yang Y-CSH, Li Z-L, Huang T-Y, Su K-W, Lin C-Y, Huang C-H, et al. Effect of estrogen on heteronemin-induced anti-proliferative effect in breast cancer cells with different estrogen receptor status. Front Cell Dev Biol. 2021;9:688607. Available from: http://dx.doi.org/10.3389/fcell.2021.688607

4. Makki J. Diversity of breast carcinoma: Histological subtypes and clinical relevance. Clin Med Insights Pathol. 2015;8. Available from: http://dx.doi.org/10.4137/cpath.s31563

5. Radu I, Scripcariu V, Panuța A, Rusu A, Afrăsânie V-A, Cojocaru E, et al. Breast sarcomas-how different are they from breast carcinomas? Clinical, pathological, imaging and treatment insights. Diagnostics (Basel). 2023;13(8):1370. Available from: http://dx.doi.org/10.3390/diagnostics13081370

6. Xiong X, Zheng L-W, Ding Y, Chen Y-F, Cai Y-W, Wang L-P, et al. Breast cancer: pathogenesis and treatments. Signal Transduct Target Ther. 2025;10(1):49. Available from: http://dx.doi.org/10.1038/s41392-024-02108-4

7. Xiao W, Zheng S, Yang A, Zhang X, Zou Y, Tang H, et al. Breast cancer subtypes and the risk of distant metastasis at initial diagnosis: a population-based study. Cancer Manag Res. 2018;10:5329–38. Available from: http://dx.doi.org/10.2147/CMAR.S176763

8. Ignatov A, Eggemann H, Burger E, Ignatov T. Patterns of breast cancer relapse in accordance to biological subtype. J Cancer Res Clin Oncol. 2018;144(7):1347–55. Available from: http://dx.doi.org/10.1007/s00432-018-2644-2

9. Obidiro O, Battogtokh G, Akala EO. Triple negative breast cancer treatment options and limitations: Future outlook. Pharmaceutics. 2023;15(7):1796. Available from: http://dx.doi.org/10.3390/pharmaceutics15071796

10. Yin L, Duan J-J, Bian X-W, Yu S-C. Triple-negative breast cancer molecular subtyping and treatment progress. Breast Cancer Res. 2020;22(1):61. Available from: http://dx.doi.org/10.1186/s13058-020-01296-5

11. Carlberg C, Muñoz A. An update on vitamin D signaling and cancer. Semin Cancer Biol. 2022;79:217–30. Available from: http://dx.doi.org/10.1016/j.semcancer.2020.05.018

12. Pike JW, Meyer MB, Lee S-M, Onal M, Benkusky NA. The vitamin D receptor: contemporary genomic approaches reveal new basic and translational insights. J Clin Invest. 2017;127(4):1146–54. Available from: http://dx.doi.org/10.1172/jci88887

13. Pike JW, Meyer MB. The vitamin D receptor: New paradigms for the regulation of gene expression by 1,25-dihydroxyvitamin D3. Endocrinol Metab Clin North Am. 2010;39(2):255–69. Available from: http://dx.doi.org/10.1016/j.ecl.2010.02.007

14. LaPorta E, Welsh J. Modeling vitamin D actions in triple negative/basal-like breast cancer. J Steroid Biochem Mol Biol. 2014;144:65–73. Available from: http://dx.doi.org/10.1016/j.jsbmb.2013.10.022

15. Blasiak J, Pawlowska E, Chojnacki J, Szczepanska J, Fila M, Chojnacki C. Vitamin D in triple-negative and BRCA1-deficient breast cancer-implications for pathogenesis and therapy. Int J Mol Sci. 2020;21(10):3670. Available from: http://dx.doi.org/10.3390/ijms21103670

16. Al-Azhri J, Zhang Y, Bshara W, Zirpoli G, McCann SE, Khoury T, et al. Tumor expression of vitamin D receptor and breast cancer histopathological characteristics and prognosis. Clin Cancer Res. 2017;23(1):97–103. Available from: http://dx.doi.org/10.1158/1078-0432.CCR-16-0075

17. Huss L, Butt ST, Borgquist S, Elebro K, Sandsveden M, Rosendahl A, et al. Vitamin D receptor expression in invasive breast tumors and breast cancer survival. Breast Cancer Res. 2019;21(1):84. Available from: http://dx.doi.org/10.1186/s13058-019-1169-1

18. Berger U, McClelland RA, Wilson P, Greene GL, Haussler MR, Pike JW, et al. Immunocytochemical determination of estrogen receptor, progesterone receptor, and 1,25-dihydroxyvitamin D3 receptor in breast cancer and relationship to prognosis. Cancer Res. 1991;51(1):239–44.

19. Thabet RH, Gomaa AA, Matalqah LM, Shalaby EM. Vitamin D: an essential adjuvant therapeutic agent in breast cancer. J Int Med Res. 2022;50(7):3000605221113800. Available from: http://dx.doi.org/10.1177/03000605221113800

20. Mosselman S, Polman J, Dijkema R. ER beta: identification and characterization of a novel human estrogen receptor. FEBS Lett. 1996;392(1):49–53. Available from: http://dx.doi.org/10.1016/0014-5793(96)00782-x

21. Sellitto A, D'Agostino Y, Alexandrova E, Lamberti J, Pecoraro G, Memoli D, et al. Insights into the role of estrogen receptor β in triple-negative breast cancer. Cancers (Basel). 2020;12(6):1477. Available from: http://dx.doi.org/10.3390/cancers12061477

22. Monaco A, Licitra F, Di Gisi M, Galasso G, Di Donato M, Giovannelli P, et al. ERβ in triple-negative breast cancer: Emerging concepts and therapeutic possibilities. Endocrines. 2021;2(3):356–65. Available from: http://dx.doi.org/10.3390/endocrines2030033

23. Treeck O, Schüler-Toprak S, Ortmann O. Estrogen actions in triple-negative breast cancer. Cells. 2020;9(11):2358. Available from: http://dx.doi.org/10.3390/cells9112358

24. Shanle EK, Zhao Z, Hawse J, Wisinski K, Keles S, Yuan M, et al. Research resource: global identification of estrogen receptor β target genes in triple negative breast cancer cells. Mol Endocrinol. 2013;27(10):1762–75. Available from: http://dx.doi.org/10.1210/me.2013-1164

25. Yang T, Li W, Huang T, Zhou J. Immunotherapy targeting PD-1/PD-L1 in early-stage triple-negative breast cancer. J Pers Med. 2023;13(3). Available from: http://dx.doi.org/10.3390/jpm13030526

26. Dey P, Wang A, Ziegler Y, Kumar S, Yan S, Kim SH, et al. Estrogen receptor beta 1: A potential therapeutic target for female triple negative breast cancer. Endocrinology. 2022;163(12). Available from: http://dx.doi.org/10.1210/endocr/bqac172

27. Reese JM, Bruinsma ES, Monroe DG, Negron V, Suman VJ, Ingle JN, et al. ERβ inhibits cyclin dependent kinases 1 and 7 in triple negative breast cancer. Oncotarget. 2017;8(57):96506–21. Available from: http://dx.doi.org/10.18632/oncotarget.21787

28. Aspros KGM, Emch MJ, Wang X, Subramaniam M, Hinkle ML, Rodman EPB, et al. Disruption of estrogen receptor beta's DNA binding domain impairs its tumor suppressive effects in triple negative breast cancer. Front Med (Lausanne). 2023;10:1047166. Available from: http://dx.doi.org/10.3389/fmed.2023.1047166

29. Revankar CM, Bologa CG, Pepermans RA, Sharma G, Petrie WK, Alcon SN, et al. A selective ligand for estrogen receptor proteins discriminates rapid and genomic signaling. Cell Chem Biol. 2019;26(12):1692-1702.e5. Available from: http://dx.doi.org/10.1016/j.chembiol.2019.10.009

30. Aspros KGM, Carter JM, Hoskin TL, Suman VJ, Subramaniam M, Emch MJ, et al. Estrogen receptor beta repurposes EZH2 to suppress oncogenic

NFκB/p65 signaling in triple negative breast cancer. NPJ Breast Cancer. 2022;8(1). Available from: http://dx.doi.org/10.1038/s41523-022-00387-0

31. Leon-Ferre RA, Goetz MP. Advances in systemic therapies for triple negative breast cancer. BMJ. 2023;381:e071674. Available from: http://dx.doi.org/10.1136/bmj-2022-071674

32. Biswas SK, Banerjee S, Baker GW, Kuo C-Y, Chowdhury I. The mammary gland: Basic structure and molecular signaling during development. Int J Mol Sci. 2022;23(7):3883. Available from: http://dx.doi.org/10.3390/ijms23073883

33. Hall MI, Suarez-Venot A, Lindvall T, Plochocki JH, Grossman A, Rodriguez-Sosa JR, et al. A reinterpretation of human breast anatomy includes all the layers of the anterior body wall. Anat Rec (Hoboken). 2024;307(11):3564–73. Available from: http://dx.doi.org/10.1002/ar.25456

34. Gupta M, Goyal N. Applied anatomy of breast cancer. In: Breast Cancer. Singapore: Springer Nature Singapore; 2022. p. 23–35.

35. Natale G, Stouthandel MEJ, Van Hoof T, Bocci G. The lymphatic system in breast cancer: Anatomical and molecular approaches. Medicina (Kaunas). 2021;57(11):1272. Available from: http://dx.doi.org/10.3390/medicina57111272

36. Leong SP, Pissas A, Scarato M, Gallon F, Pissas MH, Amore M, et al. The lymphatic system and sentinel lymph nodes: conduit for cancer metastasis. Clin Exp Metastasis. 2022;39(1):139–57. Available from: http://dx.doi.org/10.1007/s10585-021-10123-w

37. Malherbe F, Nel D, Molabe H, Cairncross L, Roodt L. Palpable breast lumps: An age-based approach to evaluation and diagnosis. S Afr Fam Pract (2004). 2022;64(1):e1–5. Available from: http://dx.doi.org/10.4102/safp.v64i1.5571

38. Sosnowska-Sienkiewicz P, Januszkiewicz-Lewandowska D, Mańkowski P. Benign and malignant breast lesions in children and adolescents - diagnostic and therapeutic approach. Front Pediatr. 2024;12:1417050. Available from: http://dx.doi.org/10.3389/fped.2024.1417050

39. Guo L, Kong D, Liu J, Zhan L, Luo L, Zheng W, et al. Breast cancer heterogeneity and its implication in personalized precision therapy. Exp Hematol Oncol. 2023;12(1):3. Available from: http://dx.doi.org/10.1186/s40164-022-00363-1

40. Marcu A, Black G, Vedsted P, Lyratzopoulos G, Whitaker KL. Educational differences in responses to breast cancer symptoms: A qualitative comparative study. Br J Health Psychol. 2017;22(1):26–41. Available from: http://dx.doi.org/10.1111/bjhp.12215

41. Winters S, Martin C, Murphy D, Shokar NK. Breast cancer epidemiology, prevention, and screening. Prog Mol Biol Transl Sci. 2017;151:1–32. Available from: http://dx.doi.org/10.1016/bs.pmbts.2017.07.002

42. Breast cancer. Mayo Clinic. [cited 2025 Jun 16]. Available from: http://mayoclinic.org/diseases-conditions/breast-cancer/symptoms-causes/syc-20352470

43. Sex hormones and risk of breast cancer in premenopausal women: a collaborative reanalysis of individual participant data from seven prospective studies. Lancet Oncol. 2013;14(10):1009–19. Available from: http://dx.doi.org/10.1016/s1470-2045(13)70301-2

44. Giordano SH. Breast cancer in men. N Engl J Med. 2018;378(24):2311–20. Available from: http://dx.doi.org/10.1056/NEJMra1707939

45. Łukasiewicz S, Czeczelewski M, Forma A, Baj J, Sitarz R, Stanisławek A. Breast cancer-epidemiology, risk factors, classification, prognostic markers, and current treatment strategies-an updated review. Cancers (Basel). 2021;13(17):4287. Available from: http://dx.doi.org/10.3390/cancers13174287

46. McGuire A, Brown JAL, Malone C, McLaughlin R, Kerin MJ. Effects of age on the detection and management of breast cancer. Cancers (Basel). 2015;7(2):908–29. Available from: http://dx.doi.org/10.3390/cancers7020815

47. Shiyanbola OO, Arao RF, Miglioretti DL, Sprague BL, Hampton JM, Stout NK, et al. Emerging trends in family history of breast cancer and associated risk. Cancer Epidemiol Biomarkers Prev. 2017;26(12):1753–60. Available from: http://dx.doi.org/10.1158/1055-9965.EPI-17-0531

48. Wu H-C, Do C, Andrulis IL, John EM, Daly MB, Buys SS, et al. Breast cancer family history and allele-specific DNA methylation in the legacy girls study. Epigenetics. 2018;13(3):240–50. Available from: http://dx.doi.org/10.1080/15592294.2018.1435243

49. Shiovitz S, Korde LA. Genetics of breast cancer: a topic in evolution. Ann Oncol. 2015;26(7):1291–9. Available from: http://dx.doi.org/10.1093/annonc/mdv022

50. Shahbandi A, Nguyen HD, Jackson JG. TP53 mutations and outcomes in breast cancer: Reading beyond the headlines. Trends Cancer. 2020;6(2):98–110. Available from: http://dx.doi.org/10.1016/j.trecan.2020.01.007

51. Corso G, Veronesi P, Sacchini V, Galimberti V. Prognosis and outcome in CDH1-mutant lobular breast cancer. Eur J Cancer Prev. 2018;27(3):237–8. Available from: http://dx.doi.org/10.1097/cej.0000000000000405

52. Kechagioglou P, Papi RM, Provatopoulou X, Kalogera E, Papadimitriou E, Grigoropoulos P, et al. Tumor suppressor PTEN in breast cancer: heterozygosity, mutations and protein expression. Anticancer Res. 2014;34(3):1387–400.

53. Chen J, Lindblom A. Germline mutation screening of the STK11/LKB1 gene in familial breast cancer with LOH on 19p. Clin Genet. 2000;57(5):394–7. Available from: http://dx.doi.org/10.1034/j.1399-0004.2000.570511.x

54. Park DJ, Lesueur F, Nguyen-Dumont T, Pertesi M, Odefrey F, Hammet F, et al. Rare mutations in XRCC2 increase the risk of breast cancer. Am J Hum Genet. 2012;90(4):734–9. Available from: http://dx.doi.org/10.1016/j.ajhg.2012.02.027

55. Hill DA, Prossnitz ER, Royce M, Nibbe A. Temporal trends in breast cancer survival by race and ethnicity: A population-based cohort study. PLoS One. 2019;14(10):e0224064. Available from: http://dx.doi.org/10.1371/journal.pone.0224064

56. Bernstein L. Epidemiology of endocrine-related risk factors for breast cancer. J Mammary Gland Biol Neoplasia. 2002;7(1):3–15. Available from: http://dx.doi.org/10.1023/a:1015714305420

57. Stordal B. Breastfeeding reduces the risk of breast cancer: A call for action in high-income countries with low rates of breastfeeding. Cancer Med. 2023;12(4):4616–25. Available from: http://dx.doi.org/10.1002/cam4.5288

58. Reeves GK, Kan S-W, Key T, Tjønneland A, Olsen A, Overvad K, et al. Breast cancer risk in relation to abortion: Results from the EPIC study. Int J Cancer. 2006;119(7):1741–5. Available from: http://dx.doi.org/10.1002/ijc.22001

59. Kim EY, Chang Y, Ahn J, Yun J-S, Park YL, Park CH, et al. Mammographic breast density, its changes, and breast cancer risk in premenopausal and postmenopausal women. Cancer. 2020;126(21):4687–96. Available from: http://dx.doi.org/10.1002/cncr.33138

60. Duffy SW, Morrish OWE, Allgood PC, Black R, Gillan MGC, Willsher P, et al. Mammographic density and breast cancer risk in breast screening assessment cases and women with a family history of breast cancer. Eur J Cancer. 2018;88:48–56. Available from: http://dx.doi.org/10.1016/j.ejca.2017.10.022

61. Hartmann LC, Sellers TA, Frost MH, Lingle WL, Degnim AC, Ghosh K, et al. Benign breast disease and the risk of breast cancer. N Engl J Med. 2005;353(3):229–37. Available from: http://dx.doi.org/10.1056/NEJMoa044383

62. Ng J, Shuryak I. Minimizing second cancer risk following radiotherapy: current perspectives. Cancer Manag Res. 2015;7:1–11. Available from: http://dx.doi.org/10.2147/CMAR.S47220

63. Zhang Q, Liu J, Ao N, Yu H, Peng Y, Ou L, et al. Secondary cancer risk after radiation therapy for breast cancer with different radiotherapy techniques. Sci Rep. 2020;10(1):1220. Available from: http://dx.doi.org/10.1038/s41598-020-58134-z

64. Hilakivi-Clarke L. Maternal exposure to diethylstilbestrol during pregnancy and increased breast cancer risk in daughters. Breast Cancer Res. 2014;16(2):208. Available from: http://dx.doi.org/10.1186/bcr3649

65. Vinogradova Y, Coupland C, Hippisley-Cox J. Use of hormone replacement therapy and risk of breast cancer: nested case-control studies using the QResearch and CPRD databases. BMJ. 2020;371:m3873. Available from: http://dx.doi.org/10.1136/bmj.m3873

66. Steingart A, Cotterchio M, Kreiger N, Sloan M. Antidepressant medication use and breast cancer risk: a case-control study. Int J Epidemiol. 2003;32(6):961–6. Available from: http://dx.doi.org/10.1093/ije/dyg155

67. Chen X, Wang Q, Zhang Y, Xie Q, Tan X. Physical activity and risk of breast cancer: A meta-analysis of 38 cohort studies in 45 study reports. Value Health. 2019;22(1):104–28. Available from: http://dx.doi.org/10.1016/j.jval.2018.06.020

68. Thune I, Brenn T, Lund E, Gaard M. Physical activity and the risk of breast cancer. N Engl J Med. 1997;336(18):1269–75. Available from: http://dx.doi.org/10.1056/NEJM199705013361801

69. Kolb R, Zhang W. Obesity and breast cancer: A case of inflamed adipose tissue. Cancers (Basel). 2020;12(6):1686. Available from: http://dx.doi.org/10.3390/cancers12061686

70. Sun L, Zhu Y, Qian Q, Tang L. Body mass index and prognosis of breast cancer: An analysis by menstruation status when breast cancer diagnosis. Medicine (Baltimore). 2018;97(26):e11220. Available from: http://dx.doi.org/10.1097/MD.0000000000011220

71. Erol A, Ho AM-C, Winham SJ, Karpyak VM. Sex hormones in alcohol consumption: a systematic review of evidence: Sex hormones in alcoholism. Addict Biol. 2019;24(2):157–69. Available from: http://dx.doi.org/10.1111/adb.12589

