Doctoral Program in Health Sciences D420

EXPRESSION PROFILE OF CELLULAR REDOX AND CALCIUM SIGNALLING-RELATED GENES: ROLE IN BREAST CANCER PROGRESSION

Doctoral thesis presented by

Ana Sofia Fonseca Ramos

Supervisors:
Professor Nuno Saraiva
Professor Pedro de la Villa Polo
Professor Ana Sofia Fernandes

Alcalá de Henares, 2024

Universidad de Alcalá
DEPARTAMENTO DE CIENCIAS BIOMEDICAS

UNIVERSIDADE LUSÓFONA
ESCOLA DE CIÊNCIAS E TECNOLOGIAS DA SAÚDE

En virtud del acuerdo de colaboracion com la Universidade Lusófona de Humanidades e Tecnologias

Programa de Doctorado en Ciencias de la Salud D420
Programa de Doutoramento em Ciências da Saúde D420

PERFIL DE EXPRESIÓN DE GENES RELACIONADOS CON LA SEÑALIZACIÓN CELULAR REDOX Y DE CALCIO: PAPEL EN LA PROGRESIÓN DEL CÁNCER DE MAMA

Tesis Doctoral presentada por

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Alcalá de Henares, 2024

Acknowledgments

After years of challenges and learning, the time has come to reflect on my academic journey and express gratitude to all those who, in one way or another, contributed to my success.

I would like to begin by thanking Professor Luís Monteiro Rodrigues, who was the first to believe that I would successfully complete this journey. Despite months filled with doubts, his unwavering faith in me made me start this journey – thank you for the challenge.

To Professor Manuel Damásio, for your trust and belief in my potential. Without your support, my time at the university would not have been possible.

My supervisors, Professor Nuno Saraiva and Professor Ana Sofia Fernandes, deserve special recognition. Their great patience at the beginning (I hadn't studied for over 10 years), allowed me to grow and gain specialized knowledge in a field I didn't even know existed: Bioinformatics. To my supervisors, I am grateful for the learning, the critical thinking, the pleasure of doing things with rigor and elegance, and the freedom to experiment, make mistakes, and try again. This freedom allowed me to seek more knowledge and new strategies for solving problems.

I extend my appreciation to Professor Pedro de la Villa Polo for his involvement in the beginning of my work, and for validating my academic journey and final thesis.

A deep sense of gratitude goes to Professor João Gregório, without whom one of my studies would have never come to life. Countless hours of discussion and boundless patience culminated in a paper with an innovative methodology.

I must add an extra note of thanks to Professor Ana Sofia Fernandes for inviting me to teach Human Genetics at the School of Health Sciences and Technologies at Universidade Lusófona. It was a crucial moment for me at a time when I felt stuck and was losing motivation. Forcing myself to motivate my students, find engaging teaching strategies, and learn how to communicate science to younger generations gave me the extra energy I needed to continue my academic journey. Similarly, Professor Nuno Saraiva encouraged me to teach Immunology at the same institution. Unbeknownst to him, this opportunity allowed me to engage with my favorite subject, which brought me immense fulfillment. This professional fulfillment, instead of making my academic life heavier and more difficult to balance, was fundamental to giving me the energy, organizational skills, and confidence in communication that I have today.

This professional fulfillment was also the result of a great understanding with the coordinators of each course. My special thanks to Professor Alexandra Nunes, Professor Bernardo Palma, and

Professor Andreia Gomes for sharing classes and assessments with me and giving me the freedom to teach “on my way.”

A word of thanks to my PhD colleagues for coffee and snacks, and for keeping me updated on what was happening at the university. A special thank you to my colleague Sandra Ferreira, the first colleague I met. We collaborated on research that resulted in two publications, and she supported me through my challenging days and frustrations, remaining a steadfast presence by my side.

To my lifelong friends, thank you for your support and for understanding my periods of isolation during this final stage. To my new friends, whom I’ve gained during this journey, discussing science with them has become a routine I want to preserve for as long as possible. The encouragement to complete this journey was crucial in helping me stay focused and instilled in me the hope that the best is yet to come.

Finally, I owe an immeasurable debt of gratitude to the light of my life, my mother. She has been my support in every sense - sometimes becomes my legs, other times my hands – always ensuring I never falter, even when everything seems too dark. My academic journey, my professional life, and my life itself, only exist because of her and for her. Words cannot fully express my gratitude for the trust, love, and invaluable advice she has provided throughout my life.

Abstract

Breast cancer (BC) remains the most prevalent cancer among women. The identification of new biological pathways, biomarkers, and therapeutic targets is essential for improving patient survival rates. The levels of reactive oxygen species (ROS) and Calcium ion (Ca^{2+}) fluxes play a crucial role in BC progression. Leveraging gene expression data from The Cancer Genome Atlas (TCGA) Breast Invasive Carcinoma (BRCA) dataset and bioinformatics tools, this study aimed to identify novel druggable targets or prognostic biomarkers based on individual or combined expression profiles of redox and Ca^{2+} signalling-related genes with impact in BC progression.

The first step was to identify and evaluate the online platforms that allow the analysis of BRCA gene expression and patient survival. The selection criteria defined were: user-friendliness, presenting global gene expression and between stages and patient survival, and providing straightforward methods for data extraction. Among the platforms assessed, GEPIA and UALCAN were deemed the most suitable for our analysis due to their robust features and ease of use.

Recognizing the critical interplay between Ca^{2+} and ROS in BC progression, we aimed to evaluate how the co-dysregulation of Ca^{2+} and ROS-associated genes influences BC prognosis. This study utilized a bioinformatics approach, using data from the TCGA BRCA, to explore the association between the expression of specific genes involved in redox (LOXL2, LOXL3, PRDX4, TXN, TXNRD1, GLRX2, GLRX3, SOD2, NOX4) and Ca^{2+} homeostasis (TRPM8, CALM2, CAMK2G, ATP2C2, PLCD1, ORAI1, STIM1) on BC patient survival. Genes were selected based on significant expression differences between normal and tumour tissues and their association with survival, measured by hazard ratios. The cumulative proportion of survival at 5 years post-diagnosis was calculated for each quartile of expression of one gene within the population expressing high (Q4) or low levels (Q1) of a second gene. Gene pairs that consistently impacted cumulative survival were further analysed through enrichment analysis, focusing on biological processes from the Gene Ontology. This analysis revealed significant correlations between the simultaneous dysregulation of specific gene pairs and BC patient survival, such as GLRX3/LOXL3, PRDX4/LOXL2, NOX4/PRDX4, TRPM8/CAM2G, ATP2C2/TRPM8, CAMK2G/STIM1, ATP2C2/GLRX2, and PLCD1/TXNRD1. These gene pairs were consistently co-dysregulated with genes associated with critical processes in BC, such as cell cycle regulation, adhesion, and cellular projection. These findings highlight the potential of these gene pairs as biomarkers or therapeutic targets in BC.

An example of redox-active proteins dysregulated in BC is the family of Lysyl oxidases. Lysyl oxidase (LOX) and LOX-like1-4 (LOXL1-4) enzymes impact cell migration and can contribute to

the metastasis of BC. In this context, understanding the differential impact of each enzyme on BC progression is essential for directing drug development. Utilizing the TCGA-BRCA database and tools like GEPIA 2.0 and TIMER2.0, the study revealed that LOXL1, LOXL2, and LOXL3 are significantly overexpressed in BC tissues. This overexpression is linked to poorer disease-free survival (DFS) and overall survival (OS), particularly in specific BC subtypes. In contrast, LOXL4 expression is notably reduced, especially in advanced stages of BC, and its downregulation is associated with worse DFS and OS in HER2+ patients. The analysis also indicated that high levels of LOXL1, LOXL2, and LOXL3 are correlated with an increase in cancer-associated fibroblasts (CAFs) and a reduction in the infiltration of immune cells, such as B cells and T cells. Additionally, there is a strong correlation between the expression of LOX, LOXL1, and LOXL2 and other extracellular matrix (ECM)-related genes, including TIMP2, PDGFR-β, MMP-2, MMP-14, FN1, ITGA5, and ZEB1. These findings suggest that while inhibiting LOXL3 may have varying effects depending on the BC subtype, targeting LOXL1 and LOXL2 could be a more effective therapeutic strategy. Conversely, inhibiting LOXL4 might not be as beneficial. This study highlights the importance of considering the specific roles of LOX family members and expression patterns in developing targeted therapies for BC.

