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Exploration of Oxidative Stress and Epigenetic Biomarkers in the Management of Breast Cancer

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Dedication

To my parents, grandparents, sisters, the entire family, and my friends

This work is for you

You have been constant pillars of support, helping me navigate life's challenges and consistently showering me with love

I love you all!

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Abstract

Breast cancer (BC) is a global and national health concern affecting women. Its molecular and prognostic heterogeneity requires the discovery of efficient biomarkers to improve the management of this disease. Epigenetic alterations can drive tumor transformation by silencing tumor suppressor genes (TSGs), and DNA hypermethylation-mediated TSG inactivation has been extensively studied in BC and could represent an early event in mammary transformation. In this respect, the role of *BRCA1* gene promoter hypermethylation in non-malignant mammary tissues, like normal adjacent tissues (NATs) and benign breast lesions (BBLs), warrants further exploration. NADPH oxidases (NOXs), recognized for their ability to generate reactive oxygen species (ROS), are overexpressed in numerous cancer types, potentially leading to oxidative stress (OS). OS is closely linked to breast malignancy by influencing the epithelial-mesenchymal transition (EMT) and angiogenesis. These processes were shown to be involved in BC by the ability of NADPH oxidase 4 (NOX4) protein to produce ROS. Actually, there is limited data regarding NOX4 and the NADPH oxidase DUOX1 proteins expression and subcellular localization in malignant breast tumors (MBTs) and their NATs.

Our research investigated the expression of NADPH oxidase proteins as oxidative markers and *BRCA1* gene promoter hypermethylation as an epigenetic marker using FFPE samples (Retrospective study). Our exploration of *BRCA1* gene promoter hypermethylation, using Methylation-Specific PCR (n=84: 48MBTs, 15NATs and 21 BBLs), revealed elevated rates in MBTs (41.67%:20/48) compared to BBLs (19.05%:4/21), aligning with previous reports on the role of this epigenetic silencing in breast malignancy. Nevertheless, its presence in BBLs suggests its potential as an early marker of genetic and epigenetic instabilities. Intriguingly, NATs, often used as controls, showed a hypermethylation rate (46.67%:7/15) comparable to MBTs. Furthermore, *BRCA1* gene promoter hypermethylation correlates with aggressive BC subtypes, including HER2-positive BC and triple-negative BC. Regarding NOX4 protein expression, the immunohistochemistry experimentation (100MBTs + 94 NATs) showed a significant upregulation of NOX4 protein in MBTs (79%:79/100) compared to NATs (51.06%:48/94). Conversely, DUOX1 protein immunostaining levels were reduced in MBTs compared to NATs. NOX4 protein subcellular localization (cytoplasm, perinuclear area, and nucleus) showed a statistically significant concordance (*Kappa*= 0.487; Cohen's kappa test) between MBTs and their paired NATs, suggesting the potential role of tumor microenvironment in regulating the subcellular localization of NOX4 protein. In addition, NOX4 protein perinuclear localization correlates with the triple-negative BC phenotype. Collectively, this translational research highlights NOX4 protein and *BRCA1* gene promoter hypermethylation in BC as potential early events driving mammary transformation and as attractive therapeutic targets of the aggressive forms of BC.

Keywords: Breast cancer (BC); Oxidative stress; Epigenetic instability; NADPH oxidases; NOX4; DUOX1; *BRCA1* gene promoter hypermethylation; Subcellular localization; Biomarker; early events; Normal adjacent tissue; Benign breast lesions

Résumé

Le cancer du sein (CS) constitue un problème de santé mondial et national qui affecte les femmes. Son hétérogénéité moléculaire et pronostique nécessite la découverte de biomarqueurs efficaces pour améliorer la gestion de la maladie. Les altérations épigénétiques peuvent favoriser la transformation tumorale en réprimant les gènes suppresseurs de tumeurs (GST), et l'inactivation des GST due à l'hyperméthylation de l'ADN a été largement étudiée dans le cancer du sein (CS) et pourrait représenter un événement précoce dans la transformation mammaire. À cet égard, le rôle de l'hyperméthylation du promoteur du gène *BRCA1* dans les tissus mammaires non malins, tels que les tissus mammaires normaux adjacents (NATs) et les lésions mammaires bénignes (BBLs), mérite une exploration plus approfondie. Les NADPH oxydases (NOX), reconnues pour leur capacité à générer des espèces réactives de l'oxygène (ROS), sont surexprimées dans de nombreux types de cancer, ce qui peut potentiellement conduire au stress oxydatif (SO). Le SO est étroitement lié à la malignité mammaire en influençant la transition épithéliale-mésenchymateuse (TEM) et l'angiogenèse. Ces processus ont été rapportés d'être impliqués dans le CS par la capacité de la protéine NADPH oxydase 4 (NOX4) à produire des ROS. Cependant, il existe peu de données concernant l'expression protéique et la localisation subcellulaire des deux protéines NOX4 et la NADPH oxydase DUOX1 dans les tumeurs mammaires malignes (MBTs) et leurs NATs.

Notre recherche a étudié l'expression des protéines NADPH oxydases comme marqueurs oxydatifs et l'hyperméthylation du promoteur du gène *BRCA1* comme marqueur épigénétique en utilisant des échantillons FFPE (Étude rétrospective). Notre exploration de l'hyperméthylation du promoteur du gène *BRCA1* a révélé des taux élevés dans les MBTs (41,67 % : 20/48) par rapport aux BBLs (19,05 % : 4/21), ce qui concorde avec les rapports antérieurs sur le rôle de ce silencing épigénétique dans la malignité mammaire. Néanmoins, sa présence dans les BBLs suggère son potentiel en tant que marqueur précoce des instabilités génétiques et épigénétiques. De manière intrigante, les NATs, souvent utilisés comme témoins, ont montré un taux d'hyperméthylation (46,67 % : 7/15) comparable à celui des MBTs. De plus, l'hyperméthylation du promoteur du gène BRCA1 est corrélée avec les sous-types agressifs du CS, notamment le sous-type HER2-positif et le sous-type triple-négatif. Concernant l'expression de la protéine NADPH oxydase 4, l'expérimentation immunohistochimique (100MBTs + 94 NATs) a révélé une augmentation significative de la protéine NOX4 dans les MBTs (79 % : 79/100) par rapport aux NATs (51,06 % : 48/94). En revanche, les niveaux de la protéine DUOX1 étaient réduits dans les MBTs par rapport aux NATs. La localisation subcellulaire de la protéine NOX4 (cytoplasme, zone périnucléaire et noyau) a montré une concordance statistiquement significative (*Kappa*= 0.487; Cohen's kappa test) entre les MBTs et les NATs, suggérant le rôle potentiel du microenvironnement tumoral dans la régulation de de la localisation subcellulaire de NOX4. De plus, la localisation périnucléaire de la protéine NOX4 est corrélée avec le phénotype du CS triple négatif. Dans l'ensemble, cette recherche translationnelle met en lumière la protéine NOX4 et l'hyperméthylation du promoteur du gène *BRCA1* dans le CS en tant qu'événements précoces potentiels favorisant la transformation mammaire et comme des cibles thérapeutiques pour les formes agressives du CS.

Mots-clés : Cancer du sein (CS) ; Stress oxydatif ; Instabilité épigénétique ; NADPH oxydases ; NOX4 ; DUOX1 ; Hyperméthylation du promoteur du gène *BRCA1* ; Localisation subcellulaire ; Biomarqueur ; Événements précoces ; Tissu normal adjacent ; Lésions bénignes du sein

Résumé Étendu

Le cancer du sein (CS) constitue un problème de santé mondial et national qui affecte les femmes. En effet, le cancer du sein est le cancer le plus fréquemment diagnostiqué chez les femmes dans le monde, représentant 24,5 % (2,3 millions) de tous les cas diagnostiqués en 2020. Il demeure la principale cause de décès liés au cancer chez les femmes dans plus de 110 pays. Au Maroc, le cancer du sein représentait 38,9 % des cas diagnostiqués par cancer chez les femmes et 24,7 % des décès liés au cancer en 2020. L'hétérogénéité moléculaire et pronostique du cancer du sein nécessite la découverte de biomarqueurs efficaces pour améliorer la gestion de la maladie. Les altérations épigénétiques peuvent favoriser la transformation tumorale en réprimant les gènes suppresseurs de tumeurs (GST), et l'inactivation des GST due à l'hyperméthylation de l'ADN a été largement étudiée dans le cancer du sein (CS) et pourrait représenter un événement précoce dans la transformation mammaire. À cet égard, le rôle de l'hyperméthylation du promoteur du géne *BRCA1* dans les tissus mammaires non malins, tels que les tissus mammaires normaux adjacents (NATs) et les lésions mammaires bénignes (BBLs), mérite une exploration plus approfondie. Les NADPH oxydases (NOX), reconnues pour leur capacité à générer des espèces réactives de l'oxygène (ROS), sont surexprimées dans de nombreux types de cancer, ce qui peut potentiellement conduire au stress oxydatif (SO). Le SO est étroitement lié à la malignité mammaire en influençant la transition épithéliale-mésenchymateuse (TEM) et l'angiogenèse. Ces processus ont été rapportés d'être impliqués dans le CS par la capacité de la protéine NADPH oxydase 4 (NOX4) à produire des ROS. Cependant, il existe peu de données concernant l'expression protéique et la localisation subcellulaire des deux protéines NOX4 et la NADPH oxydase DUOX1 dans les tumeurs mammaires malignes (MBTs) et leurs NATs.

Dans le cadre de notre étude translationnelle (rétrospective), nous avons évalué des biomarqueurs épigénétiques et oxydatifs dans différents types de tissus mammaires, en vue de d'évaluer leur prévalence et de comprendre leur corrélation avec les caractéristiques agressives du cancer du sein. Notre recherche s'est concentrée sur l'expression des protéines NADPH oxydases, servant de potentiels marqueurs oxydatifs, et sur l'hyperméthylation du promoteur du gène BRCA1, en tant que marqueur épigénétique. Pour ce faire, nous avons utilisé des échantillons de tissus mammaires fixés au formol et enrobés de paraffine (FFPE). Ces échantillons ont été analysés à l'aide de la PCR spécifique à la méthylation (pour 84 échantillons) afin d'examiner l'hyperméthylation du promoteur du gène BRCA1, et par immunohistochimie (pour 198 échantillons) pour évaluer l'expression et la localisation subcellulaire des protéines NOX4 et DUOX1.

Notre exploration de l'hyperméthylation du promoteur du gène *BRCA1* a révélé des taux élevés dans les MBTs (41,67 % : 20/48) par rapport aux BBLs (19,05 % : 4/21), ce qui concorde avec les rapports antérieurs sur le rôle de ce silencing épigénétique dans la malignité mammaire. Néanmoins, sa présence dans les BBLs suggère son potentiel en tant que marqueur précoce des instabilités génétiques et épigénétiques, et donc potentiellement impliquer dans le risque du cancer du sein chez les porteuses de ces lésions bénignes. De manière intrigante, les NATs, souvent utilisés comme témoins, ont montré un taux d'hyperméthylation (46,67 % : 7/15) comparable à celui des MBTs. Cela suggère une dérégulation des voies moléculaires au niveau de ces tissus histologiquement normaux, ce qui pourrait avoir de nombreuses implications cliniques pour la prise en charge de cette pathologie. De plus, l'hyperméthylation du promoteur du gène BRCA1 est corrélée avec les sous-types agressifs du CS, notamment le sous-type HER2-positif et le sous-type triple-négatif.

Concernant l'expression de la protéine NADPH oxydase 4, l'expérimentation immunohistochimique (100MBTs + 94 NATs) a révélé une augmentation significative de la protéine NOX4 dans les MBTs (79 % : 79/100) par rapport aux NATs (51,06 % : 48/94). En revanche, les niveaux de la protéine DUOX1 étaient réduits dans les MBTs par rapport aux NATs. L'augmentation des niveaux d'expression de la protéine NOX4, contrastée par des niveaux faibles de la protéine DUOX1 dans les échantillons tumoraux, suggère que ces deux protéines NADPH oxydases jouent des rôles différents dans le cancer du sein. Notamment, la protéine DUOX1 pourrait jouer un rôle antitumoral, un rôle similaire ayant été proposé dans plusieurs types de cancer. Nos résultats montrent que les tumeurs mammaires avec des niveaux d'expression élevés de la protéine NOX4 présentent fréquemment des caractéristiques agressives du cancer du sein comprenant un statut négatif pour le récepteur aux œstrogènes (39,74 % contre 19,05 %) ainsi la présence des métastases ganglionnaires (47,62 % contre 25 %). La localisation subcellulaire de la protéine NOX4 (cytoplasme, zone périnucléaire et noyau) a montré une concordance statistiquement significative (*Kappa*= 0.487; Cohen's kappa test) entre les MBTs et les NATs, suggérant le rôle potentiel du microenvironnement tumoral dans la régulation de de la localisation subcellulaire de NOX4. De plus, la localisation périnucléaire de la protéine NOX4 est corrélée avec le phénotype du CS triple négatif.

Dans l'ensemble, cette recherche translationnelle met en lumière la protéine NOX4 et l'hyperméthylation du promoteur du gène *BRCA1* dans le CS en tant qu'événements précoces potentiels favorisant la transformation mammaire et comme des cibles thérapeutiques pour les formes agressives du CS.

Preface

Throughout my academic journey, I have been privileged to engage in a variety of research works, contributing to several international publications:

Included publications in this thesis

1) ***Oubaddou Yassire***, *Oukabli Mohamed, Fenniche Salma, Elktaibi Abderrahim, Elochi Mohamed Reda, Al Bouzidi Abderrahmane, Qmichou Zineb, Dakka Nadia, Diorio Caroline, Richter Antje, Bakri Youssef, and Ameziane El Hassani Rabii. BRCA1 Promoter Hypermethylation in Malignant Breast Tumors and in the Histologically Normal Adjacent Tissues to the Tumors: Exploring Its Potential as a Biomarker and Its Clinical Significance in a Translational Approach.* ***Genes****, 14, 9, August* ***2023****, 1680. https://doi.org/10.3390/genes14091680.*

2) ***Oubaddou Yassire****, Ben Ali Fatima*, Oubaqui Fatima Ezzahrae*, Qmichou Zineb, Bakri Youssef, and Ameziane El Hassani Rabii. The Tumor Suppressor BRCA1/2, Cancer Susceptibility and Genome Instability in Gynecological and Mammary Cancers.* ***Asian Pacific Journal of Cancer Prevention****, 24, 9, September* ***2023****, 3139-3153. https://doi.org/10.31557/apjcp.2023.24.9.3139. *: **equal contribution**.*

3) ***Oubaddou Yassire*** *et al., 2023. Comparative Study of NOX4 and DUOX1 Protein Expression and Subcellular Localization in Breast Tumors and their Normal Adjacent Tissues: Uncovering NOX4's Role as a Biomarker for Aggressiveness and Progression of Breast Cancer (**in preparation**).*

Non-included publications in this thesis

1) *Fenniche Salma*, **Oubaddou Yassire***, Bakri Youssef, Dupuy Corine, and Ameziane El Hassani Rabii. Methods for detection of mitochondrial reactive oxygen species in senescent cells.* ***Methods in Cell Biology-Elsevier Inc****, 1st ed, October* ***2022****, 1–9. http://dx.doi.org/10.1016/bs.mcb.2022.09.011. *: **equal contribution**.*

2) *Fenniche Salma, Oukabli M,* ***Oubaddou Yassire****, Chahdi Hafsa, Damiri Amal, Alghuzlan Abir, Laraqui Abdelilah, Dakka Nadia, Bakri Youssef, Dupuy Corinne, Ameziane El Hassani Rabii. A Comparative Analysis of NOX4 Protein Expression in Malignant and Non-Malignant Thyroid Tumors.* ***Curr Issues Mol Biol****, 45, 7, July* ***2023****, 5811-5823. https://doi.org/10.3390/cimb45070367.*

Communications

1) ***Oubaddou Yassire****, Oukabli Mohamed, Fenniche Salma, Elktaibi Abderrahim, Elochi Mohamed Reda, Al Bouzidi Abderrahmane, Qmichou Zineb, Dakka Nadia, Diorio Caroline, Richter Antje, Bakri Youssef, and Ameziane El Hassani Rabii. Communicated Experimental Results entitled: Prevalence of BRCA1 Promoter Methylation in Breast Cancer of Moroccan Women, for the doctoral days at the Faculty of Sciences in Rabat.*

2) ***Oubaddou Yassire*** *et al., 2023. Comparative Study of NOX4 and DUOX1 Protein Expression and Subcellular Localization in Breast Tumors and their Normal Adjacent Tissues.* ***Noxros 2023*** *: GDR NADPH Oxydase-Stress Oxydant et Club Oxydase. 5-17 nov. 2023 Orsay (France).*

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

ADC: Antibody-drug Conjugate
AHR: Aryl Hydrocarbon Receptor
AP1: Activator Protein 1
BARD1: BRCA1 Associated RING Domain 1
BC: Breast Cancer
BETs: Bromodomain and Extra Terminal Domain Proteins
BBLs: Benign Breast Lesions
BL1: Basal-Like 1
BL2: Basal-Like 2
BRCA1: Breast Cancer 1
BRCA2: Breast Cancer 2
BRCT: BRCA1 C Terminus
CAFs: Cancer-Associated Fibroblasts
CCL21: Chemokine (C-C motif) ligand 21
CCR7: C-C Chemokine Receptor Type 7
CDK4/6: Cyclin-Dependent Kinase 4/6
CK14: Cytokeratin 14
CK17: Cytokeratin 17
CK5/6: Cytokeratin 5/6
CRE: cAMP Response Element
CREB: cAMP Response Element-Binding Protein
CRISPR-dCas9: Clustered Regularly Interspaced Short Palindromic Repeats-deactivated Cas9
CtBP: C-terminal Binding Protein
CtIP: CtBP Interacting Protein
CTCF: CCCTC-binding factor
DCIS: Ductal Carcinoma In Situ
DNMT: DNA Methyltransferases
DSBs: DNA Double-Strand Breaks
DUOX1: Dual Oxidase 1
DUOX2: Dual Oxidase 2
DUOXA1/A2: Dual Oxidase Maturation Factors 1 and 2
EGF: Epidermal Growth Factor
EGFR: Epidermal Growth Factor Receptor
EMT: Epithelial-Mesenchymal Transition
ER: Estrogen Receptor
ER^+: Estrogen Receptor Positive
ER^-: Estrogen Receptor Negative
ERa: Estrogen Receptor Alpha
ESR1: Estrogen Receptor 1
Est-2: Estrogen-responsive element 2
FA: Fibroadenomas
FAD: Flavin Adenine Dinucleotide
FGF: Fibroblast Growth Factor
FFPE: Formalin-Fixed Paraffin-Embedded

Gpx: Glutathione Peroxidase
GSH: Monomeric Glutathione
GSSG: Glutathione Disulfide
H_2O_2: Hydrogen Peroxide
HATs: Histone Acetyltransferases
HDAC1: Histone Deacetylase 1
HDACs: Histone Deacetylases
HDMs: Histone Demethylases
HER2: Human Epidermal Growth Factor Receptor 2
HIF-1α: Hypoxia-Inducible Factor 1 Alpha
HLECs: Human Lymphatic Endothelial Cells
HPF: High Power Field
HR: Homologous Recombination
IC50: Inhibitory Concentration at 50% of maximal effect
IDC: Invasive Ductal Carcinoma
ID4: Inhibitor of DNA Binding 4
ILC: Invasive Lobular Carcinoma
IM: Immunomodulator
LCIS: Lobular Carcinoma In Situ
LAR: Luminal Androgen Receptor
MAPK: Mitogen-Activated Protein Kinase
MBDs: Methyl-CpG-Binding Domain Proteins
MBTs: Malignant Breast Tumors
MCF7: A human breast cancer cell line
MDA-MB-231: A human breast cancer cell line
MSL: Mesenchymal Stem-Like
mTOR: Mammalian Target of Rapamycin
NADPH: Nicotinamide Adenine Dinucleotide Phosphate
NATs: Normal Adjacent Tissues
NOX4: NADPH Oxidase 4
NRF2: Nuclear Factor Erythroid 2-Related Factor 2
N-Ter: N-Terminal end
Nuc/Cyto: Nuclear and Cytoplasmic
$OH^{\cdot}$: Hydroxyl Radical
$O_2^{\cdot-}$: Superoxide Anion
OR: Odds Ratio
PALB2: Partner and Localizer of BRCA2
PARP: Poly (ADP-Ribose) Polymerase
PARPi: PARP inhibitor
PD-1: Programmed Death-1
PI3K: Phosphoinositide 3-Kinase
PIK3CA: Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha
PITX2: Paired-like homeodomain transcription factor 2
PR: Progesterone Receptor
PR-: Progesterone Receptor Negative
pCR: Complete Pathologic Response

PT: Phyllodes Tumors
ROS: Reactive Oxygen Species
RT-PCR: Reverse Transcription Polymerase Chain Reaction
SBR: Scarff-Bloom-Richardson
sgRNAs: Single Guide RNAs
Sp1: Specificity Protein 1
Stat3: Signal Transducer and Activator of Transcription 3
TDLU: Terminal Ductal-Lobular Unit
TETs: Ten-Eleven Translocation Enzymes
TGFβ: Transforming Growth Factor Beta
TNBC: Triple-Negative Breast Cancer
TNM: Tumor, Nodes, Metastasis
Trx: Thioredoxin
UHRF1: Ubiquitin-like with PHD and RING Finger domains 1
UNS: Unstable
VEGF: Vascular Endothelial Growth Factor
WST-1: Water Soluble Tetrazol

Bibliography and Background

I. Breast cancer

1. Mammary gland physiology and development

The mammary tissues are tubulo-alveolar exocrine glands situated in the breasts (Javed & Lteif, 2013). Their fundamental role is to produce milk, a vital newborn nutrient source during breastfeeding. The mammary glands require significant cellular plasticity to undergo multiple and distinct stages of growth, differentiation, and involution (Javed & Lteif, 2013). The development of mammary glands is identical for both males and females until puberty, when only glands in females continue to develop, exhibiting variations in structure and activity based on hormonal status, while in males, they remain rudimentary (Biswas et al., 2022).

The development of mammary glands occurs in several stages, each regulated by a specific combination of hormones and growth factors. The embryonic stage initiates the formation of primary structures from the ectoderm by two signaling pathways, Wnt and FGF (Fibroblast Growth Factor) (Watson & Khaled, 2008). The pubertal development stage leads to ductal elongation and branching within the fat pad, triggered by hormones such as estrogen and progesterone (Watson & Khaled, 2008). During pregnancy, the alveoli proliferate and differentiate under the influence of hormones like prolactin, placental lactogens, progesterone, and estrogen, preparing for milk production (Hennighausen & Robinson, 2005). Prolactin stimulates milk production by glandular cells (luminal cells), while oxytocin facilitates its secretion through the contraction of myoepithelial cells, also called basal cells (Figure 1) (Hennighausen & Robinson, 2005). The involution of mammary glands, a proapoptotic process that reduces breast volume post-lactation, involves factors such as Stat3 and TGFβ (Watson & Khaled, 2008).

In young women, the surface of the breast consists of skin with two central differentiations: the areola and the nipple (Figure 1). These are the openings for the lactiferous ducts, which originate from multiple lobes (Bistoni & Farhadi, 2015) (Figure 1). The dense perilobular connective and adipose tissues separate the lobes, forming the interlobular stroma. Within each lobe are multiple lobules, conjunctivo-epithelial compartments responsible for milk production (Figure 1) (Bistoni & Farhadi, 2015). Each lobule contains small ducts, which terminate in alveolar structures known as acini (Figure 1). The lobules are connected to the ductal system through extralobular ductules. Collectively, including the extralobular and intralobular ductules, acini, form the terminal ductal-lobular unit (TDLU) (Macias & Hinck, 2012). TDLUs are the primary sites where most breast cancers originate (Lakhani et al., 2012).

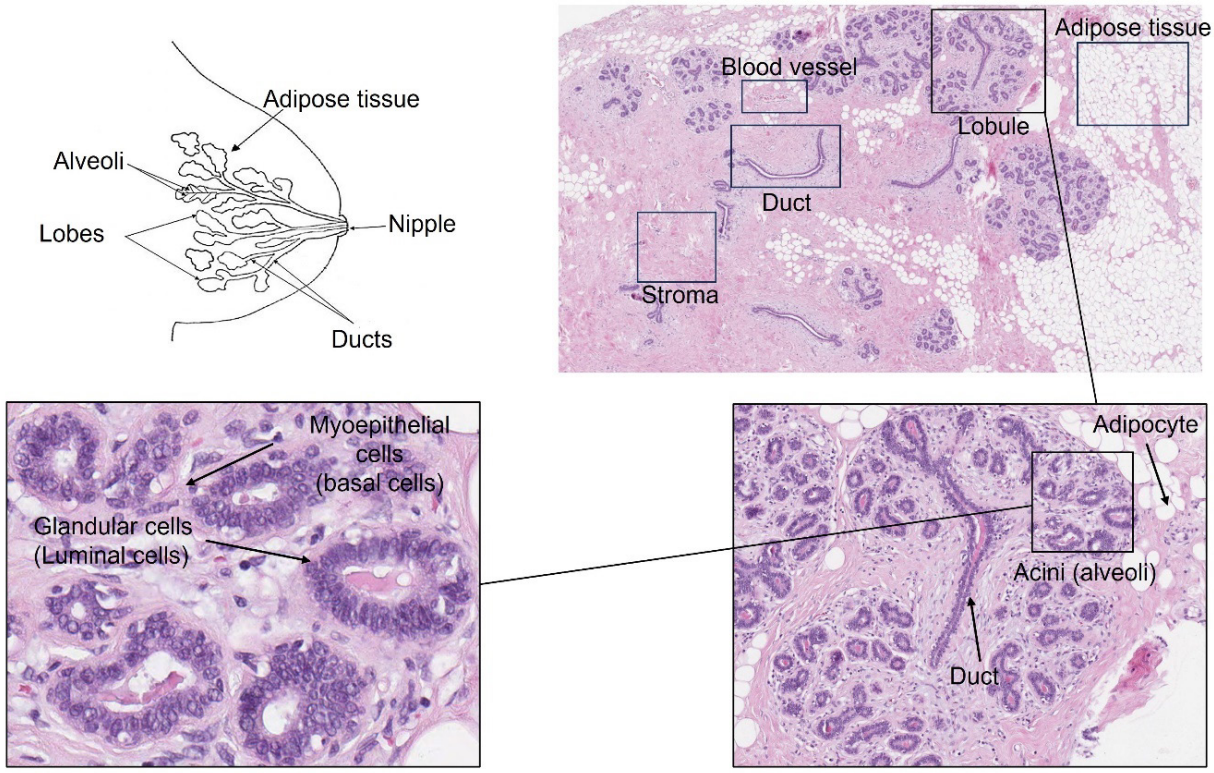


Figure 1. Histological Structure of Mammary Tissue. This figure illustrates the typical histological architecture of mammary tissue, which is primarily composed of a fundamental glandular unit known as an acinus (plural: acini) or alveolus. Each acinus is constructed from a basal layer of myoepithelial cells and a luminal layer of glandular cells. Multiple acini combine to form a lobular structure, which, along with other lobules, contributes to forming larger ducts, thereby creating mammary lobes. These lobes ultimately drain into major ducts (lactiferous ducts) that open onto the nipple of the breast. Source: We modified the images from the proteinatlas database.

2. Breast cancer epidemiology and risk factors

2.1 Statistical overview

According to a recent global study covering 185 countries (Ferlay et al., 2021; Sung et al., 2021), breast cancer was the most frequently diagnosed cancer among women in 159 of these countries, with approximately 2.3 million new cases (24.5% of all cancer diagnoses) (Figure 2a). Furthermore, breast cancer continues to be the first cause of cancer-related deaths among women in over 110 countries, with 684,995 deaths, accounting for 15.5% of all cancer deaths in 2020 (Figure 2b) (Ferlay et al., 2021; Sung et al., 2021). Intriguingly, when considering both sexes, breast cancer was identified as the most prevalent cause of cancer worldwide in 2020, accounting for 11.7% of all cases. This surpasses lung cancer, which accounted for 11.3% of global cancer incidence (Ferlay et al., 2021; Sung et al., 2021). In Morocco, breast cancer was the most commonly diagnosed cancer among women in 2020 (WHO, 2020). It accounts for 38.9% of all cancer-related females, with 11,747 new cases reported (Figure 2c). Furthermore, it is responsible for nearly a quarter (24.7%) of all cancer-related deaths in women, accounting for 3,695 deaths in 2020 in Morocco (Figure 2d) (WHO, 2020).

2.2 Trends and inequalities

Population growth and aging alone should increase breast cancer incidence and mortality by 2040, with 3 million new cases and 1 million deaths annually (Arnold et al., 2022). Globally, rates of breast cancer incidence were higher in transitioned countries (55.9 per 100,000) compared to transitioning countries (29.7 per 100,000) in 2020 (Ferlay et al., 2021; Sung et al., 2021), and this number was estimated in Morocco to be 56.4 new cases per 100,000 females (WHO, 2020). However, rates of mortality were higher in transitioning countries (15 per 100,000) compared to transitioned countries (12.8 per 100,000) in 2020 (Ferlay et al., 2021; Sung et al., 2021), with a rate of mortality reported at 17.5 deaths per 100,000 females in Morocco (WHO, 2020).

In developed countries, high levels of incidence are due to the high prevalence of hormonal and reproductive risk factors and lifestyle risk factors, including early age at menarche, fewer number of children, later age at menopause, advanced age at first birth, less breastfeeding, menopausal hormone therapy, oral contraceptives, excess body weight, alcohol intake, physical inactivity (Sung et al., 2021). In less developed countries, mortality rates are tremendous because of disease health-care systems, suffer from a weak ability to diagnose and treat breast cancer, as well as the inability to reach updated national registers, which come against the awareness of the fatality of breast cancer in those nations (Allemani et al., 2018; Anderson et al., 2008; Sung et al., 2021).

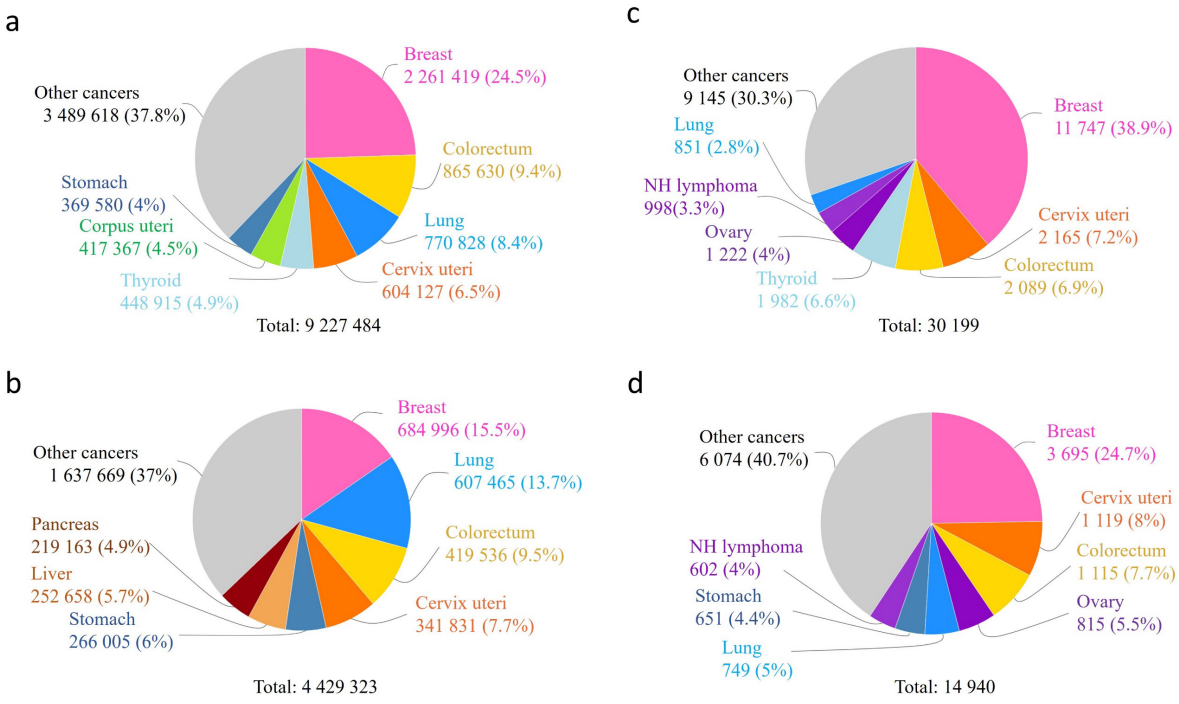


Figure 2. Global Incidence and Mortality of Breast Cancer. (a) Estimated number of new cases in 2020, in the world, in women. (b) Estimated number of deaths in 2020 in the world in women. (c) Estimated number of

new cases in 2020 in Morocco in women. (d) Estimated number of deaths in 2020 in Morocco in women. Source of the graphs: (WHO, 2020).

2.3 Key risk factors

Several factors increase the risk of breast cancer. Some of them are controllable, such as hormonal replacement therapy, physical activity, overweight, obesity, alcohol intake, and chemical exposure (Łukasiewicz et al., 2021). The uncontrollable risk factors include sex, older age, family history, genetic mutations, reproductive history, history of benign diseases, and medical radiation (Table I) (Łukasiewicz et al., 2021).

Female sex is a major risk factor for breast cancer (Łukasiewicz et al., 2021). Indeed, the susceptibility of breast cells to hormonal stimuli, such as estrogen, progesterone, and androgens, explains this sex-dependent risk. In men, breast cancer accounts for less than 1% of all cases (Collaborative group et al., 2013). Age is another inevitable risk factor for breast cancer because most breast cancer cases (80%) occur in individuals over 65 years old (Łukasiewicz et al., 2021).

Family history of breast cancer can significantly increase the risk of breast cancer, proportionally increasing with the number of first-degree relatives affected (Beral et al., 2001). This risk related to family history can be attributed to environmental, genetic, and epigenetic factors (Wu et al., 2018). Certain germline mutations in specific genes can increase women's susceptibility to breast cancer. These genes mainly play roles in DNA repair and cell cycle control pathways (Łukasiewicz et al., 2021). Among these, *BRCA1* and *BRCA2* genes have a high penetrance in predisposing individuals to breast cancer (Oubaddou et al., 2023a). Mutations in these two genes can trigger accelerated genome instability, leading to early-onset breast cancer (Oubaddou et al., 2023a). Mutations of other genes are also characterized to confer a high or moderate risk of breast cancer, including mutations of genes such as *TP53*, *CDH1*, *PTEN*, *STK11*, *ATM*, *PALB2*, *BRIP1*, and *CHEK2* (Łukasiewicz et al., 2021).

***BRCA1* and *BRCA2* Mutations in Morocco**: A study involving 163 Moroccan patients with familial breast and/or ovarian cancer identified several (27.6%) pathogenic variants in the *BRCA1* and *BRCA2* genes (Elalaoui et al., 2022). The most frequently detected mutation (33%) was the *BRCA2* mutation c.1310_1313delAAGA. Additionally, other mutations in the *BRCA1* gene, such as c.798_799delTT and c.3279delC, were reported as frequent (Elalaoui et al., 2022). A founder mutation in *BRCA1*, c.5309G>T, also known as G1770V, was identified in five independent families from Northeastern Morocco, suggesting a founder effect (Quiles et al., 2016). A recent study highlighted the recurrence (20% of familial cases) of the c.1310_1313delAAGA mutation in *BRCA2* and c.5309G>T mutation in *BRCA1* among women

in Northeastern Morocco (Melki et al., 2023). These findings underscore the importance of genetic screening for specific *BRCA1* and *BRCA2* mutations in the Moroccan population to improve disease management and screening among asymptomatic predisposed women.

Reproductive factors such as experiencing a first pregnancy at a young age, having multiple births, breastfeeding, and entering menopause early can all contribute to a decreased risk of breast cancer (Łukasiewicz et al., 2021). On the contrary, early onset of menarche and a younger age during the first menstrual cycle can increase the risk of developing this disease (Łukasiewicz et al., 2021).

Personal history of benign breast disease can be associated with an increased risk of breast cancer depending on the histology, especially in women with proliferative breast lesions with atypia with a significantly increased risk of developing breast cancer (Cote et al., 2012). Exposure to radiation for medical purposes increases the risk of breast cancer. Chest X-rays used to diagnose tuberculosis and pneumonia amplify the risk of breast cancer (OR=2.49). Similarly, radiotherapy treatment for primary cancer increases the risk of developing secondary breast cancer (OR=3.55) (John et al., 2007).

Table I. Risk Factors of Breast Cancer

Uncontrollable risk factors	Controllable risk factors
Female sex	Physical Activity
Age	Alcohol Intake
Family history of breast cancer	Smoking
Genetic mutations	Intake of Processed Food/Diet
Reproductive History	Exposure to Chemical
Personal history of benign breast disease	
Radiation therapy or chest X-rays for diagnosis	

3. Diagnosis of breast cancer

The first step in identifying breast cancer is often through breast self-examination and clinical breast examination (American Cancer Society, 2016). New breast lumps or masses are common symptoms (American Cancer Society, 2016). Mammography has emerged as a powerful tool for breast cancer screening, and it can detect tumors even when they are too small (R. A. Smith et al., 2018). Ultrasound can be an alternative to mammography, especially in women with dense breasts (Berg et al., 2012). For women at high risk (e.g. family history), magnetic resonance imaging (MRI) can be used (Kuhl et al., 2005).

After imaging, if a nodule is detected, a biopsy is recommended to reveal the malignity of cells through a microscopic observation (Gutwein et al., 2011). Further tests are performed to analyze the characteristics of tumors if cancer is found. These include tests for hormone receptor

status (ER, PR) and HER2 status, which help guide prognostic treatment decisions (Hammond et al., 2010).

4. Histological classification

Breast cancer can originate from the malignant transformation of any cell type within the breasts. This transformation most commonly affects mammary epithelial cells, forming mammary carcinomas (Lakhani et al., 2012). Sarcomas and lymphomas are rarely diagnosed within the breasts. Most breast carcinomas originate from the terminal ductal-lobular unit (TDLU) and are classified into in situ and invasive mammary carcinomas. An in situ mammary carcinoma constitutes 20 to 25% of diagnosed cancer cases, resulting from a potentially neoplastic proliferation of mammary epithelial cells (Lakhani et al., 2012). These cells are confined within the ducto-lobular structure without breaching the basal membrane (Lakhani et al., 2012).

An invasive or infiltrating mammary carcinoma results from a malignant proliferation of mammary epithelial cells, penetrating the surrounding stroma after breaching the basal membrane (Lakhani et al., 2012). In situ and invasive carcinomas are classified as ductal or lobular depending on the site of neoplastic proliferation, either at the level of the lobules or lactiferous ducts (Lakhani et al., 2012). Thus, we distinguish four main groups: i) ductal carcinoma in situ (DCIS); ii) lobular carcinoma in situ (LCIS); iii) invasive lobular carcinoma (ILC); and iv) invasive ductal carcinoma (IDC) (Lakhani et al., 2012).

Invasive ductal carcinomas (IDC) are breast cancers presenting a malignant canalicular proliferation and stromal invasion, with or without the presence of a DCIS (Lakhani et al., 2012). 40 to 75% of invasive ductal carcinomas constitute a heterogeneous group of tumors that do not present sufficient characteristics to be classified as a specific histological type. These lesions are named non-specific type ductal carcinomas (ductal NST; no specific type), also called ductal NOS (ductal NOS, not otherwise specified) (Figure 3a and 3b) (Lakhani et al., 2012). Invasive ductal carcinomas presenting a histological specificity or recognized histological features are named invasive ductal carcinomas of a specific type, such as mucinous Carcinoma (Figure 3c), invasive papillary carcinoma, medullary carcinoma, metaplastic carcinoma, and inflammatory carcinomas. Most in situ (non-invasive) mammary carcinomas are of the ductal type (Figure 3d) (Lakhani et al., 2012).

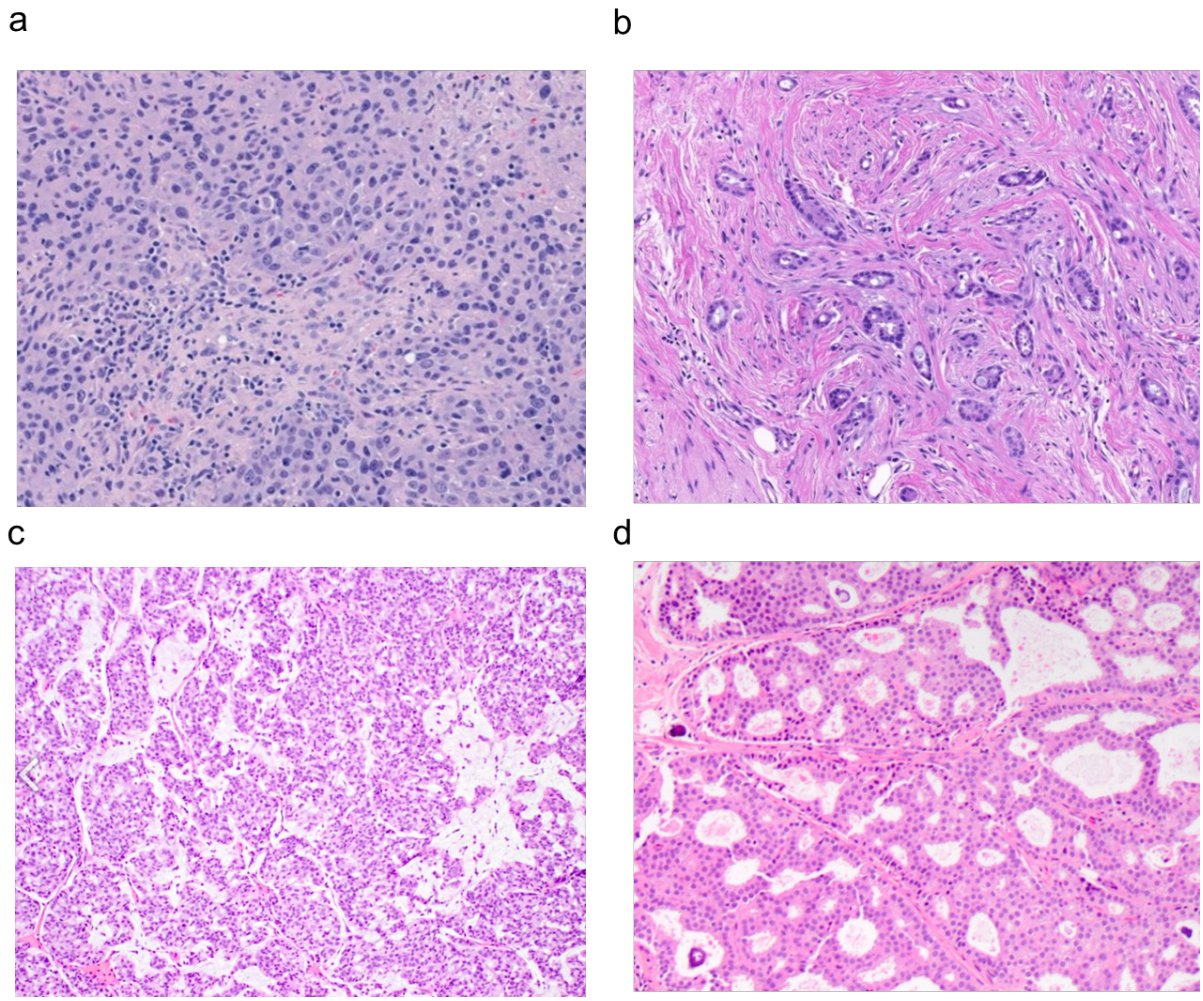


Figure 3. Histological Subtypes of Invasive Breast Carcinomas. (a) Invasive ductal carcinomas (IDC) with high histologic grade, (b) Invasive ductal carcinomas (IDC) with low histologic grade, (c) Mucinous carcinomas, (d) Ductal carcinomas in situ (*Pathology Outlines - Breast*, n.d.).

5. Benign breast lesions

Fibroadenomas (FA) are the most diagnosed benign tumors (Figure 4). They predominantly appear in women below the age of 30. Characteristics of an FA include being well-defined, isolated, growing at a slow pace, being movable, and not causing pain. While these nodules typically reach 3 cm, they can grow as large as 20 cm in diameter, especially in teenagers (Lakhani et al., 2012). Low-grade phyllodes tumors form the second most diagnosed group of benign tumors (Lakhani et al., 2012).

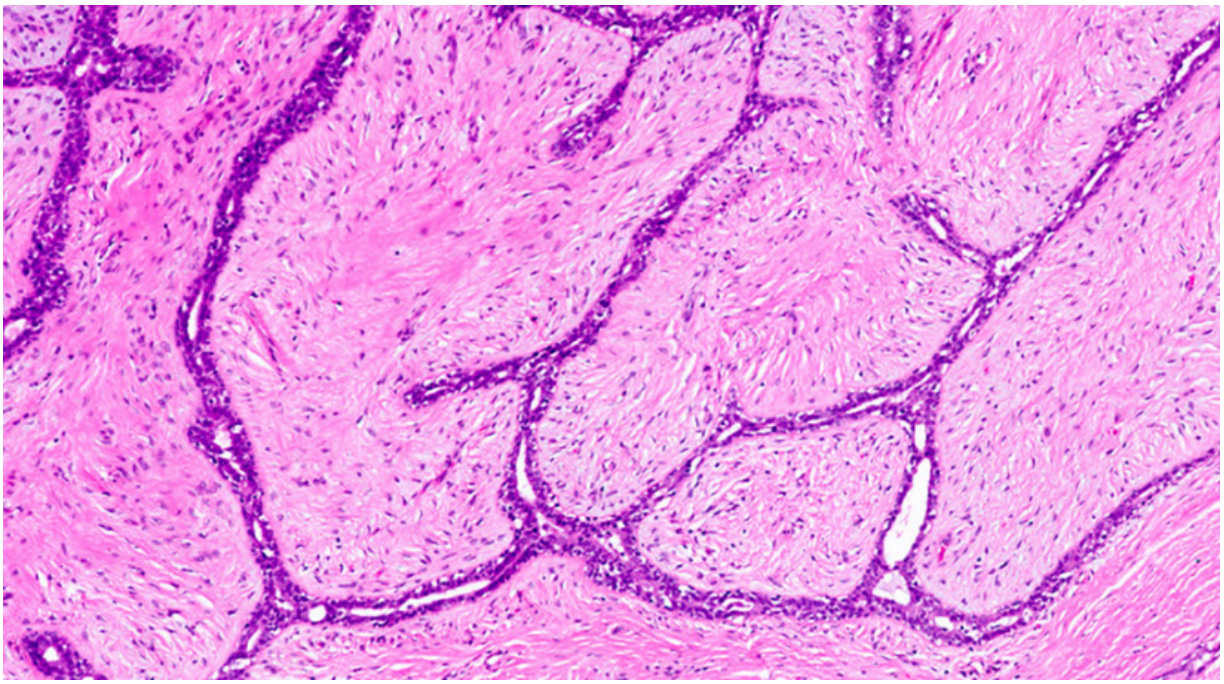


Figure 4. Microscopic Observation of a Histological Section of a Fibroadenoma of the Breast (*Pathology Outlines - Breast*, n.d.).

6. Molecular classification

Several molecular studies allow the subdivision of breast cancer into several molecular subtypes characterized by different prognoses and different therapeutic modalities (Koboldt et al., 2012; Lehmann et al., 2011; Perou et al., 2000; Prat et al., 2010; Sørlie et al., 2001). Depending on the expression of the estrogen receptor, we can distinguish between two major groups (Perou et al., 2000). Those that express the estrogen receptor (ER^+) form a " luminal " group, which has a similar expression profile to luminal epithelial cells. The second major group contains tumors with negative estrogen receptor (ER^-) expression, and it shows three distinct subgroups called "$HE2^+$", "basal-like," and "normal-like" subgroups (Perou et al., 2000; Sørlie et al., 2001).

The HER2 positive ($HER2^+$) subgroup is characterized by overexpression of the HER2 protein (Human epidermal growth factor receptor2). The "basal-like" subgroup is characterized by the lack of expression of ER and HER2 and by the expression of genes characteristic of basal-type breast cells (e.g., cytokeratins CK5/6, CK14, CK17, and often positive for EGFR) and by high proliferative activity (Perou et al., 2000; Sørlie et al., 2001). The "normal-like" class presents a triple-negative phenotype with expression profiles similar to normal mammary tissue (Perou et al., 2000; Sørlie et al., 2001).

Luminal-type breast cancers are the most common (70%), showing gene expression and prognosis heterogeneity. Two subgroups of luminal breast cancers can be distinguished: luminal A and luminal B (Sørlie et al., 2001). Luminal A breast cancers are generally low grade with an

excellent prognosis, positive for estrogen and progesterone receptors and negative for HER2 receptor, with high expression of genes related to estrogen receptor signaling and low expression of proliferation genes (Perou et al., 2000; Sørlie et al., 2001). On the other hand, luminal B tumors are of higher histological grade, have a poorer prognosis, and can be HER2 positive and/or can show a high expression of genes related to proliferation (e.g., Ki67) (Perou et al., 2000; Sørlie et al., 2001).

Ki67 is used in clinical practice to separate between the two luminal subgroups A and luminal B (Her2-) with an intermediate immunohistochemical staining intensity cutoff of 14% (Figure 5) (Prat et al., 2013). Thus, ER^+ cancers with a Ki67 equal to or greater than 14% are classified as luminal B, cancers with a Ki67 strictly less than 14%, and Her2- are classified as luminal A (Prat et al., 2013). This separation seems important from a clinical point of view because the luminal A group will likely benefit from hormonal therapy alone, while luminal B tumors could be candidates for chemotherapy (Provenzano et al., 2018). The breast cancers of the $HER2^+$ group (ER^-/PR^- and $HER2^+$) are characterized by a high histopronostic grade, strong proliferation, and a poor prognosis (Provenzano et al., 2018; Sørlie et al., 2001).