72. Liu Y, Nguyen N, Colditz GA. Links between alcohol consumption and breast cancer: a look at the evidence. Womens Health (Lond Engl). 2015;11(1):65–77. Available from: http://dx.doi.org/10.2217/whe.14.62

73. Zeinomar N, Knight JA, Genkinger JM, Phillips K-A, Daly MB, Milne RL, et al. Alcohol consumption, cigarette smoking, and familial breast cancer risk: findings from the Prospective Family Study Cohort (ProF-SC). Breast Cancer Res. 2019;21(1):128. Available from: http://dx.doi.org/10.1186/s13058-019-1213-1

74. Jones ME, Schoemaker MJ, Wright LB, Ashworth A, Swerdlow AJ. Smoking and risk of breast cancer in the Generations Study cohort. Breast Cancer Res. 2017;19(1):118. Available from: http://dx.doi.org/10.1186/s13058-017-0908-4

75. Misotti AM, Gnagnarella P. Vitamin supplement consumption and breast cancer risk: a review. Ecancermedicalscience. 2013;7:365. Available from: http://dx.doi.org/10.3332/ecancer.2013.365

76. El-Sharkawy A, Malki A. Vitamin D signaling in inflammation and cancer: Molecular mechanisms and therapeutic implications. Molecules. 2020;25(14):3219. Available from: http://dx.doi.org/10.3390/molecules25143219

77. Atoum M, Alzoughool F. Vitamin D and breast cancer: Latest evidence and future steps. Breast Cancer (Auckl). 2017;11:1178223417749816. Available from: http://dx.doi.org/10.1177/1178223417749816

78. Zhou L, Chen B, Sheng L, Turner A. The effect of vitamin D supplementation on the risk of breast cancer: a trial sequential meta-analysis. Breast Cancer Res Treat. 2020;182(1):1–8. Available from: http://dx.doi.org/10.1007/s10549-020-05669-4

79. Johns LE, Jones ME, Schoemaker MJ, McFadden E, Ashworth A, Swerdlow AJ. Domestic light at night and breast cancer risk: a prospective analysis of 105 000 UK women in the Generations Study. Br J Cancer. 2018;118(4):600–6. Available from: http://dx.doi.org/10.1038/bjc.2017.359

80. Dandamudi A, Tommie J, Nommsen-Rivers L, Couch S. Dietary patterns and breast cancer risk: A systematic review. Anticancer Res. 2018;38(6):3209–22. Available from: http://dx.doi.org/10.21873/anticanres.12586

81. Castelló A, Pollán M, Buijsse B, Ruiz A, Casas AM, Baena-Cañada JM, et al. Spanish Mediterranean diet and other dietary patterns and breast cancer risk: case-control EpiGEICAM study. Br J Cancer. 2014;111(7):1454–62. Available from: http://dx.doi.org/10.1038/bjc.2014.434

82. Videnros C, Selander J, Wiebert P, Albin M, Plato N, Borgquist S, et al. Investigating the risk of breast cancer among women exposed to chemicals: a nested case-control study using improved exposure estimates. Int Arch Occup Environ Health. 2020;93(2):261–9. Available from: http://dx.doi.org/10.1007/s00420-019-01479-4

83. Cserni G. Histological type and typing of breast carcinomas and the WHO classification changes over time. Pathologica. 2020;112(1):25–41. Available from: http://dx.doi.org/10.32074/1591-951X-1-20

84. Weigelt B, Horlings HM, Kreike B, Hayes MM, Hauptmann M, Wessels LFA, et al. Refinement of breast cancer classification by molecular characterization of histological special types. J Pathol. 2008;216(2):141–50. Available from: http://dx.doi.org/10.1002/path.2407

85. Erber R, Hartmann A. Histology of luminal breast cancer. Breast Care (Basel). 2020;15(4):327–36. Available from: http://dx.doi.org/10.1159/000509025

86. Perou CM, Sørlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. Nature. 2000;406(6797):747–52. Available from: http://dx.doi.org/10.1038/35021093

87. Sørlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A. 2001;98(19):10869–74. Available from: http://dx.doi.org/10.1073/pnas.191367098

88. Prat A, Perou CM. Deconstructing the molecular portraits of breast cancer. Mol Oncol. 2011;5(1):5–23. Available from: http://dx.doi.org/10.1016/j.molonc.2010.11.003

89. The Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. Nature. 2012;490(7418):61–70. Available from: http://dx.doi.org/10.1038/nature11412

90. Herschkowitz JI, Simin K, Weigman VJ, Mikaelian I, Usary J, Hu Z, et al. Identification of conserved gene expression features between murine mammary carcinoma models and human breast tumors. Genome Biol. 2007;8(5):R76. Available from: http://dx.doi.org/10.1186/gb-2007-8-5-r76

91. Parker JS, Mullins M, Cheang MCU, Leung S, Voduc D, Vickery T, et al. Supervised risk predictor of breast cancer based on intrinsic subtypes. J Clin Oncol. 2009;27(8):1160–7. Available from: http://dx.doi.org/10.1200/JCO.2008.18.1370

92. Gnant M, Filipits M, Greil R, Stoeger H, Rudas M, Bago-Horvath Z, et al. Predicting distant recurrence in receptor-positive breast cancer patients with limited clinicopathological risk: using the PAM50 Risk of Recurrence score in 1478 postmenopausal patients of the ABCSG-8 trial treated with adjuvant endocrine therapy alone. Ann Oncol. 2014;25(2):339–45. Available from: http://dx.doi.org/10.1093/annonc/mdt494

93. Sestak I, Cuzick J, Dowsett M, Lopez-Knowles E, Filipits M, Dubsky P, et al. Prediction of late distant recurrence after 5 years of endocrine treatment: a combined analysis of patients from the Austrian breast and colorectal cancer study group 8 and arimidex, tamoxifen alone or in combination randomized trials using the PAM50 risk of recurrence score. J Clin Oncol. 2015;33(8):916–22. Available from: http://dx.doi.org/10.1200/JCO.2014.55.6894

94. Prat A, Galván P, Jimenez B, Buckingham W, Jeiranian HA, Schaper C, et al. Prediction of response to neoadjuvant chemotherapy using core needle biopsy samples with the Prosigna assay. Clin Cancer Res. 2016;22(3):560–6. Available from: http://dx.doi.org/10.1158/1078-0432.CCR-15-0630

95. Howlader N, Altekruse SF, Li CI, Chen VW, Clarke CA, Ries LAG, et al. US incidence of breast cancer subtypes defined by joint hormone receptor and HER2 status. J Natl Cancer Inst. 2014;106(5). Available from: http://dx.doi.org/10.1093/jnci/dju055

96. Weigelt B, Geyer FC, Reis-Filho JS. Histological types of breast cancer: how special are they? Mol Oncol. 2010;4(3):192–208. Available from: http://dx.doi.org/10.1016/j.molonc.2010.04.004

97. Weigelt B, Baehner FL, Reis-Filho JS. The contribution of gene expression profiling to breast cancer classification, prognostication and prediction: a retrospective of the last decade: A commentary on microarrays in breast cancer research. J Pathol. 2010;220(2):263–80. Available from: http://dx.doi.org/10.1002/path.2648

98. Prat A, Cheang MCU, Martín M, Parker JS, Carrasco E, Caballero R, et al. Prognostic significance of progesterone receptor-positive tumor cells within immunohistochemically defined luminal A breast cancer. J Clin Oncol. 2013;31(2):203–9. Available from: http://dx.doi.org/10.1200/JCO.2012.43.4134

99. Eroles P, Bosch A, Pérez-Fidalgo JA, Lluch A. Molecular biology in breast cancer: intrinsic subtypes and signaling pathways. Cancer Treat Rev. 2012;38(6):698–707. Available from: http://dx.doi.org/10.1016/j.ctrv.2011.11.005

100. Ades F, Zardavas D, Bozovic-Spasojevic I, Pugliano L, Fumagalli D, de Azambuja E, et al. Luminal B breast cancer: molecular characterization, clinical management, and future perspectives. J Clin Oncol. 2014;32(25):2794–803. Available from: http://dx.doi.org/10.1200/JCO.2013.54.1870

101. Cheang MCU, Chia SK, Voduc D, Gao D, Leung S, Snider J, et al. Ki67 index, HER2 status, and prognosis of patients with luminal B breast cancer. J Natl Cancer Inst. 2009;101(10):736–50. Available from: http://dx.doi.org/10.1093/jnci/djp082

102. Raj-Kumar P-K, Liu J, Hooke JA, Kovatich AJ, Kvecher L, Shriver CD, et al. PCA-PAM50 improves consistency between breast cancer intrinsic and clinical subtyping reclassifying a subset of luminal A tumors as luminal B. Sci Rep. 2019;9(1):7956. Available from: http://dx.doi.org/10.1038/s41598-019-44339-4

103. Roberts SA, Lawrence MS, Klimczak LJ, Grimm SA, Fargo D, Stojanov P, et al. An APOBEC cytidine deaminase mutagenesis pattern is widespread in human cancers. Nat Genet. 2013;45(9):970–6. Available from: http://dx.doi.org/10.1038/ng.2702

104. Kuong KJ, Loeb LA. APOBEC3B mutagenesis in cancer. Nat Genet. 2013;45(9):964–5. Available from: http://dx.doi.org/10.1038/ng.2736

105. Plasilova ML, Hayse B, Killelea BK, Horowitz NR, Chagpar AB, Lannin DR. Features of triple-negative breast cancer: Analysis of 38,813 cases from the national cancer database. Medicine (Baltimore). 2016;95(35):e4614. Available from: http://dx.doi.org/10.1097/MD.0000000000004614

106. Newman LA, Reis-Filho JS, Morrow M, Carey LA, King TA. The 2014 Society of Surgical Oncology Susan G. Komen for the Cure Symposium: triple-negative breast cancer. Ann Surg Oncol. 2015;22(3):874–82. Available from: http://dx.doi.org/10.1245/s10434-014-4279-0

107. Pareja F, Geyer FC, Marchiò C, Burke KA, Weigelt B, Reis-Filho JS. Triple-negative breast cancer: the importance of molecular and histologic subtyping, and recognition of low-grade variants. NPJ Breast Cancer. 2016;2(1):16036. Available from: http://dx.doi.org/10.1038/npjbcancer.2016.36

108. Lehmann BD, Bauer JA, Chen X, Sanders ME, Chakravarthy AB, Shyr Y, et al. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. J Clin Invest. 2011;121(7):2750–67. Available from: http://dx.doi.org/10.1172/jci45014

109. Wang D-Y, Jiang Z, Ben-David Y, Woodgett JR, Zacksenhaus E. Molecular stratification within triple-negative breast cancer subtypes. Sci Rep. 2019;9(1):19107. Available from: http://dx.doi.org/10.1038/s41598-019-55710-w

110. Santonja A, Sánchez-Muñoz A, Lluch A, Chica-Parrado MR, Albanell J, Chacón JI, et al. Triple negative breast cancer subtypes and pathologic

complete response rate to neoadjuvant chemotherapy. Oncotarget. 2018;9(41):26406–16. Available from: http://dx.doi.org/10.18632/oncotarget.25413

111. Prat A, Parker JS, Karginova O, Fan C, Livasy C, Herschkowitz JI, et al. Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. Breast Cancer Res. 2010;12(5):R68. Available from: http://dx.doi.org/10.1186/bcr2635

112. Hennessy BT, Gonzalez-Angulo A-M, Stemke-Hale K, Gilcrease MZ, Krishnamurthy S, Lee J-S, et al. Characterization of a naturally occurring breast cancer subset enriched in epithelial-to-mesenchymal transition and stem cell characteristics. Cancer Res. 2009;69(10):4116–24. Available from: http://dx.doi.org/10.1158/0008-5472.can-08-3441

113. Dias K, Dvorkin-Gheva A, Hallett RM, Wu Y, Hassell J, Pond GR, et al. Claudin-low breast cancer; Clinical & pathological characteristics. PLoS One. 2017;12(1):e0168669. Available from: http://dx.doi.org/10.1371/journal.pone.0168669

114. Morel A-P, Ginestier C, Pommier RM, Cabaud O, Ruiz E, Wicinski J, et al. A stemness-related ZEB1-MSRB3 axis governs cellular pliancy and breast cancer genome stability. Nat Med. 2017;23(5):568–78. Available from: http://dx.doi.org/10.1038/nm.4323

115. Parise CA, Bauer KR, Brown MM, Caggiano V. Breast cancer subtypes as defined by the estrogen receptor (ER), progesterone receptor (PR), and the human epidermal growth factor receptor 2 (HER2) among women with invasive breast cancer in California, 1999-2004. Breast J. 2009;15(6):593–602. Available from: http://dx.doi.org/10.1111/j.1524-4741.2009.00822.x

116. Maisonneuve P, Disalvatore D, Rotmensz N, Curigliano G, Colleoni M, Dellapasqua S, et al. Proposed new clinicopathological surrogate definitions of luminal A and luminal B (HER2-negative) intrinsic breast cancer subtypes. Breast Cancer Res. 2014;16(3):R65. Available from: http://dx.doi.org/10.1186/bcr3679

117. Cheang MCU, Voduc D, Bajdik C, Leung S, McKinney S, Chia SK, et al. Basal-like breast cancer defined by five biomarkers has superior prognostic value than triple-negative phenotype. Clin Cancer Res. 2008;14(5):1368–76. Available from: http://dx.doi.org/10.1158/1078-0432.CCR-07-1658

118. Prat A, Pineda E, Adamo B, Galván P, Fernández A, Gaba L, et al. Clinical implications of the intrinsic molecular subtypes of breast cancer. Breast. 2015;24 Suppl 2:S26-35. Available from: http://dx.doi.org/10.1016/j.breast.2015.07.008

119. Vuong D, Simpson PT, Green B, Cummings MC, Lakhani SR. Molecular classification of breast cancer. Virchows Arch. 2014;465(1):1–14. Available from: http://dx.doi.org/10.1007/s00428-014-1593-7

120. Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology. 1991;19(5):403–10. Available from: http://dx.doi.org/10.1111/j.1365-2559.1991.tb00229.x

121. Bloom HJ, Richardson WW. Histological grading and prognosis in breast cancer; a study of 1409 cases of which 359 have been followed for 15 years. Br J Cancer. 1957;11(3):359–77. Available from: http://dx.doi.org/10.1038/bjc.1957.43

122. Sparano JA, Gray RJ, Makower DF, Pritchard KI, Albain KS, Hayes DF, et al. Prospective validation of a 21-gene expression assay in breast cancer. N Engl J Med. 2015;373(21):2005–14. Available from: http://dx.doi.org/10.1056/nejmoa1510764

123. Stemmer SM, Steiner M, Rizel S, Soussan-Gutman L, Ben-Baruch N, Bareket-Samish A, et al. Clinical outcomes in patients with node-negative breast cancer treated based on the recurrence score results: evidence from a large prospectively designed registry. NPJ Breast Cancer. 2017;3(1). Available from: http://dx.doi.org/10.1038/s41523-017-0034-6

124. Weiss A, Chavez-MacGregor M, Lichtensztajn DY, Yi M, Tadros A, Hortobagyi GN, et al. Validation study of the American Joint Committee on cancer eighth edition prognostic stage compared with the anatomic stage in breast cancer. JAMA Oncol. 2018;4(2):203–9. Available from: http://dx.doi.org/10.1001/jamaoncol.2017.4298

125. Abdel-Rahman O. Validation of the 8th AJCC prognostic staging system for breast cancer in a population-based setting. Breast Cancer Res Treat. 2018;168(1):269–75. Available from: http://dx.doi.org/10.1007/s10549-017-4577-x

126. Kerlikowske K, Grady D, Barclay J, Sickles EA, Eaton A, Ernster V. Positive predictive value of screening mammography by age and family history of breast cancer. JAMA. 1993;270(20):2444–50. Available from: http://dx.doi.org/10.1001/jama.1993.03510200050031

127. Shah R, Rosso K, Nathanson SD. Pathogenesis, prevention, diagnosis and treatment of breast cancer. World J Clin Oncol. 2014;5(3):283–98. Available from: http://dx.doi.org/10.5306/wjco.v5.i3.283

128. Ceber E, Mermer G, Okcin F, Sari D, Demireloz M, Eksioglu A, et al. Breast cancer risk and early diagnosis applications in Turkish women aged 50 and over. Asian Pac J Cancer Prev. 2013;14(10):5877–82. Available from: http://dx.doi.org/10.7314/apjcp.2013.14.10.5877

129. Ahmadian M, Samah AA, Redzuan M, Emby Z. Predictors of mammography screening among Iranian women attending outpatient clinics in Tehran, Iran. Asian Pac J Cancer Prev. 2012;13(3):969–74. Available from: http://dx.doi.org/10.7314/apjcp.2012.13.3.969

130. Gao Y, Reig B, Heacock L, Bennett DL, Heller SL, Moy L. Magnetic Resonance imaging in screening of breast cancer. Radiol Clin North Am. 2021;59(1):85–98. Available from: http://dx.doi.org/10.1016/j.rcl.2020.09.004

131. Narayanan D, Berg WA. Use of breast-specific PET scanners and comparison with MR imaging. Magn Reson Imaging Clin N Am. 2018;26(2):265–72. Available from: http://dx.doi.org/10.1016/j.mric.2017.12.006

132. Madjar H. Role of breast ultrasound for the detection and differentiation of breast lesions. Breast Care (Basel). 2010;5(2):109–14. Available from: http://dx.doi.org/10.1159/000297775

133. Crișan G, Moldovean-Cioroianu NS, Timaru D-G, Andrieș G, Căinap C, Chiș V. Radiopharmaceuticals for PET and SPECT imaging: A literature review over the last decade. Int J Mol Sci. 2022;23(9):5023. Available from: http://dx.doi.org/10.3390/ijms23095023

134. Almuhaideb A, Papathanasiou N, Bomanji J. 18F-FDG PET/CT imaging in oncology. Ann Saudi Med. 2011;31(1):3–13. Available from: http://dx.doi.org/10.4103/0256-4947.75771

135. Ryu JM, Kang D, Cho J, Lee JE, Kim SW, Nam SJ, et al. Prognostic impact of elevation of cancer antigen 15-3 (CA15-3) in patients with early breast cancer with normal serum CA15-3 level. J Breast Cancer. 2023;26(2):126–35. Available from: http://dx.doi.org/10.4048/jbc.2023.26.e17

136. Lauro S, Trasatti L, Bordin F, Lanzetta G, Bria E, Gelibter A, et al. Comparison of CEA, MCA, CA 15-3 and CA 27-29 in follow-up and monitoring therapeutic response in breast cancer patients. Anticancer Res. 1999;19(4C):3511–5.