The identification of redox-related genes differentially expressed in HER2+ BC compared to normal tissues, their association with patient survival, and with tumour cell infiltrate can potentially lead to the identification of new prognosis biomarkers. Using Gene Ontology and keywords related to oxidative processes, the study filtered data from the TCGA-BRCA-HER2+ database. GEPIA2 and UALCAN were employed to assess the association between the expression of redox homeostasis-related genes on patient survival and to examine differences in expression between HER2+ BC patients and normal tissues ($p<0.05$). This approach led to the identification of 55 potentially relevant redox-related genes. To explore the relationship between gene expression and cell infiltration levels in HER2+ BC patients, the TIMER2.0 tool was utilized. Out of the 55 genes identified, only six—EZH2, MAPK13, NFE2L2, HDAC2, MACROH2A1, and TSC2—have been experimentally validated in the context of HER2+ BC. However, several redox-related genes were significantly dysregulated, although their specific roles in this subtype are not fully explored. Among these, 17 genes, including TXNRD1 and EZH2, were overexpressed in HER2+ tumours, while 26 genes, such as NFE2L2 and TSC1, were downregulated and correlated with increased infiltration of macrophages and neutrophils. Additionally, ten redox-related genes demonstrated correlations with either high or low HR, suggesting an influence on tumour infiltrates. These findings underscore the potential of redox-related genes as therapeutic targets

or biomarkers in HER2+ BC, emphasizing the importance of further research to clarify their roles in cancer progression and therapy resistance.

Although experimental validation is needed, all the studies presented in this thesis identified novel potential BC biomarkers and therapeutic targets; and deepened our understanding of the interactions between redox and Ca^{2+}-related genes and other genes, and their association with tumour infiltrates and BC patient outcomes. Thus, contributing to the identification of novel potential prognosis biomarkers or druggable targets.

Keywords: Breast cancer, Redox-related genes, Calcium ion-related genes, TCGA, Patients survival

Resumen

El cáncer de mama (CM) sigue siendo el tipo de cáncer más frecuente entre las mujeres. La identificación de nuevas vías biológicas, biomarcadores y objetivos terapéuticos es esencial para mejorar las tasas de supervivencia de las pacientes. Los niveles de especies reactivas de oxígeno (ROS) y los flujos de iones de calcio (Ca^{2+}) juegan un papel crucial en la progresión del CM. Aprovechando los datos de expresión génica del conjunto de datos de carcinoma invasivo de mama (BRCA) del Atlas del Genoma del Cáncer (TCGA) y herramientas bioinformáticas, este estudio tuvo como objetivo identificar nuevos objetivos farmacológicos o biomarcadores pronósticos basados en perfiles de expresión individuales o combinados de genes relacionados con la señalización redox y de Ca^{2+} que impactan en la progresión del CM.

El primer paso fue identificar y evaluar las plataformas en línea que permiten el análisis de la expresión génica del BRCA y la supervivencia de los pacientes. Los criterios de selección definidos fueron: facilidad de uso, presentación de la expresión génica global y entre etapas, y la supervivencia de los pacientes, además de proporcionar métodos sencillos para la extracción de datos. Entre las plataformas evaluadas, GEPIA y UALCAN se consideraron las más adecuadas para nuestro análisis debido a sus características robustas y facilidad de uso.

Reconociendo la interacción crítica entre Ca^{2+} y ROS en la progresión del cáncer de mama (CM), nuestro objetivo fue evaluar cómo la co-disregulación de los genes asociados con Ca^{2+} y ROS influye en el pronóstico del CM. Este estudio utilizó un enfoque bioinformático, utilizando datos del TCGA BRCA, para explorar la asociación entre la expresión de genes específicos involucrados en el equilibrio redox (LOXL2, LOXL3, PRDX4, TXN, TXNRD1, GLRX2, GLRX3, SOD2, NOX4) y la homeostasis de Ca^{2+} (TRPM8, CALM2, CAMK2G, ATP2C2, PLCD1, ORAI1, STIM1) con la supervivencia de pacientes con CM. Los genes se seleccionaron en función de diferencias significativas de expresión entre tejidos normales y tumourales y su asociación con la supervivencia, medida mediante razones de riesgo. Se calculó la proporción acumulada de supervivencia a los 5 años después del diagnóstico para cada cuartil de expresión de un gen dentro de la población que expresaba niveles altos (Q4) o bajos (Q1) de un segundo gen. Los pares de genes que impactaron consistentemente en la supervivencia acumulada se analizaron posteriormente mediante un análisis de enriquecimiento, enfocándose en los procesos biológicos del Gene Ontology. Este análisis reveló correlaciones significativas entre la disfunción simultánea de pares de genes específicos y la supervivencia de pacientes con CM, tales como GLRX3/LOXL3, PRDX4/LOXL2, NOX4/PRDX4, TRPM8/CAM2G, ATP2C2/TRPM8, CAMK2G/STIM1, ATP2C2/GLRX2 y PLCD1/TXNRD1. Estos pares de genes se co-disregulaban consistentemente

con genes asociados con procesos críticos en el CM, como la regulación del ciclo celular, la adhesión y la proyección celular. Estos hallazgos resaltan el potencial de estos pares de genes como biomarcadores o posibles objetivos terapéuticos en el CM.

Un ejemplo de proteínas redox activas disfuncionales en el cáncer de mama (CM) es la familia de las lisis oxidasas. Las enzimas lisil oxidasa (LOX) y LOX-like1-4 (LOXL1-4) afectan la migración celular y pueden contribuir a la metástasis del CM. En este contexto, comprender el impacto diferencial de cada enzima en la progresión del CM es esencial para dirigir el desarrollo de medicamentos. Utilizando la base de datos TCGA-BRCA y herramientas como GEPIA 2.0 y TIMER2.0, el estudio reveló que LOXL1, LOXL2 y LOXL3 están significativamente sobre expresados en tejidos de CM. Esta sobreexpresión está vinculada a una menor supervivencia libre de enfermedad (DFS) y supervivencia global (OS), particularmente en subtipos específicos de CM. En contraste, la expresión de LOXL4 está notablemente reducida, especialmente en etapas avanzadas del CM, y su disminución está asociada con peores DFS y OS en pacientes con HER2+. El análisis también indicó que altos niveles de LOXL1, LOXL2 y LOXL3 están correlacionados con un aumento en los fibroblastos asociados al cáncer (CAFs) y una reducción en la infiltración de células inmunitarias, como células B y T. Además, existe una fuerte correlación entre la expresión de LOX, LOXL1 y LOXL2 y otros genes relacionados con la matriz extracelular (ECM), incluyendo TIMP2, PDGFR-β, MMP-2, MMP-14, FN1, ITGA5 y ZEB1. Estos hallazgos sugieren que, aunque inhibir LOXL3 puede tener efectos variados dependiendo del subtipo de CM, dirigirse a LOXL1 y LOXL2 podría ser una estrategia terapéutica más efectiva. Por otro lado, inhibir LOXL4 podría no ser tan beneficioso. Este estudio resalta la importancia de considerar los roles específicos de los miembros de la familia LOX y los patrones de expresión al desarrollar terapias dirigidas para el CM.

La identificación de genes relacionados con el equilibrio redox que se expresan de manera diferencial en el cáncer de mama HER2+ en comparación con tejidos normales, su asociación con la supervivencia de los pacientes y con la infiltración de células tumourales puede llevar potencialmente a la identificación de nuevos biomarcadores de pronóstico. Utilizando Gene Ontology y palabras clave relacionadas con procesos oxidativos, el estudio filtró datos de la base de datos TCGA-BRCA-HER2+. Se emplearon GEPIA2 y UALCAN para evaluar la asociación entre la expresión de genes relacionados con la homeostasis redox y la supervivencia de los pacientes, y para examinar las diferencias de expresión entre pacientes con cáncer de mama HER2+ y tejidos normales ($p<0.05$). Este enfoque llevó a la identificación de 55 genes potencialmente relevantes relacionados con el equilibrio redox. Para explorar la relación entre la expresión génica y los niveles de infiltración celular en pacientes con cáncer de mama HER2+, se utilizó la

herramienta TIMER2.0. De los 55 genes identificados, solo seis—EZH2, MAPK13, NFE2L2, HDAC2, MACROH2A1 y TSC2—han sido validados experimentalmente en el contexto del cáncer de mama HER2+. Sin embargo, varios genes relacionados con el equilibrio redox estaban significativamente disfuncionados, aunque sus roles específicos en este subtipo aún no están completamente explorados. Entre estos, 17 genes, incluidos TXNRD1 y EZH2, estaban sobre expresados en tumoures HER2+, mientras que 26 genes, como NFE2L2 y TSC1, estaban su expresados y correlacionados con una mayor infiltración de macrófagos y neutrófilos. Además, diez genes relacionados con el equilibrio redox mostraron correlaciones con HR alto o bajo, lo que sugiere una influencia en los infiltrados tumourales. Estos hallazgos subrayan el potencial de los genes relacionados con el equilibrio redox como posibles objetivos terapéuticos o biomarcadores en el cáncer de mama HER2+, enfatizando la importancia de continuar investigando para esclarecer sus roles en la progresión del cáncer y la resistencia a la terapia.