Triple-negative breast cancer (ER-/PR-/HER2-) constitutes 15% of all breast cancer subtypes. Typically, these cancers are aggressive, high-grade, frequently occurring in younger women, and associated with high recurrence rates and shorter survival compared to other breast cancer subtypes (Provenzano et al., 2018). This unfavorable prognosis is due to its heterogeneity, aggressive nature, and lack of therapeutic targets. Seven molecular subtypes within this group have been defined through genetic expression analysis: BL1 (Basal-Like 1), BL2 (Basal-Like 2), IM (Immunomodulator), MSL (Mesenchymal Stem-Like), M (Mesenchymal), LAR (Luminal Androgen Receptor), and UNS (unstable) (Lehmann et al., 2011).

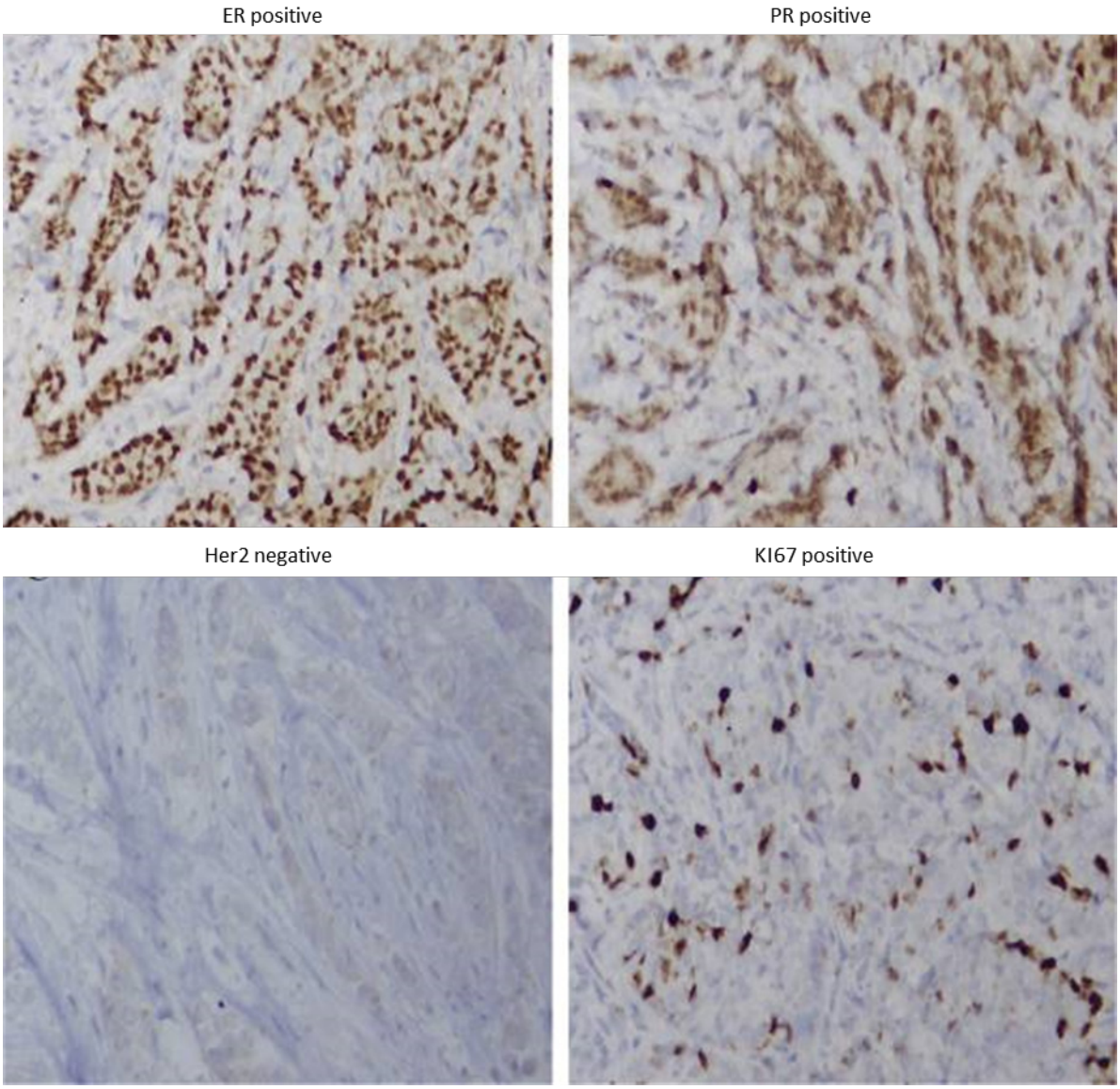


Figure 5. Example of Molecular Subtyping of an Invasive Breast Carcinoma Showing a Luminal B Subtype. Immunohistochemical analysis of ER (Estrogen Receptor), PR (Progesterone Receptor), Her2 (Human Epidermal Growth Factor 2), and ki67 protein (Orrantia-Borunda et al., 2022).

7. Prognostic factors

Prognostic factors are characteristics that accurately predict the natural progression of a disease, irrespective of any treatment interventions. They are crucial in estimating a patient's survival rate and the likelihood of disease recurrence.

7.1 Influence of age

Individuals diagnosed with cancer under the age of 40 have been associated with lower survival rates and a significant incidence of locoregional relapses (Fredholm et al., 2009). Moreover, cancer onset at a young age often indicates a poor prognosis, with a higher prevalence of triple-negative and $HER2^{+}$ cancers than older women who primarily develop luminal-type cancers (Keegan et al., 2012).

7.2 SBR grading analysis

The Scarff-Bloom-Richardson (SBR) histological grade is a widely employed tool for the histological diagnosis of invasive ductal carcinomas (ELSTON & ELLIS, 1991). This grading system assigns a prognostic score ranging from one to three to cancerous lesions (ELSTON & ELLIS, 1991). The SBR histological grade (I, II, or III) is determined based on three key characteristics of cancer cells, including the level of differentiation (tubule formation score), nuclear grade (variation of size and shape of the nucleus), as well as the count of cell divisions (mitosis score) (Table II) (ELSTON & ELLIS, 1991). Each characteristic is scaled from 1 to 3, as described in Table II. Final SBR Grade is determined as follows: Grade I (Well-differentiated): a total of 3 to 5; Grade II (Moderately differentiated): a total of 6 or 7; and Grade III (Poorly-differentiated): a total of 8 or 9.

Table II. SBR Scoring System

Feature	Score 1	Score 2	Score 3
Tubule Formation	Most (>75%) tubules form well-defined structures	Moderate (10-75%) tubule formation	Poor (<10%) tubule formation
Nuclear Pleomorphism (or grade)	Nuclei are small, regular, and uniform	Nuclei are moderate in size and variation	Nuclei are large, irregular, and vary significantly
Mitotic Rate	Few or no mitotic figures per high power field (HPF)	Moderate number of mitotic figures per HPF	Increased number of mitotic figures per HPF

7.3 Impact of nodal invasion

Cancer staging, known as TNM staging, is based on three powerful prognostic factors: tumor size (T), lymph node involvement (N), and metastasis status (M) (Weiss et al., 2018). For instance, nodal invasion is evaluated through pathological biopsied lymph node tissue examination. The level of lymph node involvement is graded on a scale, commonly ranging from N0, indicating no regional lymph node metastasis, to N3, which signifies extensive involvement (Weiss et al., 2018).

7.4 Complete pathological response

Achieving a pathologic complete response (pCR) after neoadjuvant therapies is a favorable prognostic factor (Kuerer et al., 2016).

7.5 Oncotype DX evaluation

Oncotype DX is an RT-PCR assay that measures the expression of 21 genes (sixteen cancer-related genes and five reference genes). This test evaluates the risk of recurrence among hormone receptor-positive breast cancer patients with stage I and II and negative axillary nodes. Therefore, it can predict the potential benefit of chemotherapy (Harris et al., 2007).

8. Predictive factors

On the other hand, predictive factors are characteristics that can accurately predict how a cancer will respond to a specific treatment. These factors play a crucial role in guiding treatment decisions.

8.1 Mitotic activity and Ki67

In addition to their role as prognostic factors, the mitotic index and Ki67 expression are cell proliferation markers and can predict the need for chemotherapy (Andre et al., 2005; Nishimura et al., 2010).

8.2 HER2 amplification and hormone expression

HER2 overexpression predicts the potential benefit of targeted therapies, such as trastuzumab, while hormone receptor overexpression can predict the effectiveness of hormonal therapies, such as tamoxifen or aromatase inhibitors (Abe et al., 2005; Slamon et al., 2001).

8.3 BRCA1 genetic mutations

BRCA1 germline mutations can predict the utility of Olaparib for metastatic breast cancer (Robson et al., 2017).

8.4 Triple-negative breast cancer subtype

Triple-negative breast cancers, which do not respond to hormonal therapy or trastuzumab, currently rely primarily on chemotherapy as the basis of treatment (Dent et al., 2007).

8.5 PITX2 DNA methylation patterns

Detecting DNA methylation of specific genes can also contribute to managing therapies in breast cancer. For instance, PITX2 DNA methylation can predict the outcome of adjuvant anthracycline-based chemotherapy in patients with high-risk (Davalos & Esteller, 2023).

9. Treatment approaches

Despite the significant advancements in breast cancer treatment strategies, serious challenges remain. These include resistance to certain treatments, such as hormone therapy, and the absence of therapeutic targets for triple-negative breast cancer (DeSantis et al., 2019). Nevertheless, ongoing research is pioneering various strategies to address these issues, including the development of immunotherapy, PARP inhibitors, and novel targeted therapies (Table III) (Debien et al., 2023; Iacopetta et al., 2023; Saavedra et al., 2023). For early-stage breast cancer, treatment options often involve breast-conserving surgery with radiotherapy or mastectomy. These primary treatments are frequently complemented with neoadjuvant and

adjuvant therapies, encompassing chemotherapy, radiotherapy, hormone and/or immunotherapy, and targeted therapy (Iacopetta et al., 2023).

A study in the USA showed that 50% of patients diagnosed with stage I or II breast cancer used breast-conserving surgery followed by radiation therapy, while a third opted for a mastectomy (DeSantis et al., 2019). The preference for mastectomy is growing due to concerns about recurrence. Mastectomy is more commonly chosen by younger patients and those with larger or more aggressive tumors. The practice of contralateral prophylactic mastectomy is also on the rise (DeSantis et al., 2019). Around 18% of early-stage patients were treated with chemotherapy. The administration of chemotherapy before surgery, or neoadjuvant chemotherapy, is becoming more common, especially in HER2-positive and triple-negative breast cancers (DeSantis et al., 2019). While treatment advancements for Hormone Receptor-negative/HER2-negative breast cancers have been slower, the immunotherapy drug atezolizumab offers a new treatment avenue (DeSantis et al., 2019). The majority of patients with stage IV breast cancer receive palliative care, of which 50% received chemotherapy and 28% did not, but the development of targeted systemic therapies has enhanced survival rates for metastatic disease, particularly in HR-positive and HER2-positive breast cancers (DeSantis et al., 2019). The following table represents an updated list of drugs used in targeted breast cancer therapy (American Cancer Society, 2023).

Table III. Therapeutic Approaches of Breast Cancer (An update)

Molecular subtype	Therapy Type	Drugs	Remarks
Hormone Receptor-Positive Breast Cancer	Hormone Therapy	Tamoxifen, Toremifene	Post-menopausal women with metastatic or early-stage breast cancer as adjuvant therapy
		Fulvestrant, Elacestrant	Advanced, ER-positive, Her2-negative breast cancer with ESR1 mutation
		Letrozole, Anastrozole, Exemestane	Aromatase inhibitors are used in adjuvant therapy or for metastatic cancer, particularly in post-menopausal women to decrease estrogen levels
	Targeted Therapy	Palbociclib, Ribociclib, Abemaciclib	CDK4/6 inhibitors are used in combination with hormone therapy for advanced or metastatic disease to slow cancer growth
		Everolimus	mTOR inhibitor, typically combined with Exemestane for advanced cancer resistant to first-line aromatase inhibitors
		Alpelisib	PI3K inhibitor, used with fulvestrant for advanced cancer with PIK3CA mutations after progression on or following an endocrine-based regimen
	Antibody-drug Conjugate (ADC)	Sacituzumab govitecan	Used for metastatic triple-negative breast cancer after at least two prior therapies
HER2-Positive Breast Cancer	Targeted Therapy	Trastuzumab, Pertuzumab, Margetuximab	Target the Her2 receptor; used in early-stage as adjuvant/neoadjuvant and in metastatic settings
	Antibody-drug Conjugates	Ado-trastuzumab emtansine, Fam-trastuzumab deruxtecan	ADCs that deliver cytotoxic drugs directly to HER2-positive cancer cells; used after prior HER2-targeted therapy and chemotherapy
	Kinase inhibitors	Lapatinib, Neratinib, Tucatinib	Target the Her2 receptor; used in combination with other therapies for advanced or metastatic HER2-positive disease
Triple-Negative Breast Cancer	Antibody-drug Conjugates	Sacituzumab govitecan	Targets the Trop-2 antigen; used for metastatic disease after at least two prior therapies
	Immunotherapy	Pembrolizumab	Combined with chemotherapy for PD-L1-positive, locally recurrent unresectable or metastatic disease
Breast Cancer with *BRCA* Gene Mutations	Targeted Therapy	Olaparib, Talazoparib	PARP inhibitors used for HER2-negative metastatic cancer with a germline BRCA mutation, after chemotherapy

ER: Estrogen Receptor; HER2: Human Epidermal Growth Factor Receptor 2; ESR1: Estrogen Receptor 1; CDK4/6: Cyclin-Dependent Kinase 4/6; mTOR: Mammalian Target of Rapamycin; PI3K : Phosphoinositide 3-Kinase; PIK3CA: Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha; ADC: Antibody-drug Conjugate; Top-2: Topoisomerase 2; PD-1: Programmed Death-1; *BRCA1*: Breast Cancer 1; PARP: Poly(ADP-Ribose) Polymerase.

II. The tumor suppressor *BRCA1* gene

1. Gene discovery and structure

Linkage analysis in families with hereditary breast and ovarian cancers identified chromosome 17q21 as a candidate region carrying a predisposing gene (Hall et al., 1990). Further linkage analysis refined its localization to a small chromosomal region (17q21-12) of 600 kilobases (Neuhausen et al., 1994). Shortly after, the *BRCA1* (Breast cancer gene 1) locus was first cloned (Miki et al., 1994).

The *BRCA1* gene sequence spans 81 kilobases, comprising 24 exons and 22 introns, and is transcribed into an mRNA of 7.8 kilobases. It encodes a phosphoprotein of 1863 amino acids (Y. Chen et al., 1996; T. M. Smith et al., 1996). The BRCA1 protein contains a RING domain encoded by exons 2-7 (at the N-terminal end), a BRCT domain encoded by exons 16-24 (at the C-terminal end), and a central part encoded by exons 11-13 (Figure 6) (Clark et al., 2012). Its expression and phosphorylation increase during the S and M phases of the cell cycle, during which it participates in DNA double-strand breaks (DSBs) repair and other anti-tumoral functions (Figure 6) (Clark et al., 2012; Oubaddou et al., 2023a; Ruffner et al., 1999).

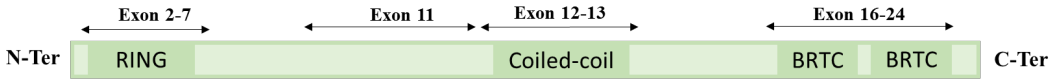


Figure 6. Protein Domains of BRCA1 and their Corresponding Exons. N-Ter: N-Terminal end; C-Ter: C-Terminal end; RING: Really Interesting New Gene; BRCT: BRCA1 C Terminus. Source: This figure represents one of our creations.

2. *BRCA1* gene silencing and functions in breast cancer

Breast cancer is a complex disease caused by multiple factors, including genetic, epigenetic, and environmental factors (Kashyap et al., 2022). Hereditary factors are associated with 5-10% of breast cancer cases, with *BRCA1* germline mutations being the most involved hereditary factor (Yoshida, 2021). Moreover, *BRCA1* gene promoter hypermethylation has been detected in 27% to 59% of sporadic breast cancer cases (90% of all breast cancer cases) (G. Sharma et al., 2009; X. Xu et al., 2009; Yoshida, 2021). Therefore, whether through genetic or epigenetic alterations, *BRCA1* gene silencing plays a substantial role in a significant portion of breast cancer. As a result, *BRCA1* is a key gene in breast tumorigenesis, and its silencing has significant clinical implications for screening, diagnosis, prognosis, and treatment of breast cancer. Since its discovery, the *BRCA1* gene has been recognized as a highly penetrant gene predisposing individuals to breast tumorigenesis (Miki et al., 1994). This predisposition is due to the involvement of this multidomain protein in a plethora of functions necessary for genome

stability (Oubaddou et al., 2023a). Therefore, any alteration of one or several BRCA1-related functions can result in genome instability, a critical step of neoplastic transformation (Roy et al., 2012; Venkitaraman, 2002).

In *BRCA1* germline mutation carriers, breast tumors often exhibit a loss of heterozygosity of the wild-type allele of the *BRCA1* gene, which signifies a loss of both alleles (Maxwell et al., 2017). This observation aligns with the 'two-hit' theory of tumorigenesis, suggesting that the loss of both alleles of a tumor suppressor gene can lead to genomic instability and subsequent neoplastic transformation (Chernoff, 2021). However, studies on human cell lines, mice, and humans have shown that a biallelic mutation of the *BRCA1* gene is lethal due to apoptosis signaling activation (Konishi et al., 2011a; Sedic et al., 2015; Venkitaraman, 2014). An additional mutation of survival genes, such as P53 and RB protein, can enable *BRCA1*-null cells to bypass apoptosis (Sedic et al., 2015). Notably, P53, Rb, and PTEN mutations are frequently observed in *BRCA1*-mutated breast tumors, with P53 and PTEN mutations estimated to precede loss of heterogenicity in 80% of *BRCA1*-associated breast tumor cases (Martins et al., 2012; J. M. Patel et al., 2020).

Emerging findings suggest an alternative mechanism that a monoallelic loss of the *BRCA1* gene is sufficient to trigger breast tumorigenesis (Oubaddou et al., 2023a). The pool of BRCA1 mRNA in *BRCA1*-mutated human mammary epithelial cells was found to decrease (almost 50%) (Sedic & Kuperwasser, 2016). This decrease may affect various BRCA1-related functions. This effect is known as *BRCA1* haploinsufficiency (haplotype insufficiency), has been shown to engender genome instability in human mammary epithelial cells through the alteration of different *BRCA1*-related functions, including double-strand breaks repair by homologous recombination, telomere stability, R-loops resolution, stalled forks resolution, cell cycle control, and oxidative stress oxidation (Figure 7a and 7b) (Oubaddou et al., 2023a). Different models used to study genomic instability induced by *BRCA1* mutations in mammary epithelial cells are detailled in our recent review (see our review in the section of "Results": (Oubaddou et al., 2023a).

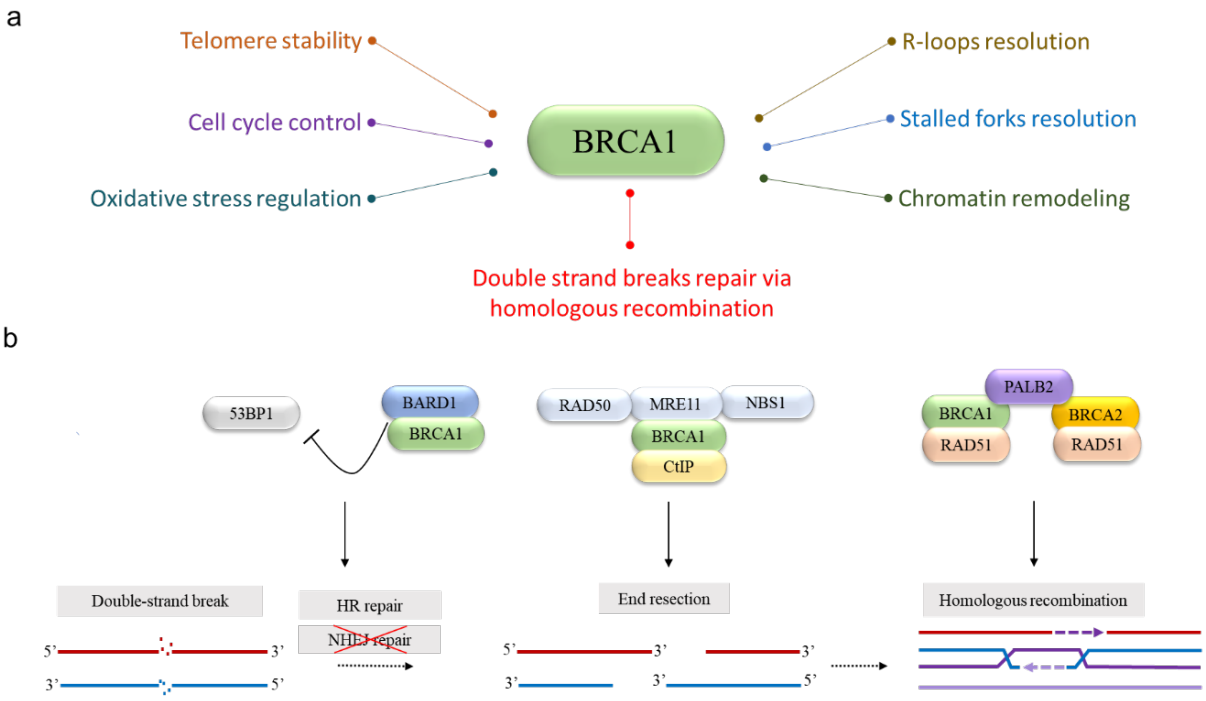


Figure 7. Anti-tumor functions of BRCA1 Protein and its Role in Homologous Recombination Repair of Double-Strand Breaks. **(a)** *BRCA1* gene is a pivotal actor for genome stability, involved in various mechanisms fundamental to maintaining genomic integrity, including R-loop resolution, stalled fork resolution, telomere stability, cell cycle control, chromatin remodeling, oxidative stress regulation, and double-strand break repair through homologous recombination. **(b)** BRCA1's role in every step of double-strand break repair via homologous recombination mechanism. The interaction of the BRCA1/BARD1 complex with 53BP1 influences the choice of homologous recombination (HR) as a mechanism of choice for double-strand breaks repair. BRCA1 protein interaction with various HR actors (CtIP, MRN complex, PALB2, and BRCA2) enables DNA end resection, RAD51 recruitment, and DNA synthesis. BRCA1: Breast Cancer 1; BARD1: BRCA1 Associated RING Domain 1; 53BP1: p53 Binding Protein 1; HR: Homologous Recombination; CtIP: CtBP Interacting Protein; MRN: Mre11-Rad50-Nbs1; PALB2: Partner and Localizer of BRCA2; BRCA2: Breast Cancer 2; RAD51: RAD51 Recombinase. Source: This figure represents one of our creations.

3. Epigenetic alterations

Epigenetic modifications are transmissible changes in gene expression without altering the DNA sequence. They are critical in managing key biological processes such as cell differentiation, embryogenesis, and cancer progression (Davalos & Esteller, 2023). The most prominent of these modifications is DNA methylation, facilitated by enzymes like DNA methyltransferases (DNMTs) and ten-eleven translocation enzymes (TETs) (Figure 8) (Davalos & Esteller, 2023). Methylation typically occurs at CpG islands (regions rich in cytosine and guanine dinucleotides), predominantly found at regulatory regions of over half human genes (Davalos & Esteller, 2023). Methylation of these CpG islands generally results in transcriptional repression and is uncommon in normal cells. However, cancer cells often exhibit promoter-associated CpG island hypermethylation, leading to the silencing of tumor suppressor genes (Davalos & Esteller, 2023).

Alongside disrupted DNA methylation, cancer also features an abnormal range of histone modifications, including acetylation, methylation, phosphorylation, ubiquitination, SUMOylation, and ADP ribosylation (Figure 8) (Davalos & Esteller, 2023). These modifications, collectively known as the histone code, affect chromatin structure and the accessibility of transcription factors, co-activators, and co-repressors, ultimately disrupting cellular homeostasis. Misinterpretation or errors in these histone modifications can lead to oncogenesis, affecting gene expression and cellular identity and significantly contributing to cancer initiation, progression, and metastasis (Davalos & Esteller, 2023).

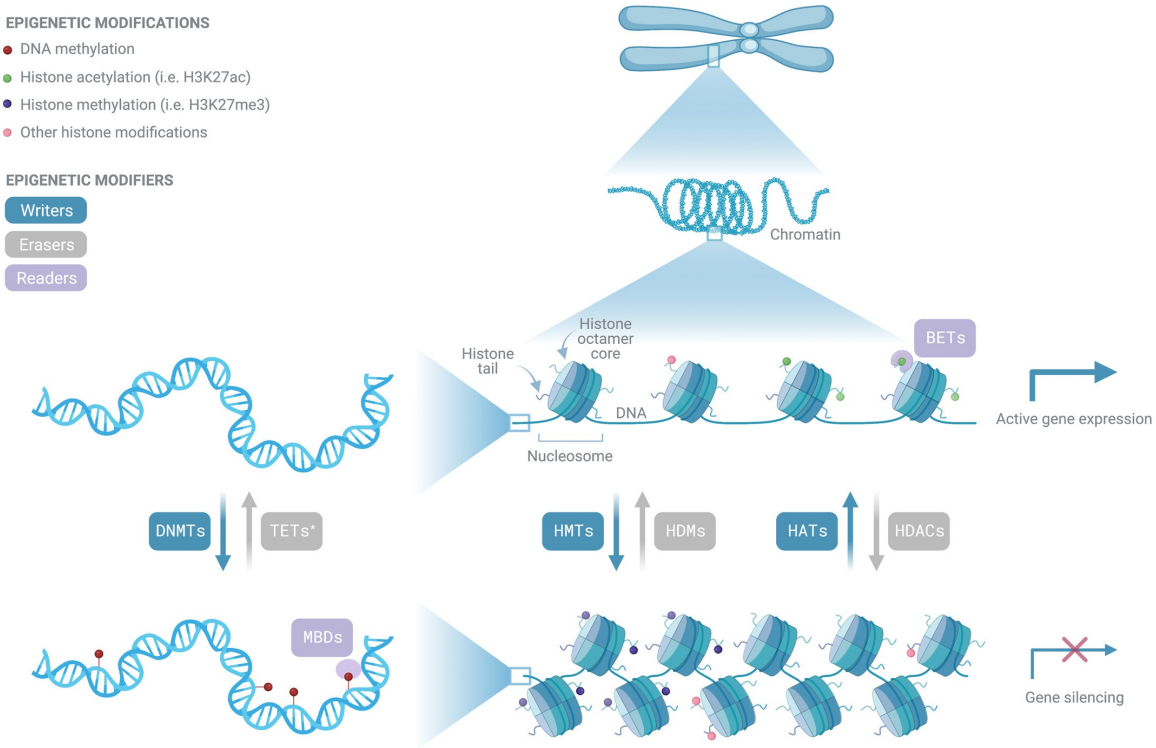


Figure 8. Epigenetic Modifications. DNA methylation and histone marks serve as key epigenetic modifications, and these are facilitated by enzymes (writers, readers, and erasers) that introduce, recognize, and remove the marks. DNA methylation is carried out by DNA methyltransferases (DNMTs) and removed by ten-eleven translocation enzymes (TETs) or through sequential cell divisions. Histone modifications, primarily acetylation and methylation, are orchestrated by a balance between histone acetyltransferases (HATs) and deacetylases (HDACs), and histone methyltransferases (HMTs) and demethylases (HDMs). The resultant epigenetic code is interpreted by proteins like methyl-CpG–binding domain proteins (MBDs) and bromodomain and extra terminal domain proteins (BETs) that bind to specific modifications. These epigenetic modifications remodel chromatin conformation, leading to transcriptional silencing or activation by recruiting other proteins. DNMTs: DNA Methyltransferases; TETs: Ten-Eleven Translocation Enzymes; HATs: Histone Acetyltransferases; HDACs: Histone Deacetylases; HMTs: Histone Methyltransferases; HDMs: Histone Demethylases; MBDs: Methyl-CpG-Binding Domain Proteins; BETs: Bromodomain and Extra Terminal Domain Proteins. Source: (Davalos & Esteller, 2023).

4. Regulation of *BRCA1* gene: epigenetic and transcriptional aspects

The *BRCA1* promoter is a TATA-less promoter with 30 CpG islands spanning positions -567bp to +44bp (Rice et al., 1998). This promoter is also characterized by a single nucleosome and a proximal sequence (from -224bp to +43bp) that includes all the essential regulatory

elements (Figure 9a) (Rice et al., 1998; C. F. Xu et al., 1997). The expression of the *BRCA1* gene is influenced by a range of agents, including genotoxic substances, hormones, developmental factors, and hypoxic conditions (Di et al., 2010; Mueller & Roskelley, 2003). A complex network of interactions involving co-repressor or co-activator complexes, promoter DNA methylation, and protein histones covalent modifications regulates *BRCA1* gene expression (Figure 9a) (Di et al., 2010; Lu et al., 2011). Key players in these processes include CtBP, P130, BRCA1 itself, and HDAC1, which maintain basal repression of the *BRCA1* gene promoter (Figure 9a) (Di et al., 2010), and estrogen, which can activate the *BRCA1* gene promoter (Di et al., 2010; Lu et al., 2011). The E2F4 and P130 or P107 complexes, E2F6, and a complex involving E2F1 and Rb also modulate the *BRCA1* promoter (Figure 9a) (Bindra & Glazer, 2006; De Siervi et al., 2010; Oberley et al., 2003).

The Estrogen Receptor alpha (ERa) and the unliganded Aromatic Hydrocarbon Receptor (AHR) are further positive modulators of *BRCA1* gene transcription, binding to the AP1 site and XREs sequence, respectively (Hockings et al., 2006; Jeffy et al., 2005). CREB protein, by binding to a cAMP response element (CRE), constitutes a crucial factor for *BRCA1* gene basal expression (Figure 9a) (Atlas et al., 2001), and oxidative stress also activates the *BRCA1* gene promoter through NRF2 transcription factor (Q. Wang et al., 2013).

In healthy tissue, a 5' segment of the *BRCA1* gene sequence, spanning 1.4 kbp, is typically demethylated and flanked by two selectively methylated DNA regions (Figure 9b) (Butcher et al., 2004). This methylation pattern is sustained by the regulatory elements CTCF (CCCTC-binding factor) and Sp1 (Figure 9b). The 5'*BRCA1* gene region contains two binding sites for CTCF and four for Sp1. By binding to the 5' regulatory region of *BRCA1*, CTCF, and Sp1 provide a safeguard against *BRCA1* gene promoter silencing by impeding the spread of aberrant DNA methylation (Figure 9b) (Butcher & Rodenhiser, 2007).

Negative modulators include the BRCA1 protein itself (Figure 9a) (De Siervi et al., 2010), which autoregulates its promoter, and the environmental pollutant TCDD, which represses *BRCA1* gene promoter activity through a cascade of events including interaction with AHR, dismissal of P300 from the *BRCA1* gene promoter, recruitment of DNMTs enzymes (1,3a, and 3b) and MBD2 protein (Figure 9a) (Papoutsis et al., 2012). Hypoxic conditions can lead to *BRCA1* gene silencing via LSD1-driven histone modifications (Lu et al., 2011). Finally, Est-2, Brg-1, and ID4 can also repress *BRCA1* gene transcription by modulating the activity of the RIBS element within the *BRCA1* gene promoter (Figure 9a) (Baker et al., 2003; Wen et al., 2012)

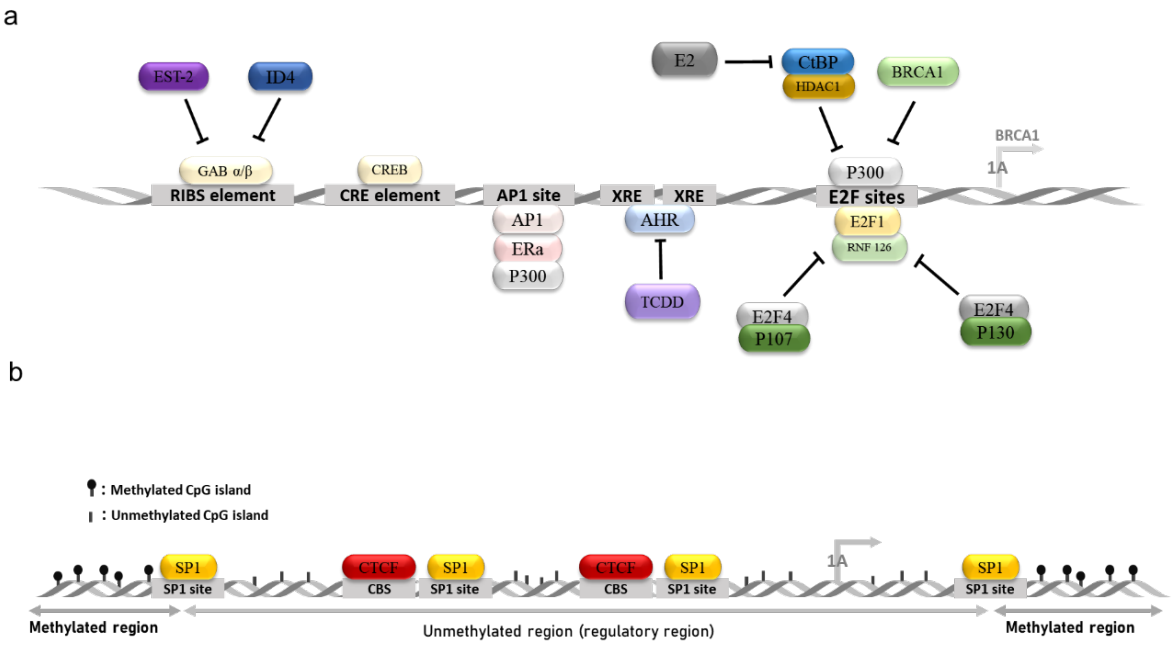


Figure 9. Regulatory Elements and Modulators of *BRCA1* Gene Expression. Est-2: Estrogen-responsive element 2; ID4: Inhibitor of DNA Binding 4; CREB: cAMP Response Element-Binding Protein; CRE: cAMP Response Element; AP1: Activator Protein 1; ERa: Estrogen Receptor Alpha; XRE: Xenobiotic Response Element; AHR: Aryl Hydrocarbon Receptor; TCDD: 2,3,7,8-Tetrachlorodibenzo-p-dioxin; E2: Estradiol; CtBP: C-terminal Binding Protein; HDAC1: Histone Deacetylase 1; CpG: Cytosine-phosphate-Guanine; Specificity Protein 1; CTCF: CCCTC-Binding Factor; CBS: CTCF Binding Site. Source: This figure represents one of our creations.

III. *BRCA1* gene promoter hypermethylation: a potential breast cancer biomarker

1. *BRCA1* gene promoter hypermethylation and its implications in breast cancer susceptibility

The *BRCA1* gene, recognized for its germline mutations leading to early onset aggressive breast tumors, often manifests as basal-like triple-negative phenotypes. However, the role of *BRCA1* gene promoter hypermethylation as a risk factor is still under investigation. Intriguingly, in cases of early-onset triple-negative breast cancer without a *BRCA1* germline mutation, approximately 50% exhibit *BRCA1* gene hypermethylation. This suggests that this epigenetic alteration might play a pivotal role in breast tumorigenesis, akin to the effects of *BRCA1* germline mutations (Glodzik et al., 2020). Furthermore, the association of *BRCA1* gene DNA hypermethylation with morphological features typical of *BRCA1*-mutated breast cancer in sporadic cases implies that genetic and epigenetic silencing of the *BRCA1* gene may converge to a similar pathological outcome (Wong et al., 2011).

Recent studies have shed light on the prevalence of *BRCA1* gene hypermethylation. For instance, 7% of newborn girls and 4.1% of healthy young women were found to have *BRCA1*

gene hypermethylation in their white blood cells (Lønning & Knappskog, 2018). Another study reported comparable rates of *BRCA1* gene promoter hypermethylation in both newborn girls (9.9%) and adult women (9.3%) (Al-Moghrabi et al., 2018). More recently, BRCA1 gene promoter hypermethylation detection in the blood was associated with a significantly increased risk for triple-negative breast cancer (Lønning et al., 2022; Prajzendanc et al., 2020).

Constitutional methylation of the *BRCA1* gene, termed epimutation, which results in the inactivation of one allele in normal mammary tissues, can mimic the effects of germline mutations, predisposing women to early-onset breast cancer. A study identified a secondary epimutation in two families with a high predisposition to breast and ovarian cancer despite lacking any previously known pathogenic variant (Evans et al., 2018). In these families, *BRCA1* DNA hypermethylation was observed across three germ layers-derived tissues: blood (mesoderm), hair follicles (ectoderm), and buccal mucosa (endoderm). Moreover, this hypermethylation co-segregated with a specific c.-107A>T 5' UTR variant within the *BRCA1* exon 1. Consequently, this epigenetic marker holds promise for enhancing risk assessment, population screening, and diagnostic procedures, potentially through non-invasive blood sample-based tools (Davalos & Esteller, 2023; Evans et al., 2018; Wong et al., 2011, 2020).

2. Therapeutic implications of *BRCA1* gene promoter hypermethylation

2.1 Predictive value for PARPi application

The alteration of BRCA1 function, originating from either genetic mutations or epigenetic modifications, impairs the homologous recombination (HR) repair mechanism. This will lead to dysfunctioning repair of double-strand breaks (DSBs), leading to tumors with consistent genomic, molecular, and histopathological characteristics. These tumors also display an increased sensitivity to specific therapeutic agents (Lord & Ashworth, 2016). Malignant breast tumors with *BRCA1* promoter hypermethylation are considered "genome phenocopies" of *BRCA1*-mutated breast tumors. They display high levels of chromosomal instability, a high rate of P53 mutations, loss of heterogeneity of the wild-type allele, and express HR deficiency signatures, a phenomenon known as "BRCAness" (Glodzik et al., 2020; Popova et al., 2012). Tumors exhibiting BRCAness have demonstrated sensitivity towards agents that stall replication forks via DNA crosslink creation (e.g., platinum salts) or block the release of essential repair proteins from DNA (such as topoisomerase poisons and PARP inhibitors) (Lord & Ashworth, 2012, 2016). Early experiments have shown that a PARP inhibitor (PARPi) agent impacts a *BRCA1* gene hypermethylated breast cancer cell line (Drew et al., 2011).

BRCA1 germline mutations have already been approved as a predictor factor for using Olaparib (a PARPi) to treat breast cancer with *BRCA1* germline mutation (Narod et al., 2017; Robson et al., 2017). Given this, investigating *BRCA1* gene promoter hypermethylation as a predictive factor is of significant interest, especially since this epigenetic mark is more common than *BRCA1* germline mutations (Glodzik et al., 2020).

2.2 Therapeutic strategies targeting *BRCA1* gene promoter hypermethylation

- Inhibitors of DNMT

Studies have demonstrated that DNMT enzymes, especially DNMT3b, are overexpressed in breast tumors (Girault et al., 2003). Moreover, increased levels of DNMT3b have been observed alongside *BRCA1* gene promoter hypermethylation and alterations in the subcellular localization of the DNA methylation boundaries, known as "CTCF," in cases of sporadic breast cancer (Butcher & Rodenhiser, 2007).

5-Azacytidine and 5-aza-2′-deoxycytidine were the first DNMT inhibitors to be discovered, and they have been approved by the FDA for the treatment of hematologic cancers (Lopez et al., 2016). Numerous clinical trials are presently assessing these nucleoside DNMT inhibitors for the treatment of solid cancers, including breast cancer (Yu et al., 2019). Once introduced into a cancer cell, 5-aza-2′-deoxycytidine gets incorporated into the DNA helix by DNA polymerases. Within CpG dinucleotides, DNMTs recognize 5-aza-dC as 2′-deoxycytidine, which inhibits the enzyme's activity and subsequently leads to its degradation by the proteasome, thereby preventing DNA methylation (Lopez et al., 2016).

Conversely, non-nucleoside DNMT inhibitors represent a newer generation of drugs developed primarily to mitigate the toxicity associated with first-generation DNMT inhibitors, although they do not exhibit the same efficiency in DNA demethylation (Yu et al., 2019). Nucleoside analogs like 5-aza-dC are not selective; they cause the degradation of different DNMT isoforms, unlike some newer-generation DNMT inhibitors, which can selectively inhibit specific isoforms (Lopez et al., 2016). This selectivity could be useful for treating cancers that overexpress a single DNMT isoform, such as breast cancer, which is known to overexpress the DNMT3b isoform (Butcher & Rodenhiser, 2007 ; Girault et al., 2003). Nanaomycin A, a non-nucleoside DNMT inhibitor, has demonstrated strong selectivity towards DNMT3b in several cancers and shown effective epigenetic reactivation of several TSGs (Figure 10) (Moreira-silva et al., 2020 ; Lopez et al., 2016). Therefore, Nanaomycin A might serve as a powerful future DNMT inhibitor, potentially reactivating the hypermethylated *BRCA1* gene by targeting DNMT3b in a range of breast cancers.

- Innovative CRISPR-dCas9-TET1 system

The CRISPR-dCas9 (d = dead) system is a modified variant of the CRISPR-Cas9 system. It lacks endonuclease activity but retains its ability to bind specific DNA sequences, thanks to single guide RNAs (sgRNAs). The fusion of the CRISPR-dCas9 system with certain epigenetic effectors creates a potent epigenome editing tool, which enables significant epigenetic modification reactions (demethylation, acetylation) within specific DNA regions (Maroufi et al., 2020).

Choudhury et al. have utilized this technique to examine the impact of demethylation on the promoter of the *BRCA1* gene, its expression, and its antiproliferative functions (Choudhury et al., 2016). This was accomplished by treating human cell lines (MCF7 cells and HeLa cells) with constructs generated by fusing the catalytic domain of Ten-Eleven Translocation dioxygenase1 (TET1CD) with deactivated Cas9, in combination with different sgRNAs specific to DNA sequences associated with CpG methylated dinucleotides of the *BRCA1* gene promoter. This treatment demonstrated high CpG demethylation efficiency and increased BRCA1 protein expression and cell apoptosis (Figure 10) (Choudhury et al., 2016). This tool can demethylate a variety of sequences in the *BRCA1* gene promoter, enabling its activation through the binding of transcription factors (Xu et al., 2010) and the displacement of certain Methyl-CpG-binding domain proteins, such as UHRF1, which has been shown to be involved in the silencing of *BRCA1* gene in sporadic breast cancers (Jin et al., 2010).

- Targeting the G9a histone methyltransferase

UHRF1, an oncogene frequently overexpressed in many cancers, facilitates the epigenetic silencing of various tumor suppressor genes, including p16INK4A (Alhosin et al., 2016). Notably, UHRF1 overexpression is associated with *BRCA1* gene promoter hypermethylation and BRCA1 mRNA reduction in sporadic breast cancers. Overexpressed UHRF1 forms a repressive complex with HDAC1, DNMT1, and G9a at the *BRCA1* gene promoter, affecting histone modifications and leading to a decrease in activating histone marks while increasing repressive marks (Jin et al., 2010). Therefore, targeting actors belonging to this complex, such as G9a inhibitors (e.g., Chaetocin), may be an option to reverse DNA methylation of the *BRCA1* gene (Figure 10) (Cao et al., 2019).

Table IV. Therapeutic Strategies Targeting *BRCA1* Gene Promoter DNA Methylation

Section	Treatment Modality	Mechanism of Action	Evidence-Base	Potential Benefits	Limitations	References
Inhibitors of DNMT	5-Azacytidine, 5-aza-2′-deoxycytidine	Inhibits DNMTs, integrated into DNA, degrades DNMTs	FDA-approved for hematologic cancers	Broad-spectrum demethylation, existing FDA approval	Lack of isoform selectivity	(Yu et al., 2019)
	Nanaomycin A	Selective inhibition of DNMT3b	Pre-clinical studies	DNMT3b-specific, potentially less toxic	Pre-clinical status	(Moreira-silva et al., 2020 ; Lopez et al., 2016)
Innovative CRISPR-dCas9-TET1 System	CRISPR-dCas9-TET1	Targeted demethylation of *BRCA1* gene promoter CpGs	Cell line studies (MCF7, HeLa)	High specificity, could reactivate silenced *BRCA1*	Technique still under development	(Choudhury et al., 2016)
Targeting the G9a Histone Methyltransferase	Chaetocin	Inhibits G9a, part of the repressive complex silencing *BRCA1*	Pre-clinical studies	Targets both DNA methylation and histone modifications	-	(Cao et al., 2019; Jin et al., 2010)

DNMT: DNA Methyltransferase; FDA: Food and Drug Administration; DNMT3b: DNA Methyltransferase 3b; CpG: Cytosine-phosphate-Guanine; G9a: A histone methyltransferase enzyme

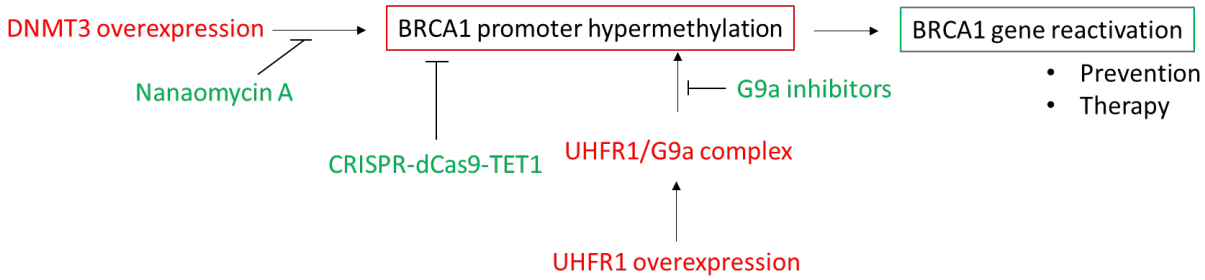


Figure 10. Strategies for Targeting *BRCA1* Gene Promoter Methylation in Breast Cancer Treatment. The elements highlighted in red are potentially linked to *BRCA1* gene promoter methylation in breast cancer. Those marked in green represent potential therapeutic approaches aimed at these tumorigenic pathways. Targeting *BRCA1* gene promoter hypermethylation could serve dual purposes: therapeutic and preventive. It could be beneficial for women already diagnosed with breast cancer and serve as a preventive measure for women at high risk. DNMT3b: DNA Methyltransferase 3b; TET1: Ten-Eleven Translocation dioxygenase1; G9a: A histone methyltransferase enzyme; UHRF1: Ubiquitin-like with PHD and RING Finger domains 1. Source: This figure represents one of our creations.

IV. Oxidative stress and breast cancer

1. An overview of oxidative stress

Oxidative stress describes a cellular imbalance where the production of reactive oxygen species (ROS) exceeds the ability of cells to neutralize their deleterious effects (Forman & Zhang, 2021). It involves elevated levels of ROS, driving damage to cellular components and leading to diverse biological consequences (Forman & Zhang, 2021; Waghela et al., 2021). Oxidative stress has been linked to various conditions, including heart disease, Alzheimer disease, and cancer (Forman & Zhang, 2021). Oppositely to oxidative stress, low levels of ROS play roles in signaling pathways to maintain physiological functions (Schieber & Chandel, 2014). A balance between the production of ROS and their elimination is key to avoiding their pathological consequences (Holmström & Finkel, 2014).

Reactive oxygen species (ROS) are highly reactive molecules originating from oxygen metabolism (Waghela et al., 2021). Major ROS include the hydroxyl radical ($OH^{\bullet}$), superoxide anion ($O_2^{\bullet-}$), and hydrogen peroxide (H_2O_2) (Brieger et al., 2012; Waghela et al., 2021). These ROS undergo various transitions between their various forms, driven by their inherent reactivity (Brieger et al., 2012). The primary pathway for ROS generation involves the reduction of molecular oxygen (O_2) to form superoxide ($O_2^{\bullet-}$), a reaction that can occur spontaneously or be facilitated by specific enzymes (Brieger et al., 2012). Superoxide can be further converted to hydrogen peroxide (H_2O_2) through the catalytic action of superoxide dismutase (SOD). This conversion is represented as $2\ O_2^{\bullet-} + 2\ H^+ \rightarrow H_2O_2 + O_2$ (Figure 11) (Brieger et al., 2012).

While superoxide has limited diffusion and a short half-life, hydrogen peroxide is more stable and can permeate cellular membranes (Figure 11) (Meitzler et al., 2019). Interactions between superoxide and nitric oxide (NO) yield another reactive species, peroxynitrite ($ONOO^-$) (Figure 11) (Brieger et al., 2012). Other peroxidase-catalyzed reactions can produce reactive species like hypochlorous acid (HOCl). Additionally, the Haber-Weiss and Fenton reactions illustrate the conversion of superoxide and hydrogen peroxide to the highly reactive hydroxyl radical ($OH^{\bullet}$) in the presence of iron catalysts (Figure 11) (Brieger et al., 2012).

The hydroxyl radical is extraordinarily reactive and can damage various biomolecules, such as amino acids, phospholipids, and DNA (Cadet & Davies, 2017). For instance, hydroxyl radicals are key initiators of lipid peroxidation, a process damaging to cell membranes and associated with various pathological conditions (Ayala et al., 2014). Furthermore, hydrogen peroxide serves a dual role; not only does it contribute to oxidative stress, but it also acts as a

signaling molecule. Due to its stability and membrane permeability, H_2O_2 plays a role in cancer signaling pathways that regulate cellular growth and survival. (Schieber & Chandel, 2014).