137. Mouttet D, Laé M, Caly M, Gentien D, Carpentier S, Peyro-Saint-Paul H, et al. Estrogen-receptor, progesterone-receptor and HER2 status determination in invasive breast cancer. Concordance between immuno-histochemistry and MapQuantTM microarray based assay. PLoS One. 2016;11(2):e0146474. Available from: http://dx.doi.org/10.1371/journal.pone.0146474

138. Onitilo AA, Engel JM, Greenlee RT, Mukesh BN. Breast cancer subtypes based on ER/PR and Her2 expression: comparison of clinicopathologic features and survival. Clin Med Res. 2009;7(1–2):4–13. Available from: http://dx.doi.org/10.3121/cmr.2009.825

139. Kim A, Lee HJ, Kim JY. Breast fine-needle aspiration cytology in the era of core-needle biopsy: what is its role? J Pathol Transl Med. 2025;59(1):26–38. Available from: http://dx.doi.org/10.4132/jptm.2024.11.01

140. Park H-L, Hong J. Vacuum-assisted breast biopsy for breast cancer. Gland Surg. 2014;3(2):120–7. Available from: http://dx.doi.org/10.3978/j.issn.2227-684X.2014.02.03

141. Aphale R, Dhar A, Chintamani, Jatoi I, Srivastava A. Why should we avoid excisional biopsy of breast lump? ASOMA guide for managing a discrete breast lump. Indian J Surg Oncol. 2023;14(1):194–8. Available from: http://dx.doi.org/10.1007/s13193-022-01687-4

142. Syed AH, Khan T. Evolution of research trends in artificial intelligence for breast cancer diagnosis and prognosis over the past two decades: A bibliometric analysis. Front Oncol. 2022;12:854927. Available from: http://dx.doi.org/10.3389/fonc.2022.854927

143. Colomer R, Aranda-López I, Albanell J, García-Caballero T, Ciruelos E, López-García MÁ, et al. Biomarkers in breast cancer: A consensus statement by the Spanish Society of Medical Oncology and the Spanish Society of Pathology. Clin Transl Oncol. 2018;20(7):815–26. Available from: http://dx.doi.org/10.1007/s12094-017-1800-5

144. Duffy MJ, Harbeck N, Nap M, Molina R, Nicolini A, Senkus E, et al. Clinical use of biomarkers in breast cancer: Updated guidelines from the European Group on Tumor Markers (EGTM). Eur J Cancer. 2017;75:284–98. Available from: http://dx.doi.org/10.1016/j.ejca.2017.01.017

145. Nasrazadani A, Thomas RA, Oesterreich S, Lee AV. Precision medicine in hormone receptor-positive breast cancer. Front Oncol. 2018;8:144. Available from: http://dx.doi.org/10.3389/fonc.2018.00144

146. Tse LA, Li M, Chan W-C, Kwok C-H, Leung S-L, Wu C, et al. Familial risks and estrogen receptor-positive breast cancer in Hong Kong Chinese women. PLoS One. 2015;10(3):e0120741. Available from: http://dx.doi.org/10.1371/journal.pone.0120741

147. Konan H-P, Kassem L, Omarjee S, Surmieliova-Garnès A, Jacquemetton J, Cascales E, et al. ERα-36 regulates progesterone receptor activity in breast cancer. Breast Cancer Res. 2020;22(1):50. Available from: http://dx.doi.org/10.1186/s13058-020-01278-7

148. Obr AE, Edwards DP. The biology of progesterone receptor in the normal mammary gland and in breast cancer. Mol Cell Endocrinol. 2012;357(1–2):4–17. Available from: http://dx.doi.org/10.1016/j.mce.2011.10.030

149. Wu J-R, Zhao Y, Zhou X-P, Qin X. Estrogen receptor 1 and progesterone receptor are distinct biomarkers and prognostic factors in estrogen receptor-positive breast cancer: Evidence from a bioinformatic analysis. Biomed Pharmacother. 2020;121(109647):109647. Available from: http://dx.doi.org/10.1016/j.biopha.2019.109647

150. Patani N, Martin L-A, Dowsett M. Biomarkers for the clinical management of breast cancer: international perspective: Biomarkers of breast cancer. Int J Cancer. 2013;133(1):1–13. Available from: http://dx.doi.org/10.1002/ijc.27997

151. Freelander A, Brown LJ, Parker A, Segara D, Portman N, Lau B, et al. Molecular biomarkers for contemporary therapies in hormone receptor-positive breast cancer. Genes (Basel). 2021;12(2):285. Available from: http://dx.doi.org/10.3390/genes12020285

152. Kohler BA, Sherman RL, Howlader N, Jemal A, Ryerson AB, Henry KA, et al. Annual Report to the nation on the status of cancer, 1975-2011, featuring incidence of breast cancer subtypes by race/ethnicity, poverty, and state. J Natl Cancer Inst. 2015;107(6):djv048. Available from: http://dx.doi.org/10.1093/jnci/djv048

153. Kontani K, Kuroda N, Hashimoto S-I, Murazawa C, Norimura S, Tanaka H, et al. Clinical usefulness of human epidermal growth factor receptor-2 extracellular domain as a biomarker for monitoring cancer status and predicting the therapeutic efficacy in breast cancer. Cancer Biol Ther. 2013;14(1):20–8. Available from: http://dx.doi.org/10.4161/cbt.22626

154. Kim H-A, Lee JK, Kim E-K, Seol H, Noh WC. Serum human epidermal growth factor receptor 2 levels as a real-time marker for tumor burden in breast cancer patients: Serum HER2 and Tissue HER2 Expression. J Surg Oncol. 2014;109(5):421–5. Available from: http://dx.doi.org/10.1002/jso.23510

155. Cheng X. A comprehensive review of HER2 in cancer biology and therapeutics. Genes (Basel). 2024;15(7):903. Available from: http://dx.doi.org/10.3390/genes15070903

156. Iqbal N, Iqbal N. Human epidermal growth factor receptor 2 (HER2) in cancers: Overexpression and therapeutic implications. Mol Biol Int. 2014;2014:852748. Available from: http://dx.doi.org/10.1155/2014/852748

157. Nishimura R, Osako T, Okumura Y, Hayashi M, Toyozumi Y, Arima N. Ki-67 as a prognostic marker according to breast cancer subtype and a predictor of recurrence time in primary breast cancer. Exp Ther Med. 2010;1(5):747–54. Available from: http://dx.doi.org/10.3892/etm.2010.133

158. de Azambuja E, Cardoso F, de Castro G Jr, Colozza M, Mano MS, Durbecq V, et al. Ki-67 as prognostic marker in early breast cancer: a meta-analysis of published studies involving 12,155 patients. Br J Cancer. 2007;96(10):1504–13. Available from: http://dx.doi.org/10.1038/sj.bjc.6603756

159. Pathmanathan N, Balleine RL, Jayasinghe UW, Bilinski KL, Provan PJ, Byth K, et al. The prognostic value of Ki67 in systemically untreated patients with node-negative breast cancer. J Clin Pathol. 2014;67(3):222–8. Available from: http://dx.doi.org/10.1136/jclinpath-2013-201793

160. Zhou S-Y, Chen W, Yang S-J, Xu Z-H, Hu J-H, Zhang H, et al. The emerging role of circular RNAs in breast cancer. Biosci Rep. 2019;39(6):BSR20190621. Available from: http://dx.doi.org/10.1042/BSR20190621

161. Tran AM, Chalbatani GM, Berland L, Cruz De Los Santos M, Raj P, Jalali SA, et al. A new world of biomarkers and therapeutics for female reproductive system and breast cancers: Circular RNAs. Front Cell Dev Biol. 2020;8:50. Available from: http://dx.doi.org/10.3389/fcell.2020.00050

162. Harris CC, Hollstein M. Clinical implications of the p53 tumor-suppressor gene. N Engl J Med. 1993;329(18):1318–27. Available from: http://dx.doi.org/10.1056/NEJM199310283291807

163. Williams AB, Schumacher B. P53 in the DNA-damage-repair process. Cold Spring Harb Perspect Med. 2016;6(5). Available from: http://dx.doi.org/10.1101/cshperspect.a026070

164. Dumay A, Feugeas J-P, Wittmer E, Lehmann-Che J, Bertheau P, Espié M, et al. Distinct tumor protein p53 mutants in breast cancer subgroups. Int J Cancer. 2013;132(5):1227–31. Available from: http://dx.doi.org/10.1002/ijc.27767

165. Petitjean A, Achatz MIW, Borresen-Dale AL, Hainaut P, Olivier M. TP53 mutations in human cancers: functional selection and impact on cancer prognosis and outcomes. Oncogene. 2007;26(15):2157–65. Available from: http://dx.doi.org/10.1038/sj.onc.1210302

166. Liu J, Zhang C, Feng Z. Tumor suppressor p53 and its gain-of-function mutants in cancer. Acta Biochim Biophys Sin (Shanghai). 2014;46(3):170–9. Available from: http://dx.doi.org/10.1093/abbs/gmt144

167. Chae BJ, Bae JS, Lee A, Park WC, Seo YJ, Song BJ, et al. P53 as a specific prognostic factor in triple-negative breast cancer. Jpn J Clin Oncol. 2009;39(4):217–24. Available from: http://dx.doi.org/10.1093/jjco/hyp007

168. Duffy MJ, Synnott NC, Crown J. Mutant p53 in breast cancer: potential as a therapeutic target and biomarker. Breast Cancer Res Treat. 2018;170(2):213–9. Available from: http://dx.doi.org/10.1007/s10549-018-4753-7

169. Wiemer EAC. The role of microRNAs in cancer: no small matter. Eur J Cancer. 2007;43(10):1529–44. Available from: http://dx.doi.org/10.1016/j.ejca.2007.04.002

170. Iorio MV, Croce CM. MicroRNA dysregulation in cancer: diagnostics, monitoring and therapeutics. A comprehensive review: MicroRNAs in cancer. EMBO Mol Med. 2012;4(3):143–59. Available from: http://dx.doi.org/10.1002/emmm.201100209

171. Adhami M, Haghdoost AA, Sadeghi B, Malekpour Afshar R. Candidate miRNAs in human breast cancer biomarkers: a systematic review. Breast Cancer. 2018;25(2):198–205. Available from: http://dx.doi.org/10.1007/s12282-017-0814-8

172. Gordon S, Martinez FO. Alternative activation of macrophages: mechanism and functions. Immunity. 2010;32(5):593–604. Available from: http://dx.doi.org/10.1016/j.immuni.2010.05.007

173. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol. 2002;23(11):549–55. Available from: http://dx.doi.org/10.1016/s1471-4906(02)02302-5

174. Biswas SK, Allavena P, Mantovani A. Tumor-associated macrophages: functional diversity, clinical significance, and open questions. Semin Immunopathol. 2013;35(5):585–600. Available from: http://dx.doi.org/10.1007/s00281-013-0367-7

175. Williams CB, Yeh ES, Soloff AC. Tumor-associated macrophages: unwitting accomplices in breast cancer malignancy. NPJ Breast Cancer. 2016;2(1). Available from: http://dx.doi.org/10.1038/npjbcancer.2015.25

176. Yang J, Li X, Liu X, Liu Y. The role of tumor-associated macrophages in breast carcinoma invasion and metastasis. Int J Clin Exp Pathol. 2015;8(6):6656–64.

177. Medrek C, Pontén F, Jirström K, Leandersson K. The presence of tumor associated macrophages in tumor stroma as a prognostic marker for breast cancer patients. BMC Cancer. 2012;12(1):306. Available from:

http://dx.doi.org/10.1186/1471-2407-12-306

178. Gwak JM, Jang MH, Kim DI, Seo AN, Park SY. Prognostic value of tumor-associated macrophages according to histologic locations and hormone receptor status in breast cancer. PLoS One. 2015;10(4):e0125728. Available from: http://dx.doi.org/10.1371/journal.pone.0125728

179. Yuan Z-Y, Luo R-Z, Peng R-J, Wang S-S, Xue C. High infiltration of tumor-associated macrophages in triple-negative breast cancer is associated with a higher risk of distant metastasis. Onco Targets Ther. 2014;7:1475–80. Available from: http://dx.doi.org/10.2147/OTT.S61838

180. Zhao X, Qu J, Sun Y, Wang J, Liu X, Wang F, et al. Prognostic significance of tumor-associated macrophages in breast cancer: a meta-analysis of the literature. Oncotarget. 2017;8(18):30576–86. Available from: http://dx.doi.org/10.18632/oncotarget.15736

181. Zhang H, Wang X, Shen Z, Xu J, Qin J, Sun Y. Infiltration of diametrically polarized macrophages predicts overall survival of patients with gastric cancer after surgical resection. Gastric Cancer. 2015;18(4):740–50. Available from: http://dx.doi.org/10.1007/s10120-014-0422-7

182. Herrera M, Herrera A, Domínguez G, Silva J, García V, García JM, et al. Cancer-associated fibroblast and M2 macrophage markers together predict outcome in colorectal cancer patients. Cancer Sci. 2013;104(4):437–44. Available from: http://dx.doi.org/10.1111/cas.12096

183. Zhang M, He Y, Sun X, Li Q, Wang W, Zhao A, et al. A high M1/M2 ratio of tumor-associated macrophages is associated with extended survival in ovarian cancer patients. J Ovarian Res. 2014;7(1):19. Available from: http://dx.doi.org/10.1186/1757-2215-7-19

184. Honkanen TJ, Tikkanen A, Karihtala P, Mäkinen M, Väyrynen JP, Koivunen JP. Prognostic and predictive role of tumour-associated macrophages in HER2 positive breast cancer. Sci Rep. 2019;9(1):10961. Available from: http://dx.doi.org/10.1038/s41598-019-47375-2

185. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. Cell. 2010;140(6):883–99. Available from: http://dx.doi.org/10.1016/j.cell.2010.01.025

186. Guthrie GJK, Charles KA, Roxburgh CSD, Horgan PG, McMillan DC, Clarke SJ. The systemic inflammation-based neutrophil-lymphocyte ratio: experience in patients with cancer. Crit Rev Oncol Hematol. 2013;88(1):218–30. Available from: http://dx.doi.org/10.1016/j.critrevonc.2013.03.010

187. Huang SH, Waldron JN, Milosevic M, Shen X, Ringash J, Su J, et al. Prognostic value of pretreatment circulating neutrophils, monocytes, and lymphocytes in oropharyngeal cancer stratified by human papillomavirus status: Leukocyte and Oropharyngeal Cancer Outcomes. Cancer. 2015;121(4):545–55. Available from: http://dx.doi.org/10.1002/cncr.29100

188. Li J, Jiang R, Liu W-S, Liu Q, Xu M, Feng Q-S, et al. A large cohort study reveals the association of elevated peripheral blood lymphocyte-to-monocyte ratio with favorable prognosis in nasopharyngeal carcinoma. PLoS One. 2013;8(12):e83069. Available from: http://dx.doi.org/10.1371/journal.pone.0083069

189. Usluogullari B, Duvan C, Usluogullari C. Use of aromatase inhibitors in practice of gynecology. J Ovarian Res. 2015;8(1):4. Available from: http://dx.doi.org/10.1186/s13048-015-0131-9

190. Proctor MJ, Morrison DS, Talwar D, Balmer SM, Fletcher CD, O'Reilly DSJ, et al. A comparison of inflammation-based prognostic scores in patients with cancer. A Glasgow Inflammation Outcome Study. Eur J Cancer. 2011;47(17):2633–41. Available from: http://dx.doi.org/10.1016/j.ejca.2011.03.028

191. Wang Y, Luo M, Chen Y, Wang Y, Zhang B, Ren Z, et al. ZMYND8 expression in breast cancer cells blocks T-lymphocyte surveillance to promote tumor growth. Cancer Res. 2021;81(1):174–86. Available from: http://dx.doi.org/10.1158/0008-5472.can-20-1710

192. Sobral-Leite M, Salomon I, Opdam M, Kruger DT, Beelen KJ, van der Noort V, et al. Cancer-immune interactions in ER-positive breast cancers: PI3K pathway alterations and tumor-infiltrating lymphocytes. Breast Cancer Res. 2019;21(1):90. Available from: http://dx.doi.org/10.1186/s13058-019-1176-2

193. Mouchemore KA, Anderson RL, Hamilton JA. Neutrophils, G-CSF and their contribution to breast cancer metastasis. FEBS J. 2018;285(4):665–79. Available from: http://dx.doi.org/10.1111/febs.14206

194. Azab B, Shah N, Radbel J, Tan P, Bhatt V, Vonfrolio S, et al. Pretreatment neutrophil/lymphocyte ratio is superior to platelet/lymphocyte ratio as a predictor of long-term mortality in breast cancer patients. Med Oncol. 2013;30(1):432. Available from: http://dx.doi.org/10.1007/s12032-012-0432-4

195. Guo W, Lu X, Liu Q, Zhang T, Li P, Qiao W, et al. Prognostic value of neutrophil-to-lymphocyte ratio and platelet-to-lymphocyte ratio for breast cancer patients: An updated meta-analysis of 17079 individuals. Cancer Med. 2019;8(9):4135–48. Available from: http://dx.doi.org/10.1002/cam4.2281

196. Jain A, Barge A, Parris CN. Combination strategies with PARP inhibitors in BRCA-mutated triple-negative breast cancer: overcoming resistance mechanisms. Oncogene. 2025;44(4):193–207. Available from: http://dx.doi.org/10.1038/s41388-024-03227-6

197. Schmid P, Rugo HS, Adams S, Schneeweiss A, Barrios CH, Iwata H, et al. Atezolizumab plus nab-paclitaxel as first-line treatment for unresectable, locally advanced or metastatic triple-negative breast cancer (IMpassion130): updated efficacy results from a randomised, double-blind, placebo-controlled, phase 3 trial. Lancet Oncol. 2020;21(1):44–59. Available from: http://dx.doi.org/10.1016/S1470-2045(19)30689-8

198. Goto W, Kashiwagi S, Asano Y, Takada K, Takahashi K, Hatano T, et al. Predictive value of lymphocyte-to-monocyte ratio in the preoperative setting for progression of patients with breast cancer. BMC Cancer. 2018;18(1):1137. Available from: http://dx.doi.org/10.1186/s12885-018-5051-9

199. Xu L, Xu P, Wang J, Ji H, Zhang L, Tang Z. Advancements in clinical research and emerging therapies for triple-negative breast cancer treatment. Eur J Pharmacol. 2025;988(177202):177202. Available from: http://dx.doi.org/10.1016/j.ejphar.2024.177202

200. Li Y, Zhang H, Merkher Y, Chen L, Liu N, Leonov S, et al. Recent advances in therapeutic strategies for triple-negative breast cancer. J Hematol Oncol. 2022;15(1):121. Available from: http://dx.doi.org/10.1186/s13045-022-01341-0

201. Nedeljković M, Damjanović A. Mechanisms of chemotherapy resistance in triple-negative breast cancer-how we can rise to the challenge. Cells. 2019;8(9):957. Available from: http://dx.doi.org/10.3390/cells8090957

202. Schmid P, Adams S, Rugo HS, Schneeweiss A, Barrios CH, Iwata H, et al. Atezolizumab and nab-paclitaxel in advanced triple-negative breast cancer. N Engl J Med. 2018;379(22):2108–21. Available from: http://dx.doi.org/10.1056/NEJMoa1809615

203. Jiang Z, Ouyang Q, Sun T, Zhang Q, Teng Y, Cui J, et al. TORCHLIGHT: A randomized, double-blind, phase III trial of toripalimab versus placebo, in combination with nab-paclitaxel(nab-P) for patients with metastatic or recurrent triple-negative breast cancer (TNBC). J Clin Oncol. 2023;41(17_suppl):LBA1013–LBA1013. Available from: http://dx.doi.org/10.1200/jco.2023.41.17_suppl.lba1013

204. Siddharth S, Sharma D. Racial disparity and triple-negative breast cancer in African-American women: A multifaceted affair between obesity, biology, and socioeconomic determinants. Cancers (Basel). 2018;10(12):514. Available from:

http://dx.doi.org/10.3390/cancers10120514

205. Xiong N, Wu H, Yu Z. Advancements and challenges in triple-negative breast cancer: a comprehensive review of therapeutic and diagnostic strategies. Front Oncol. 2024;14:1405491. Available from: http://dx.doi.org/10.3389/fonc.2024.1405491

206. Saeg F, Anbalagan M. Breast cancer stem cells and the challenges of eradication: a review of novel therapies. Stem Cell Investig. 2018;5:39. Available from: http://dx.doi.org/10.21037/sci.2018.10.05

207. Senbanjo LT, Chellaiah MA. CD44: A multifunctional cell surface adhesion receptor is a regulator of progression and metastasis of cancer cells. Front Cell Dev Biol. 2017;5:18. Available from: http://dx.doi.org/10.3389/fcell.2017.00018

208. Kristiansen G, Winzer K-J, Mayordomo E, Bellach J, Schlüns K, Denkert C, et al. CD24 expression is a new prognostic marker in breast cancer. Clin Cancer Res. 2003;9(13):4906–13.