Aunque se necesita validación experimental, todos los estudios presentados en esta tesis identificaron nuevos biomarcadores potenciales de cáncer de mama y objetivos terapéuticos, y profundizaron en nuestra comprensión de las interacciones entre los genes relacionados con el equilibrio redox y Ca^{2+} y otros genes, así como su asociación con infiltrados tumourales y los resultados de los pacientes con cáncer de mama. De este modo, se contribuye a la identificación de nuevos biomarcadores de pronóstico potenciales o posibles objetivos terapéuticos.

Palabras clave: Cáncer de mama, Genes relacionados con el equilibrio redox, Genes relacionados con el ion calcio, TCGA, Supervivencia de los pacientes.

Scientific outputs

The studies presented in this thesis allowed me to improve my bioinformatics knowledge and were or will be published in international peer-reviewed journals. Some of these works were also presented in scientific meetings, including oral and poster communication. These outputs are listed below.

A) Scientific articles

I. Ramos S, Fernandes AS, Saraiva, N. Gene expression and survival analysis in cancer research using online open access platforms: a comparative analysis. Biomedical and Biopharmaceutical Research. 2020;17(2):222-240. https://doi.org/10.19277/bbr.17.2.247
IF: NA; Q3 (Scopus)

II. Ramos S*, Ferreira S*, Fernandes AS, Saraiva, N. Lysyl Oxidases Expression and Breast Cancer Progression: A Bioinformatic Analysis. Frontiers in Pharmacology. 2022;13:883998. https://doi.org/10.3389/fphar.2022.883998
(*shared first authorship)
IF: 7.8; Q1 (Scopus)

III. Ramos S, Gregório J, Fernandes, AS, Saraiva, N. Association between Dysregulated Expression of Ca^{2+} and ROS-Related Gene Pairs and Breast Cancer Patient Survival. Submitted

IV. Luz P*, Ramos S*, Oliveira MJ, Saraiva N, Costa JG, Fernandes AS. Interaction between redox regulation, immune activation and response to treatment in HER2+ breast cancer. (* shared first authorship)
To be submitted

B) Abstracts published in international peer-reviewed journals

I. Ramos S, Ferreira S, Saraiva N, Fernandes, A. 30P Bioinformatics analysis for lysyl oxidases as therapeutic targets for breast cancer. Annals of Oncology 2021:32(Supp.6):S1355. https://doi.org/10.1016/j.annonc.2021.08.2026

II. Ferreira S, Ramos S, Saraiva N, Fernandes A. Lysyl oxidases in the pathophysiology of breast cancer: a bioinformatics approach. ID: 5055. Biomed Biopharm Res. 2021;18(2):S291-S554. https://doi.org/10.19277/bbr.18.2.265

III. Ferreira S, Ramos S, Saraiva N, Fernandes A. Lysyl oxidases in the pathophysiology of breast cancer: a bioinformatics approach. Biomedical and Biopharmaceutical Research, In Proceedings of the 56th Annual Congress of the Brazilian Society of Physiology, 2021;18(2):322-323. https://doi.org/10.19277/bbr.18.2.265

IV. Ferreira S, Ramos S, Holota S, Khyluk D, Lesyk R, Saraiva N. Fernandes AS. Targeting lysyl oxidases for breast cancer: bioinformatic analysis and biochemical evaluation of 4-thiazolidinone derivatives as LOXL2 inhibitors. FEBS Open Bio, 2022;12(Suppl.1):91. https://doi.org/10.1002/2211-5463.13440

V. Ramos AS, Fernandes AS, & Saraiva N. Simultaneous expression dysregulation of redox and calcium signalling-related genes: impact on breast cancer patient survival. FEBS Open Bio, 2022;1(Suppl.1):96. https://doi.org/10.1002/2211-5463.13440

VI. Ferreira S, Ramos S, Holota S, Khyluk D, Lesyk R, Finyuk N, dos Santos DJVA, Díaz-Lanza AM, Costa JG, Saraiva N, Fernandes AS. Lysyl Oxidase Like 2: an enzyme at the crossroad of Toxicology and Pharmacology. Toxicology Letters 2023;384(2):S92. https://doi.org/10.1016/S0378-4274(23)00482-4

VII. Ferreira S, Ramos S, Santos B, Nascimento C, Santos M, Díaz-Lanza AM, Rijo P, dos Santos DJVA, Costa JG, Leite C, Rosado C, Saraiva N, Fernandes AS. LusoModLox: in search of therapeutic lysyl oxidase inhibitors. Biomedical and Biopharmaceutical Research, In Proceedings of the 5th CBIOS Conference, 2023;20(2):8. https://doi:10.19277/bbr.20.2.320

VIII. Ramos S, Gregório J, Fernandes AS, Saraiva N. Synchronized expression dysregulation of Redox and Calcium-related genes: implications for breast cancer patient survival. Biomedical and Biopharmaceutical Research, In Proceedings of the 5th CBIOS Conference, 2023;20(2):20. https://doi:10.19277/bbr.20.2.320

C) Abstracts Published in Nationally Circulated Journals, Peer-Reviewed

I. Ferreira S, Ramos S, Holota S, Khyluk D, Lesyk R, Finyuk N, dos Santos D, Díaz-Lanza AM, Costa JG, Saraiva N, Fernandes AS. Targeting Lysyl Oxidase Like 2 to Prevent Breast Cancer Metastases. Acta Farmacêutica Portuguesa - X Congresso Iberoamericano de Ciências Farmacêuticas. 2023. ISSN: 2182-3340

D) Oral Communications

I. Ramos S, Saraiva N, Fernandes A. "Bioinformatic Databases as a tool to analyse alterations in Cell Physiology in Carcinogenesis: A comparative analysis of open access data analysis platforms." Online Presentation. Encontro Luso Brasileiro de Fisiologia, 9th, October 2020.

II. Ferreira S, Ramos S, Holota S, Khyluk, D, Lesyk R, Saraiva N, Fernandes AS. Lysyl oxidase Like 2 as a therapeutic target for breast cancer: Bioinformatics analysis and biochemical evaluation of 4-thiazolidine derivatives. Oral communication 5.3. LII Reunião da Sociedade Portuguesa de Farmacologia, 9th-11th feb 2022, Porto, Portugal

III. Ferreira S, Ramos S, Holota S, Khyluk D, Lesyk R, Finyuk N, dos Santos DJVA, Díaz-Lanza AM, Costa JG, Saraiva N, Fernandes AS. Targeting Lysyl Oxidase Like 2 to prevent breast cancer metastases. X Congresso Iberoamericano de Ciências Farmacêuticas, 26th-28th, October 2023, Coimbra, Portugal.

IV. Ferreira S, Ramos S, Holota S, Khyluk D, Lesyk R, Finyuk N, dos Santos DJVA, Díaz-Lanza AM, Costa JG, Saraiva N, Fernandes AS. Novel lysyl oxidase like 2 inhibitor decreases breast cancer cells migration. LIII Congresso da Sociedade Portuguesa de Farmacologia, 1st-3rd Feb, 2023, Coimbra, Portugal.

V. Ferreira S, Ramos S, Santos B, Nascimento C, Santos, M, Díaz-Lanza AM, Rijo P, dos Santos DJVA, Costa JG, Leite C, Rosado C, Saraiva N, Fernandes AS. LusoModLox: in search of therapeutic lysyl oxidase inhibitors. V Jornadas CBIOS, 10th November, 2023, Lisboa, Portugal.

VI. Ramos S, Gregório, J., Fernandes, A.S., & Saraiva, N. Crosstalk between expression of redox and calcium-related genes and its potential to unveil new therapeutic targets for breast cancer. LIV Congresso da Sociedade Portuguesa de Farmacologia, 7th-9th October, 2024, Lisboa, Portugal.