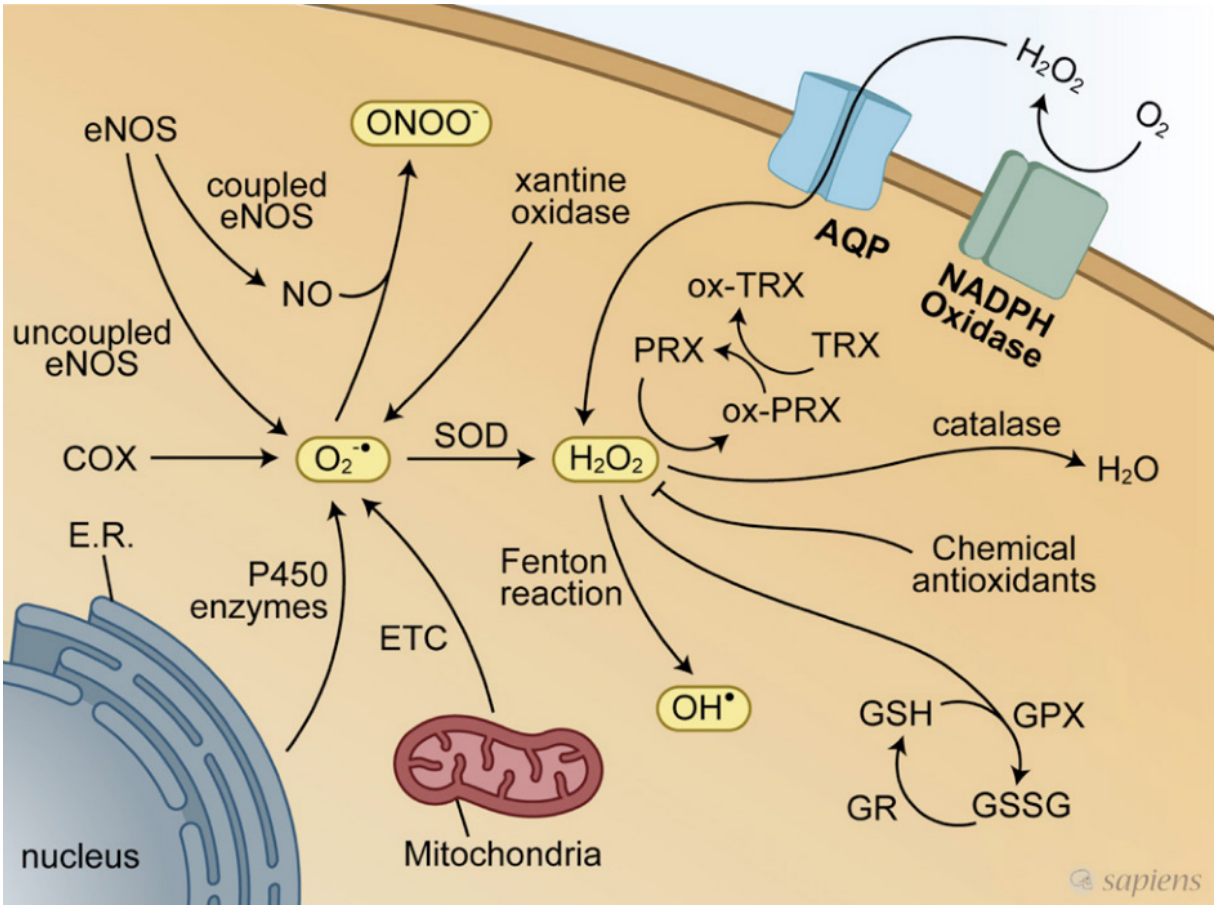


Figure 11. Overview of Reactive Oxygen Species (ROS) Formation and Neutralization. This figure summarizes the primary sources and transitions of major ROS, including hydroxyl radical ($OH^{\bullet}$), superoxide anion ($O_2^{\bullet-}$), and hydrogen peroxide (H_2O_2). The primary intracellular sources of ROS include mitochondria, NADPH oxidases, and the endoplasmic reticulum. Additional enzymes contributing to ROS production vary by cell type and include xanthine oxidase, nitric oxide synthase, and others. To maintain redox homeostasis, cells employ several antioxidant enzymatic systems, such as superoxide dismutases (SODs), glutathione peroxidase (Gpx), and catalase. $O_2^{\bullet-}$ can be further converted to H_2O_2 via the enzyme superoxide dismutase (SOD), then to water by catalase. While superoxide has a short half-life and limited mobility, H_2O_2 is more stable and membrane-permeable. Various reactions, facilitated by different enzymes and catalyzed by metal ions, produce other reactive species like the highly reactive $OH^{\bullet}$ and peroxynitrite ($ONOO^-$). ROS: Reactive Oxygen Species; $OH^{\bullet}$: Hydroxyl Radical; $O_2^{\bullet-}$: Superoxide Anion; H_2O_2: Hydrogen Peroxide; SOD: Superoxide Dismutase; $ONOO^-$: Peroxynitrite; NADPH: Nicotinamide Adenine Dinucleotide Phosphate; Gpx: Glutathione Peroxidase. Source: (Ameziane El Hassani et al., 2019).

The primary intracellular sources of ROS are the mitochondria, NADPH oxidases, and the endoplasmic reticulum (Figure 11) (K. Wang et al., 2013). Other enzymes can also contribute to ROS production, and their involvement varies depending on the cell type. These include xanthine oxidase, nitric oxide synthase, cyclooxygenases, cytochrome P450 enzymes, and lipoxygenases (Figure 11) (Holmström & Finkel, 2014). In cells, ROS levels are strictly regulated to maintain redox homeostasis. Several antioxidant enzymatic systems play a role in this regulation: Superoxide dismutases (SODs), glutathione peroxidase (Gpx), glutathione reductase (GR), peroxiredoxins, thioredoxin (Trx), and catalase (Figure 11) (K. Wang et al.,

2013). Superoxide dismutase (SOD) catalyzes the conversion of the superoxide radical into hydrogen peroxide ($O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$), as mentioned above. Catalase facilitates the breakdown of hydrogen peroxide into water and molecular oxygen ($2H_2O_2 \rightarrow 2H_2O + O_2$). Glutathione peroxidase drives the reaction between hydrogen peroxide and monomeric glutathione (GSH), producing glutathione disulfide (GSSG) and water: $H_2O_2 + 2\ GSH \rightarrow GSSG + 2H_2O$ (Figure 11). The regeneration of GSH from GSSG is mediated by glutathione reductase, utilizing NADPH (Chaudière & Ferrari-Iliou, 1999).

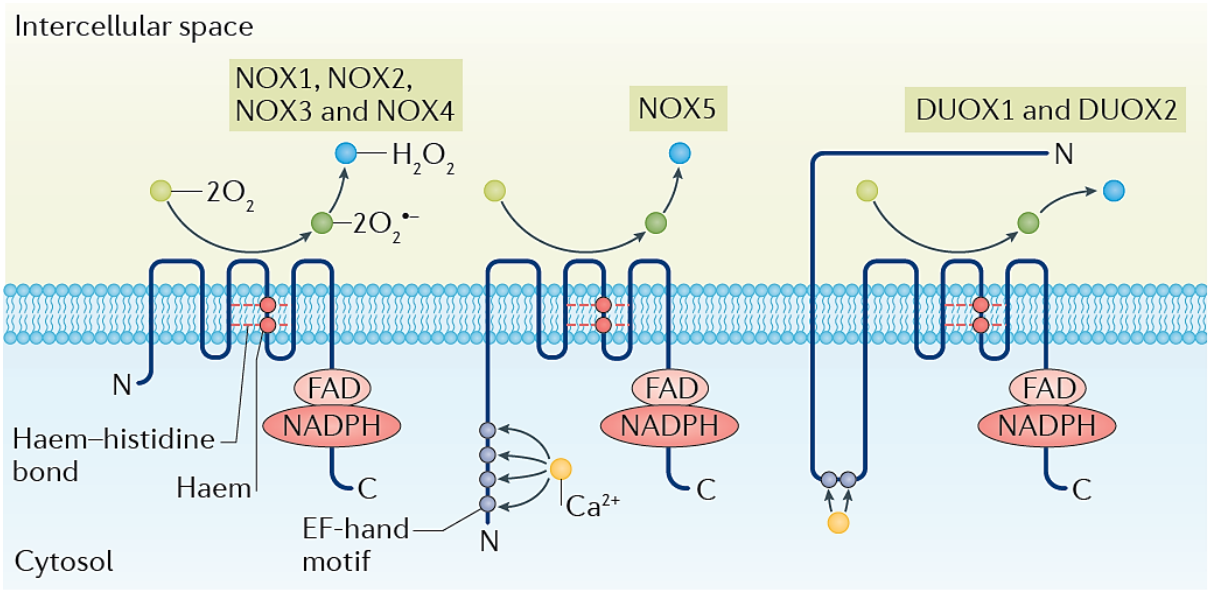
2. NADPH oxidases

2.1 Introduction and classification

The primary role of NADPH oxidases (NOX) is to produce ROS (Reactive Oxygen Species). Various extracellular stimuli can activate NOX enzymes, and their activity is strictly regulated to prevent the overproduction of ROS (Figure 12) (Ameziane-El-Hassani et al., 2016). NOX2 was the first NOX enzyme identified. NOX2 is crucial for the antimicrobial response in neutrophils because it generates hydrogen peroxide, which is essential for producing hypochlorous acid (HOCl) in neutrophils (Ameziane-El-Hassani et al., 2016). To date, seven enzymes from the NOX family have been identified. Six of these, namely NOX1, NOX3, NOX4, NOX5, DUOX1, and DUOX2 (dual oxidase 1 and 2), are responsible for ROS production in non-phagocytic cells (Figure 12) (Ameziane-El-Hassani et al., 2016).

Members of the NOX family share structural features, including six conserved transmembrane domains with heme-binding histidines (Figure 12). DUOX1 and DUOX2 have additional domains and unique heme-binding histidines. NOX enzymes also have NADPH and FAD binding sites (Bedard & Krause, 2007). NOX5, DUOX1, and DUOX2 have EF-hand motifs in their cytoplasmic domains, which allow calcium-dependent ROS production regulation (Figure 12) (Ameziane-El-Hassani et al., 2016).

NOX2 is consistently associated with the transmembrane protein $p22^{phox}$. Activating this complex requires cytosolic factors like $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ (Figure 12) (Bedard & Krause, 2007). Other NOX enzymes have different regulatory subunits and mechanisms (Figure 12). NOXA1 and NOXO1 are cytosolic factors that bind and activate NOX1/ $p22^{phox}$ and NOX3/ $p22^{phox}$ complexes. DUOXA1/A2 are membrane partners of DUOX 1 and 2, respectively (Figure 12) (Ameziane-El-Hassani et al., 2016).



Cytoplasmic partners	• NOXA1 and NOXO1 for NOX1 and NOX3 • $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$ for NOX2
Membrane partners	• $p22^{phox}$ for NOX1, NOX2, NOX3 and NOX4 • DUOXA1 for DUOX1 • DUOXA2 for DUOX2
EF-hand motifs and calcium regulation	NOX5, DUOX1 and DUOX2

Figure 12. Overview of NADPH Oxidases (NOX) and ROS Production. This figure outlines the primary regulatory mechanisms and structures of NADPH oxidases (NOX) involved in generating ROS. The NOX family comprises seven enzymes, including NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1, and DUOX2. Structural similarities and unique features among the NOX family, such as transmembrane domains, heme-binding histidines, and NADPH and FAD binding sites, are illustrated. Specific cytosolic and membrane partners for activation, including $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, NOXA1, NOXO1, DUOXA1, and DUOXA2, are also highlighted. NOX: NADPH Oxidases; ROS: Reactive Oxygen Species; NOX1, NOX2, NOX3, NOX4, NOX5: These are specific members of the NADPH Oxidases family, and they do not typically have longer names.; DUOX1, DUOX2: Dual Oxidase 1 and 2; NADPH: Nicotinamide Adenine Dinucleotide Phosphate; FAD: Flavin Adenine Dinucleotide; The 'phox' stands for 'phagocyte oxidase.'; NOXA1: NADPH oxidase activator 1; NOXO1:; NADPH oxidase organizer 1; DUOXA1, DUOXA2: Dual Oxidase Maturation Factors 1 and 2. Source: (Ameziane-El-Hassani et al., 2016)

2.2 Physiological functions of NADPH oxidases

ROS are central to various physiological functions, encompassing host defense against microbial aggressors, complex signal transduction pathways, and hormone biosynthesis (Ameziane-El-Hassani et al., 2016; Bedard & Krause, 2007). NOX enzymes are expressed in a multitude of tissues, where they regulate redox signaling to modulate several physiological processes (Table V) (Ameziane-El-Hassani et al., 2016).

Table V. Expression and Physiopathology of NADPH Oxidase Enzymes.

NOX	ROS species	Tissue expression	Function	Involvement in human diseases
NOX1	$O_2^{\bullet-}$	Digestive tract, vascular system	Host defence, blood pressure regulation, proliferation of smooth muscle (speculative)	NA
NOX2	$O_2^{\bullet-}$	Phagocytes, vascular system	Host defence (verified), signalling (speculative)	Chronic granulomatous disease
NOX3	$O_2^{\bullet-}$	Inner ear	Otoconia formation (verified)	NA
NOX4	$O_2^{\bullet-}$ H_2O_2	Kidney, thyroid, vascular system, heart, skeletal muscle, fibroblast	Vasoregulation, signalling (speculative)	NA
NOX5	$O_2^{\bullet-}$	Lymph node, testis	Spermatogenesis (speculative)	NA
DUOX1	• Immature*: $O_2^{\bullet-}$ • Mature‡: H_2O_2	Thyroid, respiratory tract, digestive tract, salivary glands, skin	Host defence, signalling (speculative)	NA
DUOX2	• Immature*: $O_2^{\bullet-}$ • Mature‡: H_2O_2	Thyroid, respiratory tract, digestive tract, salivary glands	Thyroid hormone synthesis (verified), host defence (speculative)	Congenital hypothyroidism

NOX1 is also known as mitogenic oxidase1 because its first identified function was in cell growth. NOX4 is also named Renox (renal oxidase) because it was first identified in the kidney. The DUOX enzymes are called also Thox (thyroid oxidase), because they were first identified in the thyroid gland. *Immature refers to proteins in the absence of their maturation factor. ‡ Mature refers to proteins in the presence of their maturation factor. DUOX, dual oxidase; H_2O_2, hydrogen peroxide; NA, not applicable; NOX, NADPH oxidase; $O_2^{\bullet-}$, superoxide anion. Source: (Ameziane-El-Hassani et al., 2016).

2.3 Focus on NOX4 and DUOX1 in cancer biology

Unlike other NADPH oxidase enzymes, NOX4, DUOX1, and DUOX2 carry the unique ability to produce hydrogen peroxide (H_2O_2) directly (Meitzler et al., 2019). Hydrogen peroxide, unlike other reactive oxygen species (ROS), is a relatively stable molecule. Its stability allows it to diffuse across cellular membranes and play a pivotal role in cell signaling (Meitzler et al., 2019). H_2O_2 acts as a secondary messenger, modulating the activity of various proteins and signaling pathways (Block & Gorin, 2012; Meitzler et al., 2019). For instance, it can influence the activity of protein kinases and phosphatases, which are central to many cellular processes (Lukosz et al., 2010). Redox signaling was shown to be spatially controlled by the localized generation of reactive oxygen species in specific subcellular regions (Block & Gorin, 2012; Spencer et al., 2011). Indeed, NOX4 was shown in different cellular compartments, including the nucleus, the nuclear envelope, the endoplasmic reticulum (perinuclear region), the mitochondria, and the plasma membrane (Meitzler et al., 2019). NOX4 subcellular localization seems to be an indispensable factor in studying its role in specific processes of carcinogenesis (Block & Gorin, 2012).

3. Oxidative stress in cancer development

3.1 DNA damage and mutagenesis

Oxidative stress can cause direct damage to the DNA molecule, leading to mutations. ROS, especially the hydroxyl radical, can cause various DNA lesions, including base modifications, strand breaks, and DNA-protein crosslinks (Cooke et al., 2003). If not repaired correctly, these mutations can accumulate over time and lead to oncogene activation or tumor suppressor gene

inactivation, both of which can initiate tumorigenesis (Cadet & Davies, 2017; Cooke et al., 2003). Chronic oxidative stress can also interfere with the DNA repair mechanisms, further increasing mutations and mitogenic signaling and leading to cancer (Kay et al., 2019).

3.2 Effects on tumor growth and survival

Oxidative stress can activate several signaling pathways that promote cell proliferation and survival (Schieber & Chandel, 2014). For instance, ROS can activate the MAPK and PI3K/Akt pathways, which promote cell growth and survival (Block & Gorin, 2012; Schieber & Chandel, 2014). ROS can also lead to the activation of transcription factors like NF-kB and HIF-1α, which can promote cell survival under stress conditions and induce the expression of genes that provide growth advantages to tumor cells (Block & Gorin, 2012; Schieber & Chandel, 2014). Conversely, excessive oxidative stress can trigger cell death pathways. However, cancer cells often develop mechanisms to evade ROS-induced cell death, further contributing to tumor growth and therapy resistance (Trachootham et al., 2009).

3.3 Modulation of the tumor microenvironment

The tumor microenvironment comprises various cell types, including fibroblasts, immune, and endothelial cells. ROS constitutes a pivotal factor in modulating both tumor cells and cells of the microenvironment of the tumor to promote tumor growth and survival (Aggarwal et al., 2019; Vermot et al., 2021). ROS production in endothelial cells was shown to trigger their proliferation to form new vessels within the tumor (X. Wang et al., 2021). They can also modulate angiogenesis by activating VEGF receptors (Vermot et al., 2021). ROS can induce the secretion of pro-inflammatory cytokines and chemokines, which promote cancer progression and metastasis (Aggarwal et al., 2019). Also, ROS increase in CAFs also plays a role in their activation and tumor-promoting functions (Mir et al., 2022).

4. Multifaceted roles of NOX4 in cancer

4.1 Genomic instability and NOX4

NOX4-derived ROS may affect nuclear DNA, potentially leading to DNA damage, cellular senescence, and genomic instability (Figure 13) (Block & Gorin, 2012). Indeed, the detection of NOX4 in the nuclear membrane envelope was shown to be linked to nuclear superoxide production, which could potentially directly alter nuclear DNA and proteins and thus promote genomic instability (Figure 13) (Spencer et al., 2011). H_2O_2 detection in the nucleus was produced by the perinuclear NOX4 in normal thyroid cells overexpressing the oncogene HRAS V12, causing DNA replication and DNA damage and leading to cellular senescence (Weyemi et al., 2012). Given that RAS signaling is activated in several types of cancers that do not

undergo senescence, NOX4 might play a role in causing genomic instability in these cancers (Block & Gorin, 2012).

4.2 NOX4 role on cell growth and survival

NOX4 promotes cell proliferation and cell survival by modulating the activity of protein phosphatases and transcription factors (Block & Gorin, 2012). The perinuclear localization (endoplasmic reticulum) of NOX4 was shown to spatially mediate the oxidation and subsequent inactivation of protein tyrosine phosphatase 1B (PTP B1) in the endoplasmic reticulum (Figure 13). This process was shown to regulate cell proliferation downstream of the epidermal growth factor (EGF) signaling (Figure 13) (K. Chen et al., 2008). NOX4 expression in the membrane plasma of melanoma cells promotes survival through modulation of transcription factor nuclear factor-KB (NF-KB) (Figure 13) (Brar et al., 2002). NOX4 was identified as the primary source of the increased ROS in the chronic myelogenous leukemia cells expressing Bcr-Abl. This was shown to promote survival pathway signaling through the redox inhibition of PP1a, a regulator that typically suppresses the PI3k/Akt pathway (Figure 13) (Naughton et al., 2009).

4.3 Role in angiogenesis

NOX4 was identified as a significant factor in promoting lymphangiogenesis in breast cancer (X. Wang et al., 2021). Indeed, using Human Lymphatic Endothelial Cells (HLECs), NOX4 was found to induce proliferation (cell growth), migration, anti-apoptosis (resistance to programmed cell death), tube formation, and lymphatic ring sprouting. These activities were shown to be mediated through the Nox4/ROS/ERK/CCL21 signaling pathways (Figure 13) (X. Wang et al., 2021). In ovarian cancer cells, ROS produced by NOX4 was shown to modulate the levels of Vascular Endothelial Growth Factor (VEGF) via the activation of Hypoxia-Inducible Factor 1-alpha (HIF1α) (Figure 13) (Xia et al., 2007).

4.4 NOX4 and metastasis

NOX4 was shown to be involved in the TGFβ-induced EMT process in breast epithelial cells (Figure 13). It contributes to ROS production, which seems essential for cell migration and expression of EMT markers (Boudreau et al., 2012). NOX4's modulation of fibronectin expression in breast cancer cells is believed to play a role in the formation of breast tumor cell aggregates after detachment from the tumor; these aggregates are crucial for resisting anoikis, which promotes survival and metastasis (Boudreau et al., 2012; Han et al., 2021). The NOX4 protein also regulates the release of the chemokine CCL21 by endothelial cells, which can attract tumor cells expressing CCR7 (CCL21 ligand) into lymphatic vessels, facilitating their spread and metastasis (X. Wang et al., 2021). NOX4 is overexpressed in cancer-associated

fibroblasts (CAFs) compared to normal breast fibroblasts, and this overexpression is essential for the activation and tumorigenic functions of CAFs in breast cancer (Mir et al., 2022).

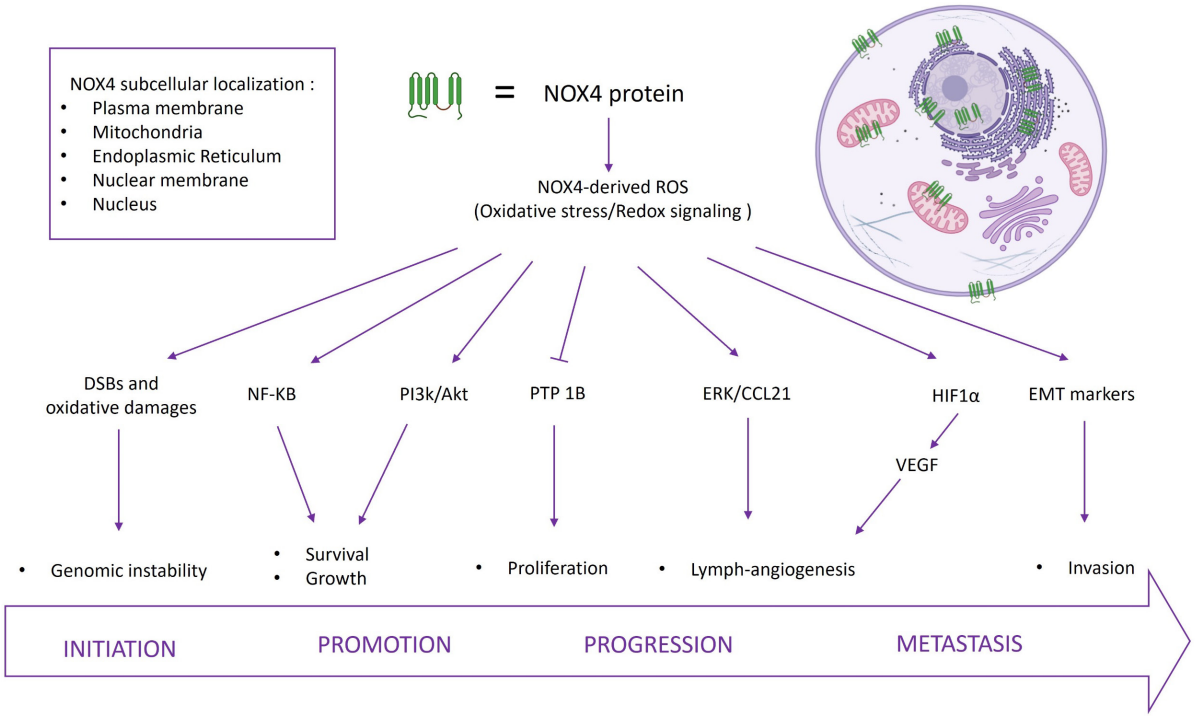


Figure 13. Multifaceted Roles of NOX4 Protein in Cancer Biology. This comprehensive figure illustrates the diverse roles of NOX4 in various cellular processes related to cancer: genomic instability, tumor growth and survival, proliferation, angiogenesis, and metastasis. NOX4: NADPH oxidase 4; ROS: Reactive Oxygen Species; NF-KB: Nuclear Factor-Kappa B; PI3k/Akt: Phosphoinositide 3-kinase/Akt pathway; VEGF: Vascular Endothelial Growth Factor; HIF1α: Hypoxia-Inducible Factor 1-alpha; EMT: Epithelial-Mesenchymal Transition; CCL21: Chemokine (C-C motif) ligand 21. Source: This figure represents one of our creations.

V. Medicinal Plants and Breast Cancer

Modern research on cancer has benefited from the traditional use of plants in developing new drugs to treat cancer (H. Wang et al., 2012). The use of plants in cancer implies their chemopreventive and therapeutic effects, primarily due to the natural components they contain. These bioactive compounds are increasingly recognized for their ability to prevent, impede, delay, or even treat cancer (H. Wang et al., 2012). Moreover, these plant-derived compounds often demonstrate fewer side effects compared to conventional chemotherapy, making them a focus for developing safer and more effective cancer treatments (Shrihastini et al., 2021). Almost 50% of anticancer drugs are derived from natural products (Naeem et al., 2022). Well-known examples of anticancer drugs derived from natural products include taxol, vinblastine, vincristine, and podophyllotoxin analogs (Naeem et al., 2022). Taxol (paclitaxel), derived from the Pacific yew tree, is used in the treatment of various cancers, including ovarian, breast, and lung cancers (Naeem et al., 2022).

In Morocco, traditional therapies against breast cancer have documented the use of 34 plant species (Alami Merrouni & Elachouri, 2021). Several components of medicinal Moroccan plants have been identified as having therapeutic effects on breast cancer (Alami Merrouni & Elachouri, 2021; El Hachlafi et al., 2022). Among these, two components stand out for their cytotoxic activities against breast cancer cell lines (Alami Merrouni & Elachouri, 2021). Firstly, Berberine, derived from Berberis species, has shown a cytotoxic effect in vitro against two triple-negative breast cancer cell lines, BT549 and MDA-MB-231 (Alami Merrouni & Elachouri, 2021). Secondly, Gingerol, from Zingiber officinale Roscoe, demonstrated cytotoxic activity against the triple-negative breast cancer cell lines MDA-MB-231 and MDA-MB-468 (Alami Merrouni & Elachouri, 2021).

The potential of medicinal plants in cancer treatment is enormous. Continued research, especially in the context of diverse flora like that of Morocco, may reveal more bioactive compounds with anticancer properties.

Objectives

Oxidative stress and epigenetic modifications are pivotal players in the complex landscape of cancer biology. My research project, "Exploration of Oxidative Stress and Epigenetic Biomarkers in the Management of Breast Cancer," examines various markers related to these two vital cellular phenomena within distinct human mammary tissues. Our initial focus is on the hypermethylation of the *BRCA1* gene promoter as an epigenetic biomarker. In addition, this work also focuses on NADPH Oxidase 4 (NOX4) and Dual Oxidase 1 (DUOX1) proteins as oxidative biomarkers.

- **Main objectives: a translational approach**

- To determine the prevalence of *BRCA1* gene promoter hypermethylation across three distinct human mammary tissue types: Malignant breast tumors, their corresponding Normal adjacent tissue, and Benign breast lesions.
- To examine the expression and subcellular localization of NOX4 and DUOX1 proteins in Malignant breast tumors and their corresponding Normal adjacent tissues.
- To compare these epigenetic and oxidative biomarkers between Malignant breast tumors and their corresponding Normal adjacent tissues.
- To explore the correlation between these epigenetic and oxidative biomarkers and the aggressive clinical and histopathological characteristics of breast cancer.

- **Progressive objectives: a cellular approach**

- To initiate the characterization of different human breast cancer cell lines to explore the potential of natural molecules from the Moroccan medicinal and aromatic plants in targeting *BRCA1* gene promoter hypermethylation and NOX4 protein in BC.

Martials and Methods

I. Translational Approach Utilizing Human Breast Tissues: Retrospective Study

In our translational research, we examined human breast tissue samples to investigate *BRCA1* promoter hypermethylation, as well as the expression and subcellular localization of NADPH oxidases NOX4 and DUOX1 (Table VI). To this end, we conducted a retrospective study utilizing formalin-fixed paraffin-embedded (FFPE) breast tissue samples, facilitated by a productive collaboration between our laboratory, Biology of Human Pathologies, and the Department of Anatomical Pathology at the Military Hospital of Instruction Mohammed V in Rabat (HMIMV-R) (Figure 13).

Figure 13. Schematic Overview of the Methodological Steps in the Retrospective Study.

Following ethical approval, we identified cases for the study from medical records and registries. The patients in our cohort were diagnosed within the same department between July 2017 and December 2019. A database was created to capture clinical and histopathological characteristics of breast cancer. Pathologists then conducted retrospective analyses on archived slides to confirm the diagnoses recorded in the medical files and registers. Ultimately, FFPE samples were chosen according to specific selection criteria. The count of samples selected for each type of analysis is detailed in the Table VI.

Table VI. Analyzed Biomarkers, Methods of Detection, and the Count of Samples.

Biomarker	Technique	FFPE samples
BRCA1 gene promoter hypermethylation	Methylation-specific PCR	MBTs (48), their NATs (15) and BBLs (21)
NOX4 protein	Immunohistochemistry	MBTs (100) and their NATs (94)
DUOX1 protein	Immunohistochemistry	MBTs (88) and their NATs (84)

II. Methylation-specific PCR (MSP) Analysis of BRCA1 Gene Promoter

Three subsets of FFPE samples were selected for the analysis of *BRCA1* promoter hypermethylation, according to specific selection criteria (Figure 14).

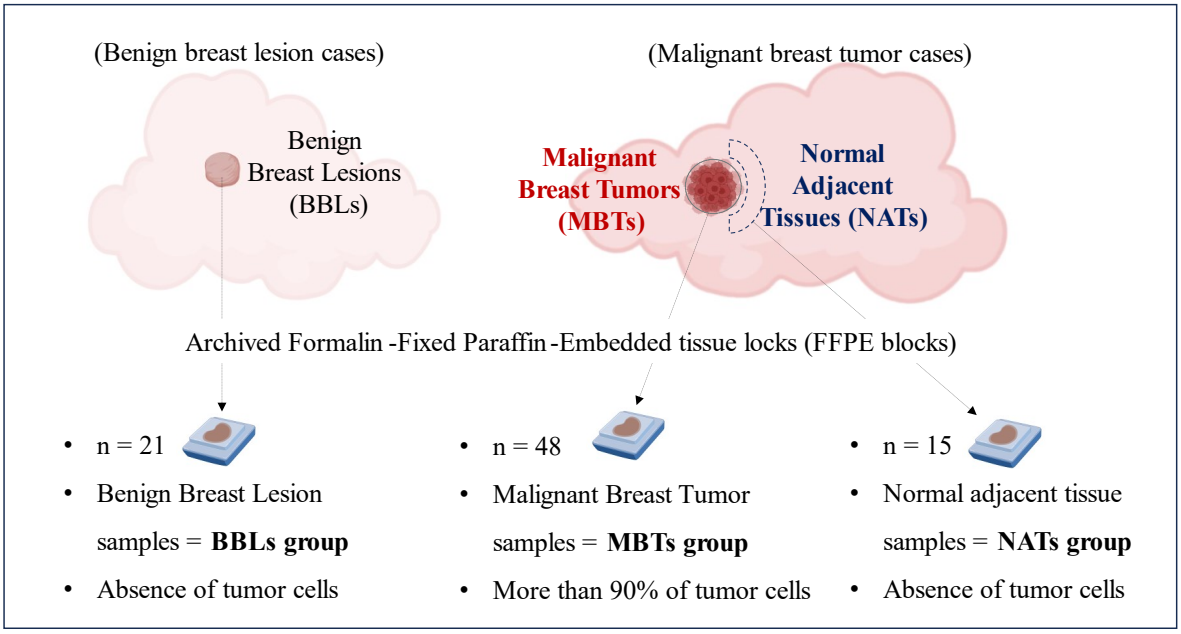


Figure 14. Sample Subsets Selected for BRCA1 Gene Promoter Hypermethylation Analysis.

We employed Methylation-Specific PCR (MSP) to assess BRCA1 gene promoter hypermethylation in selected samples. This method utilizes bisulfite treatment to convert unmethylated cytosines to uracil, whereas methylated cytosines remain unchanged. Subsequently, two pairs of primers designed to target bisulfite-converted DNA (representing unmethylated regions) and unconverted DNA (indicating methylated regions) enable differentiation between hypermethylated and unmethylated states of the DNA (Figure 15).

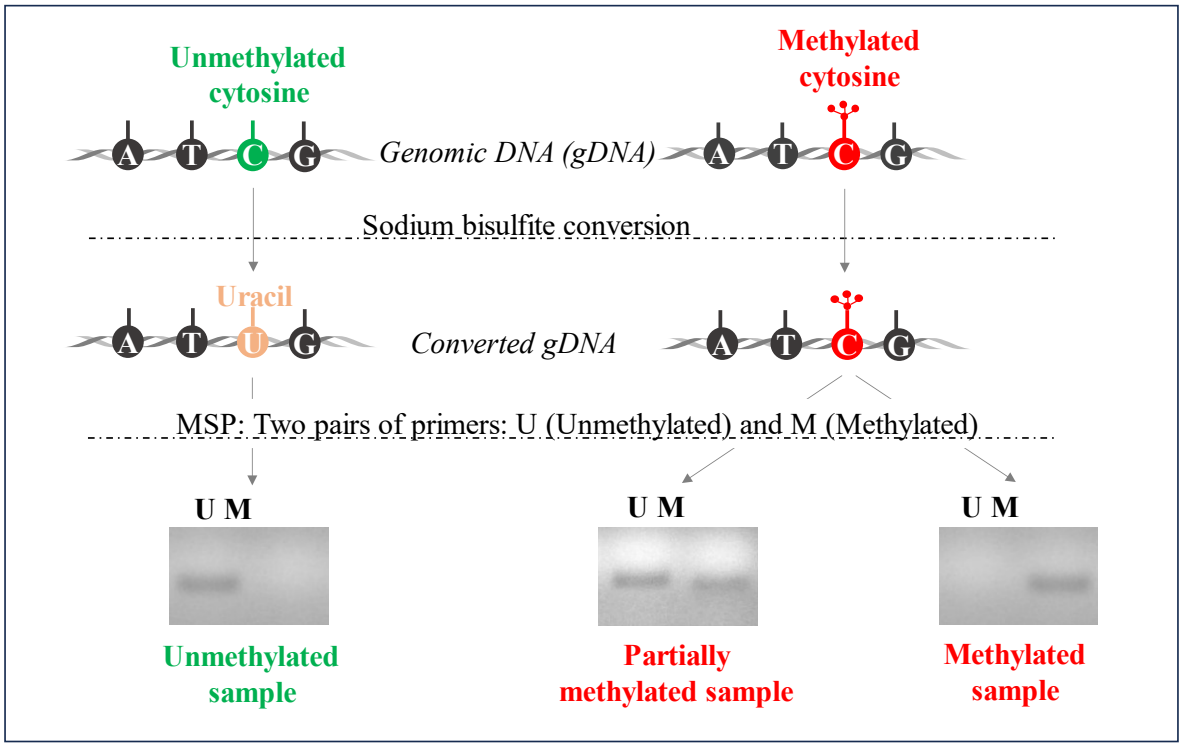


Figure 15. Analysis of DNA Methylation Patterns by Bisulfite Conversion and Methylation-Specific PCR (MSP)

Initially, we began by preparing sections from formalin-fixed paraffin-embedded (FFPE) blocks for genomic DNA extraction. We employed the QIAamp DNA FFPE Tissue Kit (Qiagen), which is optimized for FFPE samples known for their fragile DNA. Subsequently, bisulfite conversion was carried out using two kits: the Epitect Fast DNA Bisulfite Kit (Qiagen, 59826) and the EZ DNA Methylation Kit (Zymo Research, D5002). The PCR reaction was then performed using HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany). Finally, the PCR products were subjected to gel electrophoresis, and the resulting bands were visualized under UV light.

III. Immunohistochemistry Analysis of NOX4 and DUOX1 Proteins

For the evaluation of expression and subcellular localization of NOX4 and DUOX1 proteins, we employed immunohistochemistry utilizing two rabbit polyclonal antibodies: anti-NOX4 (ab154244, Abcam, Cambridge, UK) and anti-DUOX1 (Ameziane-El-Hassani et al., 2015) (Table VII). Unlike commercially available anti-DUOX1 antibodies, the antibody utilized in our study is specific to DUOX1 and does not cross-react with DUOX2, which is a homolog of DUOX1. Staining procedures were performed using Dako EnVision™ FLEX High pH 'LINK'/FLEX IHC Microscope Slides (Agilent, Santa Clara, CA, USA) on an Autostainer Link 48 device (Agilent), following the manufacturer's guidelines.

Table VII. Characterization of Antibodies Targeting NOX4 and DUOX1 proteins: Specificity and Dilution

Target	Antibody	Epitope	Optimal Dilution (Tissue Type)	Reference
NOX4 Protein	Rabbit polyclonal anti-NOX4	**Amino acids 252-564**	1:250 (FFPE human kidney)	ab154244, Abcam, Cambridge, UK
DUOX1 Protein	Rabbit polyclonal anti-DUOX1	**Amino acids 988–1011**	1:400 (FFPE Human lung)	Ameziane El Hassani R et al., Proc Natl Acad Sci U S A. 2015 Apr 21;112(16):5051-6

Pathologists assessed the expression levels using the international scoring system for immunohistochemistry. A score of 0 indicates no expression, 1 indicates weak expression, 2 indicates moderate expression, and 3 indicates strong expression (Figure 16). For the purposes of our analysis, we categorized these scores into two groups: Low Expression (scores of 0 or 1) and High Expression (scores of 2 or 3).

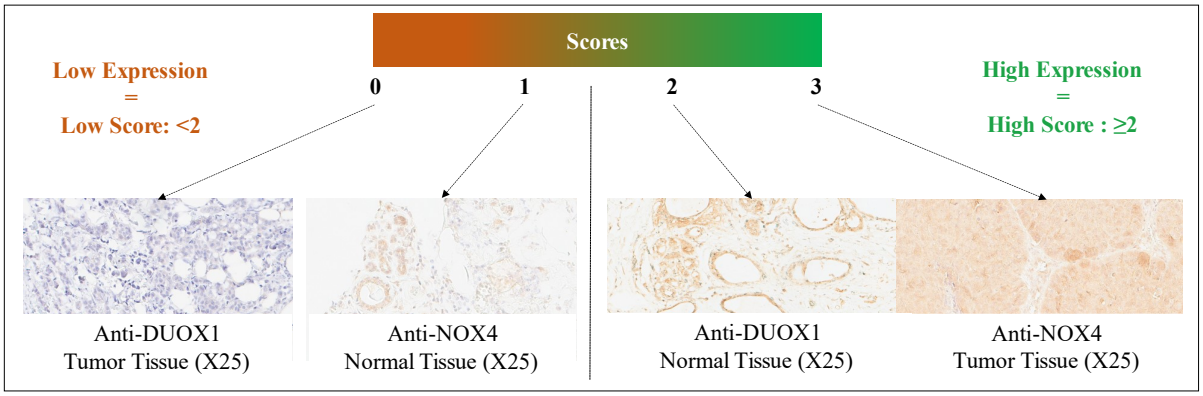


Figure 16. Immunohistochemical Staining Intensity Scores for DUOX1 and NOX4 in Breast Tissue.

Pathologists noted diverse patterns of NOX4 subcellular localization, which included a perinuclear and cytoplasmic combination, as well as a combination of nuclear and cytoplasmic localizations and exclusively cytoplasmic localization (Figure 17).

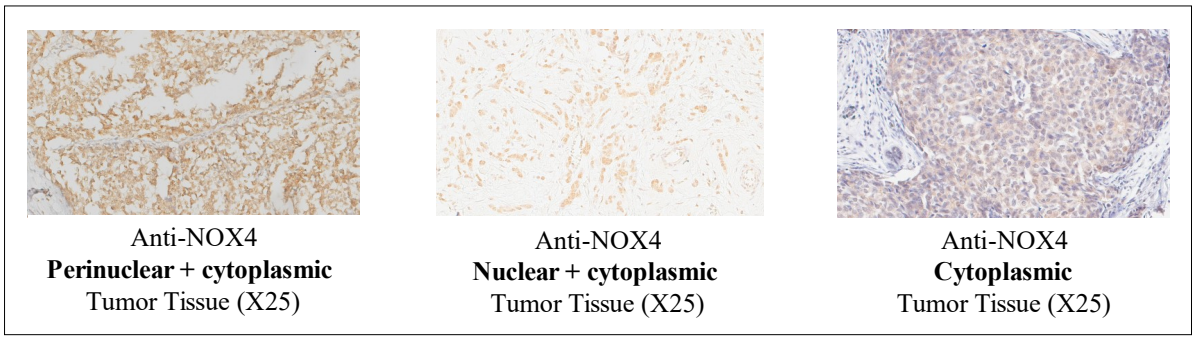


Figure 17. Immunohistochemical Localization Patterns of NOX4 in Tumor Tissue

IV. Cellular Approach Utilizing Breast Cancer Cell lines

The cellular phase of our research started by evaluating the impact of Moroccan medicinal and aromatic plants on MCF7 cells (Figure 18):

1- Initial Screening with High Dose: MCF7 cells were seeded at a density of 20 000 cells per well and treated 24 hours later with a high concentration (1 mg/ml) of six different plant extracts (P1 through P6). A control group was also included. The cell viability after treatment with these extracts was then measured using the WST-1 assay.

2- Determining IC50 Values of Efficient Plant Extracts: For extracts that demonstrated efficacy in the initial screening, a dose-response analysis was conducted to determine their IC50 values (the concentration of extract required to inhibit cell growth by 50%). This involved treating the cells with a gradient of concentrations of the effective plant extracts and then conducting the WST-1 test to assess cell viability.

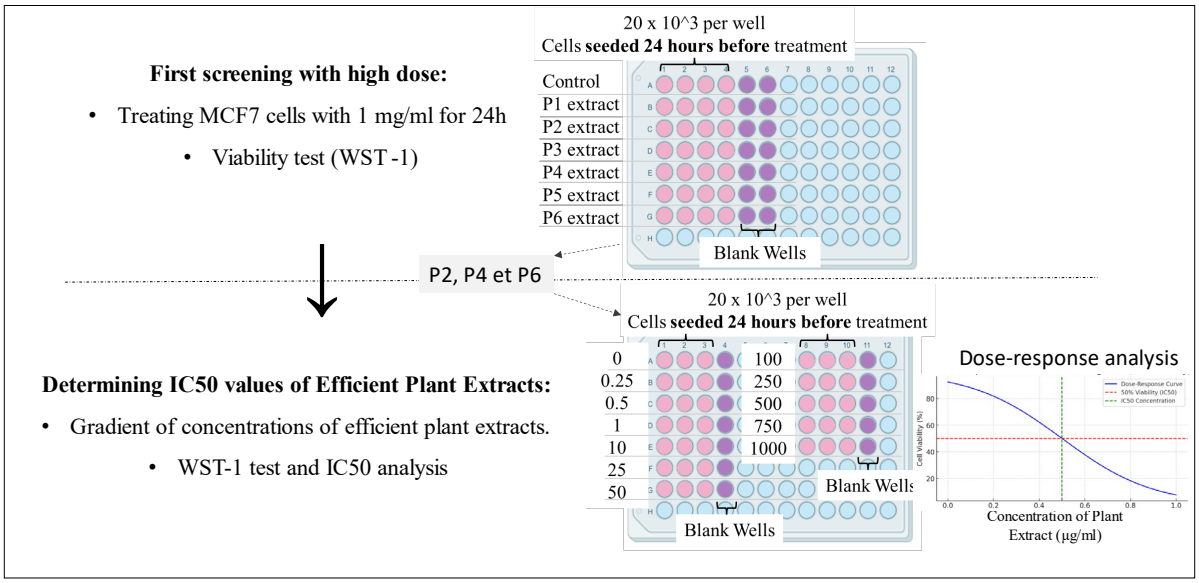


Figure 18. Experimental Process for Assessing the Cytotoxicity of Plant Extracts on MCF7 Cells: From Initial High-Dose Screening to IC50 Value Determination.

To investigate BRCA1 promoter hypermethylation and gene expression in MCF7 cells, a four-day experimental timeline was established (Figure 19). Cells were seeded at a density of 300 000 cells per well, with two wells per condition across eight plates. At specified time points, specifically on Days 1 through 4, two plates were harvested each day. From the first plate, DNA was extracted for Methylation-Specific PCR (MSP) analysis, while the second plate was utilized for RNA extraction using TRIzol reagent. The integrity of the extracted RNA was then assessed by gel electrophoresis of 1000 ng of the RNA samples.

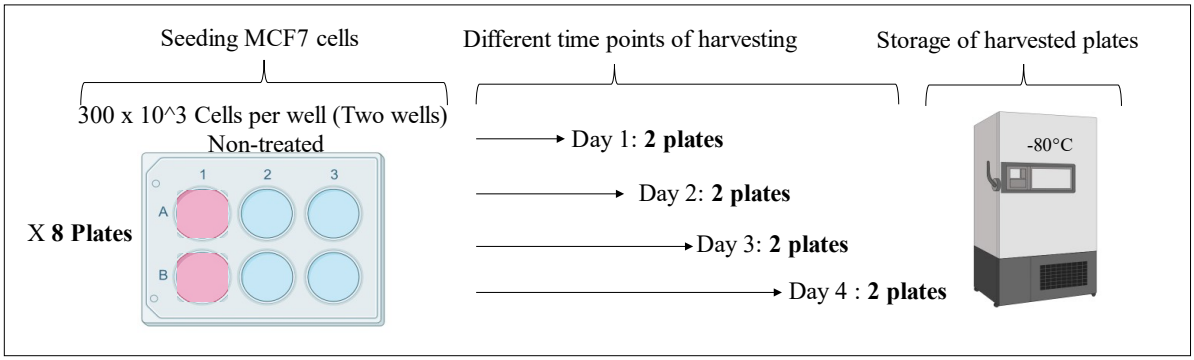


Figure 19. Sequential Harvesting Protocol for MCF7 Cells Over Four Days for BRCA1 Promoter Methylation and RNA extraction

Results

Within the 'Results' section, we detail our research findings through three pivotal articles that encapsulate the core outcomes of our study.

1. **Oubaddou Yassire***, Ben Ali Fatima*, Oubaqui Fatima Ezzahrae*, Qmichou Zineb, Bakri Youssef, and Ameziane El Hassani Rabii. The Tumor Suppressor *BRCA1/2*, Cancer Susceptibility and Genome Instability in Gynecological and Mammary Cancers. Asian Pacific Journal of Cancer Prevention, 24, 9, September 2023, 3139-3153. https://doi.org/10.31557/apjcp.2023.24.9.3139. *: equal contribution.

2. **Oubaddou Yassire**, Oukabli Mohamed, Fenniche Salma, Elktaibi Abderrahim, Elochi Mohamed Reda, Al Bouzidi Abderrahmane, Qmichou Zineb, Dakka Nadia, Diorio Caroline, Richter Antje, Bakri Youssef, and Ameziane El Hassani Rabii. *BRCA1* Promoter Hypermethylation in Malignant Breast Tumors and in the Histologically Normal Adjacent Tissues to the Tumors: Exploring Its Potential as a Biomarker and Its Clinical Significance in a Translational Approach. Genes, 14, 9, August 2023, 1680. https://doi.org/10.3390/genes14091680.

3. **Oubaddou Yassire** et al., 2023. Comparative Study of NOX4 and DUOX1 Protein Expression and Subcellular Localization in Breast Tumors and their Normal Adjacent Tissues: Uncovering NOX4's Role as a Biomarker for Aggressiveness and Progression of Breast Cancer (**in preparation**).

I. The tumor suppressor *BRCA1/2*, cancer susceptibility and genome instability in gynecological and mammary cancers

***Introduction**:* Germline mutations in the *BRCA1* and *BRCA2* genes are widely recognized for their role in predisposing individuals to breast and ovarian cancers. Recent research also suggests that mutations in these genes may increase the risk of cervical cancer. Numerous clinical, preclinical, and *in vitro* studies have explored the early events potentially leading to increased genomic instability in *BRCA1/2* mutation carriers and potentially leading to gynecomammary cancers. Our review highlights the most relevant findings of these studies.

A significant fraction of human breast and ovarian cancers associated with *BRCA1* and *BRCA2* mutations exhibit loss of heterozygosity (LOH) for these genes. This phenomenon can be linked to the "two hits" theory of tumorigenesis, suggesting that the loss of the two alleles of a tumor suppressor gene could lead to transformation. However, the tumor-suppressive functions of these genes suggest that their full inactivation could result in cell death. Other frequently observed alterations in other genes that favor cellular survival, such as those encoding *P53*, *Rb*, and *PTEN* proteins, may precede the LOH, thereby providing resistance to cell death. Intriguingly, *BRCA1* mutation-associated tumors frequently exhibit alterations in these genes, with losses in *P53* and *PTEN* reported to often precede LOH of the *BRCA1* gene.

Emerging data from human cell line studies highlighted the impact of *BRCA1* and *BRCA2* monoallelic mutations in inducing genomic instability in normal human mammary epithelial cells (HMECs). Termed as 'haploinsufficiency', this phenomenon suggests that the loss of just one allele can disrupt its intracellular protein pool, rendering it insufficient to perform all its functions. Indeed, in HMECs, *BRCA1* haploinsufficiency (*BRCA1* heterozygous) has been linked to DNA damage repair deficit, replication stress, stalled forks and R-loops accumulation, and impaired transcription. Similarly, a reduced intracellular concentration of the BRCA2 protein (BRCA2 heterozygous) in HMECs has been correlated with chromosomal instability and replication stress. In ovarian cancer, *BRCA1* haploinsufficiency has been potentially linked to impaired cell cycle control and oxidative stress damage. Finally, HPV is implicated in about 90% of cervical cancer cases. The early oncoproteins E6 and E7 from HPV have been demonstrated to directly interact and inactivate BRCA1 protein.