209. Jaggupilli A, Elkord E. Significance of CD44 and CD24 as cancer stem cell markers: an enduring ambiguity. Clin Dev Immunol. 2012;2012:708036. Available from: http://dx.doi.org/10.1155/2012/708036

210. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A. 2003;100(7):3983–8. Available from: http://dx.doi.org/10.1073/pnas.0530291100

211. So JY, Lee HJ, Smolarek AK, Paul S, Wang C-X, Maehr H, et al. A novel Gemini vitamin D analog represses the expression of a stem cell marker CD44 in breast cancer. Mol Pharmacol. 2011;79(3):360–7. Available from: http://dx.doi.org/10.1124/mol.110.068403

12. Wahler J, So JY, Cheng LC, Maehr H, Uskokovic M, Suh N. Vitamin D compounds reduce mammosphere formation and decrease expression of putative stem cell markers in breast cancer. J Steroid Biochem Mol Biol. 2015;148:148–55. Available from: http://dx.doi.org/10.1016/j.jsbmb.2014.10.016

213. Shan NL, Minden A, Furmanski P, Bak MJ, Cai L, Wernyj R, et al. Analysis of the transcriptome: Regulation of cancer stemness in breast ductal carcinoma in situ by vitamin D compounds. Cancer Prev Res (Phila). 2020;13(8):673–86. Available from: http://dx.doi.org/10.1158/1940-6207.CAPR-19-0566

214. Díaz L, Díaz-Muñoz M, García-Gaytán AC, Méndez I. Mechanistic effects of calcitriol in cancer biology. Nutrients. 2015;7(6):5020–50. Available from: http://dx.doi.org/10.3390/nu7065020

215. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646–74. Available from: http://dx.doi.org/10.1016/j.cell.2011.02.013

216. Ngo DC, Ververis K, Tortorella SM, Karagiannis TC. Introduction to the molecular basis of cancer metabolism and the Warburg effect. Mol Biol Rep. 2015;42(4):819–23. Available from: http://dx.doi.org/10.1007/s11033-015-3857-y

217. Ward PS, Thompson CB. Metabolic reprogramming: A cancer hallmark even Warburg did not anticipate. Cancer Cell. 2012;21(3):297–308. Available from: http://dx.doi.org/10.1016/j.ccr.2012.02.014

218. Scarmo S, Afanasyeva Y, Lenner P, Koenig KL, Horst RL, Clendenen TV, et al. Circulating levels of 25-hydroxyvitamin D and risk of breast cancer: a nested case-control study. Breast Cancer Res. 2013;15(1):R15. Available from: http://dx.doi.org/10.1186/bcr3390

219. Bertone-Johnson ER, Chen WY, Holick MF, Hollis BW, Colditz GA, Willett WC, et al. Plasma 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D and risk of breast cancer. Cancer Epidemiol Biomarkers Prev. 2005;14(8):1991–7. Available from: http://dx.doi.org/10.1158/1055-9965.EPI-04-0722

220. Villaseñor A, Ballard-Barbash R, Ambs A, Bernstein L, Baumgartner K, Baumgartner R, et al. Associations of serum 25-hydroxyvitamin D with overall and breast cancer–specific mortality in a multiethnic cohort of breast cancer survivors. Cancer Causes Control. 2013;24(4):759–67. Available from: http://dx.doi.org/10.1007/s10552-013-0158-4

221. Prentice RL, Pettinger MB, Jackson RD, Wactawski-Wende J, Lacroix AZ, Anderson GL, et al. Health risks and benefits from calcium and vitamin D supplementation: Women's Health Initiative clinical trial and cohort study. Osteoporos Int. 2013;24(2):567–80. Available from: http://dx.doi.org/10.1007/s00198-012-2224-2

222. Garland CF, Gorham ED, Mohr SB, Grant WB, Giovannucci EL, Lipkin M, et al. Vitamin D and prevention of breast cancer: pooled analysis. J Steroid Biochem Mol Biol. 2007;103(3–5):708–11. Available from: http://dx.doi.org/10.1016/j.jsbmb.2006.12.007

223. Finkelmeier F, Kronenberger B, Köberle V, Bojunga J, Zeuzem S, Trojan J, et al. Severe 25-hydroxyvitamin D deficiency identifies a poor prognosis in patients with hepatocellular carcinoma - a prospective cohort study. Aliment Pharmacol Ther. 2014;39(10):1204–12. Available from: http://dx.doi.org/10.1111/apt.12731

224. Garland CF, Gorham ED, Mohr SB, Garland FC. Vitamin D for cancer prevention: global perspective. Ann Epidemiol. 2009;19(7):468–83. Available from: http://dx.doi.org/10.1016/j.annepidem.2009.03.021

225. An B-S, Tavera-Mendoza LE, Dimitrov V, Wang X, Calderon MR, Wang H-J, et al. Stimulation of Sirt1-regulated FoxO protein function by the ligand-bound vitamin D receptor. Mol Cell Biol. 2010;30(20):4890–900. Available from: http://dx.doi.org/10.1128/MCB.00180-10

226. Jeon S-M, Shin E-A. Exploring vitamin D metabolism and function in cancer. Exp Mol Med. 2018;50(4):1–14. Available from: http://dx.doi.org/10.1038/s12276-018-0038-9

227. Christakos S, Dhawan P, Verstuyf A, Verlinden L, Carmeliet G. Vitamin D: Metabolism, molecular mechanism of action, and pleiotropic effects. Physiol Rev. 2016;96(1):365–408. Available from: http://dx.doi.org/10.1152/physrev.00014.2015

228. Welsh J. Vitamin D and breast cancer: Past and present. J Steroid Biochem Mol Biol. 2018;177:15–20. Available from: http://dx.doi.org/10.1016/j.jsbmb.2017.07.025

229. Segovia-Mendoza M, García-Quiroz J, Díaz L, García-Becerra R. Combinations of calcitriol with anticancer treatments for breast cancer: An update. Int J Mol Sci. 2021;22(23):12741. Available from: http://dx.doi.org/10.3390/ijms222312741

230. Matthews D, LaPorta E, Zinser GM, Narvaez CJ, Welsh J. Genomic vitamin D signaling in breast cancer: Insights from animal models and human cells. J Steroid Biochem Mol Biol. 2010;121(1–2):362–7. Available from: http://dx.doi.org/10.1016/j.jsbmb.2010.03.061

231. Welsh DJA, Kayibi KK. Corrigendum to “A linking polynomial of two matroids” [Adv. in Appl. Math. 32 (1–2) (2004) 391–419]. Adv Appl Math. 2012;49(1):78–9. Available from: http://dx.doi.org/10.1016/j.aam.2012.04.001

232. Albertson DG, Ylstra B, Segraves R, Collins C, Dairkee SH, Kowbel D, et al. Quantitative mapping of amplicon structure by array CGH identifies CYP24 as a candidate oncogene. Nat Genet. 2000;25(2):144–6. Available from: http://dx.doi.org/10.1038/75985

233. Zhalehjoo N, Shakiba Y, Panjehpour M. Gene expression profiles of CYP24A1 and CYP27B1 in malignant and normal breast tissues. Mol Med Rep. 2017;15(1):467–73. Available from: http://dx.doi.org/10.3892/mmr.2016.5992

234. Eisman JA, Macintyre I, Martin TJ, Frampton RJ, King RJ. Normal and malignant breast tissue is a target organ for 1,25-(0H)2 vitamin D3. Clin Endocrinol (Oxf). 1980;13(3):267–72. Available from: http://dx.doi.org/10.1111/j.1365-2265.1980.tb01053.x

235. Bortman P, Folgueira MAAK, Katayama MLH, Snitcovsky IML, Brentani MM. Antiproliferative effects of 1,25-dihydroxyvitamin D3 on breast cells: a mini review. Braz J Med Biol Res. 2002;35(1):1–9. Available from: http://dx.doi.org/10.1590/s0100-879x2002000100001

236. Swami S, Krishnan AV, Wang JY, Jensen K, Horst R, Albertelli MA, et al. Dietary vitamin D_3 and 1,25-dihydroxyvitamin D_3 (calcitriol) exhibit equivalent anticancer activity in mouse xenograft models of breast and prostate cancer. Endocrinology. 2012;153(6):2576–87. Available from: http://dx.doi.org/10.1210/en.2011-1600

237. Lee HJ, Paul S, Atalla N, Thomas PE, Lin X, Yang I, et al. Gemini vitamin D analogues inhibit estrogen receptor-positive and estrogen receptor-negative mammary tumorigenesis without hypercalcemic toxicity. Cancer Prev Res (Phila). 2008;1(6):476–84. Available from: http://dx.doi.org/10.1158/1940-6207.CAPR-08-0084

238. Friedrich M, Axt-Fliedner R, Villena-Heinsen C, Tilgen W, Schmidt W, Reichrath J. Analysis of vitamin D-receptor (VDR) and retinoid X-receptor α in breast cancer. Histochem J. 2002;34(1–2):35–40. Available from: http://dx.doi.org/10.1023/a:1021343825552

239. Bilani N, Elson L, Szuchan C, Elimimian E, Saleh M, Nahleh Z. Newly-identified pathways relating vitamin D to carcinogenesis: A review. In Vivo. 2021;35(3):1345–54. Available from: http://dx.doi.org/10.21873/invivo.12387

240. Chen J, Tang Z, Slominski AT, Li W, Żmijewski MA, Liu Y, et al. Vitamin D and its analogs as anticancer and anti-inflammatory agents. Eur J Med Chem. 2020;207(112738):112738. Available from: http://dx.doi.org/10.1016/j.ejmech.2020.112738

241. Koshizuka K, Koike M, Asou H, Cho SK, Stephen T, Rude RK, et al. Combined effect of vitamin D3 analogs and paclitaxel on the growth of MCF-7 breast cancer cells in vivo. Breast Cancer Res Treat. 1999;53(2):113–20. Available from: http://dx.doi.org/10.1023/a:1006123819675

242. Koshizuka K, Koike M, Kubota T, Said J, Binderup L, Koeffler HP. Novel vitamin D3 analog (CB1093) when combined with paclitaxel and cisplatin inhibit growth of MCF-7 human breast cancer cells in vivo. Int J Oncol. 1998;13(3):421–8. Available from: http://dx.doi.org/10.3892/ijo.13.3.421

243. Wang Q, Yang W, Uytingco MS, Christakos S, Wieder R. 1,25-Dihydroxyvitamin D3 and all-trans-retinoic acid sensitize breast cancer cells to chemotherapy-induced cell death. Cancer Res. 2000;60(7):2040–8.

244. Garland FC, Garland CF, Gorham ED, Young JF. Geographic variation in breast cancer mortality in the United States: a hypothesis involving exposure to solar radiation. Prev Med. 1990;19(6):614–22. Available from: http://dx.doi.org/10.1016/0091-7435(90)90058-r

245. Garland CF, Garland FC, Gorham ED, Lipkin M, Newmark H, Mohr SB, et al. The role of vitamin D in cancer prevention. Am J Public Health. 2006;96(2):252–61. Available from: http://dx.doi.org/10.2105/AJPH.2004.045260

246. Machado MRM, de Sousa Almeida-Filho B, De Luca Vespoli H, Schmitt EB, Nahas-Neto J, Nahas EAP. Low pretreatment serum concentration of vitamin D at breast cancer diagnosis in postmenopausal women. Menopause. 2019;26(3):293–9. Available from: http://dx.doi.org/10.1097/gme.0000000000001203

247. Karthikayan A, Sureshkumar S, Kadambari D, Vijayakumar C. Low serum 25-hydroxy vitamin D levels are associated with aggressive breast cancer variants and poor prognostic factors in patients with breast carcinoma. Arch Endocrinol Metab. 2018;62(4):452–9. Available from: http://dx.doi.org/10.20945/2359-3997000000062

248. Vaughan-Shaw PG, O'Sullivan F, Farrington SM, Theodoratou E, Campbell H, Dunlop MG, et al. The impact of vitamin D pathway genetic variation and circulating 25-hydroxyvitamin D on cancer outcome: systematic review and meta-analysis. Br J Cancer. 2017;116(8):1092–110. Available from: http://dx.doi.org/10.1038/bjc.2017.44

249. Estébanez N, Gómez-Acebo I, Palazuelos C, Llorca J, Dierssen-Sotos T. Vitamin D exposure and Risk of Breast Cancer: a meta-analysis. Sci Rep. 2018;8(1):9039. Available from: http://dx.doi.org/10.1038/s41598-018-27297-1

250. Song D, Deng Y, Liu K, Zhou L, Li N, Zheng Y, et al. Vitamin D intake, blood vitamin D levels, and the risk of breast cancer: a dose-response meta-analysis of observational studies. Aging (Albany NY). 2019;11(24):12708–32. Available from: http://dx.doi.org/10.18632/aging.102597

251. Hossain S, Beydoun MA, Beydoun HA, Chen X, Zonderman AB, Wood RJ. Vitamin D and breast cancer: A systematic review and meta-analysis of observational studies. Clin Nutr ESPEN. 2019;30:170–84. Available from: http://dx.doi.org/10.1016/j.clnesp.2018.12.085

252. Jiang X, O'Reilly PF, Aschard H, Hsu Y-H, Richards JB, Dupuis J, et al. Genome-wide association study in 79,366 European-ancestry individuals informs the genetic architecture of 25-hydroxyvitamin D levels. Nat Commun. 2018;9(1):260. Available from: http://dx.doi.org/10.1038/s41467-017-02662-2

253. Jiang X, Dimou NL, Al-Dabhani K, Lewis SJ, Martin RM, Haycock PC, et al. Circulating vitamin D concentrations and risk of breast and prostate cancer: a Mendelian randomization study. Int J Epidemiol. 2019;48(5):1416–24. Available from: http://dx.doi.org/10.1093/ije/dyy284

254. Jiang X, Ge T, Chen C-Y. The causal role of circulating vitamin D concentrations in human complex traits and diseases: a large-scale Mendelian randomization study. Sci Rep. 2021;11(1):184. Available from: http://dx.doi.org/10.1038/s41598-020-80655-w

255. Bouillon R, Marcocci C, Carmeliet G, Bikle D, White JH, Dawson-Hughes B, et al. Skeletal and extraskeletal actions of vitamin D: Current evidence and outstanding questions. Endocr Rev. 2019;40(4):1109–51. Available from: http://dx.doi.org/10.1210/er.2018-00126

256. Manson JE, Cook NR, Lee I-M, Christen W, Bassuk SS, Mora S, et al. Vitamin D supplements and prevention of cancer and cardiovascular disease. N Engl J Med. 2019;380(1):33–44. Available from: http://dx.doi.org/10.1056/NEJMoa1809944

257. Chandler PD, Chen WY, Ajala ON, Hazra A, Cook N, Bubes V, et al. Effect of vitamin D3 supplements on development of advanced cancer: A secondary analysis of the VITAL randomized clinical trial: A secondary analysis of the VITAL randomized clinical trial. JAMA Netw Open. 2020;3(11):e2025850. Available from: http://dx.doi.org/10.1001/jamanetworkopen.2020.25850

258. Lappe J, Watson P, Travers-Gustafson D, Recker R, Garland C, Gorham E, et al. Effect of vitamin D and calcium supplementation on cancer incidence in older women: A randomized clinical trial. JAMA. 2017;317(12):1234. Available from: http://dx.doi.org/10.1001/jama.2017.2115

259. Scragg RKR. Overview of results from the Vitamin D Assessment (ViDA) study. J Endocrinol Invest. 2019;42(12):1391–9. Available from: http://dx.doi.org/10.1007/s40618-019-01056-z

260. Arnaout A, Robertson S, Pond GR, Vieth R, Jeong A, Hilton J, et al. Randomized window of opportunity trial evaluating high-dose vitamin D in breast cancer patients. Breast Cancer Res Treat. 2019;178(2):347–56. Available from: http://dx.doi.org/10.1007/s10549-019-05392-9

261. Yaghjyan L, Colditz GA, Drake B. Vitamin D and mammographic breast density: a systematic review. Cancer Causes Control. 2012;23(1):1–13. Available from: http://dx.doi.org/10.1007/s10552-011-9851-3

262. Li Z, Wu L, Zhang J, Huang X, Thabane L, Li G. Effect of vitamin D supplementation on risk of breast cancer: A systematic review and meta-analysis of randomized Controlled Trials. Front Nutr. 2021;8:655727. Available from: http://dx.doi.org/10.3389/fnut.2021.655727

263. Boucher BJ. Why do so many trials of vitamin D supplementation fail? Endocr Connect. 2020;9(9):R195–206. Available from: http://dx.doi.org/10.1530/ec-20-0274