E) Poster communications

I. Ramos S, Saraiva N, Fernandes A. Bioinformatic Databases as a tool to analyse alterations in Cell Physiology in Carcinogenesis: A comparative analysis of open access data analysis platforms. Online Presentation. Encontro Luso Brasileiro de Fisiologia, 9th, October 2020.

II. Ramos S, Ferreira S, Saraiva N, Fernandes A. Bioinformatics analysis for Lysyl Oxidases as therapeutic targets for breast cancer. Molecular Analysis for Precision Oncology Virtual Congress 2021, 7-9th October 2021

III. Ferreira S, Ramos S, Saraiva N, Fernandes A. Lysyl Oxidases in the pathophysiology of breast cancer: a bioinformatics approach. E-poster presented in Encontro Luso Brasileiro de Fisiologia, 12th-17th, October, 2021.

IV. Ferreira, S, Ramos S, Holota S, Khyluk D, Lesyk R, Saraiva N, Fernandes AS. Lysyl oxidase like 2 as a therapeutic target for breast cancer: bioinformatics analysis and biochemical evaluation of 4-thiazolidinone derivatives. The Biochemistry Global Summit, Lisbon 2022, 9th-14th July, 2022.

V. Ramos S, Fernandes AS, Saraiva N. Expression profile of cellular redox and calcium signalling-related genes: role in breast cancer progression. The Biochemistry Global Summit, Lisbon 2022, 9th-14th July, 2022.

VI. Ramos S, Gregório J, Fernandes AS, Saraiva N. The influence of Redox and Calcium signalling-related gene expression profiles on the progression of Breast Cancer. Encontro de Ciencia 2023, 5th-7th July, 2023

VII. Ramos S, Gregório J, Fernandes AS, Saraiva N. Synchronized expression dysregulation of Redox and Calcium-related genes: implications for breast cancer patient survival. V Jornadas CBIOS, Lisboa, 10th November, 2023.

VIII. Ramos S, Gregório J, Fernandes AS, Saraiva N. Crosstalk between Expression of Redox and Calcium Signalling Genes and Its Impact on Breast Cancer Patient Survival. RSGDREAM 2023, virtual event, International Society for Computational Biology (ISCB), 28th-30th November, 2023.

IX. Ferreira S, Ramos S, Díaz-Lanza AM, Saraiva N, Rijo P, Palma BB, Fernandes AS. Exploring the Multifaceted Potential of EPCU in Targeting Tumourigenesis and Hormone Regulation. 2nd Asian Student Council Symposium, virtual event, International Society for Computational Biology (ISCB), 25th November, 2023.

X. Ramos S, Gregório J, Fernandes AS, Saraiva N. Coordinated Expression of Genes Related to Redox status and Ca^{2+} homeostasis: Implications for Breast Cancer Patient Survival. EACR Virtual Conference on Signalling Circuits in Cancer, 13th-14th February, 2024

Beyond the scope of my thesis, I was also involved in other research studies, which allowed me to develop different skills, helping to apply methodologies developed in my PhD. These studies also resulted in various articles and oral or panel communications, which are featured below.

I. Martins M, Almeida N, Nicolai M, Serrano M, Ramos S, Palmeira CM, Gerner C, Fernandes AS, Favero G, Saraiva N. TMBIM4-Mediated Transsulfuration Reprogramming Enhances Cancer Cell Survival in Starvation Conditions (manuscript in preparation)

II. Ferreira S*, Ramos S*, Díaz-Lanza AM, Rijo P, Saraiva N, Fernandes AS, Palma BB. Exploring the Multifaceted Potential of coleones and royleanones in Targeting Tumourigenesis (manuscript in preparation) (*shared first authorship)

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Chapter IV

List of Abbreviations

ADF	Adipocyte-derived fibroblasts
AKT	Protein kinase B
AJCC	American Joint Committee on Cancer
ALN	Axillary lymph node
AMPK	AMP-activated protein kinase
AP-1	Activator protein 1
APC	Adenomatous polyposis coli
ASIC1	Acid-sensing ion channel 1
ATM	Ataxia-telangiectasia mutated or ATM serine/threonine kinase
ATP2C2	ATPase Secretory Pathway Ca^{2+} Transporting 2
AXIN	Axis inhibition protein
BAD	BCL2 antagonist of cell death
BARD1	BRCA1 Associated RING Domain 1
BC	Breast cancer
BCL2	B-cell lymphoma 2
BCSC	Breast cancer stem cell
BP	Biological processes
BRCA1/2	Breast Cancer gene 1/2
BRIP1	BRCA1 Interacting Helicase 1
β-TrCP	β-transducin repeats-containing proteins
C	Clinical
Ca^{2+}	Calcium ion
CAA	Cancer-associated adipocyte
CAF	Cancer-associated fibroblast
CALM2	Calmodulin 2 (gene isoform)
CAM	Calmodulin (protein)
CAMKII	Ca^{2+}/CAM-dependent kinase
cAMP	cyclic Adenosine monophosphate
CAT	Catalase
CAV-1	Caveolin-1
CCL18	C-C motif chemokine 18
CCNE1	Cyclin E1
CDK4/6	Cyclin dependent kinase 4/6
CHECK2	Checkpoint kinase 2
ChT	Chemotherapy
CK1	Casein kinase 1

COX-2	Cyclooxygenase-2
CPG	Clinical Practice Guideline
CRAC	Ca^{2+} release-activated Ca^{2+}
c-Src	SRC Proto-Oncogene, Non-Receptor Tyrosine Kinase
CTGF	Connective tissue growth factor
CTLA4	Cytotoxic T-lymphocyte antigen 4
CXCL12	C-X-C Motif Chemokine Ligand 12
CXCR4	C-X-C Motif Chemokine Receptor 4
CuZnSOD	Copper-zinc-superoxide dismutase
DCIS	Ductal carcinoma in situ
DEPTOR	DEP-domain containing mTOR-interacting protein
DFS	Disease free survival
DKK1	Dickkopf 1
DUOX	Dual oxidase
EBC	Early breast cancer
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
eIF4E	Eukaryotic translation initiation factor 4E
EMT	Epithelial-to-mesenchymal transition
EndR	Endoplasmic reticulum
ER	Estrogen receptor
ERK1	Extracellular-regulated kinase 1
ET	Endocrine therapy
EZH2	Enhancer of Zeste 2 Polycomb Repressive Complex 2 Subunit
FAK	Focal adhesion kinase
FDR	False discovery rate
FN1	Fibronectin 1
FOXM1	Forkhead Box M1
FOXO1	Forkhead transcription factor 1
FOXO3a	Forkhead Transcription Factor O Subfamily Member 3a
FOXP3	Forkhead Box P3
FSP-1	Fibroblast specific protein-1
FZD	Frizzled receptors
GEPIA	Gene Expression Profiling Interactive Analysis
GLRX2/3	Glutaredoxin 2/3 (gene)
GO	Gene ontology
GPER	G-protein coupled estrogen receptor 1
GPX	Glutathione peroxidase

GREB1	Growth Regulating Estrogen Receptor Binding 1
GRX	Glutaredoxin (protein)
GSH	Glutathione (reduced form)
GSR	Glutathione reductase
GSK3β	Glycogen Synthase Kinase-3β
GSSG	Glutathione (oxidized form)+
GTEx	Genotype-Tissue Expression
HAS	HER2-associated sequence
HDAC2	Histone Deacetylase 2
HER2	Epidermal Growth Factor Receptor 2
HER2+	HER2 enriched
HIF	Hypoxia-inducible factor 1
HIF1α	Hypoxia-inducible factor 1-alpha
HOTAIR	HOX antisense intergenic RNA
HR	Hormone receptor
ICGC	International Cancer Genome Consortium
IFN-γ	Interferon gamma
IGF-1	Insulin Like Growth Factor 1
IGF1R	Insulin-like growth factor 1 receptor
IL	Interleukin
IP3	Inositol 1,4,5-trisphosphate
IP3R	Inositol 1,4,5-trisphosphate receptor
ITGA5	Integrin subunit alpha 5
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
KEAP1	Kelch-like ECH-associated protein 1
Ki-67	Marker of proliferation Kiel-67
LEF	Lymphoid enhancer-binding factor
LOX	Lysyl oxidase
LOXL2	LOX-like 2
LPR5/6	Low-density lipoprotein receptor-related protein 5/6
M	Metastasis
MACROH2A1	Macrohistone 2A1
MAPK	Mitogen-activated protein kinases
MCU	Mitochondrial calcium uniporter
MDM2	Mouse double minute 2 homolog
MDSC	Myeloid-derived suppressor cells
MEK	Mitogen-activated protein kinase kinase