REVIEW

Editorial Process: Submission:04/04/2023 Acceptance:09/10/2023

The Tumor Suppressor *BRCA1/2*, Cancer Susceptibility and Genome Instability in Gynecological and Mammary Cancers

Yassire Oubaddou[1], Fatima Ben Ali[1], Fatima Ezzahrae Oubaqui[1,2], Zineb Qmichou[2], Youssef Bakri[1], Rabii Ameziane El Hassani[1]*

Abstract

BRCA1 and *BRCA2* germline alterations highly predispose women to breast and ovarian cancers. They are mostly found within the TNBC (Triple-Negative Breast Cancer) and the HGSOC (High-Grade Serous Ovarian Carcinoma) subsets, known by an aggressive phenotype, the lack of therapeutic targets and poor prognosis. Importantly, there is an increased risk for cervical cancer in *BRCA1* and *BRCA2* mutation carriers that raises questions about the link between the HPV-driven genome instability and *BRCA1* and *BRCA2* germline mutations. Clinical, preclinical, and in vitro studies explained the increased risk for breast and ovarian cancers by genome instability resulting from the lack or loss of many functions related to *BRCA1* or *BRCA2* proteins such as DNA damage repair, stalled forks and R-loops resolution, transcription regulation, cell cycle control, and oxidative stress. In this review, we decipher the relationship between *BRCA1/2* alterations and genomic instability leading to gynecomammary cancers through results from patients, mice, and cell lines. Understanding the early events of *BRCA1/2*-driven genomic instability in gynecomammary cancers would help to find new biomarkers for early diagnosis, improve the sensitivity of emerging therapies such as PARP inhibitors, and reveal new potential therapeutic targets.

Keywords: *BRCA1- BRCA2-* gynecomammary cancers- breast cancer- ovarian cancer- cervical cancer- genome instability

Asian Pac J Cancer Prev, **24 (9)**, 3139-3153

Introduction

Breast, cervical, and ovarian cancers are among the most threatening diseases for women's health around the globe (Ferlay et al., 2021). The combined incidences of those gynecomammary cancers in 2020 reached 3.18 million new cases, constituting thus more than the third (34.45 %) of all diagnosed cancer cases in females in the same year (Sung et al., 2021). Indeed, even considering both sexes, breast cancer had the highest incidence rate, with 2.26 million new cases, followed by lung cancer, with 2.21 million new cases in 2020 (Sung et al., 2021). Cervical cancer came fourth (601 127 new cases), and ovarian cancer came sixth (313 959 new cases) in incidence among women in 2020. Moreover, breast, cervical, and ovarian cancers registered 1.24 million of 4.43 million deaths by cancer in women in 2020 (Sung et al., 2021). Breast cancer was the first leading cause of death by cancer in 110 countries from 185, while cervical cancer ranked as the first deathful cancer in 36 countries, mostly in sub-Saharan Africa, South-Eastern Asia, Melanesia, and South America (Sung et al., 2021). Cervical cancer occurs mainly in low-income and middle-income countries (Islam et al., 2018; Ferlay et al., 2021), while in high-income countries, it is less frequent due to improved screening and vaccination programs (Cohen et al., 2019).

Breast, ovarian, and cervical cancers constitute a distinct group of cancers because of their shared risk factors, such as feminine sex, genetics (Ring et al., 2017; Yoshida, 2021), and genome instability markers (Miyai et al., 2004; Maxwell et al., 2017). Hereditary breast and ovarian cancers constitute 10 to 15% of all breast and ovarian cancer cases (Pal et., 2005; Yoshida, 2021) and most of them are related to *BRCA1* and *BRCA2* mutations (Yoshida, 2021; Quesada et al., 2022). In sporadic breast and ovarian cancer cases, somatic mutations of *BRCA1/2* genes are less common (6.6%) (Kwong et al., 2020), *BRCA1* DNA methylation was reported in sporadic breast and ovarian cancers, respectively in 32.6% and 15% of cases (Ruscito et al., 2014; Cai et al., 2016), while there is no evidence of *BRCA2* DNA methylation in these

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cancers (Collins et al., 1997). The contribution of *BRCA1* and *BRCA2* as cancer susceptibility genes in cervical cancer remains controversial (Johannsson et al., 1999; Thompson and Easton, 2002). HPV infection (95% of cases) is the primary factor of cervical cancer development (Waggoner and Chernicky, 2004). Of note, women with HPV-associated cervical cancer are at risk of developing HPV-associated breast cancer (Lawson et al., 2016).

Since their first discovery, *BRCA1* (Chr17q) and *BRCA2* (Chr13q) genes have constituted the major known contributors to human cancer genetic susceptibility (Miki et al., 1994; Wooster et al., 1995), especially in breast and ovarian cancers (Perez-Losada et al., 2011). Germline mutations in the *BRCA1* and *BRCA2* genes have also been implicated in increasing the susceptibility to prostate cancer among men (Umarane et al., 2023). Their function in DNA damage repair by homologous recombination (HR) is widely established (Roy et al., 2012; Isono et al., 2017). Indeed, *BRCA1* multifunctional domains interact with several actors such as 53BP1, BARD1, MRN complex, PALB2, and RAD51 to facilitate the choice, initiation, and execution of HR repair machinery to resolve double-strand breaks (DSBs), which are the most dangerous DNA damages of genome integrity (Roy et al., 2012; Isono et al., 2017). Besides, *BRCA2* is indispensable for recruiting the recombinase RAD51 to DSBs foci, which constitutes the ultimate step of the HR pathway (Roy et al., 2012). Nevertheless, they play other essential functions in maintaining genome integrity, such as chromatin remodeling, R-loops resolution, and stalled forks repair (Pathania et al., 2014; Tan et al., 2017; Zhang et al., 2017; Zhang et al., 2019). Moreover, *BRCA1/2* genes play other functions in cell cycle control, transcription regulation, and oxidative stress (Rodriguez et al., 2007; Norquist et al., 2010; Chiang et al., 2019; Renaudin et al., 2021).

About 4000 and 4675 pathogenic and likely pathogenic mutations were reported in *BRCA1* and *BRCA2* genes, respectively (Clinvar-*BRCA1* gene, 2023). Deletions are the primary genetic alterations affecting both genes, followed by insertions, point mutations, duplication, and indels (Clinvar-*BRCA1* gene, 2023). The origins of genetic alterations in the *BRCA1/2* genes are widely unknown. However, the large genomic rearrangements (LGRs) of the BRCA1 gene represent 10% of all the predisposing pathogenic variants, and they are caused mainly by the high density of Alu elements (41, 5%) in the BRCA1 gene (Smith et al., 1996). Of note, Alu elements are transposable elements causing several genomic alterations and are believed to be the source of human genetic diversity (Ade et al., 2013). Also, a *BRCA1* pseudogene near 5′ of the *BRCA1* gene constitutes a recombination hot spot that can cause several LGRs (Puget et al., 2002). The frequency of a pathogenic mutation depends on the ethnic origins of each population; for example, 185delAG is a founder mutation in the Ashkenazi Jewish population, causing both breast and ovarian cancers (Struewing et al., 1995). The current review will discuss the available literature on *BRCA1/2*-driven genomic instability and cancer susceptibility in breast, ovarian, and cervical cancers.

The Tumor Suppressor BRCA1/2, Cancer Susceptibility, and Genome Instability in Breast Cancer

Background

Tumor suppressors *BRCA1* and *BRCA2* are highly penetrant genes predisposing to breast cancer (Venkitaraman, 2019). The cumulative risk of breast cancer to the age of 80 was estimated to be 72% for *BRCA1* mutation carriers and 69% for *BRCA2* mutation carriers (Kuchenbaecker et al., 2017). Moreover, contralateral breast cancer is widespread among *BRCA1* (40%) and *BRCA2* (26%) carriers after 20 years of the primary diagnosis of breast cancer (Kuchenbaecker et al., 2017). Hence, heterozygous *BRCA1* and *BRCA2* germline mutations render mammary epithelial tissue an active "field" for tumorigenic development (Venkitaraman, 2014). Human hereditary biallelic mutations of *BRCA1* and *BRCA2* are not viable, except in some rare cases when biallelic *BRCA1* or *BRCA2* carriers live with Fanconi anemia disorder manifestations associated with recurrent early onset breast and ovarian cancers (Sawyer et al., 2015; Fang et al., 2020).

BRCA1/2-associated breast tumors are frequent (~70%) in triple-negative basal-like molecular subtype (Felicio et al., 2017), known for their poor prognosis due to the lack of therapeutic targets (Ge et al., 2022). Interestingly, *BRCA1/2*-associated breast tumors show higher sensitivity toward PARP inhibitors and platinum-derived therapy (Lord and Ashworth, 2016). Understanding the early genome instability of *BRCA1/2*-associated breast tumorigenesis would improve the progress and efficiency of these therapies and reveal other potential early actors (Fu et al., 2022). In this regard, clinical, preclinical, and in vitro studies provide enormous outcomes on the link between *BRCA1/2*-associated genome instability and breast tumorigenesis (Figure 1) (Xiao et al., 2014; Vohhodina et al., 2021; Wu et al., 2022). Indeed, emerging results contradict the old paradigm of the "two-hit" theory inducing *BRCA1/2*-associated breast tumors (Sedic et al., 2015; Vohhodina et al., 2021; Sun et al., 2022), and *BRCA1* or *BRCA2* haploinsufficiency remains the plausible alternative process of tumorigenesis through the induction of an early genome instability in human mammary epithelial cells (Figure 1A) (Bhatia et al., 2014; Pathania et al., 2014; Sedic et al., 2015; Vohhodina et al., 2021; Sun et al., 2022).

Results from patients

In malignant breast tumors harboring *BRCA1* or *BRCA2* mutation, the remaining wild-type allele was found inactivated by loss of heterozygosity (LOH), respectively, in 90% and 54% of *BRCA1* and *BRCA2* -associated breast tumors (Maxwell et al., 2017). This widely observed biallelic inactivation may represent an analogy to the "two-hit" theory of accelerated tumorigenesis in familial breast tumors (Konishi et al., 2011). However, due to *BRCA1* and *BRCA2* vital tumor suppressive functions, their complete inactivation in mammary epithelial cells can cause DNA damage, leading to cell cycle arrest and apoptosis (Figure 1A) (Konishi et al., 2011; Venkitaraman, 2014; Sedic et al., 2015). For this reason, the loss of heterozygosity must be preceded by

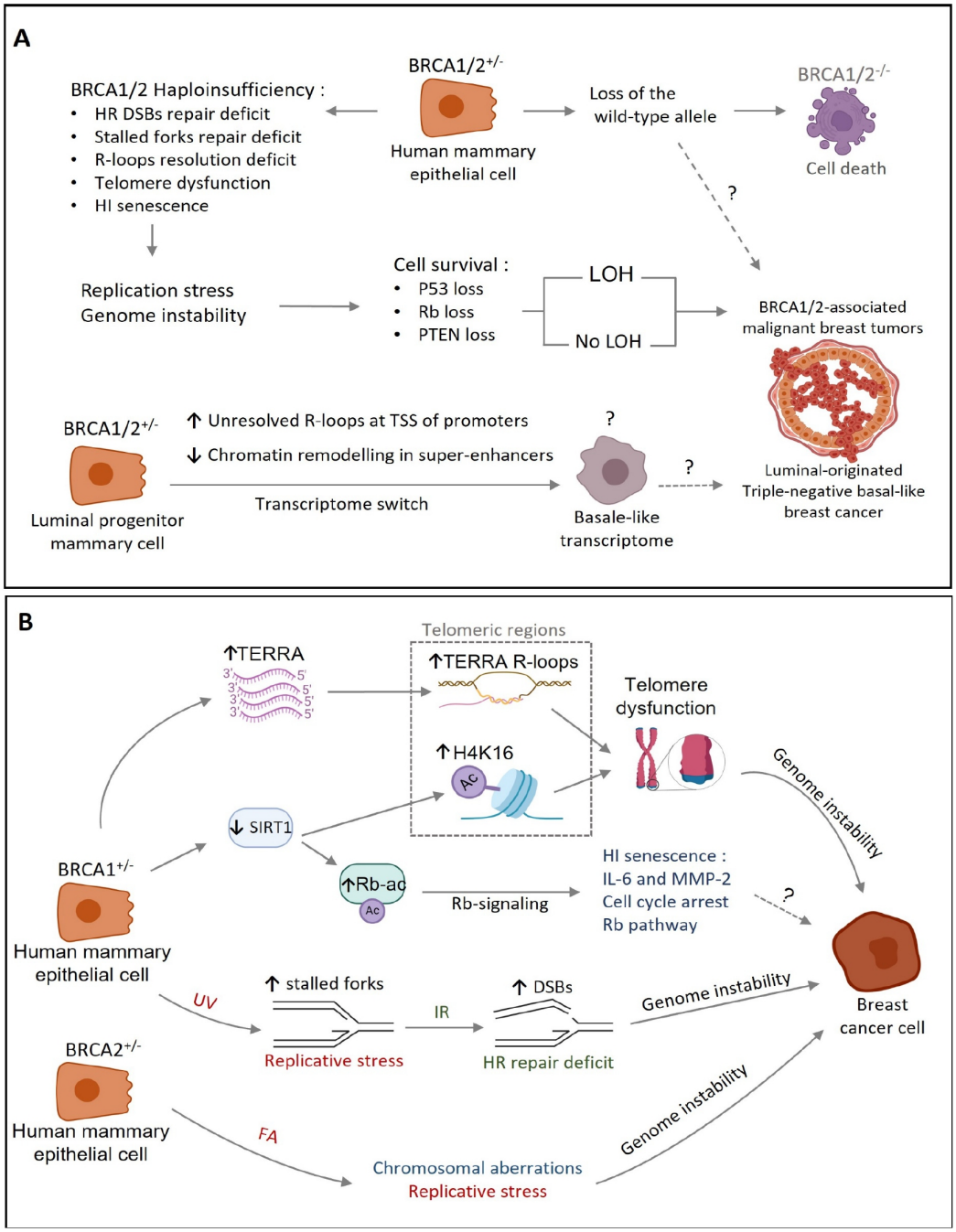

Figure 1. The Tumor Suppressor *BRCA1/2,* Cancer Susceptibility, and Genome Instability in Breast Cancer. **A** *BRCA1/2* heterozygous (*BRCA1/2*+/-) Human mammary epithelial cells (HMECs) present a *BRCA1/2* haploinsufficiency that leads to several molecular defects, including HR (homologous recombination) DSBs (double-strand breaks) repair deficit, the accumulation of unresolved stalled forks and R-loops, telomere dysfunction, and haploinsufficiency-induced senescence (HI senescence). The resulting genome instability may alter several genes involved in cell survival, such as P53, Rb protein, and PTEN. *BRCA1/2* loss of heterozygosity (LOH) may not be an early event of *BRCA1/2*-associated genome instability but rather a consequence of this later. BRCA1/2-associated breast tumors plausibly originated from *BRCA1/2* heterozygous (*BRCA1/2*+/-) luminal progenitor cells due to the role of *BRCA1/2* genes in resolving R-loops at promoters or super-enhancers of key genes of luminal differentiation, but also to the role of *BRCA1* in chromatin remodeling. **B** *BRCA1* haploinsufficiency (*BRCA1*+/- HMECs) causes TERRA-loops accumulation in telomeric and subtelomeric regions, leading to telomeric abnormalities and genome instability. *BRCA1*+/- HMECs in cell culture develop a telomeric dysfunction at early passages, then at late passages, they develop a type of senescence mediated by Rb signaling, qualified by haploinsufficiency-induced senescence (HI senescence). These consequences are correlated to a decreased expression of SIRT1 and increased acetylation of Rb protein and telomeric histones. The exposure of *BRCA1*+/- HMECs to UV (Ultras-violet) radiation induces replicative stress due to the accumulation of unresolved stalled forks, which collapse after exposure to IR (ionizing radiation), leading to DSBs accumulation and genome instability. BRCA2+/- HMECs exposed to formaldehyde (FA) show increased replication stress due to accumulated unscheduled R-loops, which may cause genome instability.

somatic alterations sustaining cell viability, such as those of the P53 or Rb genes (Sedic et al., 2015). Indeed, P53 and Rb losses occur frequently (relatively independently) in *BRCA1*-associated breast tumors (Patel et al., 2020); and P53 and PTEN losses (mutations) were estimated to precede *BRCA1* loss of heterozygosity in more than 80% of studied cases (Figure 1A) (Martins et al., 2012).

BRCA1 heterozygous (*BRCA1*+/-) mammary epithelial cells express limited amounts of functional *BRCA1* protein (Pathania et al., 2014) that does not suffice (haplotype insufficiency) to correctly perform several vital *BRCA1*-related functions, including homologous-recombination (HR) double-strand breaks (DSB) repair, stalled fork resolution, R-loop resolution, and chromatin remodeling (Figure1B) (Konishi et al., 2011; Pathania et al., 2014; Sedic et al., 2015; Zhang et al., 2017; Vohhodina et al., 2021; Sun et al., 2022). In *BRCA1* carriers, a monoallelic mutation may suffice to trigger genome instability in normal mammary epithelial cells, leading them to transformation (Sedic et al., 2015). Indeed, blood genomes analysis in family members carrying a founder *BRCA1* mutation revealed 23 deleterious mutations affecting genes implicated in tumorigenesis (e.g., BPTF and FOXP1), which are not present in non-carrier members (Xiao et al., 2014). Besides, a comparison between mother and daughter carriers revealed that daughter carriers develop de novo 9 deleterious mutations in addition to 14 deleterious mutations inherited from their mother carriers. Noteworthy, two of those deleterious mutations were found within repetitive sequences, and five were found within fragile sites, known as the most vulnerable sequences of the genome to DNA damage (Xiao et al., 2014). Another study by Litton and colleagues (2012) showed that *BRCA1/2* daughter carriers develop breast cancer earlier than their mother carriers, at 42 and 48 medians of age, respectively (Litton et al., 2012). This latter result may be explained by the accumulation of deleterious mutations in the genomes of daughter carriers (second generation), which carry more deleterious mutations (*BRCA1/2*-induced genome instability) than their mother carriers from the first generation (Xiao et al., 2014). In this model, *BRCA1/2* monoallelic mutation may promote genome instability all along the life of a *BRCA1/2* female carrier by generating potential deleterious mutations and genome instability, which increases the risk of developing malignant breast tumors.

Results from animal models

Mice heterozygous for the *BRCA1* gene do not develop spontaneous breast tumors as predisposed humans do, hypothetically due to some differences between species, such as the short lifespan in mice (Drost and Jonkers, 2009). However, biallelic inactivation of *BRCA1* or *BRCA2* genes in mice causes early embryonic lethality (Ludwig et al., 1997). Still, an additional mutation of the P53 locus can rescue embryos with *BRCA1* biallelic inactivation until adulthood (Xu et al., 2001). *BRCA1* conditional knockout in female mice (conditional in mammary tissue) carrying a P53 heterozygous mutation induced mammary tumors with the loss of the remaining wild-type P53 allele due to genome instability and the presence of similar genomic and histopathologic features to the human basal-like malignant breast tumors carrying both *BRCA1* and P53 mutations (Liu et al., 2007). However, in *BRCA1* heterozygous mice, P53 alteration did not significantly influence the levels of genome instability generated in normal breast tissues (Wu et al., 2022). Consequently, the role of P53 alterations in mammary transformation may be restricted to sustaining cell viability to overcome apoptosis after genome instability induction, at least in the mice model (Figure 1A) (Wu et al., 2022).

Reporter mice showed that homologous recombination (HR) is the primary double strand breaks repair mechanism in proliferating mammary tissue (pubertal and pregnant) compared to other tissue types (Kass et al., 2016). This reliance on HR in repairing DNA damage in proliferating mammary tissue may explain the higher risk of breast cancer conferred by *BRCA2* (or *BRCA1*) mutations in predisposed women (Kuchenbaecker et al., 2017). In humans, both *BRCA1* and *BRCA2* contribute to the resolution of R-loops, which, if accumulated, cause replicative stress and genome instability (Tan et al., 2017; Zhang et al., 2017). In mice, mammary tissue carrying *BRCA1* conditional knockout showed increased levels of R-loops in luminal cells and increased frequency of spontaneous tumors (Zhang et al., 2017). In addition, pregnant *BRCA2* homozygous mice showed reduced HR repair use in luminal and basal cells but decreased more in the luminal population (Kass et al., 2016). This specificity toward the luminal lineage of mammary tissue goes with several results generated at the human level (cell lines) in considering luminal progenitor cells as the origin of *BRCA1/2*-associated breast tumors (Figure 1A) (Sedic et al., 2015; Zhang et al., 2017).

Given that *BRCA1/2* heterozygous mutations do not cause spontaneous breast tumors in mice, the use of mice with this genetic background is valuable in studying the role of *BRCA1/2* heterozygosity in genome instability induction (Drost and Jonkers, 2009; Wu et al., 2022). Indeed, mice carrying a heterozygous *BRCA1* mutation developed early genomic instability by manifesting several genomic alterations (structural variations, copy number variations, and indels) in normal tissues from an early embryonic age (Wu et al., 2022). The breakpoints of structural variations (SVs) were enriched in repetitive sequences (54% of SVs) and multiple fragile sites of *BRCA1* heterozygous mice genomes (Wu et al., 2022). The analysis of those breakpoints revealed the use of error-prone non-homologous pathways to repair DSBs, including non-homologous end joining (492 repaired DSBs), microhomology-mediated end joining (75 repaired DSBs), and single-strand annealing with 2 repaired DSBs (Wu et al., 2022).

Results from cell lines

BRCA1 and *BRCA2* heterozygous human mammary epithelial cells express lower levels of *BRCA1* and *BRCA2* proteins, respectively (Pathania et al., 2014, Tan et al., 2017). A decreased pool of *BRCA1* or *BRCA2* proteins causes haploinsufficiency toward several *BRCA1/2* related functions, leading to genome instability in normal mammary epithelial cells of BRCA1/2 carriers (Figure

1A) (Sedic and Kuperwasser, 2016).

Exposure of immortalized (hTERT) BRCA1+/- HMECs (Human mammary epithelial cells) to UV-radiation induced the accumulation of unresolved stalled forks with no impact on homologous recombination double-strand breaks (HR DSBs) repair and other *BRCA1*-related functions (Pathania et al., 2014). When exposed to ionizing radiation, those resulting cells (pre-exposed to UV) showed decreased ability to recruit RAD51 to DSBs foci, which indicated an HR DSBs repair deficit (Figure 1B) (Pathania et al., 2014). The pool of *BRCA1* in heterozygous UV-pretreated HMECs was not sufficient to repair IR exposure consequences (DSBs), leading to DSBs accumulation and genome instability (Figure1B) (Pathania et al., 2014). These results show the limits of a decreased *BRCA1* protein intracellular pool within BRCA+/- HMECs in performing all the required functions, especially under stressful conditions (Pathania et al., 2014). Besides, the exposure of immortalized (HPV16 E6 and E7 oncogenes) *BRCA2*+/- HMECs to formaldehyde caused a decrease in the intracellular *BRCA2* protein pool, chromosomal abnormalities, and replication stress, as compared to their wild-type cells (Figure 1B) (Tan et al., 2017).

In immortalized (hTERT) *BRCA1*+/- HMECs, *BRCA1* haploinsufficiency causes a specific type of senescence, qualified by Haploinsufficiency-induced-senescence (HIS), which is mediated by Rb pathway activation (Sedic et al., 2015). Indeed, at early passages in cell culture, *BRCA1*+/- HMECs showed DNA damage response activation and multiple chromosomal abnormalities, including telomeric abnormalities. More importantly, luminal epithelial cells from breast lobules of *BRCA1* mutation carriers showed shorter telomeres than those from non-carriers. Mechanistically, this study showed that the SIRT1 intracellular pool decreased in *BRCA1*+/- HMECs, which triggers both haploinsufficiency-induced senescence and telomer dysfunction through activating acetylation of Rb protein and acetylation of telomeric histones leading them to destabilization (Figure 1B) (Sedic et al., 2015). Additionally, *BRCA1* participates in telomeric stability by regulating the long non-coding RNA TERRA (Telomeric Repeat RNA) expression and resolving TERRA-associated R-loops in telomeric and sub-telomeric regions. The accumulation of TERRA RNA and the unresolved TERRA-associated R-loops in *BRCA1*+/- HMECs (CRISPR-modified) causes telomeric abnormalities leading to genome instability (Figure 1B) (Vohhodina et al., 2021).

Most *BRCA1*-mutated malignant breast tumors are from the triple-negative basal-like subtype, known by basal mammary cell biomarkers and the negativity of estrogen and progesterone receptors (Popova et al., 2012). Besides, luminal progenitor cells are believed to be the origin of *BRCA1*-associated breast tumors (Sedic et al., 2015; Zhang et al., 2017). A transition from the luminal phenotype to the basal-like transcriptome may be the key to explaining the specific risk of breast cancer conferred by *BRCA1* haploinsufficiency (Zhang et al., 2019). Indeed, luminal mammary cells harbor increased transcriptional activity and active enhancers compared to basal mammary cells and consequently develop increased levels of DNA-RNA transcriptional secondary structures, R-loops (Gascard et al., 2015; Zhang et al., 2017; Zhang et al., 2019). R-loops promote the instability of common fragile sites due to collisions between transcription and replication (Helmrich et al., 2011). R-loops levels increased in *BRCA1*+/- luminal mature and progenitor cells compared to *BRCA1*+/- basal and stromal cells, indicating a luminal-specific *BRCA1* haploinsufficiency toward R-loops resolution (Zhang et al., 2017).

Immortalized (hTERT) *BRCA1*+/- HMECs and *BRCA1*+/- MCF10A (Immortalized non-tumorigenic HMEC line) cells show a reduced number of active super-enhancers (super-enhancer = cluster of enhancers) compared to their wild-type, *BRCA1*+/+ (Zhang et al., 2019). In both cell types (*BRCA1*+/- HMECs and *BRCA1*+/- MCF10A), the attenuation of some super-enhancers was observed and correlated with decreased expression of their downstream target genes and a decreased co-occupancy of histone H3K27 acetylation (mark of active enhancers) and bromodomain-containing protein 4 (BRD4) within these super-enhancers (Figure 1A) (Zhang et al., 2019). Consequently, *BRCA1* haploinsufficiency impacts long-distance chromatin interactions between targeted genes and their transcription enhancers (Zhang et al., 2019). Besides, depletion of *BRCA1* mRNA (siRNA) in MCF7 cells (Luminal mammary cells expressing estrogen receptors) led to decreased ESR1 (Oestrogen receptor locus) mRNA pool and increased R-loops accumulation within a super-enhancer upstream ESR1 locus (Chiang et al., 2019). Ectopic expression of RNaseH1 (RNA endonuclease, R-loops resolver) in MCF7 *BRCA1* depleted cells rescued the expression of ESR1 mRNA and reduced R-loops intensity within the upstream super-enhancer, indicating that *BRCA1* mediates the expression of this luminal biomarker through R-loops resolution within the upstream super-enhancer of ESR1 locus (Chiang et al., 2019). Therefore, *BRCA1* may mediate luminal differentiation (e.g., ESR1 locus induction), whereas *BRCA1* haploinsufficiency induces a basal-like transcriptome, consolidating the luminal origin of basal-like *BRCA1*-mutated breast tumors in *BRCA1* carriers.

The Tumor Suppressor BRCA1/2, Cancer Susceptibility, and Genome Instability in Ovarian Cancer

Ovarian cancer is a heterogeneous group of malignant neoplasms that differ in histological type, molecular features, and clinical behavior (deFazio et al., 2021). Histologically, 90% of ovarian cancer cases are epithelial, including serous, clear cell, endometrioid, and mucinous carcinomas (Weiss et al.,1977). High-grade serous ovarian carcinoma (HGSOC) is the most frequent (70% of cases) and the most aggressive ovarian cancer subtype (Bergstrom et al., 2017). The lack of specific biomarkers for the HGSOC subtype contributes to its poor survival rate (Bergstrom et al., 2017).

Women with *BRCA1/2* mutation carriers are 10 times more likely to develop ovarian cancer than non-carriers, which led to the use of prophylactic salpingo-oophorectomy (Surgical removal of the fallopian tubes and ovaries) to reduce the risk of ovarian cancer (Boyd et al., 2000).

Moreover, approximately 10-15% of ovarian cancer cases are attributable to *BRCA1/2* germline mutations (Pal et al., 2005; Cancer Genome Atlas Research N, 2011; Prat et al., 2005). This incidence can reach up to 90% of cases in hereditary ovarian cancers (Salehi et al., 2008). Features of *BRCA1/2*-associated ovarian carcinomas include high-grade phenotype, frequent TP53 mutations, copy number landscape features such as Cyclin-E amplification, and tumor suppressor Rb deletion (Quesada et al., 2022). P53 loss and loss of heterozygosity (LOH) play a controversial role in *BRCA1/2*-associated ovarian tumorigenesis (George and Shaw, 2014). There are mounting pieces of evidence for *BRCA1* haploinsufficiency toward several BRCA1-related functions in normal mammary tissue, which has been linked to genome instability induction, and this should also be elucidated in normal ovarian tissue (Sedic and Kuperwasser, 2016; George and Shaw, 2014). Notably, *BRCA1/2* mutations are the most prominent cause of homologous recombination deficiency (high genomic instability) in HGSOC, which improves response to platinum-based chemotherapy and PARP inhibitors (Mirza et al., 2016).

Results from patients

BRCA1/2-associated serous ovarian carcinomas showed a higher genomic instability than sporadic tumors. Besides, those *BRCA1/2*-associated tumors exhibited genome-wide loss of heterozygosity, which was associated with uniparental disomy (UPD). The UPD was detected in all analyzed *BRCA1/2*-associated ovarian tumors and only in 50% of sporadic tumors (Walsh et al., 2008). Besides, loss of heterozygosity (LOH) of the wild-type allele of *BRCA1* or *BRCA2* genes was widely observed among *BRCA1/2*-associated ovarian tumors (Maxwell et al., 2017). Notably, a study showed that the *BRCA1* LOH gene occurred within neoplastic lesions but was absent in precursor lesions of tubular epithelium, which was thought to be the origin of HGOSC. HGSOC are commonly characterized by P53 alterations, detected in 96% of cases (Cancer Genome Atlas Research N, 2011; Cole et al., 2016). Therefore, *BRCA1* LOH may be a consequence of genome instability driven by other alterations (Figure 2) (Paley et al., 2001; Salvador et al., 2008; Norquist et al., 2010). In this regard, P27 protein expression (Cell cycle inhibitor) was found to be significantly lower within P53 foci in the tubal epithelium of women harboring *BRCA1/2* mutation compared to non-carriers (Figure 2). The loss of P27 was higher in *BRCA1* mutation carriers compared to *BRCA2* carriers (Norquist et al., 2010). This result may explain the higher lifetime risk of ovarian carcinoma in *BRCA1* mutation carriers compared to *BRCA2* mutation carriers (Kuchenbaecker et al., 2017).

Besides its functions in HR repair and cell cycle control, *BRCA1* is involved in repairing oxidative DNA damage (Rodriguez et al., 2007). Indeed, the level of 8-oxoguanine (oxidative DNA damage) was significantly higher in DNA isolated from blood cells of *BRCA1* mutation carriers compared to non-carriers. This observed increase may result from a *BRCA1* haploinsufficiency in repairing oxidative DNA damages (Figure 2) (Dziaman et al., 2009). *BRCA1* mRNA levels showed lower levels in leukocytes of *BRCA1* mutation carriers compared to non-carriers (Chehade et al., 2016). Based on previous studies on *BRCA1* heterozygous human mammary epithelial cells, monoallelic alterations of the *BRCA1* gene may cause a haplotype insufficiency (haploinsufficiency) toward several *BRCA1* functions in normal ovarian cells of *BRCA1* carriers; thus, promoting genome instability and ovarian transformation (Figure 2).

Results from animal models

Mouse models with ovary Cre recombinase-mediated conditional inactivation of both *BRCA1* and/or p53 showed that the inactivation of both genes (*BRCA1* and P53) resulted in ovarian tumor formation in 54% of mice compared to only 5% with conditional inactivation of either gene alone (Quinn et al., 2009). Similarly, the knockout of *BRCA1* alone in murine ovarian surface epithelium (OSE) revealed no tumorigenesis in mice ovaries despite the development of significantly more premalignant changes (Clark-Knowles et al., 2007). These results suggest that *BRCA1* inactivation alone is not sufficient to promote mice ovarian cancer (Quinn et al., 2009).

The mechanism by which *BRCA1*-associated genomic instability progresses from non-transformed to transformed cancer cells in ovarian cancer is not entirely elucidated. Examining *BRCA1*+/- mouse genome throughout the developmental process from embryonic life to adulthood revealed structural variations, indels, and copy number variations that appear in early embryonic life and change dynamically throughout the developmental process. Indeed, numerous oncogenic genes and pathways, such as DNA damage repair, estrogen signaling, and oncogenesis found to be affected. Controversially, TP53 mutations showed limited contribution in early genome instability and are not required for *BRCA1*-driven genome instability in non-cancer cells (Wu et al., 2022). Additionally, the deletion or substitution of the *BRCA1* locus corresponding to the RING domain in mice showed a high degree of genomic instability and a defect in replication fork stability despite the accumulation of RAD51 at DNA damage sites (Figure 2) (Li et al., 2016).

Results from cell lines

BRCA1 is implicated in estrogen biosynthesis through regulating aromatase (enzyme converting androgen to estrogen) expression in ovarian granulosa cells (Hu et al., 2005). Besides, the over-expression of aromatase in mammary adipocytes (local production of estrogen) was associated with breast cancer development (Sasano and Harada, 1998). *BRCA1* knockdown (siRNA) in ovarian granulosa and pre-adipocytes cell lines showed a significant increase in aromatase expression (Figure 2). This finding suggests that *BRCA1* deficiency in estrogen-producing cells may contribute to tumor development in estrogen-responsive epithelial cells through the activation of proliferative pathways (Hu et al., 2005). In the presence of mitomycin (DNA-damaging agent), *BRCA1*-deficient primary mouse ovarian surface epithelial (OSE) cells revealed a higher number of centrosomes and a lack of Rad51 nuclear foci (Xing and Orsulic, 2006). Besides, in

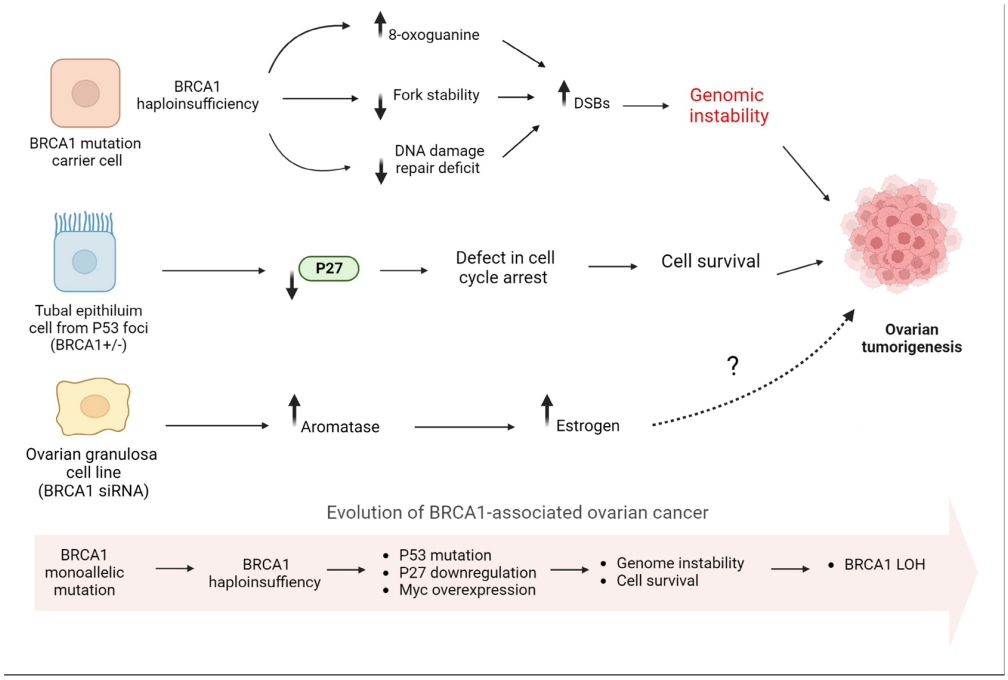

Figure 2. The Tumor Suppressor *BRCA1/2,* Cancer Susceptibility, and Genome Instability in Ovarian Cancer. *BRCA1* mutated cells isolated from *BRCA1* mutation carriers (human or mice) showed a defect in oxidative DNA damage repair (high level of 8-oxoguanine), replication fork stability, and DSBs repair, leading to DSBs accumulation and genomic instability. Tubal epithelium cells from p53 foci of heterozygous *BRCA1* mutation carriers showed a significant decrease in the expression of p27, inducing a defect in cell cycle arrest. The knockdown of the *BRCA1* gene in human ovarian granulosa cells using small interfering RNA (*BRCA1* siRNA) resulted in overexpression of aromatase, leading to increased estrogen biosynthesis. The schema at the bottom summarizes the theoretical steps of *BRCA1*-associated ovarian tumorigenesis.

those same cells, the knockout of *BRCA1* and P53 and the introduction of Myc oncogene were the minimum conditions that led to transformation (Figure 2) (Xing and Orsulic, 2006).

Bronder et al., (2021) have recently developed novel model systems of chromosomal instability (CIN) in high-grade serous ovarian cancer (HGSOC) by using CRISPR/Cas9-mediated gene editing, first to mutate TP53 and then *BRCA1* in FNE1 cells derived from non-ciliated fallopian tube epithelial cells. They demonstrated that p53 function loss was sufficient to cause subclonal karyotype alterations and global gene expression changes, affecting modules responsible for cell cycle commitment, DNA replication, G2/M checkpoint control, and mitotic spindle function (Figure 2) (Bronder et al., 2021).

The Tumor Suppressor BRCA1/2, Cancer Susceptibility, and Genome Instability in Cervical Cancer

Background

The infection with human papillomavirus (HPV) constitutes the major risk factor for cervical cancer (Bouvard et al., 2009). Indeed, HPV DNA was detected in approximately 95% of cervical malignant lesions (Waggoner and Chernicky, 2004). HPV virus was first isolated and linked to cervical cancer pathogenesis in the early 1980s (Lowndes, 2006). The genotypes of HPV that can infect the anogenital tract are classified into three groups based on their oncogenic potential: High-risk (H-R) types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82) which are associated with high-grade lesions and invasive cervical cancer (Shukla et al., 2009); Low-risk (L-R) types (HPV6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81) and probably H-R types (HPV26, 53, 66) (Bouvard et al., 2009). Persistent infection with H-R carcinogenic HPV types and the integration of the HPV genome into the host chromosome of cervical epithelial cells are key early events in the neoplastic progression of cervical malignant lesions (Williams et al., 2011).

The HPV life cycle depends on the differentiation of the host keratinocytes (Longworth and Laimins, 2004). Its genome replication occurs in three phases: establishment, maintenance, and productive replication (McBride, 2017). During the first phase, HPV infects the basal layer cells of the epithelial cervix to establish the infection (Pyeon et al., 2009). Then, the viral genome is maintained at a stable number of episomes in the host cell (Anacker and Moody, 2017). Finally, the infected basal keratinocytes divide, allowing a daughter cell to keep actively dividing in the basal line while the other starts the differentiation process, favoring the productive replication of the virus (Figure 3A) (Moody and Laimins, 2010). The replication of the viral genome is only possible due to the expression of the early oncogenes E1, E2, E6, and E7 (Nilsson et al., 2018). HPV E1 and E2 are implicated directly in the viral amplification (Bergvall et al., 2013; McBride,

2013), while E6 and E7 prevent apoptosis and allow the cell cycle entrance of the host cell (Figure 3B) (Ganguly and Parihar, 2009).

To maintain the integrity of the genome, the host cell recruits several DNA repair mechanisms that can detect genomic aberrations and repair them depending on the type of damage (Nilsson et al., 2018). The two main DDR pathways are homologous recombination (HR) and non-homologous end joining (NHEJ) (Ciccia and Elledge, 2010). Double-strand DNA breaks (DSBs) require the activation of ATM, which is recruited by the MRN complex (Mre11, Rad50, and NBS1) (Ciccia and Elledge, 2010). Activated ATM phosphorylates several downstream proteins such as Chk2, NBS1, *BRCA1*, p53, and γH2AX (Matsuoka et al., 2007; Bakkenist and Kastan, 2015). On the other hand, Single strand DNA breaks (SSBs) require the activation of ATR (Zhou and Elledge, 2000) that phosphorylates downstream effectors like Chk1, RPA, and the Fanconi Anemia (FA) pathway (Shiloh and Ziv, 2013; Hollingworth and Grand, 2015).

Many studies proved that HPV exploits the DDR pathways to replicate its genome (Hong et al., 2015; Nilsson et al., 2018; Wallace, 2020). Indeed, E1 and E2 activate the DDR pathways (Bergvall et al., 2013; McBride, 2013), while E6 and E7 interfere in the counter of downstream consequences of DDR (Vande Pol and Klingelhutz, 2013; Johnson et al., 2017). DDR pathways implicated in HPV replication include key proteins such as ATM, ATR, CHK1, CHK2, H2AX, Rad51, and *BRCA1* (Figure 3B) (Sakakibara et al., 2011; Gillespie et al., 2012; Reinson et al., 2013; Chappell et al., 2016).

Cervical cancer tumorigenesis depends mainly but not only on HPV infection (Ramachandran and Dörk, 2021). The downregulation of tumor-suppressor genes in host cells contributes to the susceptibility of this cancer (de Freitas et al., 2012). Besides, based on a Swedish report, the genetic heritability of cervical cancer accounts for 27% of its risk (Magnusson et al., 2000). Many genetic association studies evaluating the risk of cervical cancer were conducted on several candidate genes implicated in cell cycle, DNA repair, and apoptosis, such as TP53 (Hu et al., 2010), E3 ubiquitin-protein ligase MDM2 (Hu et al., 2007; Hu et al., 2010), ATM (Oliveira et al., 2012), BRIP1 (Ma et al., 2013), CDKN1A (Lima et al., 2016), CDKN2A (Thakur et al., 2012) and Fanconi Anemia genes (FANCA, FANCC, and FANCL) (Juko-Pecirep et al., 2011).

Many DDR proteins with oncogenic somatic or germline mutations were considered therapeutic targets for cervical cancer, such as ATM, *BRCA1*, PALB2, and RAD51. Therefore, the use of PARP inhibitors may be beneficial for cervical cancer patients (Qiu et al., 2022). Similarly, a case-report study showed that a patient with cervical cancer, tested (due to a family history of cancer) and found positive for a pathogenic germline *BRCA1* mutation had a positive response to the treatment with bevacizumab and PARP inhibitor 'olaparib' (Montero-Macias et al., 2021).

The DNA repair genes *BRCA1* and *BRCA2* are known to increase the risk of breast and ovarian cancers (Thompson and Easton, 2002; Mersch et al., 2015). Their implication in cervical cancer is not fully elucidated.

Results from patients

Only few studies were conducted to understand the association between *BRCA1*/2 lack or loss of function and the risk of cervical cancer. A cohort study conducted on 11847 individuals from 699 different families from Europe and North America carrying a *BRCA1* mutation showed that the risk of cervical cancer was increased by 3.72 folds (95% CI = 2.26 to 6.10, P<.001) (Thompson and Easton, 2002). On the other hand, in a Swedish cohort of 1873 carriers of *BRCA1*/*2* mutations, the risk of cervical cancer was increased in *BRCA2* mutation carriers (standardized morbidity ratio (SMR) = 4.21, 95% CI =1.15±10.79, P =0.0016) while no risk was detected in *BRCA1* mutation carriers (Johannsson et al., 1999). Similarly, another study of 1072 *BRCA1* and *BRCA2* mutation carriers in the United States showed that *BRCA2* mutations slightly increased the risk of cervical cancer (Standardized incidence ratio SIR = 4.410; 95% IC = 1.61-9.599; P = 0.006) but *BRCA1* did not show any significant association with cervical cancer (Mersch et al., 2015). The increased risk of cervical cancer by both genes *BRCA1* and *BRCA2* was observed in a research on 4405 individuals from 409 carrier families (RR=4.59, 95% CI=2.20 to 8.44, and RR=3.69, 95% CI=1.20 to 8.61; p=<0.001 respectively) (Rhiem et al., 2007). Although, these studies prove the presence of a positive correlation between the increased risk of cervical cancer and at least one of the muted BRCA genes. Correspondingly, the wild-type *BRCA1* expression in cervical cancer was correlated with survival. The protein level of *BRCA1* detected using Immunohistochemistry in 70 patients showed that the survival rate within the *BRCA1*+ group was 95.5%, while it was 76.9% within the *BRCA1*- group (Paik et al., 2021).

Results from cell lines

The implicated mechanisms of cervical cancer risk in *BRCA1*/*2* carriers are not clearly established. The HPV early oncoproteins E6 and E7, known to interact with the tumor suppressor p53 and Rb1, were found to inactivate the *BRCA1* gene in cervical cancer cell lines (SiHA, Caski, and HeLa). These oncoproteins interacted directly with *BRCA1* throughout two contact points on the *BRCA1* protein, one within an N-terminal site and the other in a C-terminal region of the *BRCA1* protein (Zhang et al., 2005). Although, another research investigating the interaction of HPV oncoproteins with DDR proteins, especially homologous recombination proteins, showed that the expression of *BRCA1*, *BRCA2*, and RAD51 were slightly increased in primary human keratinocytes (HFKs) transfected with HPV16 E6 and E7. However, DSBs repair through the HR pathway was reduced by 50%, which was correlated with the mislocalization of RAD51 away from DSBs foci (Wallace et al., 2017). HPV31 E7 was also found to increase the level of many DDR proteins, including the HR proteins *BRCA1* and Rad51 (Gillespie et al., 2012; Johnson et al., 2017). Remarkably, HPV18 E2-dependent transcription was enhanced by *BRCA1* in the C33A cervical cancer cell line (Kim et al., 2003). The co-expression of *BRCA1* and HPV18-E2 was necessary for activating the E2 binding site. These proteins form a stable complex while binding through their c-terminal region.

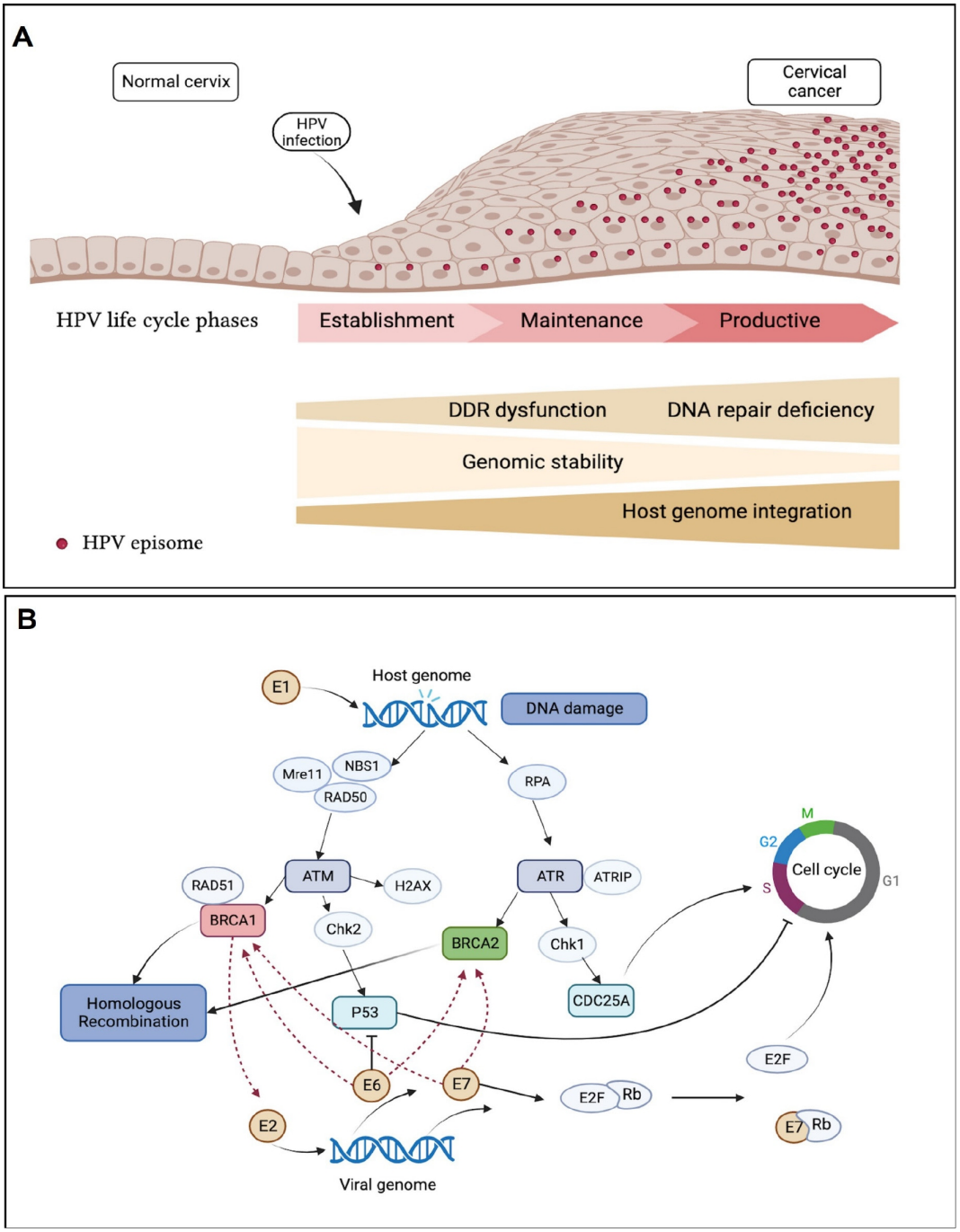

Figure 3. HPV and Genomic Instability in Cervical Cancer. **A** Model of HPV host genome integration consequences. HPV infects the cervical basal layer then starts replicating following three distinct phases (establishment, maintenance, and productive replication). During its life cycle, HPV recruits different DDR pathways and uses then in its advantage. The massive activation of the DDR pathways and the interaction of HPV oncoproteins with DDR effectors lead to the dysfunction of this mechanism. Therefore, causing a DNA repair deficiency which leads to the accumulation of DNA mutations. Furthermore, HPV integration in the host genome (e.g., chr 17q and chr 13q) leads to chromosomal aberrations. The intensity of these two phenomena, is correlated with HPV episomal copy number in the host cell. The resulting genomic instability promotes and accelerates the cervical carcinogenesis. **B** HPV oncoproteins recruit DNA damage response pathways. HPV E1 protein causes DNA damage, which activates the DDR pathway. Depending on the type of DNA damage (DSBs or SSBs), DDR leads to ATM activation via the MRN mediators (MRE11, RAD50, NSB1) and ATR activation via RPA. Once activated, these transducers lead to a signaling cascade that stimulates cell cycle arrest and the repair of the damage. In the downstream of this mechanism, E2 promotes the expression of oncoproteins E6 and E7 by hyperacetylation of the promoter region. E6 degrades p53 by ubiquitination, and E7 binds to Rb, releasing E2F and leading to the progression of the cell cycle. HPV oncoproteins also interact with BRCA1 and BRCA2 via direct protein-protein interactions. Therefore, the DNA damage remains unrepaired, and the cell escapes cell cycle arrest which allows the virus to replicate. The accumulation of unrepaired DNA damages causes genome instability and promotes cervical malignant transformation.