264. Zinser GM, Welsh J. Accelerated mammary gland development during pregnancy and delayed postlactational involution in vitamin D3 receptor null mice. Mol Endocrinol. 2004;18(9):2208–23. Available from: http://dx.doi.org/10.1210/me.2003-0469

265. Zinser G, Packman K, Welsh J. Vitamin D(3) receptor ablation alters mammary gland morphogenesis. Development. 2002;129(13):3067–76. Available from: http://dx.doi.org/10.1242/dev.129.13.3067

266. Zinser GM, Welsh J. Vitamin D receptor status alters mammary gland morphology and tumorigenesis in MMTV-neu mice. Carcinogenesis. 2004;25(12):2361–72. Available from: http://dx.doi.org/10.1093/carcin/bgh271

267. Matthews DG, D'Angelo J, Drelich J, Welsh J. Adipose-specific Vdr deletion alters body fat and enhances mammary epithelial density. J Steroid Biochem Mol Biol. 2016;164:299–308. Available from: http://dx.doi.org/10.1016/j.jsbmb.2015.09.035

268. Welsh J. Function of the vitamin D endocrine system in mammary gland and breast cancer. Mol Cell Endocrinol. 2017;453:88–95. Available from: http://dx.doi.org/10.1016/j.mce.2017.04.026

269. Campos LT, Brentani H, Roela RA, Katayama MLH, Lima L, Rolim CF, et al. Differences in transcriptional effects of 1α,25 dihydroxyvitamin D3 on fibroblasts associated to breast carcinomas and from paired normal breast tissues. J Steroid Biochem Mol Biol. 2013;133:12–24. Available from: http://dx.doi.org/10.1016/j.jsbmb.2012.08.002

70. Lopes N, Sousa B, Martins D, Gomes M, Vieira D, Veronese LA, et al. Alterations in Vitamin D signalling and metabolic pathways in breast cancer progression: a study of VDR, CYP27B1 and CYP24A1 expression in benign

and malignant breast lesions. BMC Cancer. 2010;10(1):483. Available from: http://dx.doi.org/10.1186/1471-2407-10-483

271. Murray A, Madden SF, Synnott NC, Klinger R, O'Connor D, O'Donovan N, et al. Vitamin D receptor as a target for breast cancer therapy. Endocr Relat Cancer. 2017;24(4):181–95. Available from: http://dx.doi.org/10.1530/ERC-16-0463

272. Xu H, Liu Z, Shi H, Wang C. Prognostic role of vitamin D receptor in breast cancer: a systematic review and meta-analysis. BMC Cancer. 2020;20(1):1051. Available from: http://dx.doi.org/10.1186/s12885-020-07559-w

273. Heublein S, Mayr D, Meindl A, Kircher A, Jeschke U, Ditsch N. Vitamin D receptor, Retinoid X receptor and peroxisome proliferator-activated receptor γ are overexpressed in BRCA1 mutated breast cancer and predict prognosis. J Exp Clin Cancer Res. 2017;36(1):57. Available from: http://dx.doi.org/10.1186/s13046-017-0517-1

274. Fedchenko N, Reifenrath J. Different approaches for interpretation and reporting of immunohistochemistry analysis results in the bone tissue - a review. Diagn Pathol. 2014;9(1):221. Available from: http://dx.doi.org/10.1186/s13000-014-0221-9

275. Cai H, Jiao Y, Li Y, Yang Z, He M, Liu Y. Low CYP24A1 mRNA expression and its role in prognosis of breast cancer. Sci Rep. 2019;9(1):13714. Available from: http://dx.doi.org/10.1038/s41598-019-50214-z

276. Zheng Y, Trivedi T, Lin RC, Fong-Yee C, Nolte R, Manibo J, et al. Loss of the vitamin D receptor in human breast and prostate cancers strongly induces cell apoptosis through downregulation of Wnt/β-catenin signaling. Bone Res. 2017;5(1):17023. Available from: http://dx.doi.org/10.1038/boneres.2017.23

277. Verlinden L, Verstuyf A, Convents R, Marcelis S, Van Camp M, Bouillon R. Action of 1,25(OH)2D3 on the cell cycle genes, cyclin D1, p21 and p27 in MCF-7 cells. Mol Cell Endocrinol. 1998;142(1–2):57–65. Available from: http://dx.doi.org/10.1016/s0303-7207(98)00117-8

278. Jensen SS, Madsen MW, Lukas J, Binderup L, Bartek J. Inhibitory effects of 1alpha,25-dihydroxyvitamin D(3) on the G(1)-S phase-controlling machinery. Mol Endocrinol. 2001;15(8):1370–80. Available from: http://dx.doi.org/10.1210/mend.15.8.0673

279. Lopes N, Paredes J, Costa JL, Ylstra B, Schmitt F. Vitamin D and the mammary gland: a review on its role in normal development and breast cancer. Breast Cancer Res. 2012;14(3):211. Available from: http://dx.doi.org/10.1186/bcr3178

280. Dhawan P, Weider R, Christakos S. CCAAT enhancer-binding protein α is a molecular target of 1,25-dihydroxyvitamin D3 in MCF-7 breast cancer cells. J Biol Chem. 2009;284(5):3086–95. Available from: http://dx.doi.org/10.1074/jbc.m803602200

281. Liu X, Bi L, Wang Q, Wen M, Li C, Ren Y, et al. miR-1204 targets VDR to promotes epithelial-mesenchymal transition and metastasis in breast cancer. Oncogene. 2018;37(25):3426–39. Available from: http://dx.doi.org/10.1038/s41388-018-0215-2

282. Rossdeutscher L, Li J, Luco A-L, Fadhil I, Ochietti B, Camirand A, et al. Chemoprevention activity of 25-hydroxyvitamin D in the MMTV-PyMT mouse model of breast cancer. Cancer Prev Res (Phila). 2015;8(2):120–8. Available from: http://dx.doi.org/10.1158/1940-6207.CAPR-14-0110

283. Fonseca-Filho VCN, Katayama MLH, Lyra EC, Maria DA, Basso RA, Nonogaki S, et al. Orthotopic tumorgrafts in nude mice as a model to evaluate calcitriol effects in breast cancer. Braz J Biol. 2017;77(4):856–67. Available from: http://dx.doi.org/10.1590/1519-6984.04016

284. Verma A, Cohen DJ, Schwartz N, Muktipaty C, Koblinski JE, Boyan BD, et al. 24R,25-Dihydroxyvitamin D3 regulates breast cancer cells in vitro and in vivo. Biochim Biophys Acta Gen Subj. 2019;1863(10):1498–512. Available from: http://dx.doi.org/10.1016/j.bbagen.2019.05.013

285. Vanoirbeek E, Krishnan A, Eelen G, Verlinden L, Bouillon R, Feldman D, et al. The anti-cancer and anti-inflammatory actions of $1,25(OH)_2D_3$. Best Pract Res Clin Endocrinol Metab. 2011;25(4):593–604. Available from: http://dx.doi.org/10.1016/j.beem.2011.05.001

286. Zheng W, Cao L, Ouyang L, Zhang Q, Duan B, Zhou W, et al. Anticancer activity of 1,25-(OH)2D3 against human breast cancer cell lines by targeting Ras/MEK/ERK pathway. Onco Targets Ther. 2019;12:721–32. Available from: http://dx.doi.org/10.2147/OTT.S190432

287. Weitsman GE, Koren R, Zuck E, Rotem C, Liberman UA, Ravid A. Vitamin D sensitizes breast cancer cells to the action of H2O2: mitochondria as a convergence point in the death pathway. Free Radic Biol Med. 2005;39(2):266–78. Available from: http://dx.doi.org/10.1016/j.freeradbiomed.2005.03.018

288. Reichrath J, Reichrath S, Heyne K, Vogt T, Roemer K. Tumor suppression in skin and other tissues via cross-talk between vitamin D- and p53-signaling. Front Physiol. 2014;5:166. Available from: http://dx.doi.org/10.3389/fphys.2014.00166

289. Stambolsky P, Tabach Y, Fontemaggi G, Weisz L, Maor-Aloni R, Siegfried Z, et al. Modulation of the vitamin D3 response by cancer-associated mutant p53. Cancer Cell 2010;17(3):273–85. Available from: http://dx.doi.org/10.1016/j.ccr.2009.11.025

290. Kim VN, Han J, Siomi MC. Biogenesis of small RNAs in animals. Nat Rev Mol Cell Biol. 2009;10(2):126–39. Available from: http://dx.doi.org/10.1038/nrm2632

291. Croce CM. Causes and consequences of microRNA dysregulation in cancer. Nat Rev Genet. 2009;10(10):704–14. Available from: http://dx.doi.org/10.1038/nrg2634

292. Mohri T, Nakajima M, Takagi S, Komagata S, Yokoi T. MicroRNA regulates human vitamin D receptor. Int J Cancer. 2009;125(6):1328–33. Available from: http://dx.doi.org/10.1002/ijc.24459

293. Zhang L, Stokes N, Polak L, Fuchs E. Specific MicroRNAs are preferentially expressed by skin stem cells to balance self-renewal and early lineage commitment. Cell Stem Cell. 2011;8(3):294–308. Available from: http://dx.doi.org/10.1016/j.stem.2011.01.014

294. Chen Y, Du J, Zhang Z, Liu T, Shi Y, Ge X, et al. MicroRNA-346 mediates tumor necrosis factor α-induced downregulation of gut epithelial vitamin D receptor in inflammatory bowel diseases. Inflamm Bowel Dis. 2014;20(11):1910–8. Available from: http://dx.doi.org/10.1097/MIB.0000000000000158

295. Li F, Zhang A, Shi Y, Ma Y, Du Y. 1α,25-Dihydroxyvitamin D3 prevents the differentiation of human lung fibroblasts via microRNA-27b targeting the vitamin D receptor. Int J Mol Med. 2015;36(4):967–74. Available from: http://dx.doi.org/10.3892/ijmm.2015.2318

296. Pan Y-Z, Gao W, Yu A-M. MicroRNAs regulate CYP3A4 expression via direct and indirect targeting. Drug Metab Dispos. 2009;37(10):2112–7. Available from: http://dx.doi.org/10.1124/dmd.109.027680

297. Borkowski R, Du L, Zhao Z, McMillan E, Kosti A, Yang C-R, et al. Genetic mutation of p53 and suppression of the miR-17~92 cluster are synthetic lethal in non-small cell lung cancer due to upregulation of vitamin D Signaling. Cancer Res. 2015;75(4):666–75. Available from: http://dx.doi.org/10.1158/0008-5472.CAN-14-1329

298. Liu PT, Wheelwright M, Teles R, Komisopoulou E, Edfeldt K, Ferguson B, et al. MicroRNA-21 targets the vitamin D-dependent antimicrobial pathway in leprosy. Nat Med. 2012;18(2):267–73. Available from: http://dx.doi.org/10.1038/nm.2584

299. Ji J, Zhang J, Huang G, Qian J, Wang X, Mei S. Over-expressed microRNA-27a and 27b influence fat accumulation and cell proliferation during rat hepatic stellate cell activation. FEBS Lett. 2009;583(4):759–66. Available from: http://dx.doi.org/10.1016/j.febslet.2009.01.034

300. Adlakha YK, Khanna S, Singh R, Singh VP, Agrawal A, Saini N. Pro-apoptotic miRNA-128-2 modulates ABCA1, ABCG1 and RXRα expression and cholesterol homeostasis. Cell Death Dis. 2013;4:e780. Available from: http://dx.doi.org/10.1038/cddis.2013.301

301. Guérit D, Philipot D, Chuchana P, Toupet K, Brondello J-M, Mathieu M, et al. Sox9-regulated miRNA-574-3p inhibits chondrogenic differentiation of mesenchymal stem cells. PLoS One. 2013;8(4):e62582. Available from: http://dx.doi.org/10.1371/journal.pone.0062582

302. Giangreco AA, Nonn L. The sum of many small changes: microRNAs are specifically and potentially globally altered by vitamin D3 metabolites. J Steroid Biochem Mol Biol. 2013;136:86–93. Available from: http://dx.doi.org/10.1016/j.jsbmb.2013.01.001

303. Kasiappan R, Shen Z, Tse AK-W, Jinwal U, Tang J, Lungchukiet P, et al. 1,25-Dihydroxyvitamin D3 suppresses telomerase expression and human cancer growth through microRNA-498. J Biol Chem. 2012;287(49):41297–309. Available from: http://dx.doi.org/10.1074/jbc.M112.407189

304. Min D, Lv X-B, Wang X, Zhang B, Meng W, Yu F, et al. Downregulation of miR-302c and miR-520c by 1,25(OH)2D3 treatment enhances the susceptibility of tumour cells to natural killer cell-mediated cytotoxicity. Br J Cancer. 2013;109(3):723–30. Available from: http://dx.doi.org/10.1038/bjc.2013.337

305. Alimirah F, Peng X, Gupta A, Yuan L, Welsh J, Cleary M, et al. Crosstalk between the vitamin D receptor (VDR) and miR-214 in regulating SuFu, a hedgehog pathway inhibitor in breast cancer cells. Exp Cell Res. 2016;349(1):15–22. Available from: http://dx.doi.org/10.1016/j.yexcr.2016.08.012

306. Piovan C, Palmieri D, Di Leva G, Braccioli L, Casalini P, Nuovo G, et al. Oncosuppressive role of p53-induced miR-205 in triple negative breast cancer. Mol Oncol. 2012;6(4):458–72. Available from: http://dx.doi.org/10.1016/j.molonc.2012.03.003

307. Joerger AC, Fersht AR. Structure-function-rescue: the diverse nature of common p53 cancer mutants. Oncogene. 2007;26(15):2226–42. Available from: http://dx.doi.org/10.1038/sj.onc.1210291

308. Hickman ES, Moroni MC, Helin K. The role of p53 and pRB in apoptosis and cancer. Curr Opin Genet Dev. 2002;12(1):60–6. Available from: http://dx.doi.org/10.1016/s0959-437x(01)00265-9

309. Bosco EE, Wang Y, Xu H, Zilfou JT, Knudsen KE, Aronow BJ, et al. The retinoblastoma tumor suppressor modifies the therapeutic response of breast cancer. J Clin Invest. 2007;117(1):218–28. Available from: http://dx.doi.org/10.1172/JCI28803

310. Liu X, Gu X, Sun L, Flowers AB, Rademaker AW, Zhou Y, et al. Downregulation of Smurf2, a tumor-suppressive ubiquitin ligase, in triple-negative breast cancers: involvement of the RB-microRNA axis. BMC Cancer. 2014;14(1):57. Available from: http://dx.doi.org/10.1186/1471-2407-14-57

311. Filipowicz W, Jaskiewicz L, Kolb FA, Pillai RS. Post-transcriptional gene silencing by siRNAs and miRNAs. Curr Opin Struct Biol. 2005;15(3):331–41. Available from: http://dx.doi.org/10.1016/j.sbi.2005.05.006

312. Ren Y, Han X, Yu K, Sun S, Zhen L, Li Z, et al. microRNA-200c downregulates XIAP expression to suppress proliferation and promote apoptosis of triple-negative breast cancer cells. Mol Med Rep. 2014;10(1):315–21. Available from: http://dx.doi.org/10.3892/mmr.2014.2222

313. Park S-M, Gaur AB, Lengyel E, Peter ME. The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. Genes Dev. 2008;22(7):894–907. Available from: http://dx.doi.org/10.1101/gad.1640608

314. Jang K, Ahn H, Sim J, Han H, Abdul R, Paik SS, et al. Loss of microRNA-200a expression correlates with tumor progression in breast cancer. Transl Res. 2014;163(3):242–51. Available from: http://dx.doi.org/10.1016/j.trsl.2013.11.005

315. Nassirpour R, Mehta PP, Baxi SM, Yin M-J. miR-221 promotes tumorigenesis in human triple negative breast cancer cells. PLoS One. 2013;8(4):e62170. Available from: http://dx.doi.org/10.1371/journal.pone.0062170

316. Dong G, Liang X, Wang D, Gao H, Wang L, Wang L, et al. High expression of miR-21 in triple-negative breast cancers was correlated with a poor prognosis and promoted tumor cell in vitro proliferation. Med Oncol. 2014;31(7):57. Available from: http://dx.doi.org/10.1007/s12032-014-0057-x

317. Liu H, Wang Y, Li X, Zhang Y-J, Li J, Zheng Y-Q, et al. Expression and regulatory function of miRNA-182 in triple-negative breast cancer cells through its targeting of profilin 1. Tumour Biol. 2013;34(3):1713–22. Available from: http://dx.doi.org/10.1007/s13277-013-0708-0

318. Kong W, He L, Richards EJ, Challa S, Xu C-X, Permuth-Wey J, et al. Upregulation of miRNA-155 promotes tumour angiogenesis by targeting VHL and is associated with poor prognosis and triple-negative breast cancer. Oncogene. 2014;33(6):679–89. Available from: http://dx.doi.org/10.1038/onc.2012.636

319. Augoff K, McCue B, Plow EF, Sossey-Alaoui K. miR-31 and its host gene lncRNA LOC554202 are regulated by promoter hypermethylation in triple-negative breast cancer. Mol Cancer. 2012;11(1):5. Available from: http://dx.doi.org/10.1186/1476-4598-11-5

320. Humphries B, Wang Z, Oom AL, Fisher T, Tan D, Cui Y, et al. MicroRNA-200b targets protein kinase Cα and suppresses triple-negative breast cancer metastasis. Carcinogenesis. 2014;35(10):2254–63. Available from: http://dx.doi.org/10.1093/carcin/bgu133

321. den Hollander P, Savage MI, Brown PH. Targeted therapy for breast cancer prevention. Front Oncol. 2013;3:250. Available from: http://dx.doi.org/10.3389/fonc.2013.00250

322. Yao H, He G, Yan S, Chen C, Song L, Rosol TJ, et al. Triple-negative breast cancer: is there a treatment on the horizon? Oncotarget. 2017;8(1):1913–24. Available from: http://dx.doi.org/10.18632/oncotarget.12284

323. Thakkar A, Wang B, Picon-Ruiz M, Buchwald P, Ince TA. Vitamin D and androgen receptor-targeted therapy for triple-negative breast cancer. Breast Cancer Res Treat. 2016;157(1):77–90. Available from: http://dx.doi.org/10.1007/s10549-016-3807-y

324. Chiang K-C, Yeh T-S, Chen S-C, Pang J-HS, Yeh C-N, Hsu J-T, et al. The vitamin D analog, MART-10, attenuates triple negative breast cancer cells metastatic potential. Int J Mol Sci. 2016;17(4):606. Available from: http://dx.doi.org/10.3390/ijms17040606

325. Flanagan L, Packman K, Juba B, O'Neill S, Tenniswood M, Welsh J. Efficacy of Vitamin D compounds to modulate estrogen receptor negative breast cancer growth and invasion. J Steroid Biochem Mol Biol. 2003;84(2–3):181–92. Available from: http://dx.doi.org/10.1016/s0960-0760(03)00028-1

326. Ferronato MJ, Nadal Serrano M, Arenas Lahuerta EJ, Bernadó Morales C, Paolillo G, Martinez-Sabadell Aliguer A, et al. Vitamin D analogues exhibit antineoplastic activity in breast cancer patient-derived xenograft cells. J Steroid Biochem Mol Biol. 2021;208(105735):105735. Available from: http://dx.doi.org/10.1016/j.jsbmb.2020.105735

327. Qin B, Xu B, Ji N, Yao S, Pawlish K, Llanos AAM, et al. Intake of vitamin D and calcium, sun exposure, and risk of breast cancer subtypes among black women. Am J Clin Nutr. 2020;111(2):396–405. Available from: http://dx.doi.org/10.1093/ajcn/nqz302

328. Bilinski K, Boyages J. Association between 25-hydroxyvitamin D concentration and breast cancer risk in an Australian population: an observational case-control study. Breast Cancer Res Treat. 2013;137(2):599–607. Available from: http://dx.doi.org/10.1007/s10549-012-2381-1

329. Feldman D, Krishnan AV, Swami S, Giovannucci E, Feldman BJ. The role of vitamin D in reducing cancer risk and progression. Nat Rev Cancer. 2014;14(5):342–57. Available from: http://dx.doi.org/10.1038/nrc3691

330. Richards SE, Weierstahl KA, Kelts JL. Vitamin D effect on growth and vitamin D metabolizing enzymes in triple-negative breast cancer. Anticancer Res. 2015;35(2):805–10.