MHC	Major histocompatibility complex
MMP	Matrix metalloproteinases
MnSOD	Manganese superoxide dismutase
MRE11	MRE11 Homolog, Double Strand Break Repair Nuclease
mTOR	mammalian Target of Rapamycin
mTORC1	mTOR Complex 1
N	Node
NADPH	Nicotinamide adenine dinucleotide phosphate
NBS1	Nibrin
NCX	Na^{+}/ Ca^{2+} exchanger
NFAT	Nuclear factor of activated T cells
NFkB	Nuclear factor kappa B
NFE2L2	NFE2-Like BZIP Transcription Factor 2
NK	Natural killer
NMDAR	N-methyl-D-aspartate receptor
NOX	NAPDH oxidase
Nrf2	Nuclear factor erythroid 2-related factor 2
Numb1	NUMB endocytic adaptor protein
OFS	Ovarian function suppression
ORAI	ORAI Calcium Release-Activated Calcium Modulator 1
OS	Overall survival
p53/TP53	Tumour protein 53
PALB2	Partner and Localizer of BRCA2
PARP	Poly(ADP-ribose) polymerase
PD-1	Programmed death-1
PDGFR-β	Platelet-derived growth factor receptor beta
PDKI1	Phosphoinositide-dependent kinase 1
PD-L1	Programmed cell death-ligand 1
PI3K	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase
PIK3CA	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol 3,4,5-trisphosphate
PITPNM3	Phosphatidylinositol transfer protein 3
PKA	protein kinase A
PKC	protein kinase C
PKG	GMP-dependent protein kinase
PLC	Phospholipase C
PLCD1	Phospholipase C delta 1

PMCA	Plasma-membrane Ca^{2+}-ATPase
PR	Progesterone receptor
Pro-HB-EGF	pro-Heparin-binding-epidermal growth factor
PRDX4	Peroxiredoxin 4 (gene)
PRX	Peroxiredoxin (protein)
PTEN	Phosphatase and tensin homolog
PTP	Permeability Transition Pore
PYK2	Protein tyrosine kinase 2
qRT-PCR	quantitative real-time Polymerase Chain Reaction
RAD50	RAD50 Double Strand Break Repair Protein
RAD51	RAD51 homolog 1
Ras	Rat sarcoma
ROC	Receptor-operated channels
ROS	Reactive oxygen species
RT	Radiotherapy
RYR	Ryanodine receptor
S6K1	Ribosomal Protein S6 Kinase B1
SERCA	Sarco(endo)plasmic reticulum Ca^{2+}-ATPase
SERD	Selective estrogen receptor degraders
SIRT3	Sirtuin-3
SMOC	Second-messenger-operated channels
SNAI1	Snail family transcriptional repressor 1
SOC	Store-operated channels
SOCE	Store-operated calcium entry
SOD1/2	Superoxide dismutase 1/2
SPCA	Secretory pathway Ca^{2+} ATPase
STAT3	Signal transducer and activator of transcription 3
STIM	Stromal interaction molecule
STK11	Serine/threonine kinase 11
T	Tumour
TAM	Tumour-associated macrophage
TAN	Tumour-associated neutrophile
TCF	T-cell factor
TCGA	The Cancer Genome Atlas
TCR	T cell receptor
T-DM1	Trastuzumab-emtansine
TEC	Tumour-associated endothelial cells
TGF-β	Transforming growth factor-β

TIMP-2	Tissue inhibitor of metalloproteinases 2
Treg	Regulatory T cell
Th1/2	T helper 1/2
TIL	Tumour-infiltrating lymphocyte
Tis	Tumour *in situ*
TMBIM4/6	Transmembrane B cell lymphoma 2-associated X protein inhibitor motif-containing 4/6
TME	Tumour microenvironment
TNF	Tumour necrosis factor
TNM	Tumour anatomical features, Lymph node, Metastasis
TNBC	Triple Negative Breast Cancer
TRP	Transient receptor potential ion-channels
TRPA	Transient receptor potential ion-channels ankyrin
TRPC	Transient receptor potential ion-channels canonical
TRPM	Transient receptor potential ion-channels melastatin
TRPML	Transient receptor potential ion-channels mucolipin
TRPN	Transient receptor potential ion-channels NO-mechanopotential
TRPP	Transient receptor potential ion-channels polycystin
TRPV	Transient receptor potential ion-channels vanilloid
TSC2	Tuberin
TXN	Thioredoxin
TXNRD1	Thioredoxin reductase
VEGF	Vascular endothelial growth factor
VGC	Voltage-gated channel
Wnt	Wingless-type MMTV integration site family
ZEB1	Zinc Finger E-Box Binding Homeobox 1

Chemical symbols

H_2O_2	Hydrogen peroxide
HOCl	Hypochlorous acid
$^1O_2$	Singlet oxygen
O_3	Ozone
O_2*-	Superoxide
RO	Alkoxyl
RO_2*	Peroxyl

Chapter I – General Introduction

I.1 Breast cancer

I.1.1 Breast Cancer epidemiology

Breast cancer (BC) is one of the most common types of cancer among women. In 2022, there were 2.3 million new cases reported, leading to 666,000 BC-related deaths. This accounted for approximately 11.6% of all cancer cases [1,2] (Figure 1A). The incidence rate was almost twofold higher in developed countries compared to developing countries (54.1 *versus* 30.8 per 100,000) (Figure 1B). This discrepancy might be attributed to factors such as age, reproductive history, lack of breastfeeding, hormonal imbalances, early menarche, late onset menopause, menopausal hormone therapy, and behavioural risks such as physical inactivity, excess weight, menopausal hormone therapy, oral contraceptive use, alcohol consumption, and better diagnostic resources [3,4]. However, the mortality rate was higher in developing nations due to late-stage detection and limited access to specialized care [3] (Figure 1C).

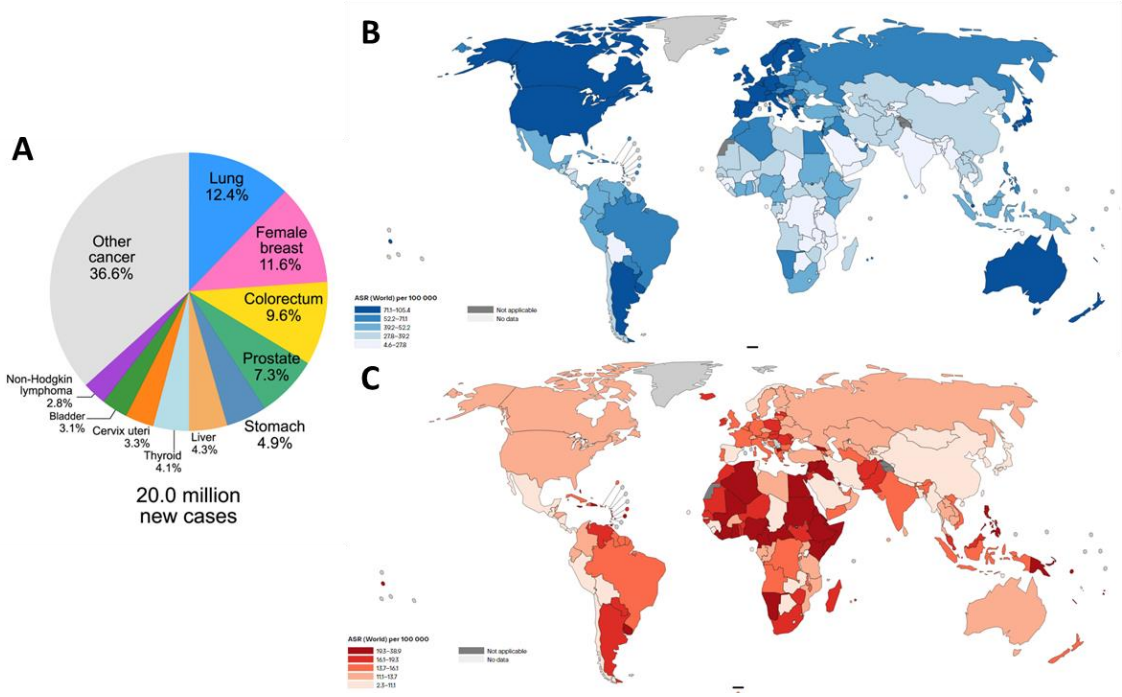

Figure 1. Incidence and Mortality Rates of Breast Cancer*. A) Relative frequency of all cancer types, highlighting the elevated incidence of breast cancer in comparison to other cancer types. B) Global incidence rates, stratified by age, revealing the substantial incidence of breast cancer, especially in high-income countries. C) Global mortality rates, categorized by age, demonstrating higher mortality rates in less economically developed nations. Source: Bray et al., CA A Cancer J Clinicians, 2024 [2].*

With existing treatments, the 5-year relative survival rate for BC patients is 86%, but in advanced stages with distant metastasis, the 5-year relative survival rate decreases to 30% [5]. Advanced BC with distant metastasis is considered incurable using current therapies [4]. These data

underscore the need to enhance our understanding of the disease mechanisms to improve early diagnosis, prognosis, and treatment. Molecular and cell biology-related research becomes pivotal in uncovering potential biomarkers, therapeutic targets, and treatments with lower toxicity.