This study also showed that Mutant c-terminal *BRCA1* reduced the activation of E2-dependent promoter even in the presence of E2 (Kim et al., 2003). The interaction of HPV oncoproteins with *BRCA1/2* proves the manipulation of DDR pathways by the virus to promote its replication. The alteration of these pathways is well-known to promote carcinogenesis by promoting DNA mutations and genomic instabilities (O'Connor, 2015).

Furthermore, in cervical cancer, losses of heterozygosity were frequently detected within chromosome 17q (containing the *BRCA1* gene) by 44% and chromosome 13q (containing the *BRCA2* gene) by 29% (Miyai et al., 2004). Similarly, another study investigating HPV integration hotspots in the host genome showed that HPV integration was detected in 15 of 21 samples (Shen-Gunther et al., 2022). In one sample, HPV integration was detected in chromosome 17q (containing the *BRCA1* gene), while in another, HPV integration was detected in chromosome 13q (containing the *BRCA2* gene) (Shen-Gunther et al., 2022). Hence, HPV genome integration within chromosomal regions containing *BRCA1* and *BRCA2* genes may be considered as an important assist in the *BRCA1/2* associated genome instability in cervical cancer.

The mutational status of BRCA1/2 in cervical cancer is not well established. Mutation in exon 11 of the *BRCA1* gene was detected in 76% of 17 precancerous lesions of the cervix. The mutation was either a complete deletion or a deletion of one or more nucleotides (Park et al., 1999). Similarly, *BRCA1* (p.T367I) and *BRCA2* (p.Q1187fs) mutations were detected each in one sample individually in a study conducted on 10 cervical cancer tissues (Xing et al., 2018). A much larger study combining the analyses of 327 squamous cell carcinomas and 86 non-squamous cell carcinomas showed that Fanconi Anemia (FA) genes *BRCA1*, *BRCA2*, and BRIP1 were mutated in 152 patients by 3.7%, 4.0%, and 2.8%, respectively (Halle et al., 2021). In another form of genomic variability, a Chinese research investigating the genetic landscape of 64 cervical cancer samples using a validated multigene next-generation sequencing (NGS) panel revealed that *BRCA1*, *BRCA2*, ATM, and TP53 gene loci had a higher frequency of copy number variations, CNVs (Qiu et al., 2022).

Overall, *BRCA1/2* may contribute to the development of cervical cancer as a co-factor to HPV either by germline mutations (cancer susceptibility) or somatic mutations (e.g., precancerous lesions). Moreover, HPV genome integration in the *BRCA1/2* genes chromosome sites may alter their expression and thus promote genomic instability, especially in *BRCA1/2* carriers. Likewise, in cervix cells of *BRCA1/2* carriers, the possible inhibitory interaction of HPV proteins with *BRCA1* and *BRCA2* and other DDR proteins may deprive those cells of essential functions such as DSBs repair through homologous recombination pathway, thus accelerating genome instability initiated by other viral mechanisms. Indeed, the DNA repair deficiency induces the accumulation of DNA mutations and chromosomal aberrations, which plausibly promote cervical carcinogenesis (Figure 3). Further investigations are needed to clarify and decipher the implication of *BRCA1/2* genes in cervical cancer susceptibility.

Conclusions

Many studies on breast and ovarian cancers and fewer on cervical cancer investigated the link between *BRCA1/2* germline mutations and genome instability-associated tumorigenesis among carriers (Table 1). Indeed, *BRCA1/2* mutations alter various vital cellular processes, which leads to the accumulation of DNA damages, mutations of critical genes, unresolved stalled forks and R-loops, telomere dysfunction, defects in cell cycle control, and increased oxidative stress. Those alterations may cause genome instability in normal cells of *BRCA1/2* mutation carriers, leading them to transformation. Notably, *BRCA1/2* alterations are one of the main contributors to HR deficiency in breast and ovarian cancers, and they constitute determinant biomarkers for the clinical use of PARP inhibitors and platinum-derived therapy. *BRCA1/2* heterozygous human, mice, and cell lines models show immense potential in establishing the link between the haplotype insufficiency of *BRCA1/2* genes and genome instability induction. *BRCA1/2* haploinsufficiency models provide strong evidence explaining *BRCA1/2*-associated genome instability compared to the "two-hit" models that use a total silencing of *BRCA1/2* genes using siRNA in cell lines or conditional mutations in mice. In cervical and uterine cancers, the increased risk among *BRCA1/2* mutation carriers and the probable interaction of HPV with these genes, either by genome integration in their chromosomal sites or direct protein interactions, suggest an implication of *BRCA1/2* in the susceptibility of this cancer. Further investigations are needed to prove *BRCA1/2*-related HR deficiency in cervical cancer, which will promote the beneficial use of PARP inhibitors in this latter.

Overall, the poor outcome of *BRCA1/2*-associated cancers renders *BRCA1/2*-driven genome instability of substantial clinical potential. Other alterations of *BRCA1/2* genes, such as DNA methylation, are frequently observed in gynecomammary cancers and may constitute another mechanism driving genome instability that needs to be investigated, especially in sporadic disease.

Abbreviations list

ATM: Ataxia-telangiectasia mutated
ATR: ataxia telangiectasia and Rad3-related protein
BPTF: bromodomain PHD finger transcription factor
BRCA1: Breast cancer susceptibility gene/protein 1
BRCA2: Breast cancer susceptibility gene/protein 2
BRD4: bromodomain-containing protein 4
BRIP1: BRCA1 Interacting Protein 1
CDKN1A/2A: Cyclin Dependent Kinase Inhibitor 1A/2A
Chk1/2: Checkpoint kinase 1/2
CI: Confidence interval
CNV: copy number variation
DDR: DNA damage response
DNA: Deoxyribonucleic acid
DSBs: double strand breaks
ESR1: Estrogen receptor locus
FA: Fanconi anemia
FANCA/C/L: Fanconi anemia complementation group A/C/L

Table 1. Pathogenic Variants Implicated in Gynecological and Mammary Cancers: Insights from Cited Models in this Review

	Breast Models	Ovary Models	Blood Models	Uterine, Risk
BRCA1	6-KB Duplication in exon 13	6-KB Duplication in exon 13	6-KB Duplication in exon 13	5385insC, exon 19
	C61G, exon 4	L598X, exon 10	185delAG, exon 2	c.4956G>A, exon 16
	E143X, exon 6	185delAG, exon 2	1294del40, exon 10	185delAG, exon 2
	4065-4068del, exon 10	1246delA, exon 11	1323delG, exon 11	
	R1203X, exon 10	2576delC, exon 10	4446C>T, exon 12	
	R1443X, exon 12	5214C>T, exon 16	IVS8+2T>A, splice mutation implicating intron 8 and exon 8	
	185delAG, exon 2			
	943ins10, exon 10			
	1100delAT, exon10			
	1135insA, exon 10			
	2530delAG, exon 10			
	2800delAA, exon 10			
	4154delA, exon10			
	4184del4, exon 10			
	5385insC, exon 19			
BRCA2	999del5, exon 9	6174delT, exon 11		T1251fs*14, exon 11
		8765delAG, exon 20		6174delT, exon 11
		5358del4, exon 11		

Pathogenic variants associated with Breast and/or Ovarian cancer risk: findings from various models in the current review, Presented in the 'Breast Models,' 'Ovary Models,' and 'Blood Models' Columns. Founder mutations increasing the risk of breast and ovarian cancers and associated with the risk of uterine cancer are presented in the "Uterine, Risk" column. The pathogenicity (Risk of breast/ovarian cancers) and the nucleotide localization of each *BRCA1/2* variant used in different models were determined using the Clinvar database (ClinVar, 2023). Alterations with unknown significance (ClinVar, 2023) for the risk of breast and/or ovarian cancers were excluded and are not mentioned in this table. The Ashkenazi founder mutation "185delAG" is one of the most used mutations in models presented in this review. The last column, "Uterine, Risk," includes *BRCA1/2* mutations increasing the risk for uterine cancer, known as founder mutations, increasing the risk of breast and/or ovarian cancers (Laitman et al., 2019).

FOXP1: forkhead box P1
GKN: Ovarian granulosa cells
HDR: homology-directed repair
HGSOC: high grad serous ovarian carcinoma
HIS: haploinsufficiency-induced-senescence
HMECs: Human mammary epithelial cells
HPV: Human papillomavirus
H-R: High risk
HR: Homologous recombination
hTERT: human telomerase reverse transcriptase
LGRs: large genomic rearrangements
LOH: loss of heterozygosity
L-R: Low risk
MCF10A: Immortalized non-tumorigenic HMECs line
MCF7: Luminal mammary cells expressing estrogen receptors
MDM2: Mouse double minute 2 homolog
MMC: mitomycin C
Mre11: Double-strand break repair protein Mre11
MRN: Mre11, Rad50, NBS1 complex
NBS1: Nijmegen breakage syndrome 1
NHEJ: non homologous end joining
NHEJ: Non-homologous end-joining
OC: ovarian cancer
OSE: ovarian epithelium surface
P53/TP53: Tumor protein P53
PARP: poly(ADP-ribose) polymerase
pRb/Rb1: Retinoblastoma protein/ gene
PTEN: phosphatase and tensin homolog
RAD50/51: DNA repair protein RAD50/RAD51 homolog 1
RNA TERRA: Telomeric Repeat RNA
RNaseH1: RNA endonuclease, R-loops resolver
RPA: Replication Protein A
SIR: Standardized incidence ratio
siRNA: small interfering RNA
SIRT: transcriptional target of BRCA1 protein
SMR: Standardized morbidity ratio
SNU251: BRCA1 mutated ovarian cell line
SSB: Single strand break
SVs: structural variations
UPD: uniparental disomy
γH2AX: Gamma Variant histone H2AX

Author Contribution Statement

R.A.E. designed the concept and scope of the manuscript/Figures/Table, and reviewed the manuscript text. Y.B. and Z.Q. have contributed to the concept of the review, and reviewed the manuscript/Figures/Table. Y.O, F.B and F.O wrote the manuscript text and prepared Figures. Y.O. prepared Table 1.

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Conflict of interest

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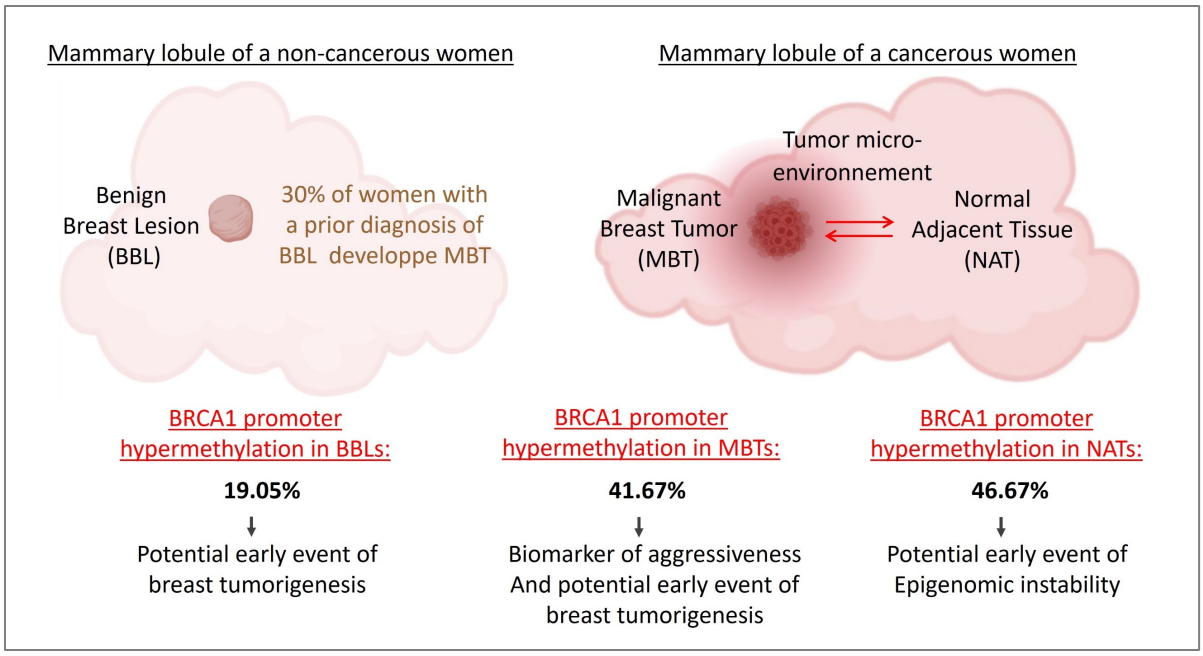
II. *BRCA1* Promoter Hypermethylation in Malignant Breast Tumors and in the Histologically Normal Adjacent Tissues to the Tumors: Exploring Its Potential as a Biomarker and Its Clinical Significance in a Translational Approach

Introduction: *BRCA1* (Breast cancer 1) is a well-recognized tumor suppressor gene linked to breast and ovarian cancers. When mutated, this gene can lead to increased genomic instability because of its diverse functions in DNA repair, resolving stalled forks and R-loops, and remodeling chromatin structure. Additionally, the role of *BRCA1* gene can be impaired by the hypermethylation of specific CpG island of its promoter. This alteration has gained more attention recently due to its broader implications in the etiology of breast tumors and could serve as a promising biomarker to improve the management of this disease. The objective of this retrospective study is to assess the prevalence of the hypermethylation of the *BRCA1* gene promoter region among different human breast tissues that include malignant breast tumors (MBTs=48), normal adjacent tissues (NATs=15), and benign breast lesions (BBLs=21). Additionally, we seek to evaluate the clinical significance of this epigenetic modification among malignant breast tumors (n=48).

Our data revealed the following rates of *BRCA1* gene promoter hypermethylation: 41.67% in MBTs, 46.67% in NATs, and 19.05% in BBLs. The increased rate among MBT samples aligns with previous scientific reports on the involvement of this epigenetic alteration in breast malignancy. Despite their histologically normal appearance, NATs show a surprisingly high rate of this epigenetic alteration, highlighting epi/genetic instabilities. Given that BBLs also exhibited this modification at a non-negligible rate, further research into the role of *BRCA1* gene promoter hypermethylation in BBL-associated breast cancer risk is necessary. Interestingly, there is a 60% concordance between the hypermethylation status in MBTs and their corresponding NATs, suggesting a "field effect" and possible early event of tumorigenesis in normal breast tissues. Furthermore, this hypermethylation in MBTs is associated with aggressive breast cancer features such as a higher Scarff-Bloom-Richardson (SBR) grade III, increased Ki67 levels, and HER2 receptor overexpression. In this context, this epigenetic silencing of *BRCA1* gene could constitute a biomarker of aggressiveness. Collectively, our findings can contribute to a better understanding of breast tumorigenesis with the aim to developpe new strategies for early detection and targeted treatment.



Graphical Abstract (Oubaddou et al., 2023b) : *BRCA1* Gene Promoter Hypermethylation in Breast Tissues and Its Clinical Implications in Breast Cancer Management. Elevated levels of *BRCA1* gene promoter hypermethylation have been detected in both malignant breast tumors (MBTs) and normal adjacent tissues (NATs), with a non-negligible rate also being found in benign breast lesions (BBLs). *BRCA1* gene promoter hypermethylation is associated with aggressive features (elevated Ki67, high mitosis score, and Her2 overexpression) of breast cancer, suggesting that this epigenetic modification could potentially be used as a biomarker of aggressiveness and a potential early event of breast tumorigenesis. In addition, the elevated rate of *BRCA1* gene promoter hypermethylation in NATs may indicate epigenomic instability and may refer to this epigenetic mark as an early event that can lead to tumor growth. The concordance in this epigenetic mark between malignant breast tumors and their normal adjacent breast tissues might suggest a form of communication within the tumor microenvironment, which could have different implications: NATs with *BRCA1* gene promoter hypermethylation (early event) could serve as a precursor to early breast tumorigenesis, and *BRCA1* gene promoter hypermethylation could be induced in normal breast tissue adjacent to an MBT, potentially increasing the risk of recurrence after surgery. It is noteworthy that *BRCA1* gene promoter hypermethylation in BBLs could be involved in the observed risk of these benign diseases leading to the development of breast cancer, as indicated in the figure "30% of women with a prior diagnosis of BBL develop MBT".

genes

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Article

BRCA1 Promoter Hypermethylation in Malignant Breast Tumors and in the Histologically Normal Adjacent Tissues to the Tumors: Exploring Its Potential as a Biomarker and Its Clinical Significance in a Translational Approach

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Abstract: The hypermethylation status of the promoter region of the breast cancer 1 (*BRCA1)*, a well-known tumor suppressor gene, has been extensively investigated in the last two decades as a potential biomarker for breast cancer. In this retrospective study, we investigated the prevalence of *BRCA1* promoter methylation in 84 human breast tissues, and we correlated this epigenetic silencing with the clinical and histopathological parameters of breast cancer. We used methylation-specific PCR (MSP) to analyze BRCA1 promoter hypermethylation in 48 malignant breast tumors (MBTs), 15 normal adjacent tissues (NATs), and 21 benign breast lesions (BBLs). The results showed that *BRCA1* promoter hypermethylation was higher in MBTs (20/48; 41.67%) and NATs (7/15; 46.67%) compared to BBLs (4/21; 19.05%). The high percentage of BRCA1 hypermethylation in the histologically normal adjacent tissues to the tumors (NATs) suggests the involvement of this epigenetic silencing as a potential biomarker of the early genomic instability in NATs surrounding the tumors. The detection of BRCA1 promoter hypermethylation in BBLs reinforces this suggestion, knowing that a non-negligible rate of benign breast lesions was reported to evolve into cancer. Moreover, our results indicated that the *BRCA1* promoter hypermethylated group of MBTs exhibited higher rates of aggressive features, as indicated by the SBR III grade (14/19; 73.68%), elevated Ki67 levels (13/16; 81.25%), and Her2 receptor overexpression (5/20; 25%). Finally, we observed a concordance (60%) in *BRCA1* promoter hypermethylation status between malignant breast tumors and their paired histologically normal adjacent tissues. This study highlights the role of BRCA1 promoter hypermethylation as a potential useful biomarker of aggressiveness in MBTs and as an early marker of genomic instability in both histological NATs and BBLs.

Keywords: BRCA1 promoter hypermethylation; breast cancers; normal adjacent tissue; benign breast lesions; methylation-specific PCR; biomarker

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1. Introduction

Breast cancer is one of the most commonly diagnosed cancers worldwide and a major cause of cancer-related deaths among women [1]. It is a complex and heterogeneous disease with a multifactorial etiology involving genetic, environmental, and lifestyle factors [2]. Mutations in the breast cancer 1 (BRCA1) gene, a well-known tumor suppressor gene, are associated with hereditary breast cancers, which constitute 5–10% of all breast cancer cases [3]. However, the majority of breast cancer cases are sporadic, with no family history of the disease, and the mechanisms underlying their development remain poorly understood [3].

Epigenetic alterations, including gene silencing through DNA hypermethylation, have emerged as crucial contributors to breast cancer development and progression [4]; BRCA1 promoter hypermethylation is the most studied epigenetic alteration in malignant breast tumors [5–7]. Interestingly, a study carried out on 237 triple-negative breast cancer (TNBC) cases indicated that the promoter hypermethylation of BRCA1 is more frequent than BRCA1 germline mutations, suggesting that both alterations can independently drive malignant breast tumorigenesis [8]. Indeed, this result is supported by the rarity of BRCA1 promoter hypermethylation in BRCA1 germline mutation carriers [9].

BRCA1 promoter hypermethylation correlates with decreased BRCA1 gene expression (epigenetic silencing), the loss of homologous recombination (HR) repair function [8,10], and, consequently, may promote genomic instability due to multiple BRCA1 gene-related functions in maintaining genome stability, such as DNA double-strand break repair, chromatin remodeling, stalled fork resolution, and R-loop resolution [11–15]. Therefore, BRCA1 DNA hypermethylation could be an early event accelerating breast tumorigenesis similarly to BRCA1 germline mutations [8]. In addition, BRCA1 promoter hypermethylation correlates with a higher risk of recurrence, reduced survival rates of breast cancer [16,17], and it can affect the response to certain chemotherapy agents and targeted therapies, such as platinum-derived therapy and PARP inhibitors [18,19]. Altogether, these results highlight the potential of exploring BRCA1 hypermethylation as a diagnostic and prognostic biomarker to improve the management of breast cancers, and further translational research is needed in this direction.

In this retrospective study, we investigated BRCA1 promoter hypermethylation in a cohort of 84 FFPE breast tissues (FFPE: formalin-fixed and paraffin-embedded) through the MSP technique (methylation-specific PCR), and we correlated it with different clinical and histopathological features of randomly selected malignant breast tumors. Our findings demonstrated a higher prevalence of BRCA1 promoter hypermethylation in malignant breast tumors (MBTs) and normal adjacent tissues (NATs) compared to benign breast lesion samples. Furthermore, we observed a concordance in the status of BRCA1 promoter hypermethylation between MBTs and their paired histologically normal adjacent tissues ('NATs'). Interestingly, BRCA1 promoter hypermethylation was associated with the aggressive characteristics of breast cancer, including Scarff–Bloom–Richardson (SBR) grade III, elevated levels of ki67 expression, the overexpression of the oncogene human epidermal growth factor receptor (Her2), and the loss of estrogen and progesterone receptor expression. Our findings can contribute to a better understanding of the role of BRCA1 promoter hypermethylation in breast tumorigenesis, with potential clinical implications for the diagnosis, prognosis, and treatment of breast cancer. In addition, our results highlight that BRCA1 promoter hypermethylation could constitute an early event of genomic instability in the histologically normal breast tissues surrounding the tumors.

2. Materials and Methods

2.1. Study Subjects

This research received ethical approval from the Ethics Committee for Biomedical Research (CERB) of the Faculty of Medicine and Pharmacy in Rabat, with approval number 52/20.

15 min, followed by 40 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 45 s, and a final extension cycle of 72 °C for 10 min, all carried out in the Thermal Cycler (ProFlex PCR System; Thermo Fisher, Waltham, MA, USA).

The PCR products were separated through gel electrophoresis (MUPID-ONE 230 V; Deutscher) using a 2% agarose gel in tris-borate EDTA X1 buffer and visualized using ethidium bromide (BET). Finally, the hypermethylation status of the BRCA1 promoter region was determined by analyzing the gel images obtained with the ENDURO™ GDS Gel Documentation system (Labnet International Inc., Edison, NJ, USA).

2.6. Inclusion/Exclusion Criteria of MSP Results

These inclusion/exclusion criteria allow us to interpret our results with rigor, reliability, and credibility. Indeed, genomic DNA extracted from FFPE tissues is known to be extensively fragmented [25–27], and the sodium bisulfite conversion can further fragilize the genomic DNA. Therefore, the MSP results strongly depend on the quality of DNA extracted from FFPE tissues and on its conversion process. To optimize our experimentations, we used a specific kit for DNA extraction from FFPE tissues, known to improve the quality of extracted genomic DNA from FFPE tissues, and two credible sodium bisulfite conversion kits.

We explored 106 FFPE human breast tissues, and we included only the results obtained from 84 FFPE samples. All MSP results (22) showing any amplification with both primers (U and M) were excluded. Note that the majority of MSP results were obtained twice with both Epitect Fast DNA Bisulfite Kit (Qiagen, 59826) and the EZ DNA Methylation kit (ZymoResearch, D5002).

2.7. Statistical Analysis

Statistical analyses and graphical visualizations were performed using GraphPad Prism8. To assess the statistical significance of various associations, different tests were employed, including Fisher's exact test, chi-square test, and unpaired *t*-test. To evaluate the concordance in BRCA1 promoter hypermethylation status between NATs and their matched MBTs, Cohen's Kappa and McNemar's tests were utilized. A *p*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Clinico-Histopathological Characteristics of Malignant Breast Tumor Patients

The median age of patients with malignant breast tumors (MBTs) was 50 years, with the youngest patient being 33 and the oldest being 70 years old (Table 1). Histologically, the majority of MBTs (41/48; 85.43%) were invasive carcinomas of no special type (NST). Additionally, 4 cases (4/48; 8.33%) were invasive medullary carcinoma, 1 case was metaplastic invasive carcinoma (1/48; 2.08%), 1 case was carcinoma ex pleomorphic adenoma of the breast (1/48; 2.08%), and 1 case was ductal in situ carcinoma with several invasive micro infiltrations (1/48; 2.08%).

We analyzed the aggressive feature data in malignant breast tumors and found that the in situ component was absent in half of the cases (21/42; 50%) (Table 1). Moreover, 37.5% (18/48) of cases displayed a tumor size greater than 3 cm, 46.15% (18/39) showed positive nodal status, and 29.17% (14/48) exhibited vascular emboli (Table 1). In addition, 55.32% (26/47) of cases were classified as SBR grade III and 64.86% (24/37) had a ki67 expression greater than or equal to 30% (Table 1). In addition, we observed that 66.67% (32/48) of cases tested negative for estrogen (ER) and progesterone (PR) receptors, and 12.50% (6/48) tested positive for Her2 receptor overexpression (Table 1). Regarding the 48 MBTs, 30 cases (30/48; 62.5%) were classified as triple-negative ($ER^-/Her2^-$), while 12 cases (12/48; 25%) were of the luminal $Her2^-$ ($ER^+/Her2^-$) subtype. The remaining cases included 4 cases (4/48; 8.33%) of the luminal $Her2^+$ ($ER^+/Her2^+$) subtype and 2 cases (2/48; 4.17%) of the $Her2^+$ "only" ($ER^-/Her2^+$) subtype (Table 1).

Table 1. Clinical and histopathological characteristics of the study population.

Characteristics		MBTs (*n* = 48)		NATs (*n* = 15)		BBLs (*n* = 21)	
		n	%	*n*	%	*n*	%
Age	Median age	50 years		48 years		22 years	
	≤47 years	19	39.58	7	46.67	19	90.48
	>47 years	29	60.42	8	53.33	2	9.52
Tumor size	≤3 cm	30	62.50	-	-	-	-
	>3 cm	18	37.50	-	-	-	-
Tubule formation score	Score 1/2	12	29.27	-	-	-	-
	Score 3	29	70.73	-	-	-	-
	Missing data	7					
Nuclear grade score	Score 1/2	21	50	-	-	-	-
	Score 3	21	50	-	-	-	-
	Missing data	6					
Mitosis score	Score 1/2	26	61.90	-	-	-	-
	Score 3	16	38.10	-	-	-	-
	Missing data	6					
SBR grading	Grade I/II	21	44.68	-	-	-	-
	Grade III	26	55.32	-	-	-	-
	Missing data	1					
Ki67 level	<30%	13	35.14	-	-	-	-
	≥30%	24	64.86	-	-	-	-
	Missing data	11					
In situ component	Absence	21	50	-	-	-	-
	Presence	21	50	-	-	-	-
	Missing data	6					
Vascular emboli	Absence	34	70.83	-	-	-	-
	Presence	14	29.17	-	-	-	-
Nodal status	Negative	21	53.85	-	-	-	-
	Positive	18	46.15	-	-	-	-
	Missing data	9					
ER/PR status	Negative	32	66.67	-	-	-	-
	Positive	16	33.33	-	-	-	-
Her2 status	Negative	42	87.50	-	-	-	-
	Positive	6	12.50	-	-	-	-
Molecular subtype	$ER^+/Her2^-$	12	25.00	-	-	-	-
	$ER^+/Her2^+$	4	8.33	-	-	-	-
	$ER^-/Her2^+$	2	4.17	-	-	-	-
	$ER^-/Her2^-$	30	62.50	-	-	-	-

MBTs: Malignant Breast Tumors; NATs: Normal Adjacent Tissues; BBLs: Benign Breast Lesions; SRB: Scarff–Bloom–Richardson; ER: Estrogen Receptor; PR: Progesterone Receptor; Her2: Human epidermal growth factor receptor 2. -: Data not analyzed (NATs) or not available (BBLs).

3.2. BRCA1 Promoter Hypermethylation in Breast Tissue Samples

In this study, we examined the BRCA1 promoter hypermethylation status in 84 human breast tissues (48 MBTs, 15 NATs, and 21 BBLs) using methylation-specific PCR. According to Esteller et al. [24], the sense of primers U binds to the 1536 bp position of the GenBank sequence "U37574," while the sense of primers M binds to the 1543 bp position of the same sequence [24] (Figure 1a). The BRCA1 regulatory region is included in a large unmethylated sequence of 1.4 kilobase pairs (Kbp) flanked by two hypermethylated regions [28]. This regulatory region is selectively maintained in normal cells unmethylated to ensure BRCA1 gene expression [28]. The targeted region of the BRCA1 promoter is located approximately between positions −45 (1536 bp in "U37574," sequence) and +39

(1619 bp in "U37574," sequence), comprising the transcriptional start site, TSS (Figure 1a). The validation of primers was conducted in the MCF-7 cell line as a human breast cancer model of Luminal A subtype 'ER^{+}, BRCA1^{wt}' [29]. Divers studies reported negative [30,31] or partial methylation of BRCA1in these cells [32,33], and our exploration reinforces the partial methylation of BRCA1 in MCF-7 cells (Figure S1). Interestingly, we observed a stable BRCA1 promoter methylation profile throughout the proliferation period of breast cancer MCF7 cells (4 days) (Figure S1). Our result highlights the stability of this epigenetic silencing in breast cancer cells independently of both the confluence state of cells and secreted cytokines in the extracellular medium over 4 days.

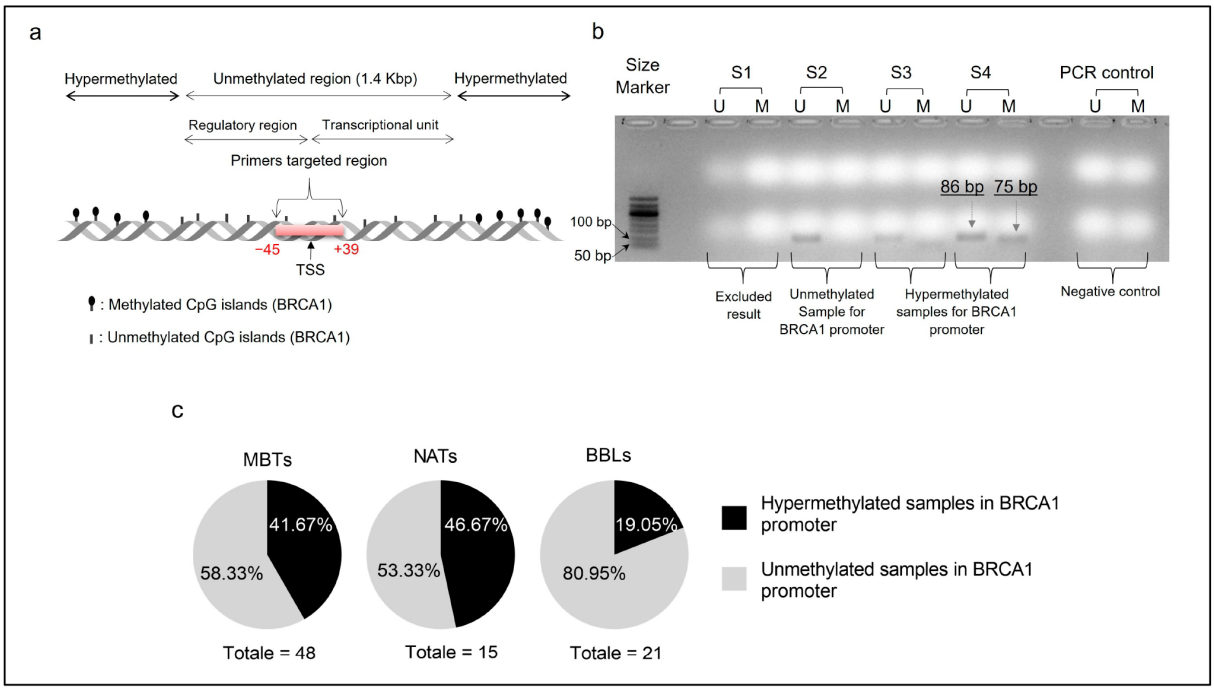

Figure 1. Results of methylation-specific PCR (MSP) analysis of the BRCA1 promoter in 84 FFPE samples. (**a**) Region targeted by primers for the MSP. The BRCA1 regulatory region is included in a large unmethylated sequence of 1.4 kilobase pairs (Kbp) flanked by two hypermethylated regions. (**b**) Electrophoresis gel image of MSP products for four malignant breast tumor samples (S1, 2,3, and 4). The PCR product "U" is 86 bp in length, while the PCR product "M" is 75 bp. (**c**) Portions of BRCA1 promoter hypermethylation and unmethylation in different breast tissue samples: Malignant Breast Tumors (48MBTs), Normal Adjacent Tissues (15NATs), and Benign Breast Lesions (21BBLs). MBTs: malignant breast tumors, NATs normal adjacent tissues to the tumor, BBLs: benign breast lesions, TSS: transcriptional start site, S: Sample, U: MSP product using pair of primers amplifying the unmethylated state of the targeted region. M: MSP product using the pair of primers M amplifying the hypermethylated state of the targeted region.

Figure 1b shows an example of MSP results of BRCA1. Sample 1 (S1) did not generate exploitable results and was, therefore, excluded from this study according to the inclusion/exclusion criteria of MSP results. Sample 2 (S2) is compatibilized as unmethylated for BRCA1, and both samples 3 and 4 (S3 and S4) are compatibilized as hypermethylated for the BRCA1 promoter (Figure 1b). Our study found BRCA1 promoter hypermethylation in 41.67% (20/48) of malignant breast tumors (MBTs) and 46.67% (7/15) of normal adjacent tissues (NATs) (Figure 1c and Table S1), strengthening a similar methylation profile between MBTs and NATs (Figure 1c and Table S1). Interestingly, we observed a non-negligible BRCA1 promoter hypermethylation of NATs compared to MBTs (Table 2). Regarding the 15 MBTs and their 15 paired NATs, 5 MBTs (33.33%) and 7 NATs (46.66%) showed BRCA1

promoter hypermethylation (Table 3). In contrast, only 19.05% (4/21) of benign breast lesions (BBLs) showed BRCA1 promoter hypermethylation (Figure 1c). Interestingly, of the 15 pairs of malignant breast tumors and their histologically normal adjacent tissues, 60% (3/5) exhibited concordance in their status of BRCA1 promoter hypermethylation, and 60% (6/10) exhibited concordance in their status of BRCA1 promoter unmethylation (Table 3).

Table 2. BRCA1 promoter hypermethylation: differences between MBTs, NATs, and BBLs.

Group vs. Group	*p*-Value	Statistical Tests
MBTs vs. NATs	0.7718	Fisher's exact test
MBTs vs. BBLs	0.0999	Fisher's exact test
NATs vs. BBLs	0.1410	Fisher's exact test

MBTs: Malignant Breast Tumors; NATs: Normal Adjacent Tissues; BBLs: Benign Breast Lesions.

Table 3. Concordance in BRCA1 promoter hypermethylation (M) and unmethylation (U) status between 15 pairs of malignant breast tumors (MBTs) and their matched normal breast adjacent tissues (NATs).

		MBTs		McNemar's Test	Cohens Test
		U	M		
NATs	U	6	2	$p = 0.6831$	Kappa = 0.182
	M	4	3		

MBTs: Malignant Breast Tumors; NATs: Normal Adjacent Tissues; U: Unmethylated; M: Hypermethylated.

3.3. Association between BRCA1 Promoter Hypermethylation and Aggressive Characteristics of Breast Cancer

Analysis of BRCA1 promoter hypermethylation status using MSP revealed two distinct groups of MBTs: BRCA1 hypermethylated group ($n = 20$) and BRCA1 unmethylated group ($n = 28$) (Table S2). In our study, we observed a positive association between BRCA1 promoter hypermethylation and the aggressive features of breast cancer. Indeed, among the MBT cases with BRCA1 promoter hypermethylation, 45% (9/20) were diagnosed at age 47 years or younger, while in the BRCA1 unmethylated group, only 35.71% (10/28) fell within this age range at diagnosis (Figure 2a). Moreover, small tumor sizes (<3 cm) are more likely to be found in the unmethylated group (71.43% U vs. 28.57% M) compared to large tumor sizes (50% U vs. 50% M) (Figure 2c). Interestingly, we found that MBTs with BRCA1 promoter hypermethylation had increased levels (≥30%) of the cell cycle (G1, S, G2, and mitosis) biomarker Ki-67 (13/16; 81.25%) compared to the BRCA1 unmethylated group (11/21; 52.38%) (Figure 2d). In addition, we observed a higher frequency of mitosis score of 3 in the BRCA1 hypermethylated group (9/17; 52.94%) compared to the BRCA1 unmethylated group (7/25; 28%) (Figure 2e). These two latter findings suggest a potential association between BRCA1 promoter hypermethylation and the proliferative phenotype of breast cancer (Figure 2d,e). The presence of a tubule formation score of 3 was more frequent in the BRCA1 hypermethylated group (15/17; 88.24%) compared to the unmethylated group of MBTs (14/24; 58.33%) (Figure 2f). Regarding the nuclear grade feature, our analysis did not reveal any differences between the two groups (Figure 2g). The mitosis score, tubule formation score, and nuclear grade provide valuable parameters for breast cancer prognosis and aggressiveness determination using the SBR grading system [34]. Indeed, an SBR grade of III was found to be more prevalent in cases within the BRCA1 promoter hypermethylated group (14/19; 73.68%) compared to cases from the BRCA1 unmethylated group (12/28; 42.86%), suggesting an association between BRCA1 promoter hypermethylation and the aggressiveness of breast cancer (Figure 2h). In addition, the absence of in situ components was more commonly observed in BRCA1 hypermethylated MBTs (11/18; 61.11%) compared to BRCA1 unmethylated MBTs (10/24; 41.67%) (Figure 2i). Furthermore, our analysis revealed that BRCA1 promoter hypermethylation did not significantly impact the presence of vascular emboli or nodal invasion features (Figure 2j,k).

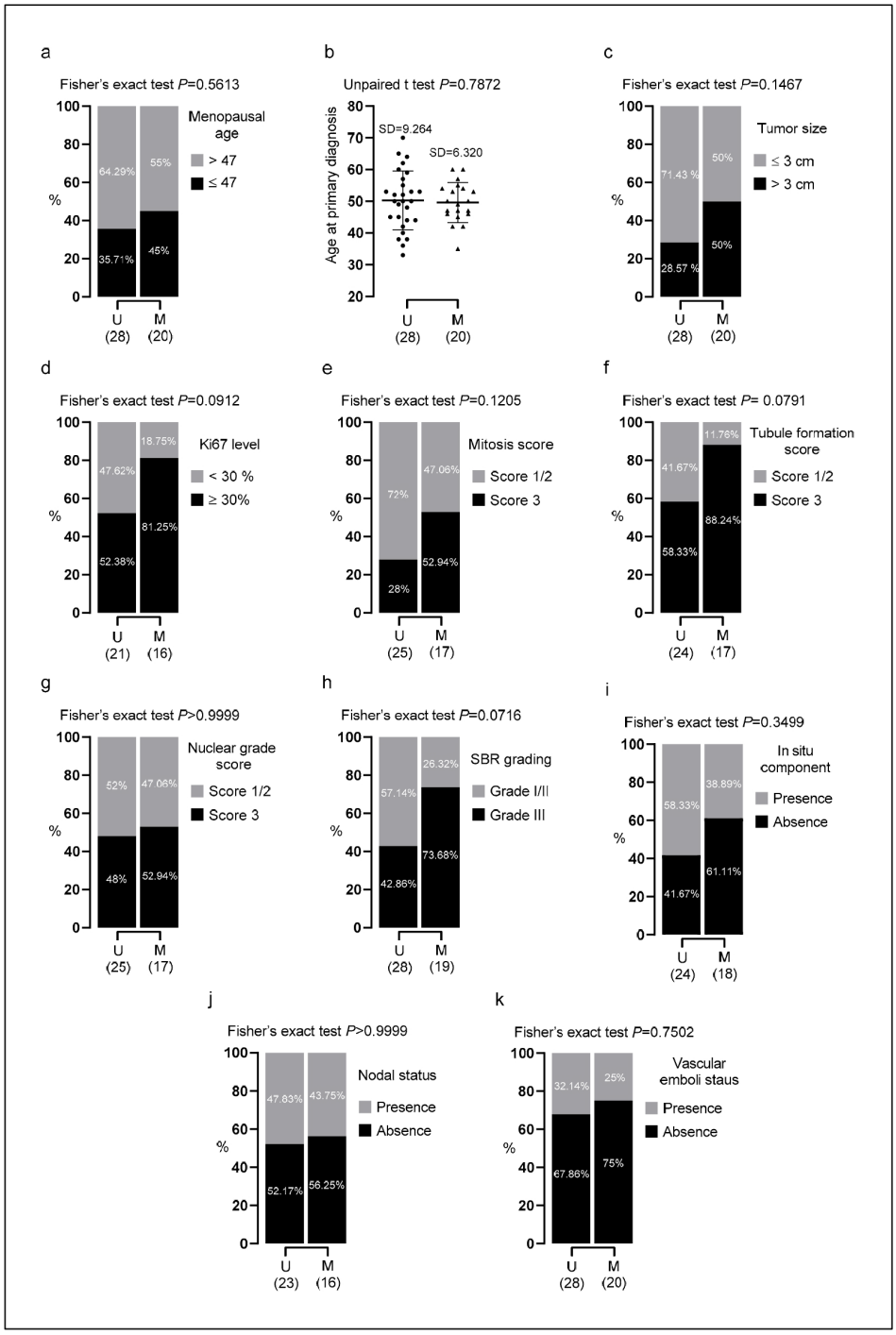

Figure 2. Association between BRCA1 promoter methylation and various clinical and histopathological characteristics of malignant breast tumors (n = 48): (**a**) menopausal age (≤47: premenopausal age; >47: postmenopausal age), (**b**) age at primary diagnosis, (**c**) tumor size, (**d**) Ki67 level, (**e**) mitosis score, (**f**) tubule formation score, (**g**) nuclear grade score, (**h**) SBR grading, (**i**) in situ component, (**j**) nodal status, (**k**) vascular emboli status. The letter "U" in each graph represents the group of BRCA1 unmethylated samples, while the letter "M" represents the group of BRCA1 hypermethylated samples. The missing data for each feature are presented in Table S2. The statistical significance was determined by GraphPad 8. SD: Standard Deviation; SRB: Scarff–Bloom–Richardson.

Breast cancers that are Her2-positive are characterized by the overexpression of the Her2 protein [35]. These tumors were shown to carry unique molecular characteristics [36]. Regardless of their hormone receptors' status, Her2-positive tumors often benefit from targeted therapies against the Her2 protein, such as trastuzumab [37]. Interestingly, Her2 overexpression was shown to have a strong prognostic value in breast cancer compared to hormone receptors (ER/PR) or nodal status [38]. Our findings indicate a pronounced prevalence of BRCA1 promoter hypermethylation in tumors with this oncogenic protein overexpression '$p = 0.0247$' (Figure 3a). Furthermore, 43.33% (13/30) of the analyzed triple-negative breast tumors exhibited BRCA1 promoter hypermethylation (Figure 3a), strengthening the role of BRCA1 promoter hypermethylation in aggressive breast cancer subtypes.

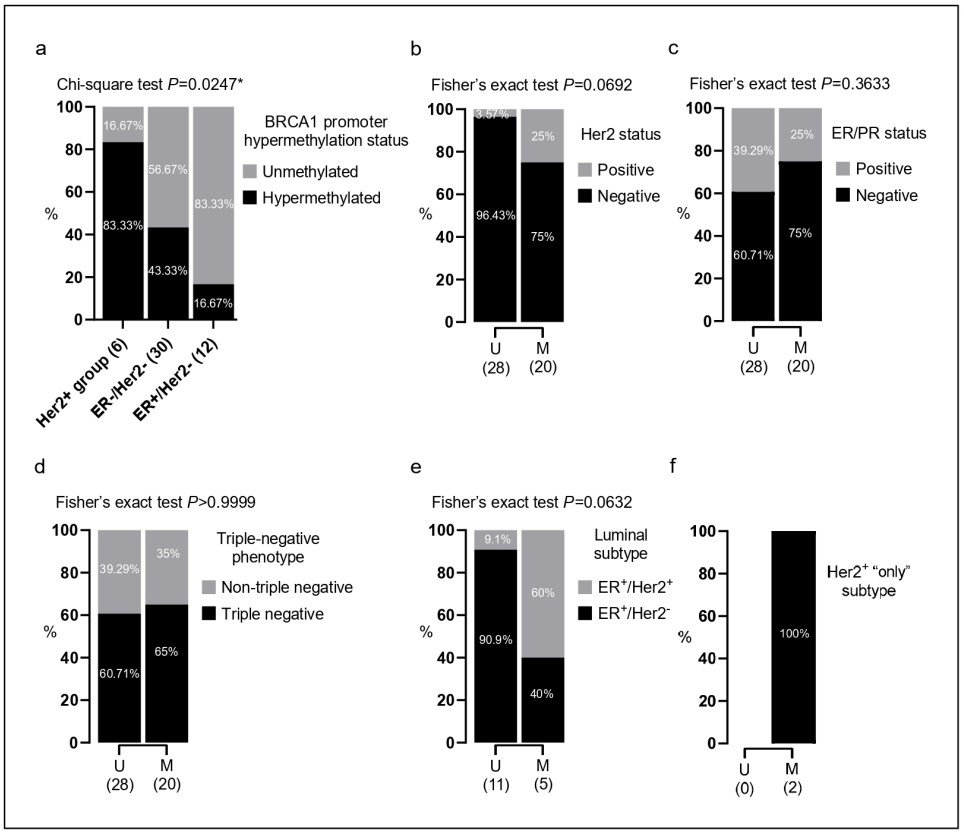

Figure 3. Association of BRCA1 promoter hypermethylation with Her2 overexpression and aggressive molecular subtypes of breast cancer: (**a**) molecular subtypes of breast cancer, (**b**) Her2 status, (**c**) ER/PR status, (**d**) triple-negative phenotype, (**e**) luminal subtype, (**f**) Her2+ "only" subtype. The letter "U" in each graph represents the group of BRCA1 unmethylated samples, while the letter "M" represents the group of BRCA1 hypermethylated samples. The missing data for each feature are presented in Table S2. The statistical significance was determined by GraphPad 8. * Statistical significance is affirmed by a *p*-value under 0.05. ER: Estrogen Receptor; PR: Progesterone Receptor; Her2: Human epidermal growth factor receptor 2.

Indeed, Her2 oncogene overexpression was more prevalent among cases with BRCA1 promoter hypermethylation (5/20; 25%) compared to the BRCA1 unmethylated group (1/28; 3.57%) (Figure 3b). Notably, Her2 receptor overexpression is known to be associated with more aggressive subtypes of breast cancer, including the luminal Her2$^+$ (ER$^+$/Her2$^+$) and Her2$^+$ "only" (ER$^-$/Her2$^+$) subtypes [39]. Consequently, the majority of cases (5/6: 83.33%) with luminal Her2$^+$ and Her2$^+$ "only" subtypes were found to be BRCA1 hypermethylated. Indeed, 100% (2/2) of Her2$^+$ "only" cases were BRCA1 hypermethylated (Figure 3f), and 60% (3/5) of luminal Her2$^+$ cases (ER$^+$/Her2$^+$) were BRCA1 hyperme-

thylated (Figure 3e and Table S2), unlike the majority of the luminal Her2$^-$ (ER$^+$/Her2$^-$) cases (10/12: 83.33), which were found BRCA1 unmethylated (Figure 3e and Table S2). More importantly, the loss of expression of estrogen (ER) and progesterone (PR) receptors was prevalent in cases with BRCA1 promoter hypermethylation (15/20; 75%) compared to the BRCA1 unmethylated group (17/28; 60.71%) (Figure 3c). Moreover, our analysis reveals a slight increase in BRCA1 promotor hypermethylation among the triple-negative (ER$^-$/Her2$^-$) subtype compared to the non-triple-negative subtype (Figure 3d).

4. Discussion

Breast cancer 'BC' is a real worldwide public health problem with an alarming incidence rate and mortality. The sporadic forms of BC, the most frequent '90–95%', remain poorly characterized, and the epigenetic instability seems to play a pivotal role in sporadic BC compared to hereditary BC [40–43]. Exploring the diagnostic biomarkers for an early detection of this disease could improve its management, and during the last decade, interesting translational studies have evaluated the potential of BRCA1 promoter hypermethylation as a useful biomarker in BC.

This study used methylation-specific PCR (MSP) to investigate BRCA1 promoter methylation status in a cohort of 84 FFPE tissues by assessing its prevalence among different types of mammary tissues and its interpretation on different clinical and histopathological features of randomly selected malignant breast tumors. BRCA1 promoter methylation rates of 41.67%, 46.67%, and 19.05% were detected, respectively, in malignant breast tumors (MBTs), normal adjacent tissues (NATs), and benign breast lesions (BBLs). This significant BRCA1 promoter hypermethylation among samples from cancerous patients is in line with the report that BRCA1 promoter hypermethylation correlates positively with a high risk of breast cancer and aggressiveness [7]. Indeed, recently, the detection of BRCA1 promoter methylation has been extensively studied as a contributor to the development and progression of breast cancer [5–7,24].