331. Hirshfield KM, Ganesan S. Triple-negative breast cancer: molecular subtypes and targeted therapy. Curr Opin Obstet Gynecol. 2014;26(1):34–40. Available from: http://dx.doi.org/10.1097/GCO.0000000000000038

332. Santos-Martínez N, Díaz L, Ordaz-Rosado D, García-Quiroz J, Barrera D, Avila E, et al. Calcitriol restores antiestrogen responsiveness in estrogen receptor negative breast cancer cells: a potential new therapeutic approach. BMC Cancer. 2014;14(1):230. Available from: http://dx.doi.org/10.1186/1471-2407-14-230

333. Zheng Z, Lang T, Huang X, Wang G, Lee RJ, Teng L, et al. Calcitriol-loaded dual-pH-sensitive Micelle counteracts pro-metastasis effect of paclitaxel in triple-negative breast cancer therapy. Adv Healthc Mater. 2020;9(12):e2000392. Available from: http://dx.doi.org/10.1002/adhm.202000392

334. Schüler-Toprak S, Häring J, Inwald EC, Moehle C, Ortmann O, Treeck O. Agonists and knockdown of estrogen receptor β differentially affect invasion of triple-negative breast cancer cells in vitro. BMC Cancer. 2016;16(1):951. Available from: http://dx.doi.org/10.1186/s12885-016-2973-y

335. Lee JS, Yost SE, Yuan Y. Neoadjuvant treatment for triple negative breast cancer: Recent progresses and challenges. Cancers (Basel). 2020;12(6):1404. Available from: http://dx.doi.org/10.3390/cancers12061404

336. Furlanetto J, Loibl S. Optimal systemic treatment for early triple-negative breast cancer. Breast Care (Basel). 2020;15(3):217–26. Available from: http://dx.doi.org/10.1159/000508759

337. Soljic M, Mrklic I, Tomic S, Omrcen T, Sutalo N, Bevanda M, et al. Prognostic value of vitamin D receptor and insulin-like growth factor receptor 1 expression in triple-negative breast cancer. J Clin Pathol. 2018;71(1):34–9. Available from: http://dx.doi.org/10.1136/jclinpath-2016-204222

338. Turner TH, Alzubi MA, Harrell JC. Identification of synergistic drug combinations using breast cancer patient-derived xenografts. Sci Rep. 2020;10(1):1493. Available from: http://dx.doi.org/10.1038/s41598-020-58438-0

339. Huss L, Gulz-Haake I, Nilsson E, Tryggvadottir H, Nilsson L, Nodin B, et al. The vitamin D receptor as a prognostic marker in Breast Cancer-A cohort study. Nutrients. 2024;16(7). Available from: http://dx.doi.org/10.3390/nu16070931

340. Veeresh PKM, Basavaraju CG, Dallavalasa S, Anantharaju PG, Natraj SM, Sukocheva OA, et al. Vitamin D3 inhibits the viability of breast cancer cells in vitro and Ehrlich ascites carcinomas in mice by promoting apoptosis and cell cycle arrest and by impeding tumor angiogenesis. Cancers (Basel). 2023;15(19). Available from: http://dx.doi.org/10.3390/cancers15194833

341. Wong FH, Palanirajan VK, Ng ESC, Tan CK, Tan ESS, Amini F. Combination of talazoparib and calcitriol enhanced anticancer effect in triple-negative breast cancer cell lines. Pharmaceuticals (Basel). 2022;15(9):1075. Available from: http://dx.doi.org/10.3390/ph15091075

342. Schneider J, Jeon YW, Suh YJ, Lim ST. Effects of ruxolitinib and calcitriol combination treatment on various molecular subtypes of breast cancer. Int J Mol Sci. 2022;23(5):2535. Available from: http://dx.doi.org/10.3390/ijms23052535

343. Vanhevel J, Verlinden L, Doms S, Wildiers H, Verstuyf A. The role of vitamin D in breast cancer risk and progression. Endocr Relat Cancer. 2022;29(2):R33–55. Available from: http://dx.doi.org/10.1530/ERC-21-0182

344. Gkotinakou I-M, Mylonis I, Tsakalof A. Vitamin D and Hypoxia: Points of interplay in cancer. Cancers (Basel). 2022;14(7):1791. Available from: http://dx.doi.org/10.3390/cancers14071791

345. Blasiak J, Chojnacki J, Pawlowska E, Jablkowska A, Chojnacki C. Vitamin D may protect against breast cancer through the regulation of long noncoding RNAs by VDR signaling. Int J Mol Sci. 2022;23(6):3189. Available from: http://dx.doi.org/10.3390/ijms23063189

346. Shan NL, Wahler J, Lee HJ, Bak MJ, Gupta SD, Maehr H, et al. Vitamin D compounds inhibit cancer stem-like cells and induce differentiation in triple

negative breast cancer. J Steroid Biochem Mol Biol. 2017;173:122–9. Available from: http://dx.doi.org/10.1016/j.jsbmb.2016.12.001

347. Zati Zehni A, Jacob S-N, Mumm J-N, Heidegger HH, Ditsch N, Mahner S, et al. Hormone receptor expression in multicentric/multifocal versus unifocal breast cancer: Especially the VDR determines the outcome related to focality. Int J Mol Sci. 2019;20(22):5740. Available from: http://dx.doi.org/10.3390/ijms20225740

348. Martínez-Reza I, Díaz L, Barrera D, Segovia-Mendoza M, Pedraza-Sánchez S, Soca-Chafre G, et al. Calcitriol inhibits the proliferation of triple-negative breast cancer cells through a mechanism involving the proinflammatory cytokines IL-1β and TNF-α. J Immunol Res. 2019;2019:6384278. Available from: http://dx.doi.org/10.1155/2019/6384278

349. Fleet JC, DeSmet M, Johnson R, Li Y. Vitamin D and cancer: a review of molecular mechanisms. Biochem J. 2012;441(1):61–76. Available from: http://dx.doi.org/10.1042/BJ20110744

350. García-Quiroz J, García-Becerra R, Santos-Cuevas C, Ramírez-Nava GJ, Morales-Guadarrama G, Cárdenas-Ochoa N, et al. Synergistic antitumorigenic activity of calcitriol with curcumin or resveratrol is mediated by angiogenesis inhibition in triple negative breast cancer xenografts. Cancers (Basel). 2019;11(11):1739. Available from: http://dx.doi.org/10.3390/cancers11111739

351. Narvaez CJ, Matthews D, LaPorta E, Simmons KM, Beaudin S, Welsh J. The impact of vitamin D in breast cancer: genomics, pathways, metabolism. Front Physiol. 2014;5:213. Available from: http://dx.doi.org/10.3389/fphys.2014.00213

352. Ditsch N, Toth B, Mayr D, Lenhard M, Gallwas J, Weissenbacher T, et al. The association between vitamin D receptor expression and prolonged overall survival in breast cancer. J Histochem Cytochem. 2012;60(2):121–9. Available from: http://dx.doi.org/10.1369/0022155411429155

353. Banwell CM, Singh R, Stewart PM, Uskokovic MR, Campbell MJ. Antiproliferative signalling by 1,25(OH)2D3 in prostate and breast cancer is suppressed by a mechanism involving histone deacetylation. Recent Results Cancer Res. 2003;164:83–98. Available from: http://dx.doi.org/10.1007/978-3-642-55580-0_5

354. Wu X, Hu W, Lu L, Zhao Y, Zhou Y, Xiao Z, et al. Repurposing vitamin D for treatment of human malignancies via targeting tumor microenvironment. Acta Pharm Sin B. 2019;9(2):203–19. Available from: http://dx.doi.org/10.1016/j.apsb.2018.09.002

355. Friedrich M, Reichert K, Woeste A, Polack S, Fischer D, Hoellen F, et al. Effects of combined treatment with vitamin D and COX2 inhibitors on breast cancer cell lines. Anticancer Res. 2018;38(2):1201–7. Available from: http://dx.doi.org/10.21873/anticanres.12340

356. Peng Z, Liu R, Li Y, Zhang Q, Cai X, Li L. Calcitriol enhances the effect of photodynamic therapy in human breast cancer. J BUON. 2016;21(5):1068–75.

357. Fuentes N, Silveyra P. Estrogen receptor signaling mechanisms. Adv Protein Chem Struct Biol. 2019;116:135–70. Available from: http://dx.doi.org/10.1016/bs.apcsb.2019.01.001

358. Couse JF, Lindzey J, Grandien K, Gustafsson JA, Korach KS. Tissue distribution and quantitative analysis of estrogen receptor-alpha (ERalpha) and estrogen receptor-beta (ERbeta) messenger ribonucleic acid in the wild-type and ERalpha-knockout mouse. Endocrinology. 1997;138(11):4613–21. Available from: http://dx.doi.org/10.1210/endo.138.11.5496

359. Jefferson WN, Couse JF, Banks EP, Korach KS, Newbold RR. Expression of estrogen receptor beta is developmentally regulated in reproductive tissues of male and female mice. Biol Reprod. 2000;62(2):310–7. Available from: http://dx.doi.org/10.1095/biolreprod62.2.310

360. Zhou Y, Liu X. The role of estrogen receptor beta in breast cancer. Biomark Res. 2020;8:39. Available from: http://dx.doi.org/10.1186/s40364-020-00223-2

361. Oueslati M, Bittaieb I, Sassi N, Jemaa AB, Gamoudi A, Rahal K, et al. ERα and ERβ co-expression: An indicator of aggressive tumors and hormonal sensitivity. Oncol Lett. 2017;14(2):1675–82. Available from: http://dx.doi.org/10.3892/ol.2017.6314

362. Madeira M, Mattar A, Logullo AF, Soares FA, Gebrim LH. Estrogen receptor alpha/beta ratio and estrogen receptor beta as predictors of endocrine therapy responsiveness-a randomized neoadjuvant trial comparison between anastrozole and tamoxifen for the treatment of postmenopausal breast cancer. BMC Cancer. 2013;13(1):425. Available from: http://dx.doi.org/10.1186/1471-2407-13-425

363. Chen P, Li B, Ou-Yang L. Role of estrogen receptors in health and disease. Front Endocrinol (Lausanne). 2022;13:839005. Available from: http://dx.doi.org/10.3389/fendo.2022.839005

364. Speirs V, Parkes AT, Kerin MJ, Walton DS, Carleton PJ, Fox JN, et al. Coexpression of estrogen receptor alpha and beta: poor prognostic factors in human breast cancer? Cancer Res. 1999;59(3):525–8.

365. Grober OMV, Mutarelli M, Giurato G, Ravo M, Cicatiello L, De Filippo MR, et al. Global analysis of estrogen receptor beta binding to breast cancer cell genome reveals an extensive interplay with estrogen receptor alpha for target gene regulation. BMC Genomics. 2011;12(1):36. Available from: http://dx.doi.org/10.1186/1471-2164-12-36

366. Hua H, Zhang H, Kong Q, Jiang Y. Mechanisms for estrogen receptor expression in human cancer. Exp Hematol Oncol. 2018;7(1):24. Available from: http://dx.doi.org/10.1186/s40164-018-0116-7

367. Miziak P, Baran M, Błaszczak E, Przybyszewska-Podstawka A, Kałafut J, Smok-Kalwat J, et al. Estrogen receptor signaling in breast cancer. Cancers (Basel). 2023;15(19). Available from: http://dx.doi.org/10.3390/cancers15194689

368. Paterni I, Granchi C, Katzenellenbogen JA, Minutolo F. Estrogen receptors alpha (ERα) and beta (ERβ): subtype-selective ligands and clinical potential. Steroids. 2014;90:13–29. Available from: http://dx.doi.org/10.1016/j.steroids.2014.06.012

369. Chang J, Liu J, Li H, Li J, Mu Y, Feng B. Expression of ERβ gene in breast carcinoma and the relevance in neoadjuvant therapy. Oncol Lett. 2017;13(3):1641–6. Available from: http://dx.doi.org/10.3892/ol.2017.5659

370. Guo L, Zhang Y, Zhang W, Yilamu D. Correlation between estrogen receptor β expression and the curative effect of endocrine therapy in breast cancer patients. Exp Ther Med. 2014;7(6):1568–72. Available from: http://dx.doi.org/10.3892/etm.2014.1634

371. Choi Y, Kim H, Pollack S. ERβ isoforms have differential clinical significance in breast cancer subtypes and subgroups. Curr Issues Mol Biol. 2022;44(4):1564–86. Available from: http://dx.doi.org/10.3390/cimb44040107

372. Hawse JR, Carter JM, Aspros KGM, Bruinsma ES, Koepplin JW, Negron V, et al. Optimized immunohistochemical detection of estrogen receptor beta using two validated monoclonal antibodies confirms its expression in normal and malignant breast tissues. Breast Cancer Res Treat. 2020;179(1):241–9. Available from: http://dx.doi.org/10.1007/s10549-019-05441-3

373. Kuiper GG, Carlsson B, Grandien K, Enmark E, Häggblad J, Nilsson S, et al. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology. 1997;138(3):863–70. Available from: http://dx.doi.org/10.1210/endo.138.3.4979

374. Correction for Stiller et al., Patterns of nucleotide misincorporations during enzymatic amplification and direct large-scale sequencing of ancient DNA. Proc Natl Acad Sci U S A. 2006;103(40):14977–14977. Available from: http://dx.doi.org/10.1073/pnas.0607610103

375. Kumar R, Zakharov MN, Khan SH, Miki R, Jang H, Toraldo G, et al. The dynamic structure of the estrogen receptor. J Amino Acids. 2011;2011:812540. Available from: http://dx.doi.org/10.4061/2011/812540

376. Swedenborg E, Power KA, Cai W, Pongratz I, Rüegg J. Regulation of estrogen receptor beta activity and implications in health and disease. Cell Mol Life Sci. 2009;66(24):3873–94. Available from: http://dx.doi.org/10.1007/s00018-009-0118-z

377. Jakacka M, Ito M, Weiss J, Chien PY, Gehm BD, Jameson JL. Estrogen receptor binding to DNA is not required for its activity through the nonclassical AP1 pathway. J Biol Chem. 2001;276(17):13615–21. Available from: http://dx.doi.org/10.1074/jbc.M008384200

378. Wang C, Li J, Ye S, Zhang Y, Li P, Wang L, et al. Oestrogen inhibits VEGF expression and angiogenesis in triple-negative breast cancer by activating GPER-1. J Cancer. 2018;9(20):3802–11. Available from: http://dx.doi.org/10.7150/jca.29233

379. De Francesco EM, Pellegrino M, Santolla MF, Lappano R, Ricchio E, Abonante S, et al. GPER mediates activation of HIF1α/VEGF signaling by estrogens. Cancer Res. 2014;74(15):4053–64. Available from: http://dx.doi.org/10.1158/0008-5472.CAN-13-3590

380. Tao S, He H, Chen Q. Estradiol induces HOTAIR levels via GPER-mediated miR-148a inhibition in breast cancer. J Transl Med. 2015;13(1):131. Available from: http://dx.doi.org/10.1186/s12967-015-0489-x

381. Song I-S, Jeong YJ, Jeong SH, Kim JE, Han J, Kim T-H, et al. Modulation of mitochondrial ERβ expression inhibits triple-negative breast cancer tumor progression by activating mitochondrial function. Cell Physiol Biochem. 2019;52(3):468–85. Available from: http://dx.doi.org/10.33594/000000034

382. Reese JM, Suman VJ, Subramaniam M, Wu X, Negron V, Gingery A, et al. ERβ1: characterization, prognosis, and evaluation of treatment strategies in ERα-positive and -negative breast cancer. BMC Cancer. 2014;14(1):749. Available from: http://dx.doi.org/10.1186/1471-2407-14-749

383. Bado I, Nikolos F, Rajapaksa G, Gustafsson J-Å, Thomas C. ERβ decreases the invasiveness of triple-negative breast cancer cells by regulating mutant p53 oncogenic function. Oncotarget. 2016;7(12):13599–611. Available from: http://dx.doi.org/10.18632/oncotarget.7300

384. Wisinski KB, Xu W, Tevaarwerk AJ, Saha S, Kim K, Traynor A, et al. Targeting estrogen receptor beta in a phase 2 study of high-dose estradiol in metastatic triple-negative breast cancer: A Wisconsin oncology network study. Clin Breast Cancer. 2016;16(4):256–61. Available from: http://dx.doi.org/10.1016/j.clbc.2016.03.005

385. Physiological Concentrations of Genistein and 17β-Estradiol Inhibit MDA-MB-231 Breast Cancer Cell Growth by Increasing BAX/BCL-2 and Reducing pERK1/2 TALITHA T. ANTICANCER RESEARCH. 2012;32:1181–92.