I.1.2 Breast Cancer Classification

Regarding disease progression, BC can be classified into *in situ* (preinvasive) and invasive carcinoma. *In situ* carcinoma remains confined to its origin site and can be categorized as ductal (more prevalent) or lobular (Figure 2). On the other hand, invasive carcinomas, including both ductal and lobular subtypes, demonstrate the ability to differentiate and infiltrate the stroma[6].

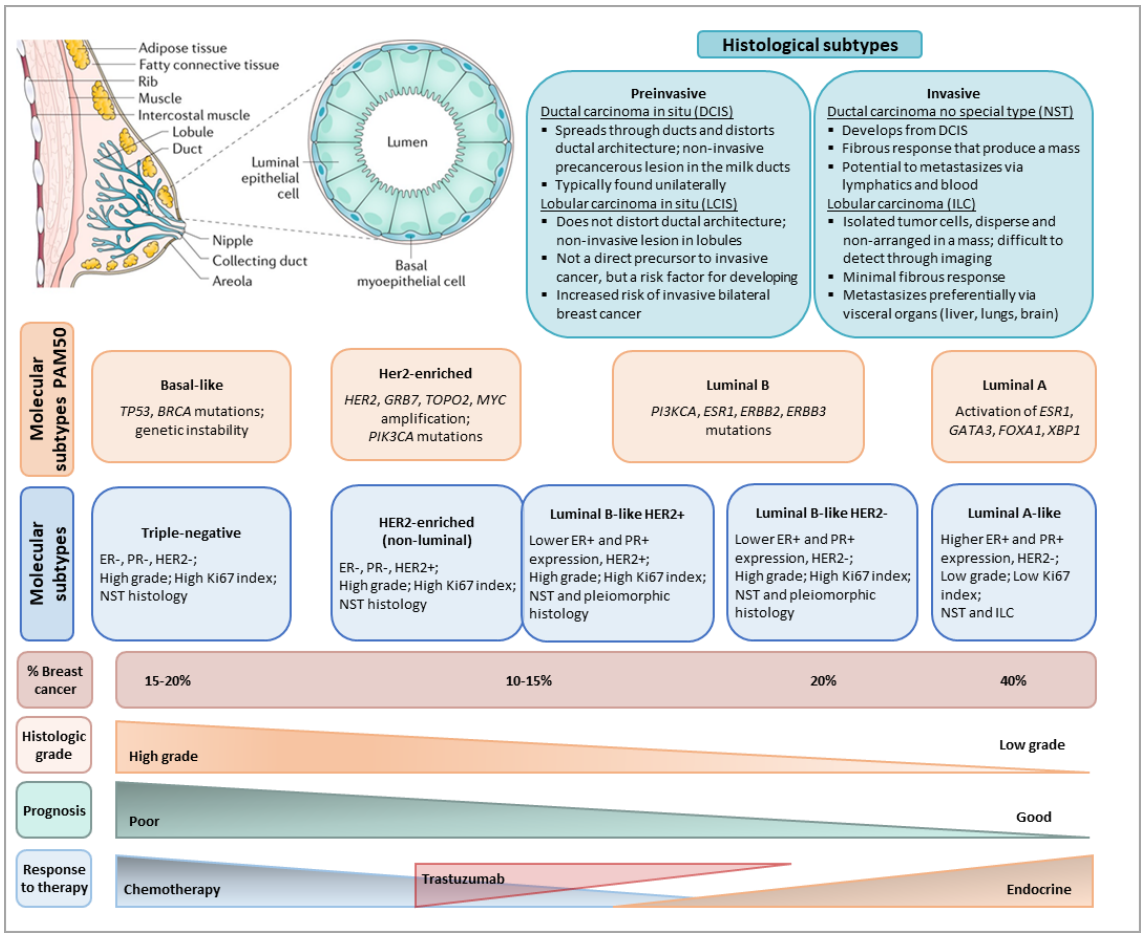

Figure 2. Breast cancer classification approaches to define characteristics and therapies*. The top section displays the structure of breast and histological subtypes, including ductal and luminal, categorized as preinvasive and invasive. PAM50 molecular subtypes (orange boxes) are based on 50 gene expression signatures. Molecular subtypes are commonly employed in clinical practice and are determined by the expression of key proteins: progesterone receptor (PR), estrogen receptor (ER), human epidermal growth factor receptor 2 (HER2), and the marker Ki67 index (blue boxes). In the bottom section, the frequency, the histologic grade, the prognosis grade, and the different therapies used are related to the outcomes of molecular subtypes. Source: Adapted from Harbeck et al, Nat Rev Dis Primers, 2019 [4] and Saha & Lukong, Front Oncol, 2022 [7]*

In the context of disease progression classification, the American Joint Committee on Cancer (AJCC) TNM staging system has been the longstanding gold standard. This system considers anatomical characteristics of the primary tumour (T; Tumour *in situ* [Tis]-T4), regional lymph node involvement (N; N0-N3), and the presence or absence of metastasis (M; M0-M1). This classification is pivotal for assessing the staging, prognosis, and the more adequate therapeutic regime of the patient (as illustrated in Figure 3 and Table 1) [8,9].

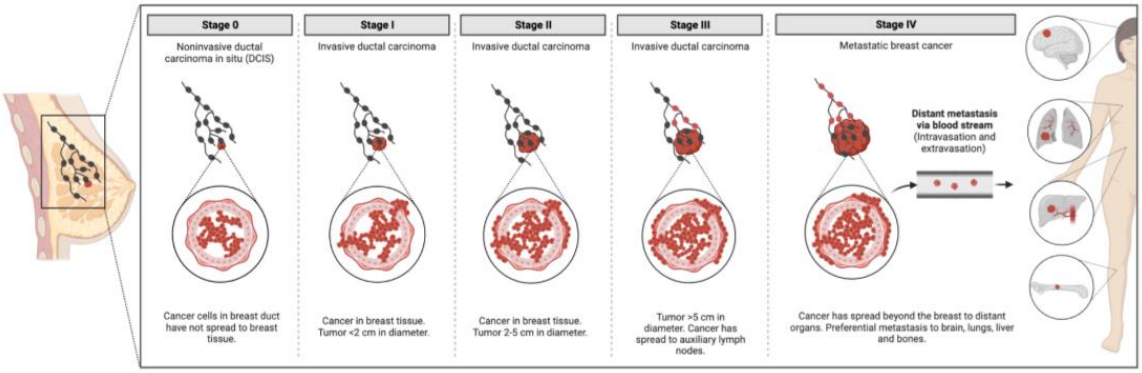

Figure 3. Breast cancer stages and their association with the classification of ductal disease progression*. The advancement of breast cancer is related to an increase in tumour size and invasion of adjacent tissues, and in the final stage, the spread to distant tissues. Source: Lazaratos, BioRender, 2020 [10]*

Table 1 – AJCC TNM staging system for breast cancer.