The primers used in this study to detect BRCA1 promoter methylation have been shown in previous research to correlate with reduced expression of the BRCA1 gene [16]. This effect has also been observed in malignant breast tumors with BRCA1 germline mutations, a genetic alteration that was found to promote genome instability in normal human mammary cells [8,11–13].

The BRCA1 promoter methylation rate of MBTs (41.67%) is in concordance with other studies using the same primers, which reported rates ranging from 27% to 59% [44,45]. Interestingly, certain histopathological features, such as ER/PR negative status, were found to be associated with BRCA1 aberrant DNA methylation [7]. Consequently, the enrichment of MBTs subset by tumors harboring an ER/PR negative status (66.67%) may contribute to enhancing the observed BRCA1 promoter methylation rate (Table 1). Indeed, BRCA1 promoter methylation, in our study, was more pronounced among ER/PR-negative MBTs than ER/PR-positive MBTs but without pointing to statistical significance (p = 0.3633) (Figure 3c).

Previous studies have suggested that BRCA1 promoter methylation and BRCA1 germline mutations are more commonly observed in triple-negative breast tumors compared to non-triple-negative breast tumors [7,46,47]. However, our results did not reflect this finding (Figure 3d), which may be due to the heterogeneity in the group of patients with an unmethylated BRCA1 promoter, which could contain potential cases with BRCA1 germline mutations. We can apply the same reasoning to question the association between the other aggressive features of the MBT group and BRCA1 promoter methylation reported in this study, especially since there are shared clinical and molecular features (BRCAness) between breast tumors associated with BRCA1 mutations and those with BRCA1 promoter methylation, as previously noted [8]. Indeed, the distribution of the analyzed MBTs according to the age at primary diagnosis revealed that the methylated group of MBTs formed a more homogeneous group of tumors compared to the unmethylated group, which exhibited greater diversity by including cases from a wider age range (standard deviation

(SD): 6.320 (M) vs. 9.264 (U)) (Figure 2b). Nevertheless, we noticed higher incidences of aggressive characteristics in the methylated group. These included a higher frequency of SBR grade III tumors, elevated levels of ki67 (≥30%), larger tumor size (>3 cm), absence of in situ component, negative ER/PR (estrogen and progesterone) receptor status, and positive Her2 receptor overexpression.

Regarding the benign breast lesions (BBLs), we reported 19.05% of BRCA1 promoter hypermethylation in the current study compared to MBTs (41.67%), and the high frequency of BRCA1 methylation in MBTs compared to BBLs is consistent with a report by Payadar et al. [48]. This aberrant DNA methylation of BRCA1 in non-cancerous mammary tissues needs a deep investigation in the aim to illustrate its early involvement in cell transformation. Some BBLs are reported to progress in breast cancer [49–51], and the proliferative character of BBLs seems to increase their risk for developing breast cancer [50]. However, the molecular mechanisms influencing this progression are still unknown. The hypermethylation of the BRCA1 promoter in BBLs could be considered as an early molecular biomarker for both genetic and epigenetic instabilities, and, consequently, patients with BBLs harboring BRCA1 hypermethylation can be considered patients at risk. Indeed, similar promoter hypermethylation is reported in 54 MBTs and 10 BBLs, suggesting that this epigenetic deregulation could be an early event in breast carcinogenesis [52].

The epigenetic silencing of a tumor suppressor gene in normal (non-cancerous) breast tissue adjacent to tumor lesions may render it a putative site of recurrent breast neoplastic transformation [53]. In the current study, a high rate of BRCA1 promoter methylation (46.67%) was observed in normal adjacent tissue samples. A previous analysis of single cells showed the presence of BRCA1 promoter methylation in normal mammary epithelial cells belonging to the histologically normal mammary adjacent tissues [54]. This disputes the risk of false-positive MSP results that may be caused by contamination by cancer cells when analyzing histologically normal adjacent tissues using the MSP technique; hence, any detected methylation in this type of tissue refers to the BRCA1 promoter methylation of normal cells. The rate of BRCA1 promoter methylation in NATs has been reported to vary depending on the distance between the normal tissue site and the tumor [55,56]. Specifically, the use of normal adjacent tissues 3 to 5 cm far from tumors showed a significant rate of BRCA1 promoter methylation, 51.90% [56], while distant normal tissues show no detection of BRCA1 promoter methylation [55].

In the current study, 60% of the MBTs and their paired NAT showed concordant BRCA1 promoter methylation status (Kappa = 0.182, p = 0.6831) (Table 3). The frequent detection of concordant aberrant DNA methylation in histologically normal adjacent tissues and tumor tissues has been attributed to various theories, including "field of cancerization", "microenvironmental factors", "BRCA1 constitutional methylation", and "age-related DNA methylation changes" [15,53,57]. According to the "field of cancerization" theory, preexisting fields of normal cells with molecular alterations (e.g., DNA methylation) may drive tumorigenesis, and some of those alterations may persist in the developed tumors [53]. Previous reports have shown that normal breast adjacent tissues carry tens of thousands of epigenetic changes, with 30% of them being shared with their matched tumors [58]. Moreover, constitutional BRCA1 promoter methylation was detected in 4–7% of newborns and young females [59], which may constitute an early driver of breast tumorigenesis. Notably, aberrant BRCA1 DNA methylation detected in the blood of females increases the risk of triple-negative breast tumors [15].

In addition to its potential diagnostic biomarker role, methylated BRCA1 could be epigenetically reactivated. One possible therapeutic approach could be epigenetic editing using CRISPR-based techniques, as it offers the potential for precision therapy and personalized medicine. This approach could enable targeted reactivation of BRCA1, specifically in tumor cells, potentially restoring its tumor-suppressive function and inhibiting cancer progression [60–62]. However, it is important to note that epigenetic editing technologies are still in the early stages of development, and their clinical application for cancer treatment is an active area of research. Further studies and advancements in this field are

needed to fully explore the potential of epigenetic editing as a therapeutic strategy for BRCA1-related cancers.

5. Conclusions

To summarize, our study shows that BRCA1 promoter methylation is a common alteration in malignant breast tumors and their adjacent normal tissues. Furthermore, tumors with this epigenetic mark were more likely to exhibit undifferentiated (score 3 of tubule formation) and hyperproliferative (score 3 of mitosis and high levels of Ki67%) phenotypes, lack of estrogen and progesterone receptors, overexpression of Her2 oncogene, and absence of in situ components. The high concordance in methylation status between tumor and normal tissues suggests that there is a consistent regulatory pathway driving BRCA1 epigenetic silencing in both types of tissues. These findings are particularly relevant given the increasing incidence of breast cancer in low- and middle-income countries (LMICs), where access to early detection and treatment options is limited, and the majority of breast-cancer-related deaths occur. The MSP technique used in this study is known for its low cost and convenience, as well as its high sensitivity to CpG methylation detection. Therefore, validating BRCA1 promoter methylation detection using this technique as a diagnostic and prognostic biomarker has the potential to improve early detection and treatment options, ultimately reducing the morbidity and mortality associated with breast cancer in LMICs.

Our results, supported by the literature, propose BRCA1 promoter hypermethylation as a potential useful biomarker for breast tumor aggressiveness, as well as an early biomarker for both BBLs at risk to progressing into cancer and NATs harboring the tumors at risk of recurrence. Normal adjacent tissues, usually used as normal control, are histologically non-cancerous but present a deep deregulation in proinflammatory pathways and genetic/epigenetic instabilities. Further translational studies exploring the relationship between BRCA1 promoter hypermethylation and the loss/decrease in its function in terms of DNA damage in both NATs and BBLs could reinforce the potential role of BRCA1 methylation as an early biomarker for patients at risk of developing cancer, as well as the clinical application of BRCA1 promoter methylation in the management of cancerous and non-cancerous breast diseases.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/genes14091680/s1, Table S1: BRCA1 promoter methylation: portions in MBTs, NATs, and BBLs. Table S2: BRCA1 promoter methylation status and its association with clinico-histopathological features in 48 malignant breast tumors. Figure S1: Gel electrophoresis image representing the hypermethylation analysis of BRCA1 promoter via MSP.

Author Contributions: Conceptualization, R.A.E.H.; methodology, R.A.E.H., S.F., Y.O., M.O., M.R.E. and A.E.; software, S.F. and Y.O.; validation, Z.Q., Y.O. and R.A.E.H.; formal analysis, Y.B., N.D., M.O., Z.Q., C.D., A.R., A.A.B. and R.A.E.H.; investigation, R.A.E.H., Y.B., M.O., A.A.B. and Y.O.; resources, R.A.E.H. and M.O.; data curation, S.F. and Y.O.; writing—original draft preparation, R.A.E.H. and Y.O.; writing—review and editing, R.A.E.H., C.D., A.R., Z.Q., Y.B., M.O. and Y.O.; visualization, R.A.E.H.; supervision, R.A.E.H., Y.B., N.D. and M.O.; project administration, R.A.E.H.; funding acquisition, R.A.E.H. and Y.B. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Archived datasets are not available due to ethical restrictions.

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Supplementary data

Table S1: *BRCA1* promoter methylation: portions in MBTs, NATs, and BBLs.

-	*n*	Portions of *BRCA1* promoter methylation	
MBTs	48	20/48	41.67%
NATs	15	7/15	46.67%
BBLs	21	4/21	19.05%

MBTs: Malignant Breast Tumors; NATs: Normal Adjacent Tissues; BBLs: Benign Breast Lesions.

Table S2: *BRCA1* promoter methylation status and its association with clinico-histopathological features in 48 Malignant Breast Tumors.

Total of cases = 48		n	Unmethylated		Methylated		Missing data
			n=28 (58.33%)	%	n=20 (41.67%)	%	
Age	≤ 47	19	10	35.71 (10/28)	9	45 (9/20)	-
	> 47	29	18	64.29 (18/28)	11	55 (11/20)	
Tumor size	≤3 cm	30	20	71.43 (20/28)	10	50 (10/20)	-
	>3 cm	18	8	28.57 (8/28)	10	50 (10/20)	
Tubule formation score	Score 1/2	12	10	41.67 (10/24)	2	11.76 (2/17)	7
	Score 3	29	14	58.33 (14/24)	15	88.24 (15/17)	
Nuclear grade score	Score 1/2	21	13	52 (13/25)	8	47.06 (8/17)	6
	Score 3	21	12	48 (12/25)	9	52.94 (9/17)	
Mitosis score	Score 1/2	26	18	72 (18/25)	8	47.06 (8/17)	6
	Score 3	16	7	28 (7/25)	9	52.94 (9/17)	
SBR grading	Grade I/II	21	16	57.14 (16/28)	5	26.32 (5/19)	1
	Grade III	26	12	42.86 (12/28)	14	73.68 (14/19)	
Ki67 level	<30 %	13	10	47.62 (10/21)	3	18.75 (3/16)	11
	≥ 30 %	24	11	52.38 (11/21)	13	81.25 (13/16)	
In situ component	Absence	21	10	41.67 (10/24)	11	61.11 (11/18)	6
	Presence	21	14	58.33 (14/24)	7	38.89 (7/18)	
Vascular emboli	Absence	34	19	67.86 (19/28)	15	75.00 (15/20)	-
	Presence	14	9	32.14 (9/28)	5	25.00 (5/20)	
Nodal status	Negative	21	12	52.17 (12/23)	9	56.25 (9/16)	9
	Positive	18	11	47.83 (11/23)	7	43.75 (7/16)	
ER/PR status	Negative	32	17	60.71 (17/28)	15	75 (15/20)	-
	Positive	16	11	39.29 (11/28)	5	25 (5/20)	
Her2 status	Negative	42	27	96.43 (27/28)	15	75 (15/20)	-
	Positive	6	1	3.57 (1/28)	5	25 (5/20)	
Molecular subtypes	$ER^+/PR^+/Her2^-$	12	10	35.71 (10/28)	2	10 (2/20)	-
	$ER^+/PR^+/Her2^+$	4	1	3.57 (1/28)	3	15 (3/20)	
	$ER^-/PR^-/Her2^+$	2	0	0.00	2	10 (2/20)	
	$ER^-/PR^-/Her2^-$	30	17	60.71 (17/28)	13	65 (13/20)	

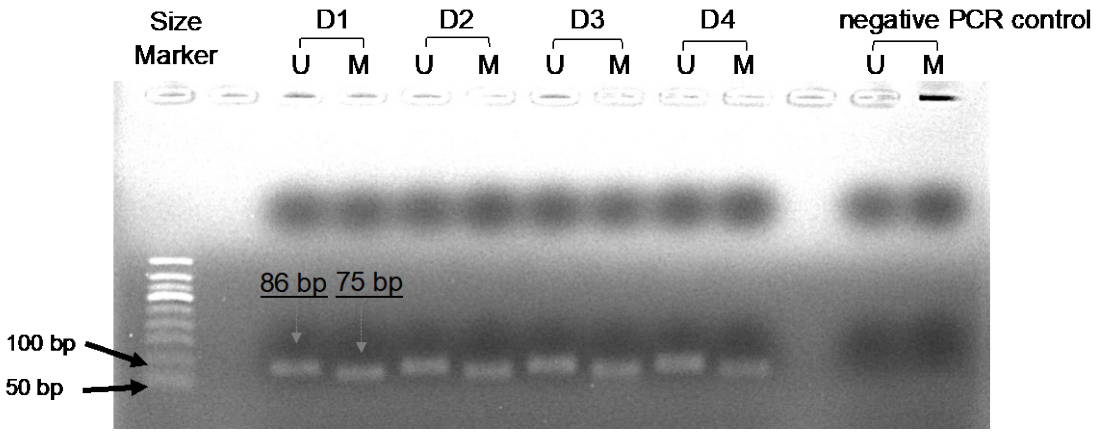
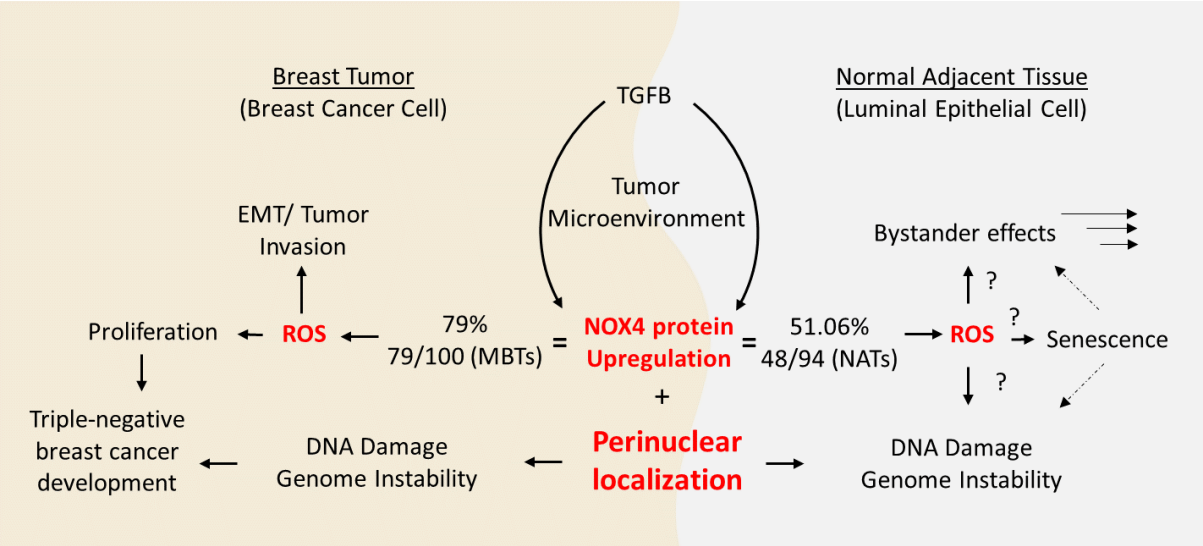


Figure S1: Gel electrophoresis image representing the hypermethylation Analysis of BRCA1 promoter via MSP. This analysis was conducted using MCF7 cultured cells, seeded at an initial concentration of 300 x 10^3 cells per well (plates of six wells). Genomic DNA was extracted at D1, D2, D3 and D4 (D:Day). U: MSP product using pair of primers amplifying the unmethylated state of the targeted region. M: MSP product using the pair of primers M amplifying the hypermethylated state of the targeted region.

III. Comparative Study of NOX4 and DUOX1 Protein Expression and Subcellular Localization in Breast Tumors and their Normal Adjacent Tissues: Uncovering NOX4's Role as a Biomarker for Aggressiveness and Progression of Breast Cancer (Article in preparation)

Introduction: NADPH oxidase enzymes are recognized as cellular sources of reactive oxygen species (ROS), and their expression was found to be dysregulated in many malignancies, potentially leading to increased levels of ROS and oxidative stress. Among NADPH oxidases, the NOX4 (NADPH oxidase 4) protein is known for its roles in epithelial-mesenchymal transition and angiogenesis in breast cancer. However, the clinical implications of its subcellular localization in breast tumorigenesis are not thoroughly examined. Furthermore, there is a lack of data on DUOX1 (another NADPH oxidase member) protein expression and subcellular localization in malignant breast tumors. In this regard, our study aimed to investigate the prevalence, clinical significance, and subcellular localization of NOX4 and DUOX1 proteins in breast tumors and their corresponding normal adjacent tissues using FFPE samples from breast cancer patients.

Utilizing immunohistochemistry on 198 human breast tissues, our analysis revealed a pronounced upregulation of NOX4 protein in 79% of malignant breast tumors (79/100) compared to their corresponding normal adjacent tissues (51.06%: 48/94). Conversely, DUOX1 expression was more prominent in the histologically normal adjacent tissues than in the tumors, suggesting a potential tumor-suppressive function. Notably, malignant breast tumors with increased NOX4 protein expression were more likely to exhibit positive nodal status and negative expression of estrogen receptor. Our findings also displayed that NOX4 exhibited diverse localization patterns in both tissue types, spanning the cytoplasm, perinuclear region, and nucleus. Notably, a statistically significant concordance in NOX4 protein subcellular localization was observed between malignant tumors and their corresponding normal tissues (*Kappa*= 0.487). Furthermore, the perinuclear presence of NOX4 in malignant tumors was correlated significantly with the triple-negative phenotype (p=0.0276) and a higher tumor grade (p=0.0249). This association is particularly significant because the triple-negative subtype is recognized for its aggressive nature, largely attributed to the rarity of therapeutic targets compared to other subtypes. Overall, the findings we share in this article motivate deeper exploration into the role of the NOX4 protein in breast cancer biology.



Graphical abstract: Comparative analysis of NOX4 protein levels and subcellular localization in malignant breast tumors (MBTs) and their normal adjacent tissues (NATs). We assessed NOX4 protein expression and subcellular localization using immunohistochemistry on FFPE breast cancer samples. We observed a significant upregulation of NOX4 protein expression in cancerous samples compared to the surrounding normal tissue samples, which aligns with NOX4 protein involvement with breast malignancy. Moreover, the similar subcellular localization of NOX4 protein in both tissue types, indicated by a *Kappa* statistic of 0.487, suggests a potential shared regulatory mechanism, possibly influenced by the tumor microenvironment factor TGFβ. This shared regulation might explain the increased expression of NOX4 protein in NATs. The increased expression and the similar subcellular localization of NOX4 protein in both cancerous and normal adjacent tissues reflect broader implications of this oxidative marker in breast cancer development and progression. The established role of NOX4 protein in promoting epithelial-mesenchymal transition (EMT) and invasion in human breast cancer cell lines was represented in our study by a high rate of nodal invasion in MBTs with elevated NOX4 protein expression. A higher rate of Ki67 overexpression was also observed in MBTs with elevated NOX4 protein expression, suggesting its role in promoting a proliferative phenotype in breast cancer. Furthermore, a correlation between NOX4 protein overexpression and the triple-negative breast cancer phenotype was evident, suggesting a potential role of this oxidative marker in the development of this aggressive breast cancer subtype. The overexpression of NOX4 protein in normal mammary cells warrants further investigation into its roles in inducing DNA damage, senescence, and bystander effects. Such research could be pivotal in improving recurrence assessment post-surgery and identifying early biomarkers for breast tumorigenesis. Beyond its significant association with the triple-negative phenotype of breast cancer and SBR grade III, the perinuclear localization of NOX4 protein in MBTs and NATs may be implicated in DNA damage, potentially leading to genome instability and serving as an early driver of breast tumorigenesis.

Comparative Study of NOX4 and DUOX1 Protein Expression and Subcellular Localization in Breast Tumors and their Normal Adjacent Tissues: Uncovering NOX4's Role as a Biomarker for Aggressiveness and Progression of Breast Cancer

Oubaddou Yassire et al., 2024 (In preparation)

Abstract:
NADPH oxidases, especially the NOX4 protein, play a pivotal role in cancer pathogenesis. While NOX4 is recognized for its roles in breast tumorigenesis processes like epithelial-mesenchymal transition, angiogenesis, and metastasis, its clinical implications and subcellular localization in breast tumorigenesis remain underexplored. Our retrospective analysis investigated the prevalence and clinical implications of NOX4 and DUOX1 protein expression and subcellular localization in malignant breast tumors (MBTs) and their normal adjacent tissues (NATs). Utilizing immunohistochemistry on 198 human breast tissues (104 MBTs and 94 NATs), we found a pronounced ($p<0.0001$) upregulation of NOX4 protein in 79% of MBTs (79/100) compared to 51.06% in NATs (48/94). Conversely, DUOX1 protein was predominantly expressed in NATs over MBTs ($p=0.0096$). MBTs with elevated NOX4 levels were enriched with estrogen-negative status (39.74% vs. 19.05%) and positive nodal invasion (47.62% vs. 25%). We observed diverse patterns of NOX4 protein subcellular localization in MBTs and their NATs, spanning the cytoplasm, perinuclear region, and nucleus. The significant concordance in NOX4 protein subcellular localization patterns between MBTs and their corresponding NATs (*Kappa*= 0.487) suggests a potential interplay between these tissues in regulating the subcellular localization of NOX4. Additionally, the perinuclear presence of NOX4 protein in MBTs exhibited a marked correlation with the triple-negative phenotype ($p=0.0276$) and SBR grade III ($p=0.0249$). In conclusion, our findings highlight the pivotal role of NOX4 protein in breast tumorigenesis, offering valuable insights for future diagnostic and therapeutic strategies in breast cancer.

Keywords: NADPH oxidases; NOX4; DUOX1; Breast cancer; Immunohistochemistry; Normal adjacent tissue; Subcellular localization; Triple-negative breast cancer

1. Introduction:

Breast cancer is a global health concern affecting women (Sung et al., 2021). It is a complex and heterogeneous pathology characterized by many factors, including genetic, epigenetic, lifestyle, and environment (Koboldt et al., 2012; Łukasiewicz et al., 2021). This pathology is categorized into several molecular subtypes, each defined by unique characteristics that differ in prognosis, diagnosis, and treatment responsiveness (Koboldt et al., 2012; Lehmann et al., 2011; Perou et al., 2000; Prat et al., 2010; Sørlie et al., 2001). Indeed, a particularly aggressive form of the disease is triple-negative breast cancer (TNBC), which constitutes up to 15% of all breast cancer cases (Kim et al., 2022; Koboldt et al., 2012). TNBC lacks estrogen receptor expression and Her2 receptor overexpression, rendering it untargetable by hormone or anti-HER2 targeted therapies (DeSantis et al., 2019; Kim et al., 2022). Those tumors' complexity and poor prognosis require ongoing research on the molecular signaling pathways involved in their development and progression to reveal new personalized therapeutic targets (DeSantis et al., 2019; Kim et al., 2022).

Reactive oxygen species (ROS) are highly reactive molecules derived from oxygen. Aberrant amounts of ROS have been observed in several pathologies, including cancer (Vermot et al., 2021; Waghela et al., 2021). The major reactive oxygen species are hydroxyl radical ($OH^{\bullet}$), superoxide anion ($O_2^{\bullet-}$), and hydrogen peroxide (H_2O_2) (Waghela et al., 2021). At a physiological level, ROS are byproducts of oxygen metabolism and have roles in homeostasis and signaling (Sies & Jones, 2020). NADPH oxidase enzymes are considered one of the major sources of ROS. This family consists of seven enzymes, specifically NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1, and DUOX2 (Ameziane-El-Hassani et al., 2016).

In cancer, ROS carry a dual function (Aggarwal et al., 2019). At moderate concentrations, ROS trigger survival signaling pathways, including MAPK/ERK1/2, p38, c-Jun N-terminal kinase (JNK), and PI3K/Akt, while at high concentrations, they induce apoptosis (Aggarwal et al., 2019). In particular, hydrogen peroxide (H_2O_2) plays a role in signal transduction due to its stability and ability to diffuse through organelles and cellular membranes (Harrison & Selemidis, 2014; Meitzler et al., 2019). NOX4, DUOX1, and DUOX2 are known to primarily produce H_2O_2 (Meitzler et al., 2019). Interestingly, H_2O_2 produced by DUOX1 causes genomic instability and transformation of radio-treated thyroid cells (Ameziane-El-Hassani et al., 2015). NOX4 is the most overexpressed NOX member in malignancies and is consequently the most studied member of NOXs to carry functions in cancer biology, including breast cancer (Azouzi et al., 2017; Eun et al., 2019; Lin et al., 2017; X. Wang et al., 2021). NOX4 overexpression was associated with a worse prognosis in various solid malignancies, including breast cancer (Eun et al., 2019; Lin et al., 2017; Ma et al., 2021; X. Wang et al., 2021). An upregulation of NOX4 was correlated with reduced survival and increased proliferation markers in patients with P53-mutated tumors (Ma et al., 2021). In breast cancer, increased levels of NOX4 protein have been linked to nodal invasion and a higher histological grade (X. Wang et al., 2021).

Contrary to other NOXs, NOX4 is regulated solely at the transcriptional level and is constitutively active within cells due to its unique structure (Guo & Chen, 2015; Siuda et al., 2012). Therefore, NOX4 protein levels are directly related to ROS production (Fenniche et al., 2023). NOX4 has been detected in various subcellular localizations, including the nucleus, endoplasmic reticulum, perinuclear vesicles, mitochondria, and plasma membrane (Meitzler et al., 2019). Foor splice variants of the NOX4 (NOX4A) protein have been identified in a lung cancer cell line: NOX4B, C, D, and E. Isoforms B and C are believed to lack the function of ROS production. Isoforms D and E are ROS producers lacking the transmembrane domains but preserve the other two domains. D and E splice variants were shown to produce the same levels of ROS as the prototype isoform NOX4A (Goyal et al., 2005). Prior research indicates that the NOX4 protein is present in various breast cancer-associated cells, playing a pivotal role in promoting tumor growth, proliferation, EMT, angiogenesis, and metastasis (Boudreau et al., 2012; Graham et al., 2010; Kim et al., 2022; Liu et al., 2022; Mir et al., 2022; X. Wang et al., 2021). Such cells include cancer cells, the endothelial cells of vessels irrigating the tumor, and cancer-associated fibroblasts (CAFs) (Graham et al., 2010; Mir et al., 2022; X. Wang et al., 2021). All these findings show the importance of investigating NOX4 and DUOX1 protein expression and subcellular localization in breast tumors.

Using immunohistochemistry, this study investigated the expression and subcellular localization of NOX4 and DUOX1 proteins in malignant breast tumors (MBTs=104) and their corresponding normal adjacent tissues (NATs=94). NOX4 protein expression was significantly higher in MBTs compared to NATs, with 79% of tumor tissues showing elevated expression. Conversely, DUOX1 protein expression was notably reduced in tumor tissues. Subcellular localization patterns of NOX4 in both tissue types revealed three primary patterns: cytoplasmic, combined perinuclear and cytoplasmic, and combined nuclear and cytoplasmic. Interestingly, there was a significant concordance in NOX4 subcellular localization between MBTs and NATs.

Additionally, high levels of NOX4 protein were enriched with aggressive breast cancer features, including negative estrogen receptor status and nodal invasion. Notably, a significant association was identified between the perinuclear subcellular localization of NOX4 protein in MBTs and NATs and the triple-negative breast cancer subtype, suggesting its possible involvement in the evolution and progression of this prevalent and aggressive breast cancer subtype. Additionally, the analysis of NOX4's subcellular localization in NATs and MBTs and its expression levels offers clinical insights into the tumor microenvironment's role in modulating NOX4's documented functions in cancer, particularly breast cancer.

These findings highlight the significance of the NOX4 protein as a potential diagnostic, prognostic, and therapeutic biomarker for breast cancer.

2. Materials and methods:

2.1. Study subjects

This study was ethically approved by the Ethics Committee for Biomedical Research (CERB) at the Faculty of Medicine and Pharmacy in Rabat, under the approval number 52/20.

We conducted a retrospective analysis of 198 samples from breast cancer patients diagnosed at the Department of Anatomical Pathology in the Military Hospital of Instruction Mohammed V in Rabat (HMIMV-R) between July 2017 and December 2019. Our cohort comprised 104 malignant breast tumor samples and 94 of their normal adjacent tissues. These samples underwent histological classification based on the World Health Organization (WHO) classification guidelines (Lakhani et al., 2012). Additionally, experienced pathologists from the HMIMV-R performed a retrospective validation of the diagnoses. Furthermore, we compiled a detailed database from patient medical records to gather clinicopathological information. Any data not found in these records is considered unavailable and categorized as missing in Table 1, and Tables S3 and S4 (Supplementary materials).

2.2. Immunohistochemical staining of NOX4 and DUOX1 enzymes.

The immunohistochemical evaluation of NOX4 utilized a rabbit polyclonal anti-NOX4 antibody (ab154244, Abcam, Cambridge, UK) that recognizes the aa 252-564 region of Human NADPH oxidase 4. Immunostaining for DUOX1 was performed using a rabbit polyclonal anti-DUOX1 antibody ((Ameziane-El-Hassani et al., 2015); Eurogentec, Belgium), which was specifically generated against a unique segment of the DUOX1 protein, spanning amino acid residues 988–1011. The specificity of this antibody for DUOX1 ensures that it does not cross-react with DUOX2, thereby providing a reliable method for the exclusive detection and visualization of DUOX1 in the analyzed samples (Ameziane-El-Hassani et al., 2015). Staining procedures were performed using Dako EnVision™ FLEX High pH 'LINK'/FLEX IHC Microscope Slides (Agilent, Santa Clara, CA, USA) on an Autostainer Link 48 device (Agilent), following the manufacturer's guidelines.

2.3. Scores definition of NOX4 and DUOX1 intensity of staining:

Experienced pathologists assigned two categories for NOX4 or DUOX1 expression: A low score (<2) indicates either an absence or low expression; A high score (≥2) signifies medium or high expression. Typically, trained pathologists use a scoring system for staining intensity as follows: Negative (0), Weak (1), Moderate (2), and Strong (3). For the purpose of our study, we grouped the scores into two categories: scores <2 (including 0 and 1) and scores ≥2 (including 2 and 3). We noted heterogeneous immunostaining for the NOX4 or DUOX1 proteins in certain instances. When this occurred, we adopted the highest score if it appeared in a significant portion of the tumor area. However, if the score was confined to a small, localized tumor region, it was not factored into the final score definition.

2.4. Subcellular localization determination:

The expertise of the pathologists involved in this study allowed them to differentiate between various subcellular localizations under high microscopic magnification. Sometimes, distinguishing between staining in the nucleus and the perinuclear region is particularly challenging. While proteins in the nucleus appear as distinct dots, those in the perinuclear region form a ring around the nucleus. Even when this perinuclear staining seems to cover the nucleus, a clear luminous gap remains within the nucleus, indicating an unstained nucleus. The blue counterstain (Hematoxylin) further enhances this distinction.

2.5. Selection of samples for NOX4 and DUOX1 protein expression and subcellular analysis:

We began our study with 208 samples for NOX4 analysis, consisting of 109 Malignant Breast Tumors (MBTs) and 99 Normal Adjacent Tissues (NATs). After immunostaining and microscopic examination, 10 samples (5 MBTs and 5 NATs) were considered non-exploitable due to the poor quality of FFPE blocks and were thus excluded. This left us with 104 MBTs and 94 NATs for further analysis. NOX4 protein expression status was exploitable in 100 MBTs and 94 NATs. DUOX1 protein was examined in 88 MBTs and 84 NATs.

We investigated the protein subcellular localization of NOX4 and DUOX1 in the exploitable samples. For samples that lacked NOX4 or DUOX1 staining (score 0), determining subcellular localization was impossible, leading to their exclusion from the subcellular localization counts. Specifically, for NOX4, 4 MBTs and 8 NATs were excluded, leaving observable subcellular localization in 96 MBTs and 87 NATs. For NOX4's subcellular localization, we identified three primary patterns and a minor group of 7 samples (5 MBTs and 2 NATs). To maintain statistical integrity, this minor group was excluded from further analysis. Thus, 91 MBTs and 85 NATs, which exhibited the three main NOX4 subcellular patterns, were considered for subsequent analysis.

2.6. Statistical analysis

Statistical analyses and the creation of graphics were performed with GraphPad Prism8. A range of tests were used to determine the statistical relevance of various correlations, such as the Fisher's exact test, Chi-square test, and Unpaired t-test. The agreement of NOX4 subcellular localization between normal adjacent tissues (NATs) and their corresponding malignant breast tissues (MBTs) was assessed using Cohen's Kappa test. Any *p*-value below 0.05 signified a statistical significance.

3. Results:

3.1. Characteristics of the study population

This retrospective study includes a cohort of 198 samples (104MBTs and 94NATs) taken from 104 patients diagnosed with malignant breast tumors. The collected clinical and histopathological data provided a broad spectrum for the subsequent analysis. At the time of diagnosis, 38.83% (40/103) of the patients were aged 47 years or below (Table 1). About 36.27% (37/102) of tumors had sizes larger than 3 cm. About 62.5% (55/88) of tumors displayed a tubule formation score of 3, and 40.45% (36/89) showed a nuclear grade score of 3 (Table 1). The mitosis score was predominantly 1 and 2, observed in 71.91% (64/89) of the cases, while a score of 3 was less common (28.09%; 25/89). In terms of SBR grading, 63.37% (64/101) of the tumors were classified as Grade I or II, and 36.63% (37/101) as Grade III (Table 1). Ki67 level was less than 30% "< 30 %" in 55.13% (43/78) of the cases and 30% or more "≥ 30 %" in 44.87% (35/78) of the cases. Vascular emboli were found in 36.63% (37/101) of the cases, and 44.58% (37/83) of cases showed a nodal invasion (Table 1). About 34.95% (36/103) of the cases lack the expression of estrogen receptor, while 65.05% (67/103) of cases have shown a positive status of this expression (Table 1). The Her2 status was predominantly negative (89.8%: 88/98) and positive in only 10.2% (10/98) cases. Finally, when considering the molecular subtype, 66.34% (67/103) of the cases were breast tumors of the luminal breast cancer (Positive for estrogen receptor), 30% (30/103) were triple-negative breast cancer (Negative for both estrogen receptor and Her2 receptor), and only 3.96% (4/103) of Her2-enriched breast tumors (Negative for estrogen receptor and positive for Her2 receptor) (Table 1).

Table 1: Characteristics of the study population

Clinical and histopathological features		*Malignant breast tumors (n=104)*	
		n	*%*
Age	≤ 47 years	40	38.83 (40/103)
	> 47 years	63	61.17 (63/103)
	Missing data	1	
Tumor size	≤ 3 cm	65	63.73 (65/102)
	> 3 cm	37	36.27 (37/102)
	Missing data	2	
Tubule formation score	Score 1/2	33	37.50 (33/88)
	Score 3	55	62.50 (55/88)
	Missing data	16	
Nuclear grade score	Score 1/2	53	59.55 (53/89)
	Score 3	36	40.45 (36/89)
	Missing data	15	
Mitosis score	Score 1/2	64	71.91 (64/89)
	Score 3	25	28.09 (25/89)
	Missing data	15	
SBR grading	Grade I/II	64	63.37 (64/101)
	Grade III	37	36.63 (37/101)
	Missing data	3	
Ki67 level	< 30 %	43	55.13 (43/78)
	≥ 30 %	35	44.87 (35/78)
	Missing data	26	
Ki67 level	< 30 %	43	55.13 (43/78)
	≥ 30 %	35	44.87 (35/78)
	Missing data	26	
Vascular emboli	Absence	64	63.37 (64/101)
	Presence	37	36.63 (37/101)
	Missing data	3	
Nodal status	Negative	46	55.42 (46/83)
	Positive	37	44.58 (37/83)
	Missing data	21	
ER status	Negative	36	34.95 (36/103)
	Positive	67	65.05 (67/103)
	Missing data	1	
Her2 status	Negative	88	89.80 (88/98)
	Positive	10	10.20 (10/98)
	Missing data	6	
Molecular subtype	TNBC	30	29.70 (30/101)
	Luminal BC	67	66.34 (67/101)
	Her2-enriched	4	3.96 (4/101)
	Missing data	3	

3.2. NOX4 and DUOX1 expression levels and subcellular localization in malignant breast tumors (MBTs) and their normal adjacent tissues (NATs)

The current study aimed to investigate the expression levels and subcellular localization of NOX4 and DUOX1 proteins in breast tumor tissues and their corresponding normal adjacent tissues (NAT) using immunohistochemistry (Figure 1a & 1b). The specificity of the anti-NOX4 antibody and its dilution choice (1:250) was confirmed using human kidney tissues, while anti-DUOX1 specificity and dilution choice (1:400) were validated on human lung tissues (Data not shown). Both kidney and lung tissues are known to express NOX4 and DUOX1 proteins, respectively (Ameziane-El-Hassani et al., 2015, 2016). Interestingly, nearly all the examined FFPE blocks contained areas of tumor and normal

adjacent tissue. This enabled us to conduct a unified IHC analysis on a single slide for each pair of MBT and NAT, as illustrated in Figures 1a and 1b.

3.3. Levels of expression of NOX4 and DUOX1 proteins in MBTs and NATs

In our investigation, we observed the expression of NOX4 and DUOX1 in cancer cells located in tumor regions and in luminal epithelial cells in the surrounding normal areas, as shown in the highly magnified images in Figure 2d. Our results demonstrated that NOX4 protein expression was significantly higher in both malignant breast tumors and normal adjacent tissues than DUOX1 protein expression (Figure 1c & 1d & Table S1). Indeed, NOX4 protein expression was significantly increased (p<0.0001) in tumor tissues (79% showed a score ≥2: 79/100), compared to normal adjacent tissues (51.06% showed a score ≥2: 48/94) (Figure 1c & Table S1). In contrast, DUOX1 protein expression was significantly decreased (p= 0.0096) in breast tumor tissues (94.32% showed a low score (<2): 83/88), compared to normal adjacent tissues (80.95% showed a low score (<2): 68/84) (Figure 1d & Table S1).

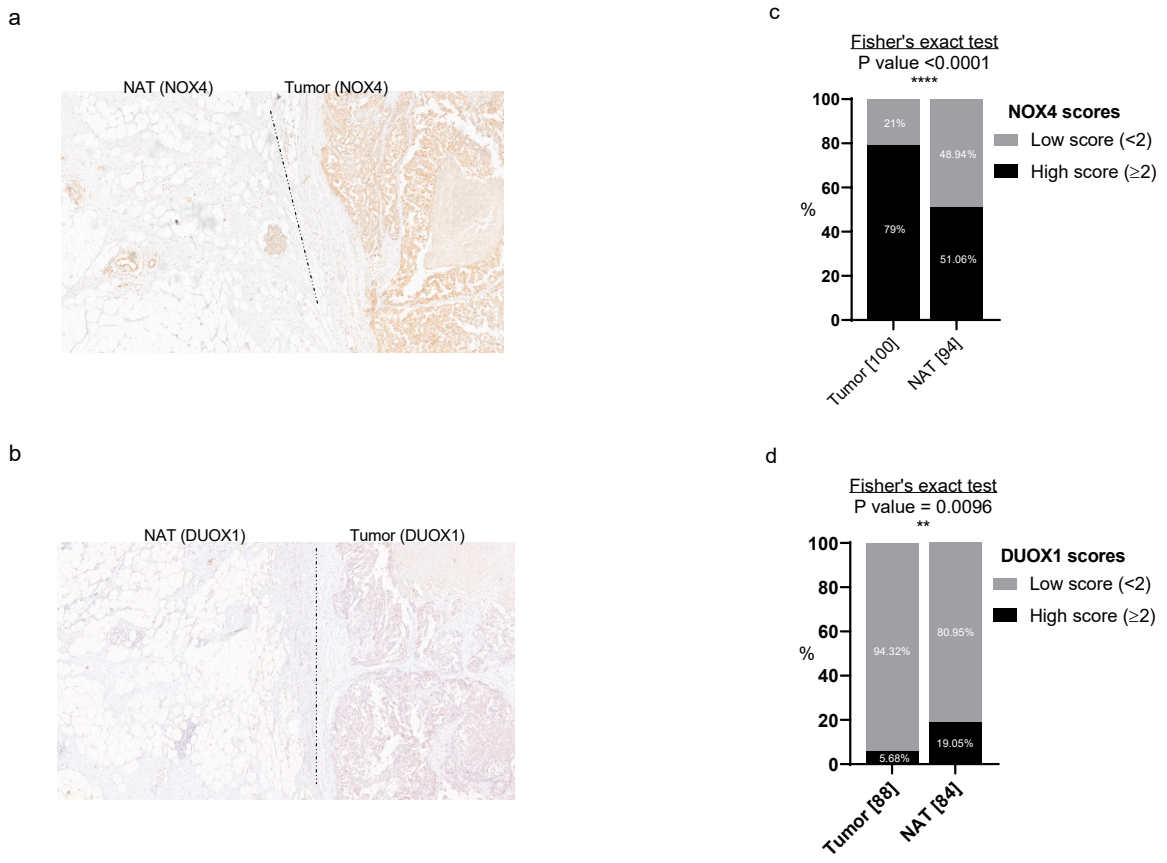


Figure 1: Immunohistochemical analysis of NOX4 and DUOX1 protein expression in breast tumor and normal adjacent tissues. a) Representative example of NOX4 expression in a breast tumor and its normal adjacent tissue (NAT). b) Representative example of DUOX1 expression in a breast tumor and its normal adjacent tissue (NAT). c) Comparative analysis of NOX4 protein expression between breast tumor tissues (100 Tumors) and their corresponding normal adjacent tissues (94 NAT). c) Comparative analysis of DUOX1 protein expression between breast tumor tissues (88 Tumors) and their corresponding normal adjacent tissues (84 NAT). *The statistical significance is affirmed by a *P*-value under 0.05. NOX4: NADPH Oxidase 4; DUOX1: Dual Oxidase 1; NAT: Normal Adjacent Tissue

3.4. Subcellular localization of NOX4 protein in breast cancer cells and luminal epithelial cells:

The subcellular localization of NOX4 and DUOX1 proteins was examined in malignant breast tumors (MBTs) and their normal adjacent tissues (NATs). Our results demonstrated that NOX4 protein exhibited diverse subcellular localizations in both tissue types (Figure 2a & Table S2). The three major

staining patterns were: i) cytoplasmic (Cyto) localization, which was observed in 41.76% (38/91) of tumor samples and 42.35% (36/85) of NAT samples, ii) a combination of perinuclear and cytoplasmic (Peri/Cyto) localization, observed in 28.57% (26/91) of tumor samples and 24.71% (21/85) of NAT samples, and iii) a combination between nuclear and cytoplasmic (Nuc/Cyto) localization, observed in 29.67% (27/91) of tumor samples and 32.94% (28/85) of NAT samples (Figure 2a and Table S2). DUOX1 primarily displayed cytoplasmic staining, with only one pair of tissues (Tumor and its corresponding NAT) exhibiting a combined perinuclear and cytoplasmic pattern of DUOX1 subcellular localization (Figure 2b). In addition, tumors with a cytoplasmic NOX4 subcellular localization showed lower scores (<2), accounting for 23.68% (9/38) of the samples, compared to those with a Peri/Cyto localization (7.69%: 2/26) (Figure 2c).

Beyond the slight similarity in rates of NOX4 subcellular localizations observed between tumors and NATs (Figure 2a), we observed a significant concordance (*Kappa*= 0.487, indicating a moderate agreement) in these localizations between both types of tissue (Figure 2d and Table 2). Specifically, 63.64% (21/33) of tumor samples demonstrating NOX4 cytoplasmic localization had corresponding NATs exhibiting the same pattern (Figure 2d and Table 2). Furthermore, 56% (14/25) of tumors with a combined perinuclear and cytoplasmic localization had corresponding NATs showing the same localization (Figure 2d and Table 2), and 81.82% (18/22) of tumors with a combined nuclear and cytoplasmic localization had corresponding NATs with the same pattern (Figure 2d and Table 2). These findings raise crucial questions about the relationship between tumors and NATs in regulating the subcellular localization of NOX4 protein.

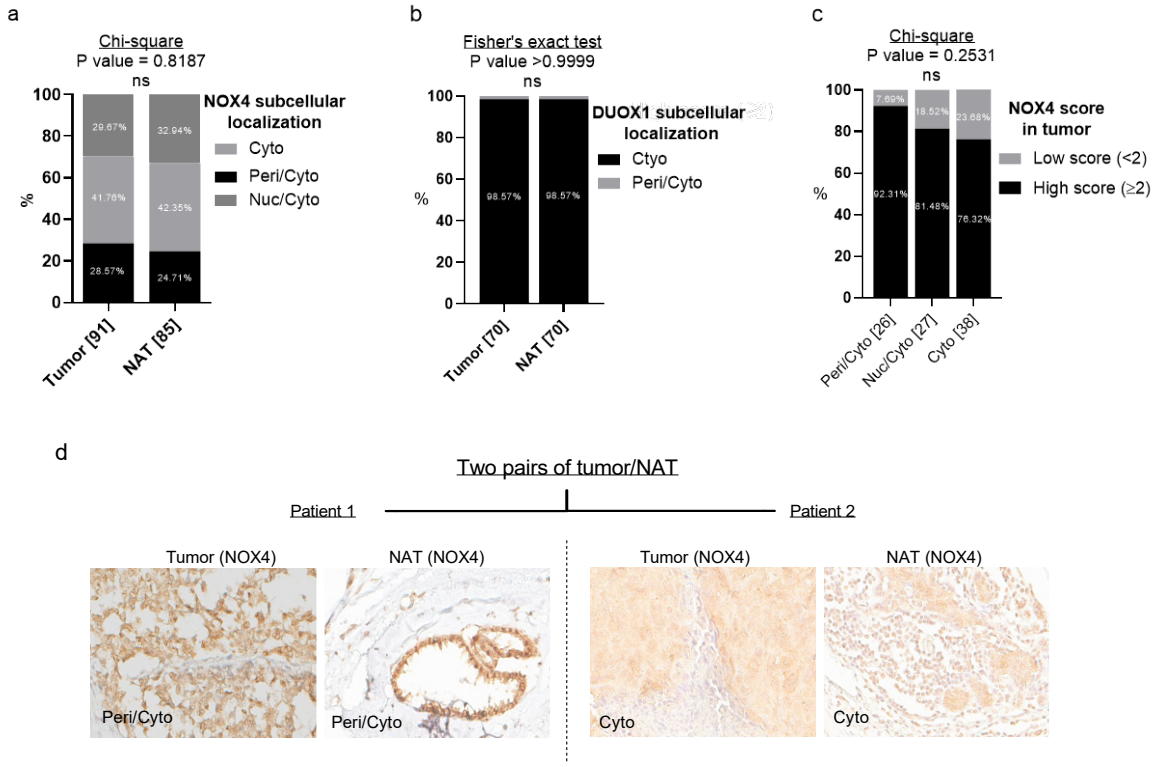


Figure 2: NOX4 and DUOX1 proteins subcellular localization in breast tumor and normal adjacent tissues. a) Subcellular localization patterns of NOX4 protein in breast tumors (n=91) and their normal adjacent tissues (n=85). b) Subcellular localization patterns of DUOX1 protein in breast tumors (n=70) and their normal adjacent tissues (n=70). c) Subcellular localization of NOX4 varies depending on its intensity score of expression. d) Representative images of two pairs of tumor and NAT showing a concordant subcellular localization of NOX4 protein. *The statistical significance is affirmed by a *P*-value under 0.05. NOX4: NADPH Oxidase 4; DUOX1: Dual Oxidase 1; NAT: Normal Adjacent Tissue.

Table 2: Concordance in subcellular localizations of NOX4 protein between breast tumor tissues and their corresponding normal adjacent tissues (NATs).

Tumor	NAT	n	Concordance	Discordance	Testing agreement
Cyto	Cyto	21	63.64% (21/33)	36.36% (12/33)	*Kappa*= 0.487 (Kappa between 0.41 and 0.60: Moderate agreement)
	Peri/cyto	7			
	Nuc/Cyto	5			
Peri/Cyto	Cyto	8	56% (14/25)	44% (11/25)	
	Peri/cyto	14			
	Nuc/Cyto	3			
Nuc/Cyto	Cyto	4	81.82% (18/22)	18.18% (4/22)	
	Peri/cyto	0			
	Nuc/Cyto	18			

3.5. Association between levels of expression of NOX4 protein and aggressive characteristics of breast cancer

A significant majority of breast tumor samples (79%:79/100) showed high levels of NOX4 protein (≥2), suggesting a possible widespread role of this NADPH oxidase in breast tumorigenesis and progression (Figure 1b and Table S3). Our analysis showed that among all aggressive features of breast cancer, the negative status (39.74% vs. 19.05% in low NOX4 score) and the presence of nodal invasion (47.62% vs. 25% in low NOX4 score) were more likely to exhibit increased levels of NOX4 protein (high levels of NOX4; ≥2).

About 42.31 % (33/78) of cases with high levels of NOX4 (≥2) were diagnosed at an early age (≤ 47), compared to 28.57% (6/21) of cases with low NOX4 levels (<2) diagnosed within this age range (Figure 3a). Furthermore, 49.15% (29/59) of tumors with high levels of NOX4 (≥2) showed increased levels of the proliferation biomarker, ki67 protein, compared to tumors with low NOX4 protein levels (<2), which exhibited this feature in 31.25% (5/16) of cases (Figure 3c). A slight increase in mitosis score of 3 was observed among tumors with high NOX4 protein levels (30.43%: 21/69), compared to those with low NOX4 levels (18.45%: 3/16) (Figure 3e). Oppositely, nuclear grade, SBR grading, and tumor size did not seem to be affected by NOX4 levels of expression (Figures 4b & 4c, Figure 3b). Notably, nodal invasion was more prevalent in tumors with high NOX4 protein levels (47.62%: 30/63), compared to tumors with low levels of this protein (25%: 4/16) (Figure 4f). In contrast, we noticed only a marginal difference in the status of vascular emboli between tumors with high and low levels of NOX4 protein (Figure 4e).