386. Zhao J, Jiang CQ, Lam TH, Liu B, Cheng KK, Kavikondala S, et al. Genetically predicted 17β-estradiol and systemic inflammation in women: a separate-sample Mendelian randomisation analysis in the Guangzhou Biobank Cohort Study. J Epidemiol Community Health. 2014;68(8):780–5. Available from: http://dx.doi.org/10.1136/jech-2013-203451

387. Mann S, Laucirica R, Carlson N, Younes PS, Ali N, Younes A, et al. Estrogen receptor beta expression in invasive breast cancer. Hum Pathol. 2001;32(1):113–8. Available from: http://dx.doi.org/10.1053/hupa.2001.21506

388. Harvey JM, Clark GM, Osborne CK, Allred DC. Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. J Clin Oncol. 1999;17(5):1474–81. Available from: http://dx.doi.org/10.1200/JCO.1999.17.5.1474

389. Ross DS, Zehir A, Brogi E, Konno F, Krystel-Whittemore M, Edelweiss M, et al. Immunohistochemical analysis of estrogen receptor in breast cancer with ESR1 mutations detected by hybrid capture-based next-generation sequencing. Mod Pathol. 2019;32(1):81–7. Available from: http://dx.doi.org/10.1038/s41379-018-0116-5

390. Thomsen C, Nielsen S, Nielsen BS, Pedersen SH, Vyberg M. Estrogen receptor-α quantification in breast cancer: Concordance between immunohistochemical assays and mRNA-in situ hybridization for ESR1 gene. Appl Immunohistochem Mol Morphol. 2020;28(5):347–53. Available from: http://dx.doi.org/10.1097/PAI.0000000000000760

391. Mur C, Martínez-Carpio PA, Fernández-Montolí ME, Ramon JM, Rosel P, Navarro MA. Growth of MDA-MB-231 cell line: different effects of TGF-beta(1), EGF and estradiol depending on the length of exposure. Cell Biol Int. 1998;22(9–10):679–84. Available from: http://dx.doi.org/10.1006/cbir.1998.0306

392. Zambo KDA, Nagandla H, Cap KC, Phillips A, Nikolos F, Qian W, et al. Abstract 5550: Crosstalk of estrogen receptor B with tumor microenvironment

signaling explains tumor suppressor actions in TNBC. Cancer Res. 2024;84(6_Supplement):5550–5550. Available from: http://dx.doi.org/10.1158/1538-7445.am2024-5550

393. Mukhopadhyay UK, Oturkar CC, Adams C, Wickramasekera N, Bansal S, Medisetty R, et al. TP53 status as a determinant of pro- vs anti-tumorigenic effects of estrogen receptor-beta in breast cancer. J Natl Cancer Inst. 2019;111(11):1202–15. Available from: http://dx.doi.org/10.1093/jnci/djz051

394. Das GM, Mukhopadhyay UK, Bansal S, Wickramasekera N, Medisetty R, Swetzig WM, et al. Abstract 3465: p53 status as a determinant of estrogen receptor beta function in breast cancer. Cancer Res. 2015;75(15_Supplement):3465–3465. Available from: http://dx.doi.org/10.1158/1538-7445.am2015-3465

395. Scarpetti L, Oturkar CC, Juric D, Shellock M, Malvarosa G, Post K, et al. Therapeutic role of tamoxifen for triple-negative breast cancer: Leveraging the interaction between ERβ and mutant p53. Oncologist. 2023;28(4):358–63. Available from: http://dx.doi.org/10.1093/oncolo/oyac281

396. Lei S, Fan P, Wang M, Zhang C, Jiang Y, Huang S, et al. Elevated estrogen receptor β expression in triple negative breast cancer cells is associated with sensitivity to doxorubicin by inhibiting the PI3K/AKT/mTOR signaling pathway. Exp Ther Med. 2020;20(2):1630–6. Available from: http://dx.doi.org/10.3892/etm.2020.8809

397. Austin D, Hamilton N, Elshimali Y, Pietras R, Wu Y, Vadgama J. Estrogen receptor-beta is a potential target for triple negative breast cancer treatment. Oncotarget. 2018;9(74):33912–30. Available from: http://dx.doi.org/10.18632/oncotarget.26089

398. Wimberly H, Han G, Pinnaduwage D, Murphy LC, Yang XR, Andrulis IL, et al. ERβ splice variant expression in four large cohorts of human breast cancer patient tumors. Breast Cancer Res Treat. 2014;146(3):657–67. Available from: http://dx.doi.org/10.1007/s10549-014-3050-3

399. Yan M, Rayoo M, Takano EA, kConFab Investigators, Fox SB. Nuclear and cytoplasmic expressions of ERβ1 and ERβ2 are predictive of response to therapy and alters prognosis in familial breast cancers. Breast Cancer Res Treat. 2011;126(2):395–405. Available from: http://dx.doi.org/10.1007/s10549-010-0941-9

400. Nagandla H, Thomas C. Estrogen signals through ERβ in breast cancer; What we have learned since the discovery of the receptor. Receptors (Basel). 2024;3(2):182–200. Available from: http://dx.doi.org/10.3390/receptors3020010

401. Prognostic significance of full-length estrogen receptor beta expression in stage I-III triple negative breast cancer Erin K Shanle. Am J Transl Res. 2015;7(7):1246–59.

402. Takano EA, Younes MM, Meehan K, Spalding L, Yan M, Allan P, et al. Estrogen receptor beta expression in triple negative breast cancers is not associated with recurrence or survival. BMC Cancer. 2023;23(1):459. Available from: http://dx.doi.org/10.1186/s12885-023-10795-5

403. Samanta S, Sharma VM, Khan A, Mercurio AM. Regulation of IMP3 by EGFR signaling and repression by ERβ: implications for triple-negative breast cancer. Oncogene. 2012;31(44):4689–97. Available from: http://dx.doi.org/10.1038/onc.2011.620

404. Salahuddin A, Ghanem H, Omran GA, Helmy MW. Epigenetic restoration and activation of ERβ: an inspiring approach for treatment of triple-negative breast cancer. Med Oncol. 2022;39(10):150. Available from: http://dx.doi.org/10.1007/s12032-022-01765-1

405. Smart E, Hughes T, Smith L, Speirs V. Estrogen receptor β: putting a positive into triple negative breast cancer? Horm Mol Biol Clin Investig. 2013;16(3):117–23. Available from: http://dx.doi.org/10.1515/hmbci-2013-0042

406. Tremblay A, Tremblay GB, Labrie F, Giguère V. Ligand-independent recruitment of SRC-1 to estrogen receptor beta through phosphorylation of activation function AF-1. Mol Cell. 1999;3(4):513–9. Available from: http://dx.doi.org/10.1016/s1097-2765(00)80479-7

407. Nilsson S, Gustafsson J-Å. Estrogen receptors: therapies targeted to receptor subtypes. Clin Pharmacol Ther. 2011;89(1):44–55. Available from: http://dx.doi.org/10.1038/clpt.2010.226

408. Cittelly D, Contreras-Zarate MJ, ALvarez-Eraso K, Tesic V, Tsuji N, Chafee L, et al. Abstract GS5-07: Estradiol represses anti-tumoral immune response to promote progression of triple-negative breast cancer brain metastases. Cancer Res. 2023;83(5_Supplement):GS5-07-GS5-07. Available from: http://dx.doi.org/10.1158/1538-7445.sabcs22-gs5-07

409. van Barele M, Heemskerk-Gerritsen BAM, Louwers YV, Vastbinder MB, Martens JWM, Hooning MJ, et al. Estrogens and progestogens in triple negative breast cancer: Do they harm? Cancers (Basel). 2021;13(11):2506. Available from: http://dx.doi.org/10.3390/cancers13112506

410. Cho N. Imaging features of breast cancer molecular subtypes: state of the art. J Pathol Transl Med. 2021;55(1):16–25. Available from: http://dx.doi.org/10.4132/jptm.2020.09.03

411. Magaki S, Hojat SA, Wei B, So A, Yong WH. An introduction to the performance of immunohistochemistry. In: Methods in Molecular Biology. New York, NY: Springer New York; 2019. p. 289–98.

412. Ebata A, Suzuki T, Shoji-Harada N, Hamanaka Y, Miyashita M, Iwabuchi E, et al. Immunolocalization of cytoplasmic ER in ER-negative breast carcinoma as a potent favorable prognostic predictor. Acta Histochem Cytochem. 2023;56(4):59–66. Available from: http://dx.doi.org/10.1267/ahc.23-00016

413. Nigjeh SE, Yeap SK, Nordin N, Kamalideghan B, Ky H, Rosli R. Citral induced apoptosis in MDA-MB-231 spheroid cells. BMC Complement Altern Med. 2018;18(1):56. Available from: http://dx.doi.org/10.1186/s12906-018-2115-y

414. Pan L, Li J, Xu Q, Gao Z, Yang M, Wu X, et al. HER2/PI3K/AKT pathway in HER2-positive breast cancer: A review. Medicine (Baltimore). 2024;103(24):e38508. Available from: http://dx.doi.org/10.1097/MD.0000000000038508

415. Stoica GE, Franke TF, Moroni M, Mueller S, Morgan E, Iann MC, et al. Effect of estradiol on estrogen receptor-alpha gene expression and activity can be modulated by the ErbB2/PI 3-K/Akt pathway. Oncogene. 2003;22(39):7998–8011. Available from: http://dx.doi.org/10.1038/sj.onc.1206769

416. Yan S, Wang J, Chen H, Zhang D, Imam M. Divergent features of ERβ isoforms in triple negative breast cancer: progress and implications for further research. Front Cell Dev Biol. 2023;11:1240386. Available from: http://dx.doi.org/10.3389/fcell.2023.1240386

417. Malloy PJ, Feldman D. Inactivation of the human vitamin D receptor by caspase-3. Endocrinology. 2009;150(2):679–86. Available from: http://dx.doi.org/10.1210/en.2008-1217

418. Huang Y, Hong W, Wei X. The molecular mechanisms and therapeutic strategies of EMT in tumor progression and metastasis. J Hematol Oncol. 2022;15(1):129. Available from: http://dx.doi.org/10.1186/s13045-022-01347-8

419. Gilad LA, Bresler T, Gnainsky J, Smirnoff P, Schwartz B. Regulation of vitamin D receptor expression via estrogen-induced activation of the ERK 1/2 signaling pathway in colon and breast cancer cells. J Endocrinol. 2005;185(3):577–92. Available from: http://dx.doi.org/10.1677/joe.1.05770

420. Tito C, Masciarelli S, Colotti G, Fazi F. EGF receptor in organ development, tissue homeostasis and regeneration. J Biomed Sci. 2025;32(1):24. Available from: http://dx.doi.org/10.1186/s12929-025-01119-9

421. Wee P, Wang Z. Epidermal growth factor receptor cell proliferation signaling pathways. Cancers (Basel). 2017;9(5):52. Available from: http://dx.doi.org/10.3390/cancers9050052

422. Trujillo V, Marín-Luevano P, González-Curiel I, Rodríguez-Carlos A, Ramírez-Reyes M, Layseca-Espinosa E, et al. Calcitriol promotes proangiogenic molecules in keratinocytes in a diabetic foot ulcer model. J Steroid Biochem Mol Biol. 2017;174:303–11. Available from: http://dx.doi.org/10.1016/j.jsbmb.2017.10.013

423. Masuda H, Zhang D, Bartholomeusz C, Doihara H, Hortobagyi GN, Ueno NT. Role of epidermal growth factor receptor in breast cancer. Breast Cancer Res Treat. 2012;136(2):331–45. Available from: http://dx.doi.org/10.1007/s10549-012-2289-9

424. Srivastava N, Usmani SS, Subbarayan R, Saini R, Pandey PK. Hypoxia: syndicating triple negative breast cancer against various therapeutic regimens. Front Oncol. 2023;13:1199105. Available from: http://dx.doi.org/10.3389/fonc.2023.1199105

425. Lewis-Wambi JS, Jordan VC. Estrogen regulation of apoptosis: how can one hormone stimulate and inhibit? Breast Cancer Res. 2009;11(3):206. Available from: http://dx.doi.org/10.1186/bcr2255

426. Bajbouj K, Sahnoon L, Shafarin J, Al-Ali A, Muhammad JS, Karim A, et al. Vitamin D-mediated anti-cancer activity involves iron homeostatic balance disruption and oxidative stress induction in breast cancer. Front Cell Dev Biol. 2021;9:766978. Available from: http://dx.doi.org/10.3389/fcell.2021.766978

427. Wang W, Smith R 3rd, Burghardt R, Safe SH. 17 beta-Estradiol-mediated growth inhibition of MDA-MB-468 cells stably transfected with the estrogen receptor: cell cycle effects. Mol Cell Endocrinol. 1997;133(1):49–62. Available from: http://dx.doi.org/10.1016/s0303-7207(97)00142-1

428. Swami S, Krishnan AV, Peng L, Lundqvist J, Feldman D. Transrepression of the estrogen receptor promoter by calcitriol in human breast cancer cells via two negative vitamin D response elements. Endocr Relat Cancer. 2013;20(4):565–77. Available from: http://dx.doi.org/10.1530/ERC-12-0281

429. Manente AG, Pinton G, Zonca S, Cilli M, Rinaldi M, Daga A, et al. Intracellular lactate-mediated induction of estrogen receptor beta (ERβ) in biphasic malignant pleural mesothelioma cells. Oncotarget. 2015;6(28):25121–34. Available from: http://dx.doi.org/10.18632/oncotarget.4486

430. Murphy LC, Peng B, Lewis A, Davie JR, Leygue E, Kemp A, et al. Inducible upregulation of oestrogen receptor-beta1 affects oestrogen and tamoxifen

responsiveness in MCF7 human breast cancer cells. J Mol Endocrinol. 2005;34(2):553–66. Available from: http://dx.doi.org/10.1677/jme.1.01688

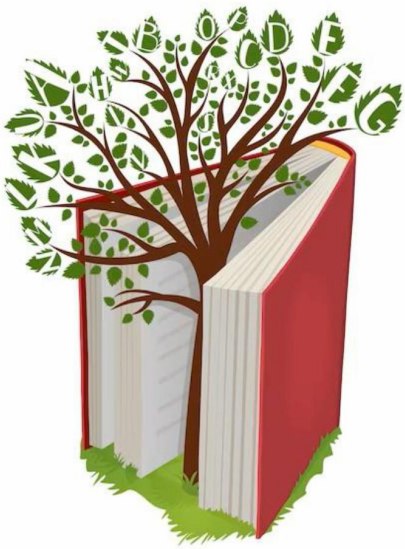
431. Segovia-Mendoza M, Díaz L, González-González ME, Martínez-Reza I, García-Quiroz J, Prado-Garcia H, et al. Calcitriol and its analogues enhance the antiproliferative activity of gefitinib in breast cancer cells. J Steroid Biochem Mol Biol. 2015;148:122–31. Available from: http://dx.doi.org/10.1016/j.jsbmb.2014.12.006

432. Khode V, Patil S, Kaveeshwar V, Ruikar K, Bargale A, E S, et al. Ubiquitin mediated degradation of EGFR by 17 β-estradiol in triple negative MDA-MB-231 (TNBC) breast cancer cells line. Curr Mol Med. 2022;22(5):449–57. Available from: http://dx.doi.org/10.2174/1566524021666210729144713

433. Ben-Shoshan M, Amir S, Dang DT, Dang LH, Weisman Y, Mabjeesh NJ. 1alpha,25-dihydroxyvitamin D3 (Calcitriol) inhibits hypoxia-inducible factor-1/vascular endothelial growth factor pathway in human cancer cells. Mol Cancer Ther. 2007;6(4):1433–9. Available from: http://dx.doi.org/10.1158/1535-7163.MCT-06-0677

434. Chimento A, De Luca A, Avena P, De Amicis F, Casaburi I, Sirianni R, et al. Estrogen receptors-mediated apoptosis in hormone-dependent cancers. Int J Mol Sci. 2022;23(3):1242. Available from: http://dx.doi.org/10.3390/ijms23031242

435. Van Themsche C, Parent S, Leblanc V, Descôteaux C, Simard A-M, Bérubé G, et al. VP-128, a novel oestradiol-platinum(II) hybrid with selective anti-tumour activity towards hormone-dependent breast cancer cells in vivo. Endocr Relat Cancer. 2009;16(4):1185–95. Available from: http://dx.doi.org/10.1677/ERC-09-0113



ANNEXURES

Intuitional Ethical Certificate

Azadi Ka Amrit Mahotsav

BLDE
(DEEMED TO BE UNIVERSITY)
Declared as Deemed to be University u/s 3 of UGC Act, 1956
Accredited with 'A' Grade by NAAC (Cycle-2)
The Constituent College
SHRI B. M. PATIL MEDICAL COLLEGE, HOSPITAL & RESEARCH CENTRE, VIJAYAPURA

BLDE (DU)/IEC/ 631-C/2022-23 28/5/2022

INSTITUTIONAL ETHICAL CLEARANCE CERTIFICATE

The Ethical Committee of this University met on 27th May, 2022 at 11.00 a.m. scrutinizes the Synopsis/ Research Projects of Post Graduate Student / Under Graduate Student /Faculty members of this University /**Ph.D. Student** College from ethical clearance point of view. After scrutiny, the following original/ corrected and revised version synopsis of the thesis/ research projects has been accorded ethical clearance.

Title: "Vitamin D3 mediated regulation of hormone receptors in the pathogenesis of triple negative breast cancer".

Name of the Principal Investigator: Mr.Shankaramurthy K.N., Ph.D Scholar (JRF), Dept. of Biochemistry.

Dr. Santoshkumar Jeevanagi
Chairperson
IEC, BLDE (DU),
VIJAYAPURA
Chairman,
Institutional Ethical Committee,
BLDE (Deemed to be University)
Vijayapura

Dr.Akram A. Naikwadi
Member Secretary
IEC, BLDE (DU),
VIJAYAPURA
MEMBER SECRETARY
Institutional Ethics Committee
BLDE (Deemed to be University)
Vijayapura-586103, Karnataka

Following documents were placed before Ethical Committee for Scrutinization

- Copy of Synopsis/Research Projects
- Copy of inform consent form
- Any other relevant document

Smt. Bangaramma Sajjan Campus, B. M. Patil Road (Sholapur Road), Vijayapura - 586103, Karnataka, India.
BLDE (DU): Phone: +918352-262770, Fax: +918352-263303, Website: www.bldedu.ac.in, E-mail:office@bldedu.ac.in
College: Phone: +918352-262770, Fax: +918352-263019, E-mail: bmpmc.principal@bldedu.ac.in

PRESENTATIONS

RAMAIAH Medical College | A Constituent College, Ramaiah University of Applied Sciences

National Health Conference & Medical Expo

Certificate of Participation

MR SHANKARA MURTHY K N

Has participated as a Delegate in the

63rd Annual Conference of National Academy of Medical Sciences held on 6th to 8th October 2023 at Ramaiah Medical College, Bengaluru.