Anatomical Staging Group	Sub-category	TNM	Features
Stage 0		Tis N0 M0	Ductal carcinoma in situ or Paget disease of the nipple
Stage I	A	T1 N0 M0	Tumour ≤20 mm at its greatest dimension
	B	T0 or T1 N1mi M0	No tumour or tumour ≤20 mm at its greatest dimension Micrometastasis to regional lymph nodes
Stage II	A	T0 or T1 N1 M0	No tumour or tumour ≤20 mm at its greatest dimension Limited metastasis to regional lymph nodes
		T2 N0 M0	Tumour >20 mm but ≤50 mm at its greatest dimension No metastasis
	B	T2 N1 M0	Tumour >20 mm but ≤50 mm at its greatest dimension Limited metastasis to regional lymph nodes
		T3 N0 M0	Tumour >50 mm at its greatest dimension No metastasis
Stage III	A	T0, T1 T2, or T3 N2 M0	Tumour of any size Moderate metastasis to regional lymph nodes
		T3 N1 M0	Tumour >50 mm at its greatest dimension Limited metastasis to regional lymph nodes
	B	T4 N0, N1, or N2 M0	Tumour (any size) with extension to chest wall and/or skin Limited or moderate metastasis to regional lymph nodes
	C	Any T N3 M0	Tumour presence Significant metastasis to regional lymph nodes
Stage IV		Any T Any N M1	Distant metastasis

Legends: TNM – Tumour anatomical features, Lymph node, Metastasis; Tis – Tumour *in situ*; N1mi – lymph node with micrometastasis
Source: Adapted from Kalli et al., *RadioGraphics*, 2018 [8], Giuliano et al., *CA A Cancer J Clinicians*, 2017[9], Teichgraeber et al., *American Journal of Roentgenology*, 2021 [11]

In the 8th edition of the AJCC system, biological factors were introduced alongside the traditional TNM anatomic factors. Due to the importance of providing a more precise and adaptable staging system, these biological factors encompass tumour grade, proliferation rate, immunochemistry markers, and gene expression prognostic panels [9,11]. Furthermore, lobular carcinoma *in situ*

was excluded from the staging system due to its characterization as a benign tumour with an associated risk of carcinoma development but lacking metastatic potential. However, anatomic staging maintains its global relevance, ensuring worldwide accessibility, while biomarkers serve as a complementary layer for prognosis assessment [9,11].

Immunohistochemistry markers, namely the expression of estrogen receptor (ER), progesterone receptor (PR), and Human epidermal growth factor receptor 2 (HER2) are commonly used to categorize BC into five intrinsic molecular subtypes: Luminal A, Luminal B-like HER2-, Luminal B-like HER2+, HER2-enriched (HER2+), and Triple Negative Breast Cancer (TNBC). Luminal A, characterized by ER+ and/or PR+ and HER2-, exhibits the highest incidence and the most favourable prognosis [12]. Conversely, TNBC subtype, lacking expression of ER, PR, and HER2, has the lowest incidence and the worst prognosis, carrying a higher risk of death within 5 years of diagnosis compared to other subtypes [12,13] (Figure 2).

The PAM50, a 50-gene signature developed by Perou et al. [14], effectively differentiates molecular subtypes through quantitative Real-Time Polymerase Chain Reaction (qRT-PCR), serving as a reliable alternative to full microarray analysis. It classifies tumours into four intrinsic subtypes: Luminal A, Luminal B, HER2-enriched, and Basal-like [15,16] (Figure 2). Additionally, low Claudin expression identifies a rare subtype that shares certain gene expression features and poor prognosis with the Basal-like subtype [17]. Integrating the PAM50 gene set into the molecular classification significantly enhanced the predictive ability for relapse risk compared to models based solely on clinical variables like tumour size, node status, and histologic grade. The combination of both approaches significantly increased the specificity of prognosis and relapse risk prediction [15,16].

The molecular progression of breast cancer depends strongly on two pathways linked to ER expression and tumour grade. The low-grade-like pathway as defined by Harbeck et al. [4] is characterized by a gene expression signature marked by genes associated with the ER phenotype (e.g. ERα), diploid or near-diploid karyotypes, and includes low tumour-grade tumours such as the Luminal A and certain Luminal B subtypes. Luminal B subtypes are more aggressive than Luminal A, due to higher expression of genes involved in cell proliferation and cell cycle, such as Aurora kinase, and to a higher rate of Tumour protein 53 (p53) mutations [18]. Conversely, the high-grade-like is defined by Harbeck et al. as an expression signature [4] involving more genes tied to the cell cycle and cell proliferation (e.g., *TP53*, Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha [*PIK3CA*], *HER2*). The HER2+ subtype and TNBC subtypes align with these pathways [4,19,20] (Figure 2). The HER2+ BC subtype does not express

ERα and PR receptors, but overexpresses HER2 receptors, leading to the overactivation of signalling pathways related to cell proliferation and cell survival, such as Rat sarcoma (Ras)/Mitogen-activated protein kinases (MAPK) or Phosphatidylinositol-4,5-Bisphosphate 3-Kinase (PI3K)/Protein kinase B (AKT). TNBC does not express ERα, PR, and HER2, but expresses keratin 5, keratin 17, integrin-B4, laminin, and overexpresses MYC proto-oncogene (MYC), Cyclin-dependent kinase 6 (CDK6), Cyclin E1 (CCNE1), genes related to cell proliferation. Additionally, it inhibits the expression of Breast cancer gene 2 (BRCA2), Phosphatase and tensin homolog (PTEN), and Mouse double minute 2 homolog (MDM2), genes that are related to DNA repair, and increases the mutation rate of TP53 [18].

The marker of proliferation Kiel-67 (Ki-67) protein, frequently employed as a biomarker, aids in determining cell proliferation rates within tumours due to its presence in various cell cycle phases except for the resting phase. The Ki-67 index indicates the proportion of cells displaying Ki-67 protein. A percentage lower than 15% signifies low proliferation, while values exceeding 30% indicate higher proliferation (Figure 2). This index can support therapy decisions, particularly for ER+ and HER2- BC [4,21,22].

While tissue biopsy remains the gold standard for detecting and characterizing breast tumours and determining histology, molecular subtype, prognosis, and treatment strategy, it falls short of capturing the full genomic landscape heterogeneity of the tumour [7]. To counteract this limitation, the emerging concept of liquid biopsy has gained traction. Liquid biopsy involves isolating DNA or microRNA from circulating breast tumour cells in the patient's blood. This approach has the potential to capture tumour heterogeneity, but also to detect early-stage BC, monitor treatment effectiveness and resistance, and assess relapse risk. However, in certain cases, liquid biopsies may not be as sensitive as immunohistochemistry requiring an initial histologic diagnosis obtained through tissue biopsy. This underscores the need for further research and improvements in BC diagnostics [23].

I.1.3 Breast Cancer Tumourigenesis

The genetic background of women significantly influences the risk of BC, with inherited DNA mutations often underlying familial cancer patterns. Comprehensive genetic analysis, including multigene panels and whole exome sequencing, allows for the assessment of functional mutations, paving the way for innovative clinical trial strategies [24].

Previous studies have estimated that inherited BC accounts for 5-10% of cases, with only 4-5% showing mutations in high-penetrant genes [25,26]. The *BRCA1* and *BRCA2* genes encode proteins that participate in DNA repair, transcription control, and cell cycle responses to DNA damage, and are the most commonly mutated genes associated with hereditary cases of BC [24,25]. Women with specific *BRCA1* mutations face a 55–65% lifetime risk of developing BC, while those with *BRCA2* mutations have a 45% lifetime risk. On average, women with *BRCA1* or *BRCA2* mutations have a roughly 70% chance of developing BC by age 80 [26]. These mutations also increase the likelihood of earlier BC diagnosis, and occurrence in both breast and ovarian cancers [26].

Other high-penetrance inherited mutations include *TP53* in Li-Fraumeni syndrome, Serine/threonine kinase 11 (*STK11*) in Peutz-Jeghers syndrome, and *PTEN* in Cowden syndrome, all functioning as tumour suppressor genes [25,27]. More frequent but less penetrant mutations in genes such as Checkpoint kinase 2 (*CHECK2*), Ataxia-telangiectasia mutated (*ATM*), Partner and localizer of BRCA2 (*PALB2*), BRCA1 interacting helicase 1 (*BRIP1*), MRE11 homolog, double-strand break repair nuclease-RAD50 double-strand break repair protein nibrin (*MRE11-RAD50-NBS1*) complex, RAD51 homolog 1 (*RAD51*), and BRCA1 associated RING domain 1 (*BARD1*), that are mostly involved in regulating various cellular pathways associated with DNA repair, are identified in some familial BC cases [25,27]. These inherited changes when combined with intrinsic genetic alterations in tumours, can impact therapeutic response through drug metabolism alterations. Personalized therapy is particularly relevant for these patients, offering potential strategies to all patients with poor responses to traditional therapeutics [24].