Tumors with high levels of NOX4 (<2) protein had a prevalent negative estrogen receptor expression status (39.74%: 31/78), compared to tumors with low NOX4 levels (<2), with only 4 cases (19.9%) out of 21 (Figure 5a). This observation could be attributed to an overrepresentation of tumors with high NOX4 levels among triple-negative (TNBC) and Her2-enriched breast cancer subtypes, typically lacking estrogen receptor expression. Indeed, NOX4 protein levels were more pronounced within tumors with TNBC phenotype (32.90%: 25/76), while in tumors with low NOX4 levels, the TNBC phenotype was less presented (19.10%: 4/21) (Figure 5c). Moreover, all Her2-enriched breast cancer tumors displayed high NOX4 protein levels (100%: 4/4) (Figure 5d). In contrast, the luminal breast cancer (positive for estrogen receptor) phenotype was more presented among tumors with low levels of NOX4 (80.95%: 17/21), compared to tumors with high levels of this protein (61.84%: 47/76) (Figure 5e).

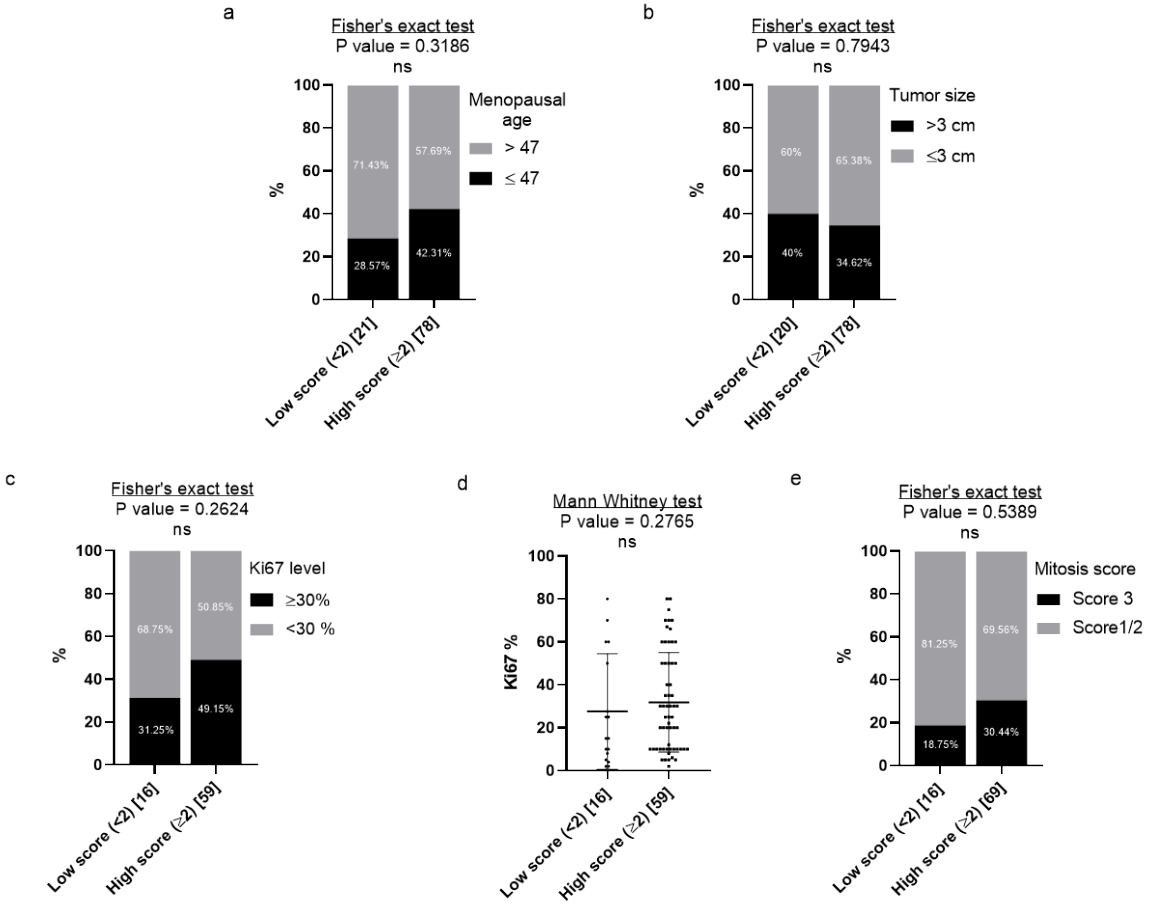


Figure 3: Association between NOX4 expression levels (low score (<2) or high score (≥2)) in tumor samples and various clinical and histopathological characteristics of malignant breast tumors: (a) Menopausal age, (b) Tumor size, (c and d) ki67 level, (e) Mitosis score. The missing data for each feature are presented in Table S3. The statistical significance was determined by GraphPad 8. *The statistical significance is affirmed by a *P*-value under 0.05.A

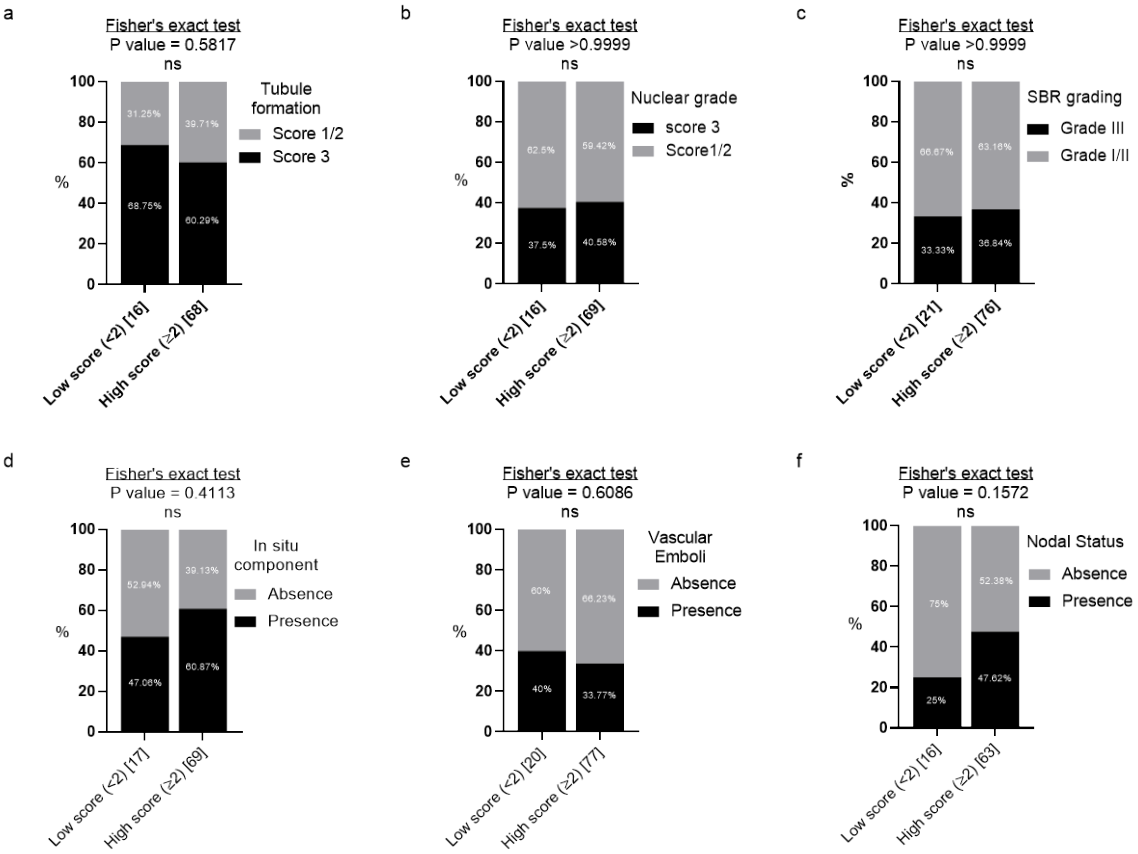


Figure 4: Association between NOX4 expression levels (low score (<2) or high score (≥2)) in tumor samples and various clinical and histopathological characteristics of malignant breast tumors: (a) Tubule formation score, (b) Nuclear grade, (c) SBR grading, (d) In situ component status, (e) vascular emboli, (f) Nodal status. The missing data for each feature are presented in Table S3. The statistical significance was determined by GraphPad 8. *The statistical significance is affirmed by a *P*-value under 0.05. SRB: Scarff-Bloom-Richardson.

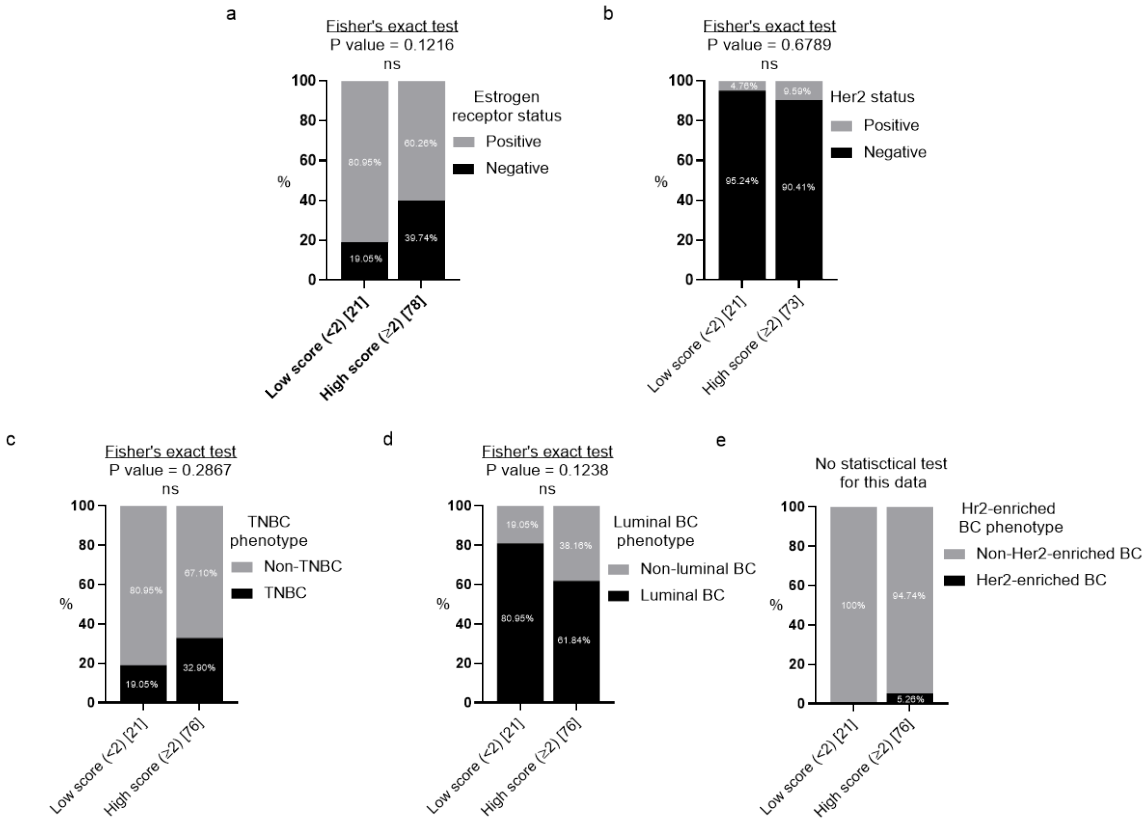


Figure 5: Association between NOX4 expression levels (low score (<2) or high score (≥2)) in tumor samples and various molecular biomarkers and subtypes: (a) Estrogen receptor status, (b) Her2 status, (c) Triple-negative breast cancer (TNBC) phenotype, (d) Luminal breast cancer phenotype, (e) Her2-enriched breast cancer phenotype. The missing data for each feature are presented in Table S3. The statistical significance was determined by GraphPad 8. *The statistical significance is affirmed by a *P*-value under 0.05. Her2: Human epidermal growth factor receptor 2; ER: Estrogen Receptor.

3.6. Association between subcellular localizations of NOX4 protein and aggressive characteristics of breast cancer

Tumor samples were categorized into three groups based on the major observed NOX4 protein subcellular localization patterns in malignant breast tumors. The most prevalent category exhibited cytoplasmic NOX4 localization (Cyto, 41.76%: 38/91), followed by a category with both nuclear and cytoplasmic localization (Nuc/Cyto, 29.67%: 27/91). The third category exhibited a combination of perinuclear and cytoplasmic localization (Peri/Cyto, 28.57%: 26/91).

The Peri/Cyto category was significantly associated with aggressive features of breast cancer, including SBR grade III (p=0.0249), triple-negative phenotype (p=0.0276), and non-luminal BC subtype (p=0.0418). This group also showed a positive trend towards other aggressive characteristics such as high levels of ki67 protein and mitosis score of 3, and vascular emboli presence. Conversely, the Nuc/Cyto group was significantly enriched with the lower SBR grades (I and II) (p=0.0249) and the luminal BC phenotype (p=0.0418), and non-triple negative BC phenotype (p=0.0276), along with positive trend awards other good prognosis features such as a mitosis score of 1 and 2 and lower Ki67 levels. Tumors with exclusively cytoplasmic subcellular localization showed intermediate rates of aggressive breast cancer features, falling between the other two groups.

Tumors with Peri/Cyto localization were more likely to be diagnosed at an early age (50%: 13/26), compared to those with cytoplasmic staining (40.54%: 15/37) or Nuc/Cyto localization (37.04%: 10/27) (Figure 6a). Moreover, tumors with Peri/Cyto localization were more likely to exhibit higher rates of

proliferation markers, including high ki67 levels and a mitosis score of 3 (42.86%: 9/21) (Figure 6b & 6e). In contrast, tumors with Nuc/Cyto localization showed low ki67 levels and a low rate of mitosis score of 3 (12.5%: 3/24) (Figure 6b & 6e). Similarly, a tubule formation score of 3 and a nuclear grade of 3 were more pronounced among tumors with Peri/Cyto localization, with rates of 71.43% (15/21) and 52.38% (11/21), respectively (Figure 6c & 6d). Interestingly, SBR grade of III was significantly (p=0.0249) more common in tumors displaying Peri/Cyto localization (56%: 14/25), followed by cytoplasmic localization (36.84%: 24/38) and less pronounced in tumors with Nuc/Cyto pattern (19.23%: 5/26) (Figure 6f).

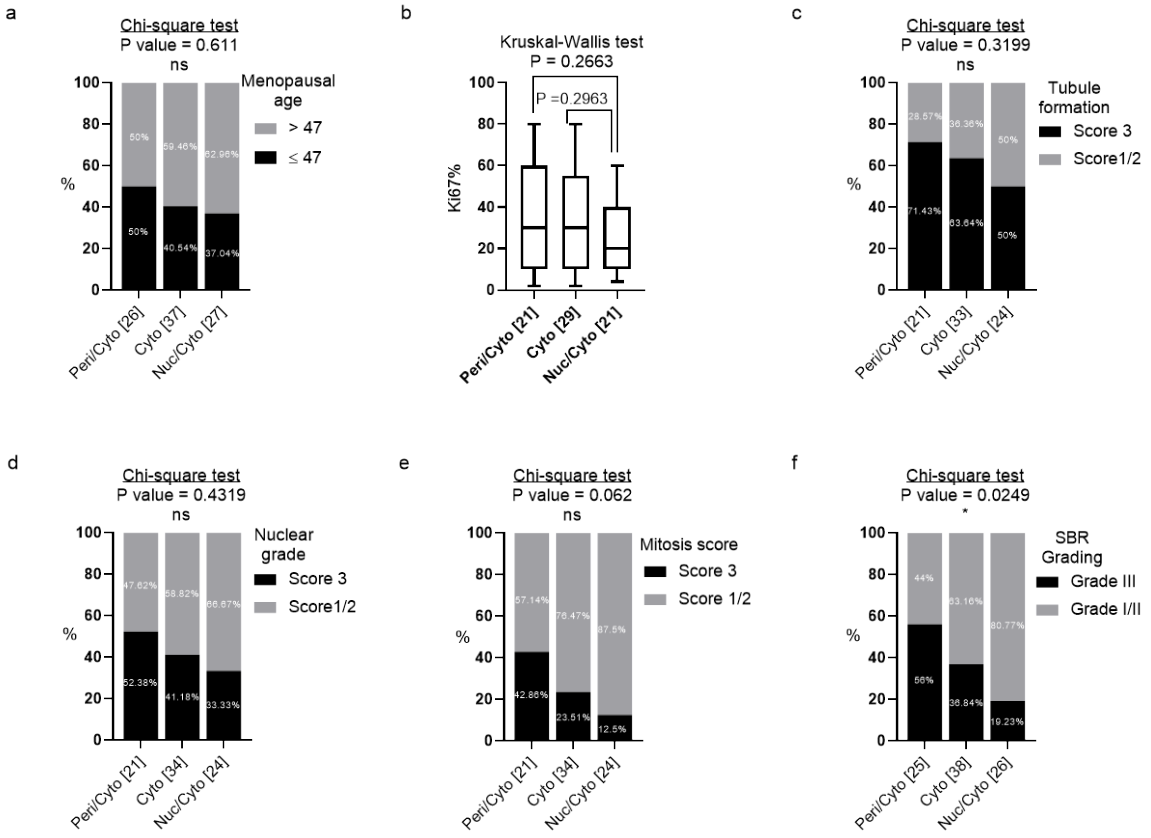


Figure 6: Correlation between NOX4 subcellular localizations and various clinical and histopathological features of breast cancer: (a) Menopausal age, (b) ki67 levels, (c) Tubule formation score, (d) Nuclear score, (e) Mitosis score, (f) SBR grading. The missing data for each feature are presented in Table S4. The statistical significance was determined by GraphPad 8. *The statistical significance is affirmed by a *P*-value under 0.05. Cyto: Cytoplasmic staining; Peri/Cyto: Perinuclear and Cytoplasmic; Nuc/Cyto: Nuclear and Cytoplasmic; SRB: Scarff-Bloom-Richardson.

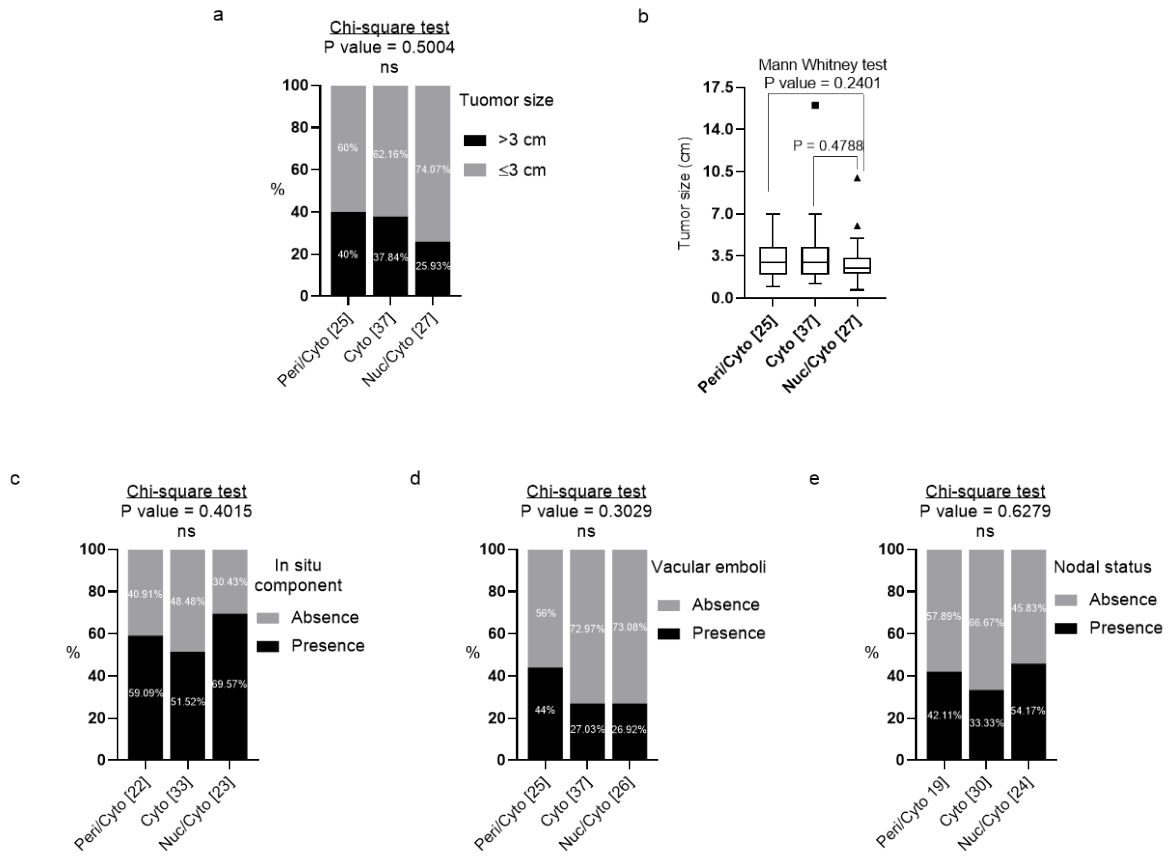


Figure 7: Correlation between NOX4 subcellular localizations and various clinical and histopathological features of breast cancer: (a and b) Tumor size, (c) In situ component, (d) Vascular emboli, (e) Nodal status. The missing data for each feature are presented in Table S4. The statistical significance was determined by GraphPad 8. *The statistical significance is affirmed by a *P*-value under 0.05. Cytoplasmic localization; Peri/Cyto: Perinuclear and Cytoplasmic localization; Nuc/Cyto: Nuclear and Cytoplasmic localization.

We also revealed crucial insights by analyzing the expression data of the two molecular biomarkers in malignant breast tumors: the Her2 receptor and the estrogen receptor. Indeed, a significant proportion of tumors with Peri/Cyto localization (53.85%: 14/26) exhibited a lack of estrogen receptor expression, followed by the group of tumors with cytoplasmic localization (36.84%: 14/38), and the lack of estrogen expression was the least common among tumors with Nuc/Cyto localization, where only 23.08% (6/26) of cases showed this feature (Figure 8a). Her2 receptor overexpression (n=8) was present exclusively in tumors with Peri/Cyto localization (4 cases) and tumors with Nuc/Cyto localization (4 cases) (Figure 8b).

Interestingly, the Peri/Cyto group was significantly associated with the TNBC phenotype (p=0.0276), while the Nuc/Cyto localization was significantly correlated with the luminal breast cancer phenotype (p=0.0418). The cytoplasmic took an intermediate position between the other two groups, Peri/Cyto and Nuc/Cyto (Figure 8c and 8d). Indeed, 46.16% (12/26) of tumors with Peri/Cyto localization showed a TNBC phenotype, followed by the cytoplasmic localization with 35.14% (13/37), then the least rate of TNBC tumors were among the group of Nuc/Cyto localization with only 12% (3/25) of cases (Figure 8c). On the other hand, the luminal breast cancer phenotype was more pronounced among the group of Nuc/Cyto localization, accounting for 80% (20/25), followed by cytoplasmic localization with 64.86% (24/37), and least common among the group of Peri/Cyto localization (46.15%: 12/26) (Figure 8d).

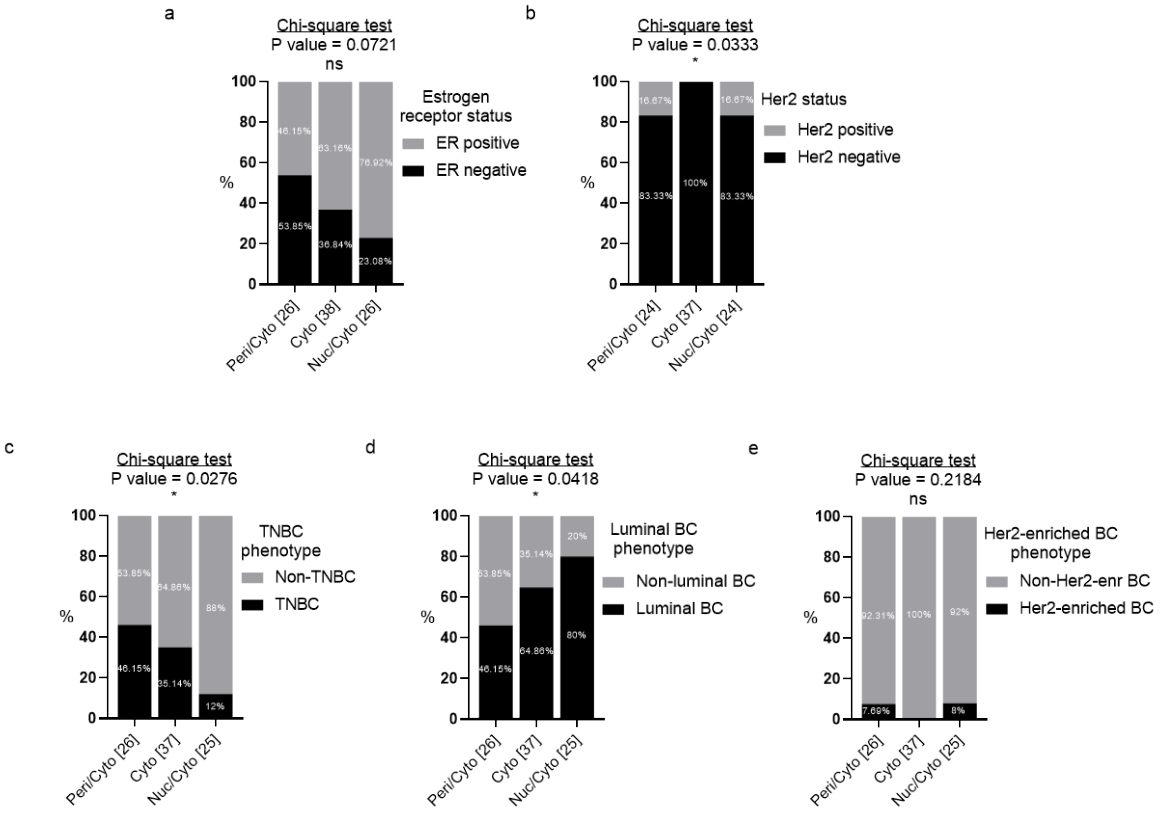


Figure 8: Correlation between NOX4 subcellular localizations and various molecular biomarkers and subtypes: (a) Estrogen receptor status, (b) Her2 receptor status, (c) TNBC phenotype, (d) Luminal breast cancer phenotype, (e) Her2-enriched breast cancer phenotype. The missing data for each feature are presented in table S4. The statistical significance was determined by GraphPad 8. *The statistical significance is affirmed by a *P*-value under 0.05. Cyto: Cytoplasmic localization; Peri/Cyto: Perinuclear and Cytoplasmic localization; Nuc/Cyto: Nuclear and Cytoplasmic localization; Her2: Human epidermal growth factor receptor 2; TNBC: Triple-Negative Breast Cancer.

3.7. Subcellular localization in normal adjacent tissue as a complementary biomarker for TNBC characterization

Considering the observed significant concordance in subcellular localization between tumors and their corresponding normal adjacent tissues (Table 2), along with the association between Peri/Nuc localization in tumors and the triple-negative breast cancer (TNBC) phenotype, we asked a question. If Peri/Nuc subcellular localization can be detected in normal adjacent tissues corresponding to tumors with a non-Peri/Cyto localization, it might also be associated with the TNBC phenotype. If confirmed, this association could serve as a supplementary biomarker, enhancing the characterization of breast cancers, particularly those of the TNBC subtype.

Remarkably, we found a significant association (p= 0.0191) between the detection of Peri/Cyto localization in NATs matched to the non-Peri/Cyto group of tumors and the TNBC phenotype (Figure 9). Indeed, the TNBC phenotype was observed in 71.4% (5/7) of the Peri/Cyto localization NATs group, compared to only 28.57% (4/24) in the cytoplasmic localization group of NATs and 16.67% in the Nuc/Cyto group of NATs.

Overall, the Peri/Cyto localization feature overlap in tumors and NATs corresponds to 60.71% (17/28) of all analyzed triple-negative breast cancers. This feature could have potential implications in managing this aggressive group of tumors. However, further clinical analysis and mechanistic studies are needed to clarify these implications.

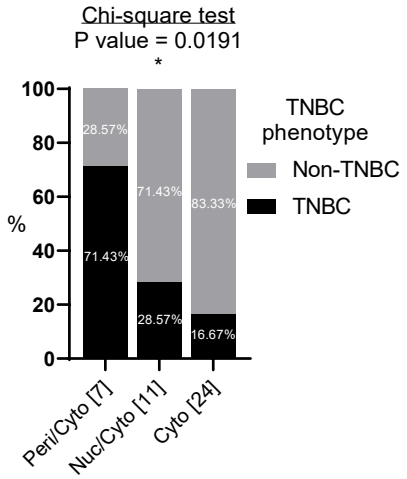


Figure 9: Correlation between Peri/Cyto localization in adjacent normal tissues and TNBC henotype in corresponding tumors exhibiting non-Peri/Cyto localization. *The statistical significance is affirmed by a *P*-value under 0.05.

4. Discussion:

The protein expression and subcellular localization analyses of NOX4 and DUOX1 in a larger group of MBTs and their matched NATs could offer a more comprehensive understanding of the actual protein activity in these tissues. Our retrospective analysis investigated the prevalence and clinical implications of NOX4 and DUOX1 protein expression and subcellular localization in malignant breast tumors (MBTs) and their normal adjacent tissues (NATs). Utilizing immunohistochemistry on 198 breast tissues, we found a pronounced ($p<0.0001$) upregulation of NOX4 protein in MBTs compared to NATs. Conversely, DUOX1 protein was predominantly expressed in NATs over MBTs ($p=0.0096$). The significant increase of NOX4 protein expression we observed in MBTs (79%:79/100) may indicate a widespread role of this NADPH oxidase in breast cancer. Few studies have analyzed NOX4 protein expression in malignant breast tumors. Notably, two prior studies reported elevated protein levels in breast tumor samples with rates of 89% (16/18) and 49.38% (40/81), respectively (Meitzler et al., 2017; X. Wang et al., 2021). A separate study reported moderate to high NOX4 mRNA expression in a set of 14 malignant breast tumors (Juhasz et al., 2009), which is consistent with the protein expression data from our research and prior investigations (Meitzler et al., 2017; X. Wang et al., 2021).

Our study is the first to evaluate NOX4 protein expression in a substantial number of malignant breast tumors (n=100) and their corresponding histologically normal adjacent tissues (n=94). A previous study by Juhasz et al. (2009) utilized mRNA expression analysis and identified elevated NOX4 levels in both MBTs and their corresponding NATs (Juhasz et al., 2009). However, they found no significant difference between the two tissue types (Juhasz et al., 2009). In another study on NOX4 protein expression, similar levels were shown between MBTs (89%:16/18) and their corresponding NATs (87.5%:7/8) (Meitzler et al., 2017). Oppositely to our study, these previous studies on mRNA and protein have shown no difference in the expression of NOX4 between MBTs and NATs, likely due to the limited sample sizes they used (Juhasz et al., 2009; Meitzler et al., 2017). Our study used a larger set of samples and detected a significant difference ($p<0.0001$) in NOX4 protein expression between the two tissue types.

Despite observing significant differences in NOX4 protein expression between MBTs and NATs, an upregulation of NOX4 protein in over 51.06% (48/94) of the histologically normal adjacent tissues may be considered remarkable and may have interesting clinical implications (discussed below), especially

since the histologically normal breast adjacent tissues adjacent to the tumors were reported to be molecularly abnormal (Gadaleta et al., 2022).

This is the first study to generate the DUOX1 protein expression status of malignant breast tumors and their NATs. An earlier study observed a notable increase in DUOX1 mRNA levels in normal adjacent breast tissues (n=10) compared to their corresponding breast tumors, where DUOX1 was largely suppressed (Fortunato et al., 2018). This finding aligns with our results. DUOX1 silencing has been documented in other types of cancers, including lung cancer (Luxen et al., 2008). These results may suggest a potential anti-tumorigenic role of this protein in breast tissue.

The consistent NOX4 subcellular localization between MBTs and NATs (*Kappa*= 0.487), along with the increased levels of NOX4 protein in NATs (51.06%: 48/94), raise questions about the interplay between NOX4 protein regulation in MBTs and their corresponding NATs. One potential explanation for these observations could be the existence of a shared diffusible factor in the tumor microenvironment (Gadaleta et al., 2022). This factor might regulate NOX4 protein expression and its subcellular localization in both tumor and adjacent normal epithelial cells. A plausible candidate microenvironmental factor is TGFβ, which was shown to induce NOX4 expression and ROS production in both the human breast cancer cell line, MDA-MB-231, and in the normal epithelial cell line, MCF10A. Interestingly, the overexpression of NOX4 mRNA in the normal human breast epithelial cell line, MCF12A, was shown to induce cellular senescence, anti-apoptotic effects, and tumorigenic phenotypes that include anchorage-independent growth and invasion (Graham et al., 2010). A prior study showed that exposure of normal cells to TGFβ induced senescence through the SMAD3/NOX4/ROS pathway (Hubackova et al., 2012). These findings suggest that the overexpression of NOX4 protein in the histologically normal breast tissue adjacent to the tumors may initiate a TGFβ-mediated state of senescence in some cells. Previous research has indicated that senescent cells, even those originating from normal human epithelial cells, can bypass cell cycle arrest, accumulating genomic instability that may lead to malignant transformation (P. L. Patel et al., 2016; Romanov et al., 2001). Notably, these senescent cells can secrete a range of proinflammatory molecules, termed SASP, encompassing TGFβ (Hubackova et al., 2012). *In vitro* studies have shown that TGFβ, when released by these cells, plays a pivotal role in initiating and sustaining senescence in adjacent (bystander) normal cells, mediated through the SMAD/NOX4/ROS pathway (Hubackova et al., 2012). These bystander effects may spread through a large surface of the histologically normal adjacent tissue, leading to an increased risk of recurrence in surgery sites. This holds clinical significance since 15% of post-surgical breast tumors recur within 10 years (Gadaleta et al., 2022). Notably, TGFβ, released by the tumor microenvironment and potentially by senescent cells near the tumors, could influence a pivotal aspect of tumor biology: EMT (epithelial-mesenchymal transition) in breast cancer cells (Boudreau et al., 2012). This process is regulated by the TGFβ/SMAD3/NOX4/ROS pathway in breast cancer cells, revealing the broad implication of this pathway that may constitute a potential therapeutic target (Boudreau et al., 2012). Therefore, our study highlights the clinical evidence on the prior findings that support the involvement of tumor microenvironment in NOX4 protein expression and its role in breast tumorigenesis.

Subcellular localization of NADPH oxidase enzymes such as NOX4 plays a pivotal role in tumorigenesis. For instance, NOX4 has been identified in diverse subcellular localizations, such as the perinuclear area, endoplasmic reticulum, mitochondria, nucleus, and plasma membranes (Meitzler et al., 2019). The clinical implications of studying this characteristic in MBTs and NATs remain largely unexplored. Our research seeks to fill this gap by investigating the exploitable results of 91 MBTs alongside their matched 85 NATs with known subcellular localization of NOX4. Our investigation noted that the primary expression of NOX4 and DUOX1 proteins was within the cancer cells and the luminal cells of adjacent normal regions. Indeed, DUOX1 was observed mainly to localize within the cytoplasm of both MBTs and their NATs.

In contrast, NOX4 displayed three primary subcellular localizations in both tissue types, with relatively similar rates between MBTs and NATs: cytoplasmic localization (41.76%MBTs and 42.35%NATs), a combined perinuclear and cytoplasmic localization (28.57%MBTs and 24.71%NATs); a combined nuclear and cytoplasmic localization (29.67%MBTs and 32.94%NATs). The presence of different splice variants of NOX4 protein might account for the diverse NOX4 subcellular localizations we observed, especially that these variants have previously shown various subcellular localizations in different cell types (Anilkumar et al., 2013; K. Chen et al., 2008; Goyal et al., 2005; Moloney et al., 2017; Szanto, 2022). Currently, the ability of the antibody used in this study to detect different NOX4 splice variants is unknown, presenting an unresolved question that needs to be investigated.

Among all aggressive features of breast cancer, the negative status of estrogen receptor (39.74% vs. 19.05%) and the presence of nodal invasion (47.62% vs. 25%) were more likely to exhibit increased levels of NOX4 protein. The increased nodal invasion among tumors overexpressing NOX4 protein was previously reported (X. Wang et al., 2021). In this regard, the role of NOX4 in breast tumor invasion and metastasis was supported by many reports (Boudreau et al., 2012; Mir et al., 2022; X. Wang et al., 2021).

The presence of perinuclear localization in MBT samples was significantly associated with aggressive features, including SBR grade III (p=0.0249) and triple-negative phenotype (p=0.0276), with additional tendencies towards high ki67 protein levels and vascular emboli presence. Conversely, the presence of nuclear staining in MBT samples was associated with lower SBR grades (I/II) (p=0.0249) and luminal BC phenotype (p=0.0418), indicating a more favorable prognosis. Notably, tumors with exclusive cytoplasmic localization exhibited intermediate aggressive characteristics, bridging the gap between the two primary groups. Given the significant concordance (*Kappa*= 0.487) in NOX4 subcellular localization we found between MBTs and their NATs, analyzing the clinical significance of this feature among NATs samples is questionable. Indeed, the presence of NOX4 perinuclear localization in whether MBTs or NATs samples was observed in 60.71% (17/28) of all analyzed TNBC cases. These findings suggest a potential contribution of NOX4 protein perinuclear localization to the development/progression of the TNBC molecular subtype of breast cancer and, therefore, it could constitute a potential therapeutic target. Two recent studies have shown a potential role of NOX4 in the proliferation of triple-negative breast cancer cells, suggesting a plausible role in the early stages of development of this aggressive subtype of breast cancer (Kim et al., 2022; Liu et al., 2022). Prior research reported that TGFβ mediated-NOX4'roles in breast cancer could depend on P53 alteration (Boudreau et al., 2014; Ma et al., 2021). Notably, a recent study on a murine model showed that P53 mutations may be essential for the growth and survival of triple-negative breast cancer (Dibra et al., 2023). Therefore, the NOX4's perinuclear localization could present a promising therapeutic target for TNBC. Nevertheless, further studies are needed to uncover NOX4 subcellular localization's role in TNBC biology.

5. Conclusions:

Our study has identified the NOX4 protein as a promising diagnostic biomarker for breast cancer, distinguishing malignant breast tumors (MBTs) from normal adjacent tissues (NATs). Elevated levels of NOX4 protein were associated with aggressive breast cancer characteristics, notably negative estrogen receptor status and nodal invasion. The study of NOX4's subcellular localization in both NATs and MBTs, in conjunction with its expression levels, has provided valuable clinical insights into the role of the tumor microenvironment in modulating NOX4's known functions in oncogenesis, especially in breast cancer. A significant finding was the association between the perinuclear localization of NOX4 protein in MBTs and the triple-negative breast cancer subtype. This association indicates NOX4's potential involvement in the development and progression of this particularly aggressive and common breast cancer variant. These findings suggest its potential utility in the prognosis and therapy of breast

cancer. The findings provide clinical evidence for further research into the clinical applications of NOX4 in breast cancer diagnosis, treatment, and management.

Abbreviations list:

BC: Breast Cancer
CAFs: Cancer-Associated Fibroblasts
CERB: Ethics Committee for Biomedical Research
Cyto: Cytoplasmic
DUOX1: Dual oxidase 1
DUOX2: Dual oxidase 2
EMT: Epithelial-Mesenchymal Transition
ER: Estrogen Receptor
FFPE: Formalin-Fixed Paraffin-Embedded
H_2O_2: Hydrogen Peroxide
Her2: Human Epidermal Growth Factor Receptor 2
HMIMV-R: Military Hospital of Instruction Mohammed V in Rabat
IHC: Immunohistochemistry
JNK: c-Jun N-terminal kinase
MAPK/ERK1/2: Mitogen-Activated Protein Kinase/Extracellular Signal-Regulated Kinase 1/2
MBTs - Malignant Breast Tumors
MCF10A: A normal human breast epithelial cell line
MCF12A: A normal human breast epithelial cell line
MDA-MB-231 – A breast cancer cell line
NADPH: Nicotinamide Adenine Dinucleotide Phosphate
NATs: Normal Adjacent Tissues
Nuc/Cyto: Combination of Nuclear and Cytoplasmic
$OH^{\bullet}$: Hydroxyl Radical
$O2^{\bullet-}$: Superoxide Anion
Peri/Cyto: Combination of Perinuclear and Cytoplasmic
PI3K/Akt: Phosphoinositide 3-kinase/Protein kinase B
ROS: Reactive Oxygen Species
SASP: Senescence-Associated Secretory Phenotype
SBR: Scarff-Bloom-Richardson (a grading system for breast tumors)
TGFβ: Transforming Growth Factor Beta
TNBC: Triple-Negative Breast Cancer
WHO: World Health Organization
NOX1, NOX2, NOX3, NOX4, NOX5: Different isoforms of NADPH Oxidase

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Supplementary data

Table S1: NOX4 and DUOX1 proteins expression in malignant breast tumors and their normal adjacent tissues (NATs).

NADPH oxidase protein	Tissue type	n	low score (<2)		High score (≥2)		*P*-values
NOX4	Tumor	100	21	21% (21/100)	79	79% (79/100)	<0.0001
	NAT	94	46	48.94% (46/94)	48	51.06% (48/94)	
DUOX1	Tumor	88	83	94.32% (83/88)	5	5.68% (5/88)	0.0096
	NAT	84	68	80.95% (68/84)	16	19.05% (16/84)	

Table S2: The major NOX4 subcellular localization patterns in breast tumor and normal adjacent tissues (NATs).

Tissue type	n	Cytoplasmic		Perinuclear/Cytoplasmic		Nuclear/Cytoplasmic		*P*-value
Tumor	91	38	41.76% (38/91)	26	28.57% (26/91)	27	29.67% (27/91)	0.8187
NAT	85	36	42.35% (36/85)	21	24.71% (21/85)	28	32.94% (28/85)	

Table S3: Association between NOX4 expression levels in malignant breast tumors and clinical and histopathological features of breast cancer.

Clinical and histopathological features		NOX4 scores in tumor				*P*-value
		<2		≥2		
		n	%	n	%	
Age	≤ 47	6	28.57% (6/21)	33	42.31% (33/78)	0.3186
	> 47	15	71.43% (15/21)	45	57.69% (45/78)	
	Missing data	1				
Tumor size	≤ 3 cm	12	60% (12/20)	51	65.38% (51/78)	0.7943
	> 3 cm	8	40% (8/20)	27	34.62% (27/78)	
	Missing data	2				
Tubule formation score	Score 1/2	5	31.25% (5/16)	27	39.71% (27/68)	0.5817

	Score 3	11	68.75% (11/16)	41	60.29% (41/68)	
	Missing data		16			
Nuclear grade	Score 1/2	10	62.5% (10/16)	41	59.42% (41/69)	>0.9999
	Score 3	6	37.5% (6/16)	28	40.58% (28/69)	
	Missing data		15			
Mitosis score	Score 1/2	13	81.25% (13/16)	48	69.56% (48/69)	0.5389
	Score 3	3	18.75% (3/16)	21	30.44% (21/69)	
	Missing data		15			
SBR grading	Grade I/II	14	66.67% (14/21)	48	63.16% (48/76)	>0.9999
	Grade III	7	33.33% (7/21)	28	36.84% (28/76)	
	Missing data		3			
Ki67 level	12	11	68.75% (11/16)	30	50.85% (30/59)	0.2624
	≥ 30 %	5	31.25% (5/16)	29	49.15% (29/59)	
	Missing data		25			
In situ component	Absence	9	52.94% (9/17)	27	39.13% (27/69)	0.4113
	Presence	8	47.06% (8/17)	42	60.87% (42/69)	
	Missing data		14			
Vascular emboli	Absence	12	60% (12/20)	51	66.23% (51/77)	0.6086
	Presence	8	40% (8/20)	26	33.77% (26/77)	
	Missing data		3			
Nodal status	Negative	12	75% (12/16)	33	52.38% (33/63)	0.1572
	Positive	4	25% (4/16)	30	47.62% (30/63)	
	Missing data		21			
ER status	Negative	4	19.05% (4/21)	31	39.74% (31/78)	0.1216
	Positive	17	80.95% (17/21)	47	60.26% (47/78)	
	Missing data		1			
Her2 status	Negative	20	95.24% (20/21)	66	90.41% (66/73)	0.6789
	Positive	1	4.76% (1/21)	7	9.59% (7/73)	
	Missing data		6			
Molecular subtypes	Luminal BC	17	80.95% (17/21)	47	61.84% (47/76)	-
	TNBC	4	19.05% (4/21)	25	32.9% (25/76)	
	Her2-enriched BC	0	0%	4	5.26% (4/76)	
	Missing data		3			

Table S4: Association between NOX4 subcellular localization in cancerous cells and clinical and histopathological features of breast cancer.

Clinical and histopathological features		NOX4 subcellular localization in tumor						*P*-value
		Cytoplasmic		Perinuclear & cytoplasmic		Nuclear & cytoplasmic		
		n	%	n	%	n	%	
Age	≤ 47	15	40.54% (15/37)	13	50% (13/26)	10	37.04% (10/27)	0.611
	> 47	22	59.46% (22/37)	13	50% (13/26)	17	62.96% (17/27)	
	Missing data				10			
Tumor size	≤3 cm	23	62.16% (23/37)	15	60% (15/25)	20	74.07% (20/27)	0.5004
	>3 cm	14	37.84% (14/37)	10	40% (10/25)	7	25.93% (7/37)	
	Missing data				11			
Tubule formation score	Score 1/2	12	36.36% (12/33)	6	28.57% (6/21)	12	50% (12/24)	0.3199
	Score 3	21	63.64% (21/33)	15	71.43% (15/21)	12	50% (12/24)	
	Missing data				22			
Nuclear grade score	Score 1/2	20	58.82% (20/34)	10	47.62% (10/21)	16	66.67% (16/24)	0.4319
	Score 3	14	41.18% (14/34)	11	52.38% (11/21)	8	33.33% (8/24)	
	Missing data				21			
Mitosis score	Score 1/2	26	76.47% (26/34)	12	57.14% (12/21)	21	87.5% (21/24)	0.062
	Score 3	8	23.53% (8/34)	9	42.86% (9/21)	3	12.5% (3/24)	
	Missing data				21			
SBR grading	Grade I/II	24	63.16% (24/38)	11	44% (11/25)	21	80.77% (21/26)	0.0249*
	Grade III	14	36.84% (24/38)	14	56% (14/25)	5	19.23% (5/26)	
	Missing data				11			

Ki67 level	12	12	41.38% (12/29)	10	47.62% (10/21)	13	68.42% (13/19)	0.176
	≥ 30 %	17	58.62% (17/29)	11	52.38% (11/21)	6	31.58% (6/19)	
	Missing data				31			
In situ component	Absence	16	48.48% (16/33)	9	40.91% (9/22)	7	30.43% (7/23)	0.4015
	Presence	17	51.52% (17/33)	13	59.09% (13/22)	16	69.57% (6/23)	
	Missing data				22			
Vascular emboli	Absence	27	72.97% (27/37)	14	56% (14/)	19	73.08% (19/26)	0.3029
	Presence	10	27.03% (10/37)	11	44% (11/)	7	26.92% (7/26)	
	Missing data				12			
Nodal status	Absence	20	66.67% (20/30)	11	57.89% (11/19)	13	45.83% (13/24)	0.6279
	Presence	10	33.33% (10/30)	8	42.11% (8/19)	11	54.17% (11/24)	
	Missing data				27			
ER status	Negative	14	36.84% (14/38)	14	53.85% (14/26)	6	23.08% (6/26)	0.0721
	Positive	24	63.16% (24/38)	12	46.15% (12/26)	20	76.92% (20/26)	
	Missing data				10			
Her2 status	Negative	37	100% (37/37)	20	83.33% (20/24)	20	83.33% (20/24)	0.0333*
	Positive	0	0.00%	4	16.67% (4/24)	4	16.67% (4/24)	
	Missing data				15			
Molecular subtypes	TNBC	13	35.14% (13/37)	12	46.16% (12/26)	3	12% (3/25)	0.0387*
	Luminal BC	24	64.87% (24/37)	12	46.15% (12/26)	20	80% (20/25)	
	Her2-enriched	0	0%	2	7.69% (2/26)	2	8% (2/25)	
	Missing data				12			

General Discussion

Breast cancer is the most frequently diagnosed cancer among women worldwide, accounting for 24.5% (2.3 million) of all cancer diagnoses (Ferlay et al., 2021; Sung et al., 2021). It remains the leading cause of cancer-related deaths for women in over 110 countries (Ferlay et al., 2021; Sung et al., 2021). In Morocco, breast cancer represented 38.9% of female cancer diagnoses and 24.7% of cancer-related deaths in 2020 (*Cancer Today*, n.d.). By 2040, due to population growth and aging, breast cancer cases and deaths are expected to rise to 3 million and 1 million, respectively (Arnold et al., 2022). The search for new biomarkers is essential for better management of this prevalent and heterogeneous disease. Biomarkers for early detection of BC can improve treatment outcomes, especially for early onset BC, which often displays a worse prognosis and a TNBC phenotype (Canfield et al., 2017; Felicio et al., 2017; Li et al., 2017). Moreover, biomarkers can help in risk assessment, enabling better monitoring and prevention strategies (Li et al., 2017; Łukasiewicz et al., 2021). Another aspect is the identification of molecular biomarkers that are associated with key processes of BC development and progression, such as genetic or epigenetic instabilities or EMT, facilitating the development of personalized therapies and improving prognosis assessment (Boudreau et al., 2012; Glodzik et al., 2020; Lønning et al., 2022; Oubaddou et al., 2023a). Both epigenetic instability and oxidative stress are linked to the complex landscape of breast cancer and may contain several significant biomarkers warranting exploration.