Prof. S K Sarin, President of NAMS

Dr. Umesh Kapil, Secretary of NAMS

Prof. O P Kharbanda, Chief Mentor-Organizing Committee, NAMSCOM 2023

Dr. Shalini C Nooyi, Organizing Chairperson, NAMSCOM 2023

Dr. Nanda Kumar B S, Organizing Secretary, NAMSCON 2023

Dr. Ashwini C Appaji, Organizing Secretary, NAMSCON 2023

Dr. Lalitha K, Scientific Committee Chairperson, NAMSCON 2023

CERTIFICATE OF APPRECIATION NAMSCON 2023

National Forensic Sciences University
Knowledge | Wisdom | Fulfilment
An Institution of National Importance
(Ministry of Home Affairs, Government of India)

Karnataka Institute for DNA Research
(An Autonomous Institute under Department of Higher Education Government of Karnataka)

Certificate

of Participation

International Conference on Recent Trends in DNA Forensics

This is to certify that

Mr. Shankaramurthy K N

participated in the International Conference on Recent Trends in DNA Forensics held at National Forensic Sciences University, Dharwad, Karnataka, India on 29th & 30th November, 2023.

Organizing Secretary
Dr. Saroj P. Babar

Convenor
Prof. (Dr.) Manjunath Ghate

Certificate No.: NFSU-DH/CON/RTDNAF/23010

JSS Academy of Higher Education & Research
(Deemed to be University), Accredited 'A+' Grade by NAAC & Rank 34 by NIRF, Gov. of India
Division of Biochemistry, School of Life Sciences, Mysuru
Mysuru – 570015, Karnataka, India

Certificate of Appreciation

This is to certify that

Prof./ Dr. /Mr./ Ms. Shankaramurthy K. N

has participated as a DELEGATE in the DST-SERB sponsored International Conference on Genetics and Epigenetics of Cancer, organized by the Division of Biochemistry, School of Life Sciences, Mysuru, JSS Academy of Higher Education & Research on 30th & 31st October 2023.

Dr. D. Guru Kumar
Organizing Secretary

Dr. M.V.S.S.T. SubbaRao
Co-Convener

Dr. K.A. Raveesha
Convener

Dr. B. Manjunatha
Registrar

RESEARCH TO PUBLICATION

Certificate of completion

This is to certify that

Shankarmurthy K N

Completed the health research methodology and publishing programme

Research to Publication

On

5th May 2022

Research to Publication was developed by BMJ and UCSF and brought to students in association with BLDE (Deemed to be University).
BMJ and UCSF have assigned 200 hours of CPD/CME credit for this programme.

Fiona Godlee
Editor in Chief, The BMJ

Jennifer R. Grandis
Associate Vice Chancellor of Clinical and Translational Research, Director of Clinical and Translational Science Institute

BMJ

UCSF

CERTIFICATE

PROUDLY PRESENTED TO

Shankaramurthy K N

Peer Review 101: Build Expertise, Become a Peer Reviewer

Sep 24, 2024

Date of Completion

Taylor and Francis Group

Organizer

Scopus

ELSEVIER

Certificate of Attendance

This is to certify that

SHANKARAMURTHY K N

has attended the following FDP

Author Workshop and Scopus Metrics Analysis

at BLDE (DU), Vijayapura on 28th to 30th June, 2021

Presented by Vikram Sudhakar, Dr. Ishima Badhwar and Sameer Gupta, Health Solutions, Elsevier

Umang Gupta
Marketing Director, India
RELX India Pvt. Ltd.

Prof. Aravind V Patil
Principal
BLDE (Deemed to be University)

BLDE (DEEMED TO BE UNIVERSITY)

SHRI B. M. PATIL MEDICAL COLLEGE, HOSPITAL & RESEARCH CENTRE, VIJAYAPURA

RESEARCH AND DEVELOPMENT CELL

In Association With

IQAC & CAMR

CERTIFICATE
OF APPRECIATION

This is to certify that

Mr. Shankaramurthy K N

has participated in Workshop on
"Human Stem Cells and their Applications" organized by R & D Cell in association with IQAC & CAMR, BLDE(Deemed to be University), on 24th and 25th August 2023

Dr. Lata Mullur
Director/Co-Ordinator, IQAC, BLDE(DU)

Dr. M. M. Patil
Director, R & D Cell, BLDE (DU)

Dr. R. V. Kulkarni
Registrar, BLDE (DU)

ZYIRN RESEARCH CONSULTANCY SERVICES
Your success is our business

CERTIFICATE
OF PARTICIPATION

Proudly Presented To:

Mr. Shankaramurthy K N

In recognition of your Participation at the Training Workshop on

Good Laboratory Practices (GLP) for Non-clinical Laboratory Studies

ORGANIZED BY
Zyirn Research Consultancy Services
by Dr. Imran Khan, Expert GLP Consultant and Trainer

IN ASSOCIATION WITH
Research & Development Cell & Centre for Advanced Medical Research (CAMR)
BLDE (Deemed to be University)
Shri B. M. Patil Medical College, Hospital and Research Centre,
on 19th April 2024 at Vijayapura, Karnataka.

GLP topics covered during the one-day Onsite GLP Training Program are
1. Introduction to Principles of GLP covering Ten Primary Elements of GLP
2. Responsibilities of Test Facility Management
3. Roles and Responsibilities of Study Director and Study Personnel
4. Quality Assurance Unit and GLP including the Responsibilities of QAU
5. The Conduct and Performance of the Study under GLP
6. Apparatus, Materials, and Reagents used under GLP

19.04.2024	8 hrs	Dr. Imaran Khan, Expert GLP Consultant, Director, Operations	Dr. M.M. Patil, Director R & D, BLDE (DU)
Issued on	Training Hours		

BLDE
(DEEMED TO BE UNIVERSITY)
SHRI B. M. PATIL MEDICAL COLLEGE, HOSPITAL & RESEARCH CENTRE, VIJAYAPURA-586103
Accredited with 'A' Grade by NAAC (Cycle-2)

SCIENTIFIC ACADEMIC AND RESEARCH SOCIETY (SARS)
(Registered Under Karnataka Societies Registration Act 1960 with Society No. BIJ-S106-2014-15 dated 02-06-2014)

CERTIFICATE

THIS IS TO CERTIFY THAT

Mr Shankaramurthy KN MSc in Biochemistry

has presented in the **Paper Presentation**. Entitled **Vitamin D Receptor Status in Triple-Negative Breast Cancer Tumors.** at the Scientific Academic And Research Society (SARS) held on **28th December 2023**

PRESIDENT | SESSION CHAIRPERSON | CHAIRMAN | SECRETARY

PUBLICATIONS

Cureus
Part of SPRINGER NATURE

Open Access Original Article

Published via BLDE (Deemed to be University)

Immunohistochemical Expression of Vitamin D Receptors (VDRs) and Estrogen Receptor Beta 1 (ERβ1) in Molecular Subtypes of Triple-Negative Breast Cancer Tumors: A Cross-Sectional Study

Shankaramurthy K N [1], Basavaraj B. Devaranavadagi [1], Indira A. Hundekari [1]

1. Biochemistry, Bijapur Lingayat District Educational (BLDE) Shri B.M. Patil Medical College Hospital and Research Centre, Vijayapura, IND

Corresponding author: Indira A. Hundekari, indira.hundekari@bldedu.ac.in

Received 11/17/2024
Review began 01/01/2025
Review ended 01/07/2025
Published 01/18/2025

DOI: 10.7759/cureus.77637

Abstract

Introduction

Breast cancer (BC) is still the most common malignancy among women globally, and triple-negative breast cancer (TNBC) presents major therapeutic and management issues due to its aggressive nature. Recent studies suggest that the vitamin D receptor (VDR) and estrogen receptor beta 1 (ERβ1) play crucial roles in regulating TNBC progression. Increased expression of VDR and ERβ1 has been linked to tumor suppression, highlighting their potential to impact cancer progression via various signaling pathways. This study analyzes VDR and ERβ1 expressions in TNBC subtypes to discover potential therapeutic targets and improve treatment outcomes for this challenging BC subtype.

Method

This cross-sectional study analyzed 30 invasive ductal carcinoma (IDC) cases of TNBC subtypes using formalin-fixed paraffin embedding (FFPE) tissues. Immunohistochemistry assessed cytoplasmic and nuclear VDR and ERβ1 expression, scoring staining intensity and extent, categorized as negative/low, moderate, or high expression.

Results

High VDR and ERβ1 expressions were analyzed across molecular subtypes of TNBC to explore their therapeutic potential, particularly in TNBC. In TNBC, a high VDR expression was observed in the cytoplasm (n = 10, 33.3%) and the nucleus (n = 2, 6.6%), with statistical significance ($p < 0.042$). Luminal A cases demonstrated high VDR expression in the cytoplasm (n = 6, 20%) and the nucleus (n = 2, 6.6%) ($p < 0.042$), while luminal B exhibited high VDR expression exclusively in the cytoplasm (n = 4, 13.3%) ($p < 0.042$). In HER2-enriched, high VDR expression was confined to the nucleus (n = 3, 10%) ($p < 0.042$). ERβ1 expression patterns in TNBC showed moderate cytoplasmic expression (n = 9, 50%) and high cytoplasmic expression (n = 1, 5.5%), with statistical significance ($p < 0.025$). By contrast, luminal A displayed moderate cytoplasmic expression (n = 3, 16.6%) and high cytoplasmic expression (n = 5, 27.7%) ($p < 0.025$). These findings suggest that VDR and ERβ1 exhibit subtype-specific expression patterns, with significant expression in TNBC, indicating their potential as therapeutic targets.

Conclusion

VDR and ERβ1 expressions differ between TNBC subtypes, indicating their potential as targeted therapies, particularly in TNBC.

Categories: General Surgery, Pathology, Oncology
Keywords: breast cancer, erβ1, immunoreactivity, tnbc, vdr

Introduction

Globally, breast cancer (BC) is the most prevalent cancer diagnosed in women and the primary cause of cancer-related mortality [1]. Triple-negative breast cancer (TNBC) is one of the most perilous kinds of BC. The lack of important receptors, including the human epidermal growth factor receptor 2 (HER2), progesterone receptor (PR), and estrogen receptor (ER), makes treatment extremely difficult [2]. About 15% to 20% of all invasive BCs are TNBC, which is linked to a higher risk of distant metastases and early death, usually within three to five years after diagnosis [3]. The majority of TNBC patients are young, premenopausal women. Patients with TNBC still have a poor prognosis despite advances in treatment options such as radiation therapy, chemotherapy, and surgery [4]. Experimental studies suggest that patients with TNBC often have significantly lower serum 25-hydroxyvitamin D (25(OH)D) levels [5]. This potential involvement is further highlighted by findings showing that high vitamin D receptor (VDR) expression

How to cite this article
K N S, Devaranavadagi B B, Hundekari I A (January 18, 2025) Immunohistochemical Expression of Vitamin D Receptors (VDRs) and Estrogen Receptor Beta 1 (ERβ1) in Molecular Subtypes of Triple-Negative Breast Cancer Tumors: A Cross-Sectional Study. Cureus 17(1): e77637. DOI 10.7759/cureus.77637

Cureus
Part of SPRINGER NATURE

Open Access Original Article

Published via BLDE (Deemed to be University)

Targeting ERβ1-Positive Triple-Negative Breast Cancer: Molecular Effects of Calcitriol and 17β-Estradiol

Shankaramurthy K N [1], Praveenkumar Shetty [2], Basavaraj Devaranagadi [1], Indira A. Hundekari [1]

1. Biochemistry, Bijapur Lingayat District Educational (BLDE) Shri B.M. Patil Medical College Hospital and Research Centre, Vijayapura, IND 2. Biochemistry, K S Hegde Medical Academy, NITTE (Deemed to be University), Mangaluru, IND

Corresponding author: Indira A. Hundekari, indira.hundekari@bldedu.ac.in

Received 02/24/2025
Review began 03/18/2025
Review ended 04/13/2025
Published 04/24/2025

DOI: 10.7759/cureus.82934

Abstract

Background

Breast cancer is the most common malignancy in women. Triple-negative breast cancer (TNBC) is characterized by the absence of estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2, contributing to its aggressive nature, limited treatment options, and poor prognosis. Emerging evidence highlights estrogen receptor beta 1 (ERβ1) as a potential tumor suppressor in TNBC, influencing key oncogenic pathways such as cell proliferation, survival, angiogenesis, and apoptosis. In this regard, calcitriol (active vitamin D) and 17β-estradiol have been identified as key regulators of tumor behavior. Calcitriol shows strong anti-proliferative and pro-apoptotic effects, while the ability of 17β-estradiol to modulate tumor progression through ERβ1 signaling is context-dependent. This study aims to investigate the individual and combined effects of calcitriol and 17β-estradiol in ERβ1-expressing MDA-MB-468 TNBC cells, with a focus on their role in regulation of tumor progression, angiogenesis, and apoptosis. The findings provide novel insights into the potential therapeutic utility of targeting ERβ1 in TNBC.

Methodology

The MDA-MB-468 TNBC cells were treated with calcitriol (1-5 µM) and/or 17β-estradiol (100-500 nM). The effect on cell viability was assayed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. At the same time, immunoblot analysis investigated the time-dependent manner of ERβ1, epidermal growth factor receptor (EGFR), vascular epithelial growth factor (VEGF), and caspase-3.

Results

Both calcitriol and 17β-estradiol substantially decreased TNBC cell viability, with the highest level of cytotoxicity observed at 24 and 32 hours, respectively. The combination increased the amount of cell death. Immunoblots revealed lasting downregulation of ERβ1, EGFR, VEGF, and caspase-3 after calcitriol treatment. In comparison, 17β-estradiol demonstrated biphasic regulatory behavior for ERβ1, where ERβ1 was first downregulated, then partially recovered. The combination therapies produced more significant ERβ1 downregulation and heightened suppression of EGFR and VEGF, further enhancing their effects on TNBC progression.

Conclusions

This study aims to investigate the individual and combined effects of calcitriol and 17β-estradiol in ERβ1-expressing MDA-MB-468 TNBC cells, with a focus on their role in the regulation of tumor progression, angiogenesis, and apoptosis.

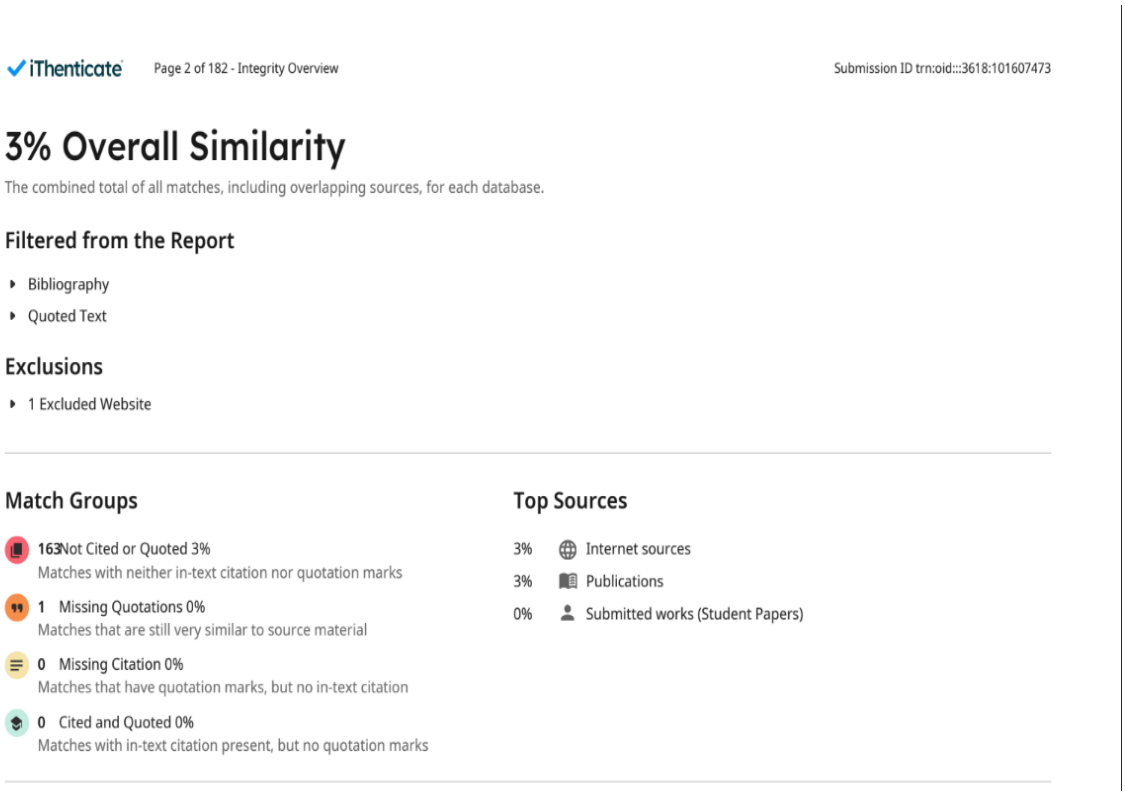
Categories: Pathology, Medical Education, Oncology
Keywords: 17β-estradiol, breast cancer, calcitriol, caspase 3, cytotoxicity, egfr, erβ1, hormone-based therapy, tnbc, vegf

Introduction

Breast cancer (BC) is the most frequently diagnosed cancer in women globally [1]. Among its subtypes, triple-negative breast cancer (TNBC) is the most aggressive, comprising 15-20% of BC cases [2]. TNBC is characterized by a lack of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) expression, consequently making TNBC the most challenging subtype to treat due to a lack of targeted treatment options [3]. This is more common in younger women, African Americans, and those with *BRCA1* mutations [4]. Currently, chemotherapy is the standard treatment, but the high rate of recurrences, the onset of chemoresistance, and poor survival indicate an urgent need for new therapeutic options [5,6]. Recently, estrogen receptor beta 1 (ERβ1) has emerged as a potential tumor suppressor in TNBC and regulates oncogenic pathways essential for proliferation, survival, angiogenesis, and apoptosis

How to cite this article
K N S, Shetty P, Devaranagadi B, et al. (April 24, 2025) Targeting ERβ1-Positive Triple-Negative Breast Cancer: Molecular Effects of Calcitriol and 17β-Estradiol. Cureus 17(4): e82934. DOI 10.7759/cureus.82934

PLAGIARISM CERTIFICATE



iThenticate Page 2 of 182 - Integrity Overview Submission ID trn:oid:::3618:101607473

3% Overall Similarity

The combined total of all matches, including overlapping sources, for each database.

Filtered from the Report

- Bibliography
- Quoted Text

Exclusions

- 1 Excluded Website

Match Groups

163 Not Cited or Quoted 3%
Matches with neither in-text citation nor quotation marks

1 Missing Quotations 0%
Matches that are still very similar to source material

0 Missing Citation 0%
Matches that have quotation marks, but no in-text citation

0 Cited and Quoted 0%
Matches with in-text citation present, but no quotation marks

Top Sources

3% Internet sources
3% Publications
0% Submitted works (Student Papers)