The first primary aim of our research is to comprehensively explore the expression and subcellular localization of the NOX4 and DUOX1 proteins in malignant breast tumors (MBTs) and their corresponding normal adjacent tissues (NATs). Moreover, we aim to investigate the hypermethylation of the *BRCA1* gene promoter across three distinct human mammary tissue types, including MBTs, their corresponding NATs, and benign breast lesions (BBLs).

A pivotal part of our objective is to compare the status of these oxidative and epigenetic biomarkers (Table VIII) between MBTs and their associated NATs. This will offer insight into potential molecular abnormalities present in histologically normal breast tissues neighboring the tumors. Additionally, a crucial goal is to understand the relationship between these biomarkers (Table VIII) and the aggressive clinical and histopathological features of breast cancer. Grasping this relationship can provide important clinical insights regarding the potential influence of these biomarkers on breast cancer management.

Table VIII. Overview of the Studied Biomarkers: Detection Techniques and Measured Variables

Biomarker	Technique	Measured Variables	FFPE samples
***BRCA1* gene promoter hypermethylation**	Methylation-specific PCR	**The status of *BRCA1 gene* promoter hypermethylation** (Methylated or not)	**MBTs, their NATs and BBLs**
NOX4 protein	Immunohistochemistry	- **Expression levels** (High or low) - **Subcellular localization** (Cytoplasm; nucleus; perinuclear region)	**MBTs and their NATs**
DUOX1 protein	Immunohistochemistry	- **Expression levels** (High or low) - **Subcellular localization** (Cytoplasm; nucleus; perinuclear region)	**MBTs and their NATs**

To enhance our understanding of the role of the *BRCA1* gene in breast cancer tumorigenesis, we reviewed the literature and published an exhaustive article review detailing the mechanisms of genomic instability and *BRCA1/2*-associated cancer susceptibility in gynecological and mammary tumors. Our review summarizes key findings of several studies on clinical, preclinical, and human *in vitro* models (Oubaddou et al., 2023a).

The tumor suppressor BRCA1/2, cancer susceptibility and genome instability in gynecological and mammary cancers

We reviewed recent literature and collected relevant data regarding the role of *BRCA1* and *BRCA2* genes in triggering early genome instability in gynecological and mammary cancers. Our analysis emphasized data from human studies, animal models (specifically mice), and human cell line models. We directed our attention to three predominant female cancer types: breast cancer (BC), ovarian cancer (OC), and cervical cancer (CC). In 2020, the total number of new cases of these gynecomammary cancers was 3.18 million, representing over a third (34.45%) of all female cancer diagnoses (Sung et al., 2021). In 2020, out of 4.43 million cancer-related deaths in women, breast, cervical, and ovarian cancers accounted for 1.24 million (Sung et al., 2021). While *BRCA1* and *BRCA2* gene mutations are well-known for increasing susceptibility to BC and OC, limited literature links them to cervical cancer. However, emerging evidence highlights the potential role of *BRCA1* and *BRCA2* genes in HPV-associated genome instability (Shen-Gunther et al., 2022; Y. Zhang et al., 2005).

The frequently observed loss of heterozygosity (LOH) of the wild-type allele of *BRCA1* and *BRCA2* genes in BC and OC aligns with the 'two-hit' theory, suggesting that the loss of the second allele of a tumor suppressor gene can drive early genome instability and thus trigger

malignant transformation (Maxwell et al., 2017). However, the inherent tumor suppressive functions of *BRCA1* and *BRCA2* genes can result in apoptosis (Konishi et al., 2011b; Sedic et al., 2015; Venkitaraman, 2014). Notably, alterations in cell survival genes such as *P53*, *Rb*, and *PTEN* are commonly detected in BC of *BRCA1* mutation carriers, suggesting that these alterations might be required for cell survival after the complete inactivation of the *BRCA1* gene (Martins et al., 2012; J. M. Patel et al., 2020; Sedic et al., 2015). Moreover, *P53* mutations are frequently detected in *BRCA1* mutation-associated OC (Cole et al., 2016). More than that, *PTEN* and *P53* were shown to precede LOH of *BRCA1* in BC evolution among carriers, reinforcing their role in promoting cell survival in breast transformation (Martins et al., 2012). In parallel, data from human cell lines, notably human mammary epithelial cell lines carrying *BRCA1* or *BRCA2* heterozygous mutations, exhibit a diminished level of their corresponding proteins (Pathania et al., 2014; Tan et al., 2017). This could give rise to a *BRCA1* haploinsufficiency, impairing many functions necessary for genome integrity (Konishi et al., 2011b; Pathania et al., 2014; Sedic et al., 2015; Sun et al., 2022; Vohhodina et al., 2021; X. Zhang et al., 2017). In this model, a single mutation in the *BRCA1* or *BRCA2* genes might be sufficient to initiate an early genome instability, potentially resulting in driver mutations that favor cell survival and oncogenic transformation.

Human, mouse, and human cell line models with *BRCA1/2* heterozygosity highlight a strong link between haploinsufficiency of these genes and increased genome instability. Analyses of blood genomes in both humans and mice carrying the *BRCA1* mutation revealed potential oncogenic changes, suggesting early genome instability (Sun et al., 2022; Xiao et al., 2014). In mice, this instability correlated with increased DNA repair via the NHEJ mechanism, referring to a deficiency in HR (Sun et al., 2022). Human cell line models have been key in understanding the complexity of mechanisms governing *BRCA1* haploinsufficiency-associated genome instability. For instance, *BRCA1* haploinsufficiency in *BRCA1* heterogenous HMECs (Human mammary epithelial cells) is associated with deficits in DNA damage repair, replicative stress, telomeric instability, accumulation of stalled forks and R-loops, and impaired chromatic remodeling and transcription (Konishi et al., 2011b; Pathania et al., 2014; Sedic et al., 2015; Sun et al., 2022; Vohhodina et al., 2021; X. Zhang et al., 2017). Likewise, decreased cellular levels of the BRCA2 protein (*BRCA2* heterozygous) in HMECs are linked to chromosomal instability and replication stress (Tan et al., 2017). In the context of ovarian cancer, *BRCA1* haploinsufficiency is potentially linked to impaired cell cycle regulation and oxidative DNA damage (Dziaman et al., 2009; Norquist et al., 2010).

BRCA1 Promoter Hypermethylation in Malignant Breast Tumors and in the Histologically Normal Adjacent Tissues to the Tumors: Exploring Its Potential as a Biomarker and Its Clinical Significance in a Translational Approach

BRCA1 (Breast cancer 1) is a well-recognized tumor suppressor gene that predisposes individuals to breast and ovarian cancers (Oubaddou et al., 2023a). Mutations in this gene are thought to promote accelerated genome instability in carriers due to its multifaceted roles in DNA damage repair, resolution of stalled forks and R-loops, and chromatin remodeling (Oubaddou et al., 2023a).

Another factor contributing to *BRCA1* gene dysfunction is the inactivating hypermethylation of its promoter (P. Sharma et al., 2014). This could result in genome instability, thereby enhancing the risk of breast cancer initiation and progression (Glodzik et al., 2020; Lønning et al., 2022; L. Zhang & Long, 2015). Our study investigated this epigenetic modification across three mammary tissue types: malignant breast tumors (MBTs), their normal adjacent tissues (NATs), and benign breast lesion samples (BBLs). The examination of tissues adjacent to tumors is gaining research focus due to the potential molecular abnormalities they may harbor (Gadaleta et al., 2022). Furthermore, benign breast lesions can elevate breast cancer risk (Figueroa et al., 2021), and the epigenetic silencing of the tumor suppressor *BRCA1* gene could be a potent initiating event of malignancy in these tissues.

We utilized methylation-specific PCR (MSP) to analyze the *BRCA1* gene promoter methylation status across 84 FFPE mammary tissue samples. The primers we used for detecting BRCA1 gene promoter hypermethylation were first introduced by Esteller (Esteller, 2000). We aim through this analysis to determine the prevalence of this epigenetic alteration among various mammary tissue types and understand its correlation with breast cancer (BC) aggressive features. Notably, the detected *BRCA1* gene promoter hypermethylation rates were 41.67% in MBTs, 46.67% in NATs, and 19.05% in BBLs (Figure 1c, page 67).

The significant prevalence (41.67%) of *BRCA1* gene promoter hypermethylation in MBTs suggests the involvement of this epigenetic alteration in breast malignancy. The epigenetic silencing of the *BRCA1* gene by DNA methylation may constitute an early event of genome instability, leading to early-onset sporadic forms of breast cancer (Glodzik et al., 2020). Furthermore, this high rate does not exclude its potential as a separate marker for tumor aggressiveness, originating during the late stages of tumorigenesis (L. Zhang & Long, 2015). The hypermethylation rate of the *BRCA1* gene promoter in MBTs (41.67%) aligns with other studies using the same primers, which have reported rates between 27% and 59% (G. Sharma et al., 2009; X. Xu et al., 2009).

The percentage (46.67%) of NATs exhibiting *BRCA1* promoter hypermethylation is comparable to MBTs (41.67%), raising pertinent questions about the nature of the tumor microenvironment and its influence on surrounding tissues (Gadaleta et al., 2022). This may also refer to a pre-cancerous stage where NATs with an epigenetic alteration could be precursors for malignant transformation (Gadaleta et al., 2022; Lønning et al., 2022). A concordance of 60% between paired MBTs and NATs in *BRCA1* gene promoter hypermethylation status supports these roles suggested for *BRCA1* gene promoter hypermethylation in NATs. This consistent abnormal DNA methylation can be linked to theories like "field of cancerization" or "tumor microenvironment influence" (Gadaleta et al., 2022; Teschendorff et al., 2016).

The lower prevalence of *BRCA1* gene promoter hypermethylation in BBLs (19.05%) compared to MBTs and NATs reinforces the potential association of *BRCA1* gene promoter hypermethylation with malignant transformation. A similar difference between BBLs and MBTs was previously reported, reinforcing our findings (Parrella et al., 2004). However, the incidence of this epigenetic alteration in BBLs is not negligible, highlighting its possible role as an early event of transformation that may explain the observed risk for breast cancer among BBL cases (Figueroa et al., 2021). Therefore, the hypermethylation of the *BRCA1* gene promoter in BBLs might serve as an early molecular biomarker indicating both genetic and epigenetic instabilities.

The group of MBTs with hypermethylation seemed more homogenous in age distribution (Standard deviation: 6.320) compared to the group without this epigenetic alteration (Standard Deviation: 9.264), which shows a more heterogenous distribution (Figure 2b, page 69). The presence of cases with *BRCA1* gene mutations within this group mostly causes this heterogeneity. Significantly, both germline mutations of the *BRCA1* gene and promoter hypermethylation have been identified as independent factors driving malignant breast tumorigenesis. This is supported by the rarity of *BRCA1* gene promoter hypermethylation in *BRCA1* germline mutation carriers (Glodzik et al., 2020; Vos et al., 2018). It is crucial to consider this heterogeneity when investigating the association between *BRCA1* gene promoter hypermethylation and the aggressive features of randomly selected breast cancer cases, as the case in our study. Thus, even without definitive statistical significance linking aggressive features with *BRCA1* gene promoter hypermethylation status, the emerging trends remain clinically pertinent (discussed below). Consequently, further research should consider checking for germline mutations to ensure more accurate and unbiased results.

Younger (age ≤47) breast tumor cases were more frequent (45% vs. 35.71%) among the hypermethylated group (homogenous for age distribution), suggesting a correlation of this

epigenetic alteration with early-onset breast cancer (Figure 2a, page 69). This aligns with a recent study indicating that about 50% of young women diagnosed with triple-negative breast cancer, who do not have a *BRCA1* germline mutation, carry the hypermethylation of *BRCA1* gene promoter (Glodzik et al., 2020). This finding emphasizes the potential role of this epigenetic alteration as an early event accelerating genome instability. Moreover, new findings suggest that detecting this epigenetic marker in blood could increase the risk of triple-negative breast cancer in the general population (Lønning et al., 2022). This later study strengthen the potential of using blood tests to identify this epigenetic marker for breast cancer risk assessment (Davalos & Esteller, 2023).

Recently, *BRCA1* germline mutations have been validated as predictors for the use of the PARP inhibitor Olaparib, a treatment approach targeting breast tumors exhibiting the so-called BRCAness phenotype (Glodzik et al., 2020; Popova et al., 2012). Malignant breast tumors with *BRCA1* promoter hypermethylation are considered "genome phenocopies" of *BRCA1*-mutated breast tumors due to their exhibition of the BRCAness phenotype, potentially presenting similar prognosis and therapeutic responses (Glodzik et al., 2020; Popova et al., 2012). Interestingly, early experiments demonstrated that a PARP inhibitor (PARPi) affects a breast cancer cell line with *BRCA1* gene hypermethylation (Drew et al., 2011). Therefore, *BRCA1* gene silencing through *BRCA1* promoter hypermethylation or *BRCA1* gene germline mutations could offer a very powerful useful marker in PARP inhibitors (e.g., Olaparib) prediction. This must improve the treatment of a significant portion of breast cancer cases, especially those with TNBC phenotype.

The elevated levels of the Ki-67 biomarker (81.25%), SBR grade III (73.68%), and other aggressive features in the hypermethylated group of our analysis indicate a correlation between *BRCA1* gene promoter hypermethylation and tumor aggressiveness (Figure 2d, h, page 69). Additionally, the significant correlation between *BRCA1* gene promoter hypermethylation and Her2 overexpression or triple-negative tumors highlights the vast heterogeneity of breast cancer and shows the potential of *BRCA1* gene promoter hypermethylation as a marker for aggressive tumor subtypes (Figure 3a, page 70). Targeting this epigenetic alteration in these tumors, especially the TNBC subtype, may improve their prognosis. Different strategies were used to reverse DNA methylation and thereby restore *BRCA1* gene function, which may have various implications in preventing or treating breast cancer. Among these approaches we found DNMT inhibitors, natural compounds, histone methyltransferase inhibitors, and the innovative CRISPR-dCas9-TET1 System (Al-Yousef et al., 2020; Cao et al., 2019; Choudhury et al., 2016; Donovan et al., 2019; Moreira-silva et al., 2020; Papoutsis et al., 2012; Yu et al., 2019).

Comparative Study of NOX4 and DUOX1 Protein Expression and Subcellular Localization in Breast Tumors and their Normal Adjacent Tissues: Uncovering NOX4's Role as a Biomarker for Aggressiveness and Progression of Breast Cancer

By analyzing the protein expression and subcellular localization of NOX4 and DUOX1 in a larger set of malignant breast tumors (MBTs) and their corresponding normal adjacent tissues (NATs), we can increase our understanding of the actual protein activity within these tissues, potentially uncovering new biomarkers to enhance disease management. While NOX4 protein is known for its roles in epithelial-mesenchymal transition (EMT) and angiogenesis in breast cancer, its subcellular localization and clinical implications in breast tumorigenesis are not thoroughly examined. Furthermore, there is a lack of data on DUOX1 protein expression and subcellular localization in malignant breast tumors.

Using immunohistochemistry on 198 human mammary samples (104 MBTs and 98 NATs), we explored the prevalence, clinical significance, and subcellular localization of NOX4 and DUOX1 proteins in MBTs and their corresponding NATs. The optimal immunostaining conditions using anti-NOX4 and anti-DUOX1 antibodies were respectively determined in human kidney tissue (1: 250), and human lung tissue (1: 400). Most of the analyzed FFPE blocks had both tumor and normal adjacent tissue sections, allowing for a combined IHC analysis on one slide for every MBT and NAT pair. After immunostaining, NOX4 protein expression status was exploitable in 100 MBTs and 94 NATs. Furthermore, DUOX1 protein expression was exploitable in 88 MBTs and 84 NATs.

NOX4 protein expression was significantly upregulated in MBTs (79% with a score ≥2) compared to NATs (51.06% with a score ≥2) ($p<0.0001$) (Figure1a, page 86). In contrast, DUOX1 protein expression was lower in breast tumor tissues (94.32% showed a low score) than in NATs (80.95% showed a low score) ($p=0.0096$) (Figure 1d, page 86). The significantly higher expression of NOX4 in MBTs, contrasted with the reduced levels of DUOX1, indicates distinct roles for these proteins in breast cancer pathology.

Aligning with our findings, two prior investigations have reported NOX4 protein levels to be elevated in breast tumor samples at rates of 89% and 49.38%, respectively (Meitzler et al., 2017; X. Wang et al., 2021). Moderate to high NOX4 mRNA expression was reported in a set of breast tumor samples (Juhasz et al., 2009), which is consistent with the protein expression data (Meitzler et al., 2017; X. Wang et al., 2021). The comparison between mRNA and protein expression in the context of NOX4 is crucial, given that its regulation predominantly occurs at the transcriptional level (Siuda et al., 2012). Moreover, due to its unique structure, NOX4 is constitutively active within cells and consequently potentially produces ROS at sites of protein

expression (Guo & Chen, 2015). However, the specific functions of NOX4 protein within cells may be influenced by factors such as its subcellular localization (discussed below), as well as other factors such the membrane partner of NOX4 protein called $P22^{phox}$ (Ameziane-El-Hassani et al., 2016).

Our research is the first to evaluate NOX4 protein expression in a significant cohort of malignant breast tumors (n=100) and their corresponding histologically normal adjacent tissues (n=94). A previous study conducted by Juhasz et al (2009) utilized mRNA expression analysis and identified elevated NOX4 levels in both MBTs and their corresponding NATs (Juhasz et al., 2009). However, they did not find any difference between the two tissue types (Juhasz et al., 2009). In another study on NOX4 protein expression, similar levels were shown between MBTs (89%:16/18) and their corresponding NATs (87.5%:7/8) (Meitzler et al., 2017). In contrast to these findings, our study detected a significant difference ($p<0.0001$) in NOX4 protein expression between MBTs and NATs, possibly because we analyzed a larger cohort. Despite observing this significant difference, an upregulation of NOX4 protein in over 51.06% (48/94) of the histologically normal adjacent tissues may be considered remarkable and may have interesting clinical implications (discussed below), especially since the histologically normal breast adjacent tissues adjacent to the tumors were reported to be molecularly abnormal (Gadaleta et al., 2022).

Our research is the first to characterize the expression of the DUOX1 protein in malignant breast tumors and their NATs. A previous study reported a significant increase in DUOX1 mRNA levels in normal adjacent breast tissues (n=10) compared to their corresponding breast tumors, where DUOX1 expression was predominantly decreased (Fortunato et al., 2018), which aligns with our results. Our results, supported by the existing literature, suggest a potential anti-tumorigenic role of this protein in breast tissue. DUOX1 epigenetic silencing has been documented in human lung cancer, where it was proposed as a tumor suppressor gene (Luxen et al., 2008). Supporting this, DUOX1 silencing in lung cancer cell lines was shown to promote epithelial-mesenchymal transition (Little et al., 2016). An epigenetic silencing of DUOX1 has also been reported in human hepatocellular carcinoma (Ling et al., 2014). Patients with cervical cancer had higher mRNA levels of DUOX1, which associated with better overall and disease-free survival, suggesting a possible tumor suppressive role for this type of cancer (Cho et al., 2019). However, DUOX1's role in tumorigenesis remains controversial and may depend on external factors such as radiation exposure. For example, elevated DUOX1 expression was noted several days after radiation exposure and was linked to persistent DNA damage and cell cycle arrest in human normal thyroid cells (Ameziane-El-Hassani et al., 2015).

Moreover, overexpression of the DUOX1 protein has been detected in radiation-induced thyroid tumors, reinforcing its role in radio-induced tumorigenesis (Ameziane-El-Hassani et al., 2015).

Our results indicate that breast tumors with elevated NOX4 protein levels often exhibit aggressive features, including negative estrogen receptor status (39.74% vs. 19.05% for low NOX4) and nodal invasion (47.62% vs. 25% for low NOX4) (Figure 4f, 5a, pages 90 and 91). The association between high NOX4 levels and the TNBC subtype was evident (32.90% vs. 19.10% for low NOX4) (Figure 5c, page 91). The increased nodal invasion among breast tumors overexpressing NOX4 protein was previously reported (M. Wang et al., 2021).

The role of NOX4 protein in breast tumor invasion and metastasis was supported by many reports (Boudreau et al., 2012; Mir et al., 2022; X. Wang et al., 2021). Indeed, in breast cells, NOX4 was shown to be instrumental in the TGFβ-induced epithelial-mesenchymal transition (EMT) process, which is crucial for cell migration and EMT marker expression (Boudreau et al., 2012). It was also shown to modulate fibronectin expression (driving tumor cells metastasis), and regulate chemokine release from endothelial cells, which favors tumor cell dissemination (Boudreau et al., 2012; Han et al., 2021; X. Wang et al., 2021).

NOX4 was documented to be overexpressed in several solid malignancies, including the prostate cancer, glioblastoma, liver cancer, melanoma, and colorectal cancer (Lin et al., 2017). Additionally, NOX4's involvement extends to other cancer types, including lung cancer, renal cell cancer, gastric cancer, pancreatic cancer, and ovarian cancer (Gong et al., 2022). NOX4 protein has been identified in diverse subcellular localizations, including the endoplasmic reticulum (human vascular endothelial cells), mitochondria (murine fibroblast cell line), nucleus (human vascular endothelial cells), nuclear envelope (murine hepatic cells), and plasma membrane in M1619 melanoma cells (Brar et al., 2002; K. Chen et al., 2008; Graham et al., 2010; Meitzler et al., 2019; Spencer et al., 2011). Indeed, subcellular localization of NADPH oxidase enzymes such as NOX4 plays a pivotal role in various carcinogenic processes. NOX4 produces hydrogen peroxide (H_2O_2), considered as intracellular secondary messenger in various signaling pathways (Block & Gorin, 2012; Meitzler et al., 2019). Interestingly, ROS-associated signaling functions were shown to be spatially controlled by the subcellular localization of ROS production (Block & Gorin, 2012; Spencer et al., 2011). For instance, in the perinuclear region, specifically the endoplasmic reticulum, NOX4 protein has been observed to oxidize and subsequently inactivate protein tyrosine phosphatase 1B (PTP B1), influencing cell proliferation via the epidermal growth factor (EGF) signaling pathway (K. Chen et al., 2008). Additionally, NOX4 protein presence in the nuclear envelope might impact nuclear DNA and

proteins, thus potentially causing genomic instability (Spencer et al., 2011). NOX4 protein expression in the membrane plasma was shown to promote cell survival through modulation of transcription factor nuclear factor-KB (NF-KB) (Brar et al., 2002). Interestingly, a study has been shown that NOX4 protein is essential for maintaining mitochondrial ROS in thyroid tumor cells during hypoxia, stabilizing HIF1α and thus promoting glycolysis and cancer cell proliferation (Tang et al., 2018).

The clinical implications of studying the subcellular localization of NADPH oxidases in malignant breast tumors and their NATs remain largely unexplored. We observed that DUOX1 predominantly localized in the cytoplasm, whereas NOX4 protein exhibited diverse subcellular localizations in both MBTs and their NATs (Figure 2a, page 87). Indeed, in MBTs, 41.76% exhibited NOX4 cytoplasmic localization (Cyto group of tumors), 28.57% had a combined NOX4 perinuclear and cytoplasmic localization (Peri/Cyto group of tumors), and 29.67% had a combined NOX4 nuclear and cytoplasmic localization (Nuc/Cyto group of tumors). These rates were relatively similar for NATs. We also observed a significant concordance in NOX4 protein subcellular localization between MBTs and their corresponding NATs (*Kappa*= 0.487) (Table 2, page 88) (Figure 2d, page 87). The concordant NOX4 protein subcellular localization between tumors and NATs highlights its potential as a reliable biomarker. The existence of different splice variants of NOX4 protein might account for the diverse NOX4 protein subcellular localizations we observed, especially since these variants have previously shown various subcellular localizations in different cell types (Anilkumar et al., 2013; K. Chen et al., 2008; Goyal et al., 2005; Moloney et al., 2017; Szanto, 2022). The antibody (ab154244, Abcam, Cambridge, UK) used in this study is not capable of distinguishing between different variants of NOX4, implying that further research is necessary (Fenniche et al., 2023).

The concordant NOX4 protein subcellular localization between MBTs and NATs (*Kappa*= 0.487), suggests shared mechanisms regulating NOX4 protein expression and subcellular localization in MBTs and their matched NATs. This may be attributed to a common diffusible element within the tumor microenvironment (Gadaleta et al., 2022) that governs NOX4 protein subcellular localization in both malignant and adjacent normal cells. TGFβ is a probable candidate, having previously been identified to modulate NOX4 protein expression and ROS generation in both the MDA-MB-231 (triple-negative human breast cancer cell line) and the MCF10A (normal human breast epithelial cell line) (Boudreau et al., 2012). Interestingly, elevated NOX4 mRNA levels in the MCF12A (normal human breast epithelial cell line) have been linked to cellular senescence and tumorigenic behaviors (Graham et al., 2010). Moreover, a previous study has shown that TGFβ can induce senescence in normal cells via the

SMAD3/NOX4/ROS pathway (Hubackova et al., 2012), suggesting that NOX4 overexpression in adjacent normal breast tissue could trigger a TGFβ-driven senescent state in certain cells. Such senescent cells, even those originating from normal human epithelial cells, can bypass cell cycle arrest, accumulating genomic instability that may lead to malignant transformation (P. L. Patel et al., 2016; Romanov et al., 2001). Notably, these senescent cells can secrete a range of proinflammatory molecules, termed SASP, encompassing TGFβ itself and the reactive oxygen species (Fenniche et Oubaddou al., 2022; Hubackova et al., 2012). Furthermore, in *vitro* studies have shown that TGFβ when released by these cells, plays a pivotal role in initiating and sustaining senescence in adjacent (bystander) normal cells through the SMAD/NOX4/ROS pathway (Hubackova et al., 2012). These bystander effects may spread through a large surface of the histologically normal adjacent tissue, leading to an increased risk of recurrence in surgery sites. This holds clinical significance since 15% of post-surgical breast tumors recur within 10 years (Gadaleta et al., 2022). Notably, TGFβ, released by the tumor microenvironment and potentially by senescent cells near the tumors, could influence a pivotal aspect of tumor biology: EMT (epithelial-mesenchymal transition) in breast cancer cells (Boudreau et al., 2012). This process was reported to be regulated by the TGFβ/SMAD3/NOX4/ROS pathway in breast cancer cells, revealing the broad implication of this pathway that may constitute a potential therapeutic target in breast cancer (Boudreau et al., 2012). Therefore, our findings highlight the clinical relevance of prior research, emphasizing the role of the tumor microenvironment in NOX4 protein expression/subcellular localization and its significance in breast cancer development.

The presence of perinuclear localization in MBT samples was significantly associated with aggressive features such as SBR grade III (p=0.0249) and triple-negative phenotype (p=0.0276) (Figure 6f, 8c, pages 92 and 94). This pattern also trended towards higher ki67 protein levels and the presence of vascular emboli. On the other hand, nuclear staining in MBT samples correlated with lower SBR grades (I/II) (p=0.0249) and luminal BC phenotype (p=0.0418), indicating a more favorable prognosis (Figure 6f, 8d, pages 92 and 94). Notably, tumors with exclusive cytoplasmic localization exhibited intermediate aggressive characteristics, bridging the gap between the two primary groups. Given the marked concordance (*Kappa*= 0.487) in NOX4 protein subcellular localization between MBTs and NATs, examining the clinical relevance of this feature in NATs can provide interesting insights (Figure 9, page 95). Indeed, the presence of NOX4 protein perinuclear localization in MBTs and/or NATs samples was observed in 60.71% (17/28) of all analyzed TNBC cases. These findings suggest a potential contribution of NOX4 protein perinuclear localization to the development/progression of the

TNBC molecular subtype of breast cancer. Therefore, the NOX4's perinuclear localization could present a promising therapeutic target for TNBC. Nevertheless, further studies are needed to uncover NOX4 protein subcellular localization's role in TNBC biology.

Conclusions and Perspectives

In this research on breast cancer, we thoroughly investigated NOX4 and DUOX1 protein expression and subcellular localization and the *BRCA1* gene promoter hypermethylation using various mammary tissues. Our findings showed differential statuses of these markers and the potential of NOX4 protein regulation and *BRCA1* gene promoter hypermethylation in predicting the aggressiveness of breast cancer. Their status in normal adjacent tissues adjacent to the tumors highlights a deep dysregulation of several Redox signaling pathways and epigenetic regulation pathways, highlighting the broad potential implications these tissues could present. For instance, our research highlights the importance of understanding the tumor microenvironment and its potential influence on breast cancer progression. Our findings highlight NOX4 protein subcellular localization and *BRCA1* gene promoter hypermethylation in BC as potential early events of epi/genetic instabilities in mammary transformation. In addition, our findings could contribute to improving diagnostic tools, targeted treatments, and more effective early detection strategies for breast cancer.

In exploring *BRCA1* gene promoter hypermethylation across diverse mammary tissues, we elucidated its pivotal role in breast malignancy. The elevated prevalence of this epigenetic alteration in MBTs compared to BBLs highlights its association with breast tumorigenesis. Nonetheless, the presence of this feature in a non-negligible fraction of BBLs suggests its potential as an early marker for genetic and epigenetic instabilities, elucidating the associated risks of breast cancer among BBL cases. Remarkably, while NATs are considered as typical controls, in our study, they exhibited hypermethylation rates similar to those of MBTs. While these tissues are histologically non-cancerous, they display profound deregulation in proinflammatory pathways and genetic/epigenetic instabilities. The concordant identification of this epigenetic marker in paired MBT and NAT possibly indicates the influence of the tumor microenvironment. Such findings highlight the significance of detecting these alterations in NATs, especially considering their broader clinical implications. Younger MBT cases were more frequent among the hypermethylated group (homogenous for age distribution), suggesting a correlation with early-onset breast cancer. Our findings, supported by existing literature, propose *BRCA1* gene promoter hypermethylation as a potentially useful biomarker for breast cancer aggressiveness. Further translational studies exploring the relationship between *BRCA1* promoter hypermethylation and the loss/decrease in its function in terms of DNA damage in both NATs and BBLs could reinforce the potential role of *BRCA1* gene promoter hypermethylation as an early biomarker for patients at risk of developing breast cancer, as well as the clinical application of *BRCA1* gene promoter hypermethylation in the management of cancerous and non-cancerous breast diseases.

Our research discovered a significant upregulation of NOX4 protein in malignant breast tumors when compared with their normal adjacent tissues, highlighting its potential pathological significance. Conversely, DUOX1 protein levels were reduced in tumor tissues, suggesting its possible tumor-suppressive role in mammary tissues. The subcellular localization of NOX4, a determinant factor of NOX4-specific functions in cancer cells, was explored, revealing varied localizations within both tumor and normal adjacent tissues, with a significant concordance between them. Interestingly, the perinuclear localization of NOX4 protein in breast tumor samples was linked to aggressive cancer features, while nuclear staining suggested a more favorable prognosis. Interestingly, the significant correlation between perinuclear staining and the TNBC phenotype suggests this marker as a potential therapeutic target. Moreover, our analysis also showed the potential of normal adjacent tissues in analyzing NOX4 protein subcellular localization as a biomarker, manifesting a significant correlation with the TNBC phenotype. This research also suggests the influence of the tumor microenvironment, possibly mediated by factors like TGFβ, on NOX4 protein expression and localization in both malignant and normal adjacent cells. Such findings emphasize the significance of the tumor microenvironment in breast cancer development and recurrence, with implications for therapeutic interventions targeting the NOX4 pathway. Overall, these findings provide a comprehensive understanding of the role of NOX4 and DUOX1 in breast cancer, highlighting the need for further studies to elucidate the clinical implications and therapeutic potential of these proteins.

Among our results of my thesis, we discovered that tissues adjacent to breast tumors exhibit significant molecular changes. Specifically, these tissues show epigenetic silencing of the *BRCA1* gene, an upregulation of NOX4 protein, and similar subcellular localizations of NOX4 protein between these normal tissues and their matched tumors. These findings are particularly significant considering that *BRCA1* gene promoter hypermethylation is a recognized marker of epigenetic instability, and the activity of NOX4 protein is associated with genetic instability through DNA damage. These findings suggest that the histologically normal tissues neighboring breast tumors are undergoing profound molecular alterations. These alterations involve several pathways related to epigenetic and genetic instabilities, implying their potential role in the initiation and progression of breast tumorigenesis. This provides more understanding on the interplay between breast tumors and their adjacent tissues, which are typically used as control samples in cancer research, raising important questions about the suitability of these tissues as controls. It is crucial to investigate the molecular abnormalities in these adjacent tissues, especially their relationship with the diverse clinical and histopathological characteristics of

tumors. Such research will not only enhance our understanding of breast cancer but also will guide us to more effective management of breast cancer and potentially other forms of cancer.

Perspectives

Our translational research highlights NOX4 protein subcellular localization and *BRCA1* gene promoter hypermethylation as potential targets for breast cancer therapy. Particularly, the perinuclear localization of NOX4 exhibits a strong correlation with triple-negative breast cancer (TNBC) cases either detected in MBTs or NATs. Furthermore, NOX4 protein subcellular localization potentially modulates EMT in breast cancer through the TGFβ signaling pathway. Parallelly, the aberrant methylation detected in the *BRCA1* gene promoter was also linked to aggressive breast cancer subtypes, including TNBC and BC overexpressing Her2 receptor. Therefore, targeting these markers may influence some specific carcinogenic processes, such as proliferation and EMT, which can lead to anti-tumoral effects.

Moroccan medicinal and aromatic plants, constitute a tremendous source of natural components with potential anti-cancerous effects. As our research progresses, we focus on assessing the potential therapeutic effects of Moroccan medicinal and aromatic plant extracts on the identified epigenetic and oxidative markers using human breast cancer cell lines. It is essential to highlight that this aim is still in its early phases. At present, our attention is directed towards characterizing human breast cancer cell lines for their status on the different biomarkers we identified towards the translational approach (Figure 20). This involves analyzing the impact of plant extracts on NOX4 protein expression, its subcellular localization, and the *BRCA1* gene promoter hypermethylation status across diverse human breast cancer cell lines, including MCF7 luminal cell line, MDA-MB-231 triple-negative cell line, and Her2 positive cell line.

We initiated this objective by first characterizing the MCF7 cell line by determining their reactions to different plant extracts (Figure 22) as well as characterizing their status of *BRCA1* gene promoter hypermethylation (Figure 20). We also extracted the whole mRNA to analyze the expression of several genes, including the *BRCA1* gene and NOX4 (Figure 21).

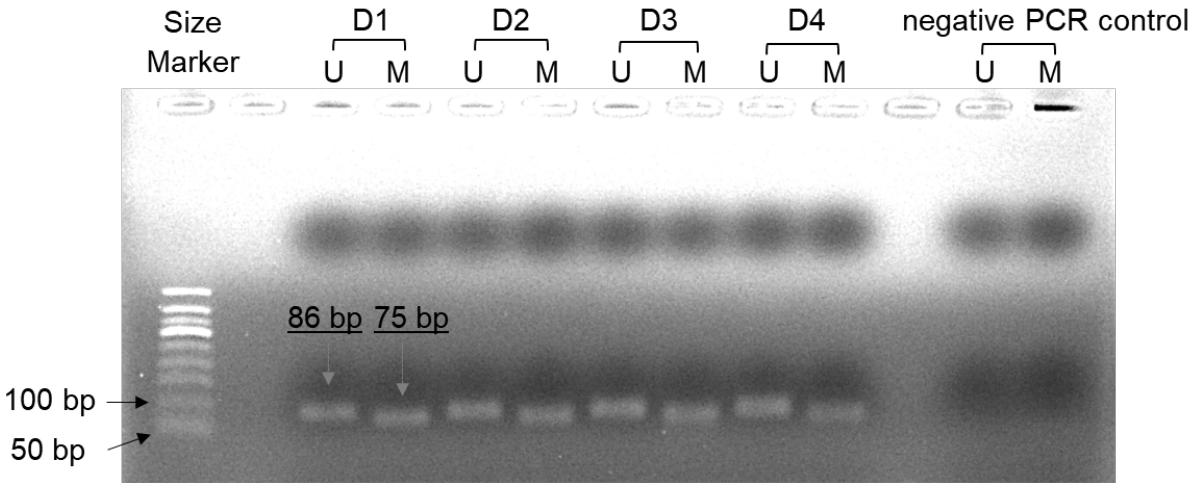

Figure 20. Gel Electrophoresis Image Representing the Hypermethylation Analysis of *BRCA1* Gene Promoter via MSP in the MCF7 Cell Line. This analysis was conducted using MCF7 cultured cells, seeded at an initial concentration of 300 x 103 cells per well (plates of six wells). Genomic DNA was extracted at D1, D2, D3 and D4 (D: Day). U: MSP product using pair of primers amplifying the unmethylated state of the targeted region. M: MSP product using the pair of primers M amplifying the hypermethylated state of the targeted region.

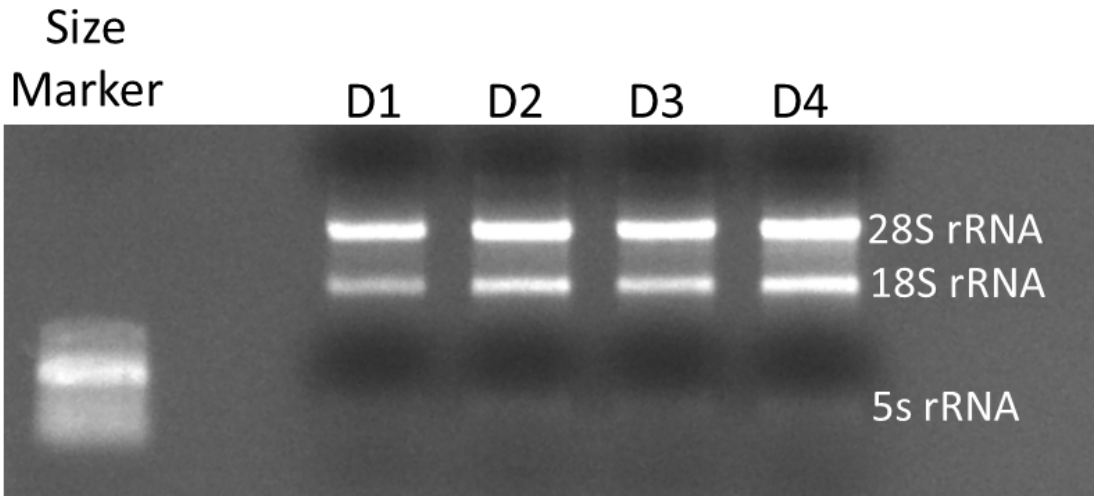

Figure 21. Gel Electrophoresis Image Representing the Migration of 1000 ng of mRNA Extracted from MCF7 Cells. This analysis was conducted using MCF7 cultured cells, seeded at an initial concentration of 300 x 103 cells per well (plates of six wells). The whole mRNA was extracted at D1, D2, D3 and D4 (D: Day). The integrity of the RNA is indicated by the clear 28S and 18S rRNA bands.

MSP analysis of DNA extracted from MCF7 cells demonstrated a stable *BRCA1* gene promoter hypermethylation profile throughout the four-day proliferation period (Figure 20). Prior studies have demonstrated varying statuses of this epigenetic alteration in MCF7 cells (Y. Chen et al., 2009; Donovan et al., 2019; Magdinier et al., 2000; J. Xu et al., 2010), and our research confirms that *BRCA1* gene promoter is partially methylated in MCF7 cells (Oubaddou et al., 2023b). This epigenomic background of MCF7 cells presents a hallmark for assessing the potential influence of Moroccan medicnal and aromatic plant extracts on breast cancer's epigenetic instability.

The reactions of the MCF7 cell line to various plant extracts were explored using the WST-1 test, a cell viability and proliferation metric. Six distinct plant extracts were added at a concentration of 1mg/ml to treat cultured MCF7 cells for 24 hours. Of these, extracts from plants No. 2, No. 4, and No. 6 exhibited pronounced anti-proliferative effects, measured by the WST-1 test (Figure 22a). Using a dose-response analysis, we then aimed to determine the IC50 (effective concentration at 50% of maximal effect) for these promising extracts. Different plant extract concentrations were used, spanning from 0 to 1 mg (Figure 22b, c and d). A derived IC50 value of 622.8 µg/ml was determined for plant extract 2, highlighting its potential therapeutic efficacy. Furthermore, for plant extract N°6 an IC50 value of 744.8 µg/ml was identified. However, despite initial anti-proliferative effects at 1mg/ml, we observed no response in the subsequent tests for plant extract N°4.

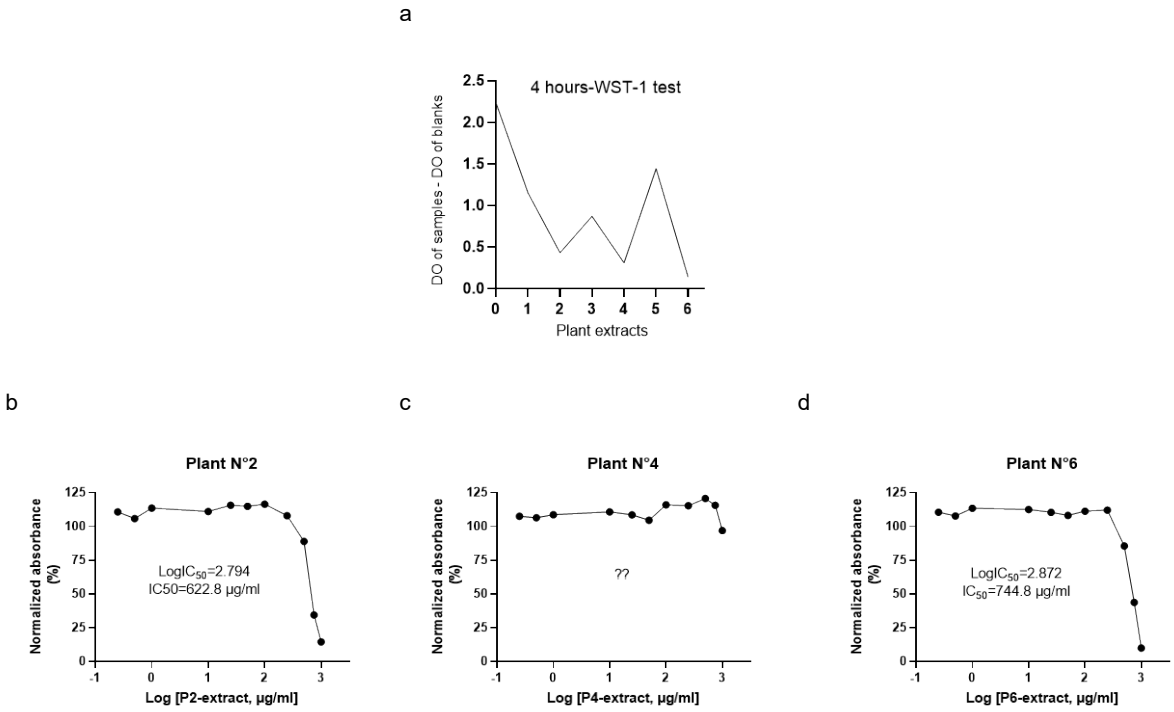

Figure 22: Impact of Medicinal and Aromatic Moroccan Plant Extracts on the Proliferation of MCF7 Cells. (a) Demonstrates the anti-proliferative effects of six distinct plant extracts on MCF7 cells. (b-d) Dose-Response Curves depicting the MCF7 cell response to varying concentrations of extracts from Plant No. 2, No. 4, and No. 6, respectively. Each curve includes the respective IC50 value, highlighting the concentration at which 50% inhibitory effect is observed.

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Abstract

Breast cancer is a global and national health concern affecting women. Its molecular and prognostic heterogeneity requires the discovery of efficient biomarkers to enhance disease management. Epigenetic alterations can drive tumor transformation by silencing tumor suppressor genes (TSGs). DNA hypermethylation-mediated TSG inactivation has been extensively studied in BC and could represent an early event in mammary transformation. In this respect, the role of BRCA1 promoter hypermethylation in non-malignant mammary tissues, like normal adjacent tissues (NATs) and benign breast lesions (BBLs), warrants further exploration. NADPH oxidases (NOXs), recognized for their ability to generate reactive oxygen species (ROS), are overexpressed in numerous cancer types, potentially leading to oxidative stress (OS). OS is closely linked to breast malignancy by influencing the epithelial-mesenchymal transition (EMT) and angiogenesis. These processes were shown to be involved in BC by the ability of NADPH oxidase 4 (NOX4) to produce ROS. Notably, there is limited data regarding NOX4 and DUOX1 (Dual oxidase 1) protein expression and subcellular localization in malignant breast tumors (MBTs) and their NATs.

Our research investigated these gaps by analyzing FFPE samples from various mammary tissues using Methylation-Specific PCR (84 Samples) and immunohistochemistry (198 Samples). Our exploration of BRCA1 promoter hypermethylation revealed elevated rates in MBTs (41.67%:20/48) compared to BBLs (19.05%:4/21), aligning with previous reports on its role in breast malignancy. Nevertheless, its presence in BBLs suggests its potential as an early marker of genetic and epigenetic instabilities. Intriguingly, NATs, often used as controls, showed a hypermethylation rate (46.67%:7/15) comparable to MBTs. Furthermore, BRCA1 promoter hypermethylation correlates with aggressive BC subtypes, including Her2-positive BC and triple-negative BC. Our data showed a significant upregulation of NOX4 protein in MBTs (79%:79/100) compared to NATs (51.06%:48/94). Conversely, DUOX1 levels were reduced in MBTs. NOX4 subcellular localization (cytoplasm, perinuclear area, and nucleus) was concordant (*Kappa*= 0.487) between MBTs and NATs, suggesting the potential role of tumor microenvironment in regulating NOX4, possibly through factors like TGFβ. Interestingly, NOX4 perinuclear localization correlates with the triple-negative BC phenotype. This translational research highlights NOX4 subcellular localization and BRCA1 promoter hypermethylation in BC as potential early events driving mammary transformation and as attractive therapeutic targets of the aggressive forms of BC.

Keywords: Breast cancer (BC); Oxidative stress; Epigenetic instability; NADPH oxidases; NOX4; DUOX1; BRCA1 promoter hypermethylation; Subcellular localization; Biomarker; early events; Normal adjacent tissue; Benign breast lesions

Résume

Le cancer du sein (CS) constitue un problème de santé mondial et national qui affecte les femmes. Son hétérogénéité moléculaire et pronostique nécessite la découverte de biomarqueurs efficaces pour améliorer la gestion de la maladie. Les altérations épigénétiques peuvent favoriser la transformation tumorale en réprimant les gènes suppresseurs de tumeurs (GST), et l'inactivation des GST due à l'hyperméthylation de l'ADN a été largement étudiée dans le cancer du sein (CS) et pourrait représenter un événement précoce dans la transformation mammaire. À cet égard, le rôle de l'hyperméthylation du promoteur du gène BRCA1 dans les tissus mammaires non malins, tels que les tissus mammaires normaux adjacents (NATs) et les lésions mammaires bénignes (BBLs), mérite une exploration plus approfondie. Les NADPH oxydases (NOX), reconnues pour leur capacité à générer des espèces réactives de l'oxygène (ROS), sont surexprimées dans de nombreux types de cancer, ce qui peut potentiellement conduire au stress oxydatif (SO). Le SO est étroitement lié à la malignité mammaire en influençant la transition épithéliale-mésenchymateuse (TEM) et l'angiogenèse. Ces processus ont été rapportés d'être impliqués dans le CS par la capacité de la protéine NADPH oxydase 4 (NOX4) à produire des ROS. Cependant, il existe peu de données concernant l'expression protéique et la localisation subcellulaire des deux protéines NOX4 et la NADPH oxydase DUOX1 dans les tumeurs mammaires malignes (MBTs) et leurs NATs.

Notre recherche a étudié l'expression des protéines NADPH oxydases comme marqueurs oxydatifs et l'hyperméthylation du promoteur du gène BRCA1 comme marqueur épigénétique en utilisant des échantillons FFPE (Etude rétrospective). Notre exploration de l'hyperméthylation du promoteur du gène BRCA1 a révélé des taux élevés dans les MBTs (41,67 % : 20/48) par rapport aux BBLs (19,05 % : 4/21), ce qui concorde avec les rapports antérieurs sur le rôle de ce silencing épigénétique dans la malignité mammaire. Néanmoins, sa présence dans les BBLs suggère son potentiel en tant que marqueur précoce des instabilités génétiques et épigénétiques. De manière intrigante, les NATs, souvent utilisés comme témoins, ont montré un taux d'hyperméthylation (46,67 % : 7/15) comparable à celui des MBTs. De plus, l'hyperméthylation du promoteur du gène BRCA1 est corrélée avec les sous-types agressifs du CS, notamment le sous-type HER2-positif et le sous-type triple-négatif. Concernant l'expression de la protéine NADPH oxydase 4, l'expérimentation immunohistochimique (100MBTs + 94 NATs) a révélé une augmentation significative de la protéine NOX4 dans les MBTs (79 % : 79/100) par rapport aux NATs (51,06 % : 48/94). En revanche, les niveaux de la protéine DUOX1 étaient réduits dans les MBTs par rapport aux NATs. La localisation subcellulaire de la protéine NOX4 (cytoplasme, zone périnucléaire et noyau) a montré une concordance statistiquement significative (Kappa= 0.487; Cohen's kappa test) entre les MBTs et les NATs, suggérant le rôle potentiel du microenvironnement tumoral dans la régulation de de la localisation subcellulaire de NOX4. De plus, la localisation périnucléaire de la protéine NOX4 est corrélée avec le phénotype du CS triple négatif. Dans l'ensemble, cette recherche translationnelle met en lumière la protéine NOX4 et l'hyperméthylation du promoteur du gène BRCA1 dans le CS en tant qu'événements précoces potentiels favorisant la transformation mammaire et comme des cibles thérapeutiques pour les formes agressives du CS.

Mots-clés : Cancer du sein (CS) ; Stress oxydatif ; Instabilité épigénétique ; NADPH oxydases ; NOX4 ; DUOX1 ; Hyperméthylation du promoteur du gène BRCA1 ; Localisation subcellulaire ; Biomarqueur ; Événements précoces ; Tissu normal adjacent ; Lésions bénignes du sein