However, inherence is not the primary factor of BC development. Key markers, including ER, PR, and HER2 play pivotal roles, with the ER and HER2 signalling pathways being the principal drivers of cell proliferation and survival in the majority (85%) of BC cases [28,29].

I.1.3.1 Estrogen signalling pathways

The estrogen signalling pathways dysregulation associated with ER and PR receptor positivity, occur in nearly two-thirds of all BC [18]. ER plays a pivotal role in regulating breast tumour growth. Three distinct ER receptors have been identified in BC: two nuclear receptors, ERα and ERβ, and a G-protein coupled estrogen receptor 1 (GPER) that triggers non-genomic effects of estrogens in the cytosol (Figure 4) [30].

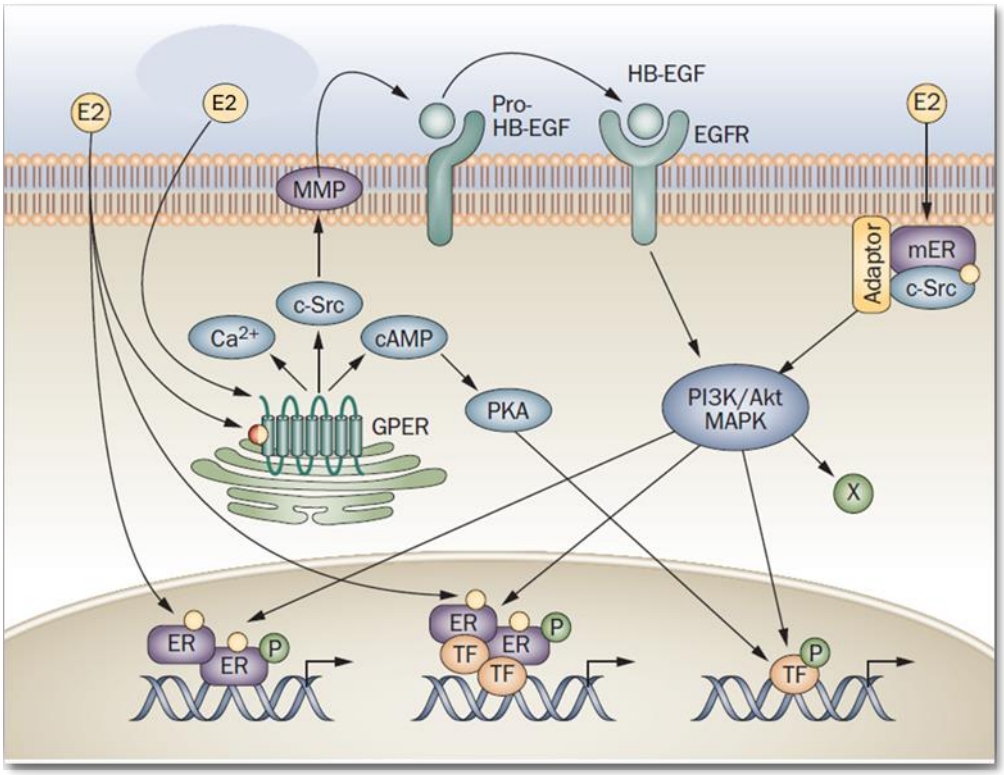

Figure 4. Example of Estrogen-associated signalling pathways. *Endogenous estrogens like 17β-estradiol (E2) activate estrogen receptors ERα, ERβ, and G-protein-coupled ER (GPER). They trigger nuclear ERs to bind gene promoters or interact with transcription factors. At the membrane, ERs interact with proteins like SRC Proto-Oncogene (c-Src), activating Phosphatidylinositol-4,5- Bisphosphate 3-Kinase (PI3K)/Protein kinase B (Akt) and Mitogen-activated protein kinases (MAPK) pathways. GPER activation leads to cyclic Adenosine monophosphate (cAMP) production, Ca^{2+} release, and Matrix metalloproteinase (MMP) activation. MMPs release Heparin-binding-epidermal growth factor (HB-EGF), transactivating Epidermal growth factor receptor (EGFR) and activating MAPK and PI3K-Akt pathways for gene regulation. E2 affects gene transcription through ER phosphorylation (P) or Transcription factor (TF) interactions in gene promoters. ER - Estrogen receptor; pro-HB-EGF - pro-Heparin-binding-epidermal growth factor. Source: Adapted from Prossnitz & Barton, Nature Rev Endocrinol, 2011 [31]*

ERα, the predominant hormonal receptor, undergoes activation upon estrogen binding, translocating to the nucleus and interacting with coactivators [4,26]. ERα also participates in non-genomic effects through membrane G proteins, acting as a second messenger of PI3K/AKT or Ras/MAPK pathways, thereby regulating cell proliferation and survival [18]. ERα promotes BC growth by interacting with cyclin D1, sustaining mitogenic effects critical for cell cycle progression [32]. Cyclin 1, an important activator of Cyclin-dependent kinases (CDK) 4 and 6, plays a key role in regulating the transition from G1 to S phase, connecting with breast tumourigenesis and endocrine therapy resistance [33,34]. Erα also activates genes like *MYC*, Forkhead box M1 (*FOXM1*), Growth regulating estrogen receptor binding 1 (*GREB1*), B-cell lymphoma 2 (*BCL2*), Insulin-like growth factor 1 (*IGF-1*), and C-X-C motif chemokine ligand 12 (*CXCL12*), contributing to increased tumour cell proliferation and elevated DNA damage risk in response to estrogens [4,18].

ERβ, a nuclear receptor homologous to ERα, exhibits various isoforms, complicating the understanding of its function. Its differential distribution in human breast tissues and controversial roles have been discussed [30]. Some authors argue for its role as a tumour suppressor, depending on factors such as the tissue studied (e.g. mammary gland), cell context, transcriptional coactivators, and ERα expression levels [35]. Other authors have observed that ERβ is differentially expressed in different BC subtypes, including ERα negative and TNBC cases [36,37]. Additionally, certain ERβ isoforms are associated with risk factors for poor prognosis [38].

GPER, an ER-associated receptor controls several signalling pathways governing the extra-nuclear and non-genomic effects of estrogens. This protein has well-established roles in activating the MAPK and PI3K pathways, contributing to cell proliferation and survival. In recent years, additional signalling events associated with estrogen binding to GPER were identified and include: (i) the inactivation of the transcription factor Forkhead transcription factor O subfamily member 3a (FOXO3a) via Akt kinase, (ii) the activation of the Hippo pathway, resulting in increased Connective tissue growth factor (CTGF) expression and enhanced metastasis, and (iii) induction of HOX antisense intergenic RNA (HOTAIR) expression, contributing to the heightened metastatic potential of BC cells [30].

It is also important to highlight the role of progestin and PR in BC. Despite progestin being implicated in inducing BC growth, the presence of PR in breast tumours serves as an independent marker indicating a favourable prognosis. Notably, the loss of PR in ER+ tumours is associated with a diminished response to endocrine therapies. This paradox in the dual role of PR can be explained by associating PR as a major driver of mammary tumour growth and its interaction with unliganded Erα, exerting genomic effects and leading to the assembly of transcriptional complexes governing BC growth [39]. Tumours that retain PR are often more responsive to hormonal therapies, like Tamoxifen, targeting BC-ER^{+} [39]. In practical terms, blocking ERα function inhibits coordinated PR rapid effects and hampers transcriptional effects, consequently impeding PR-mediated proliferation. Therefore, the intricate interplay between PR and ERα in BC cells sensitive to endocrine therapy highlights the significance of understanding their coordinated actions for devising effective therapeutic strategies.

I.1.3.2 HER2 signalling pathways

HER2 (also known as ERBB2) belongs to the Epidermal growth factor receptor (EGFR) family, functioning as a receptor tyrosine kinase with extracellular transmembrane, and intracellular

domains [40,41]. Lacking a ligand, HER2 relies on heterodimerization with another family member or homodimerization with itself to exert be activated [42,43]. HER2 overexpression, driven by HER2 gene amplification, prompts spontaneous dimerization, inducing tyrosine residue phosphorylation in the intracellular domain, and activation of various downstream signalling pathways, such as PI3K/AKT/mammalian target of rapamycin (mTOR), Ras/Mitogen-activated protein kinase kinase (MEK)/MAPK (Figure 5) and Signal transducer and activator of transcription 3 (STAT3), associated with cell proliferation, differentiation, survival, and with angiogenesis [29,40,42]. These pathways are strongly correlated with breast tumourigenesis [29,41,44].

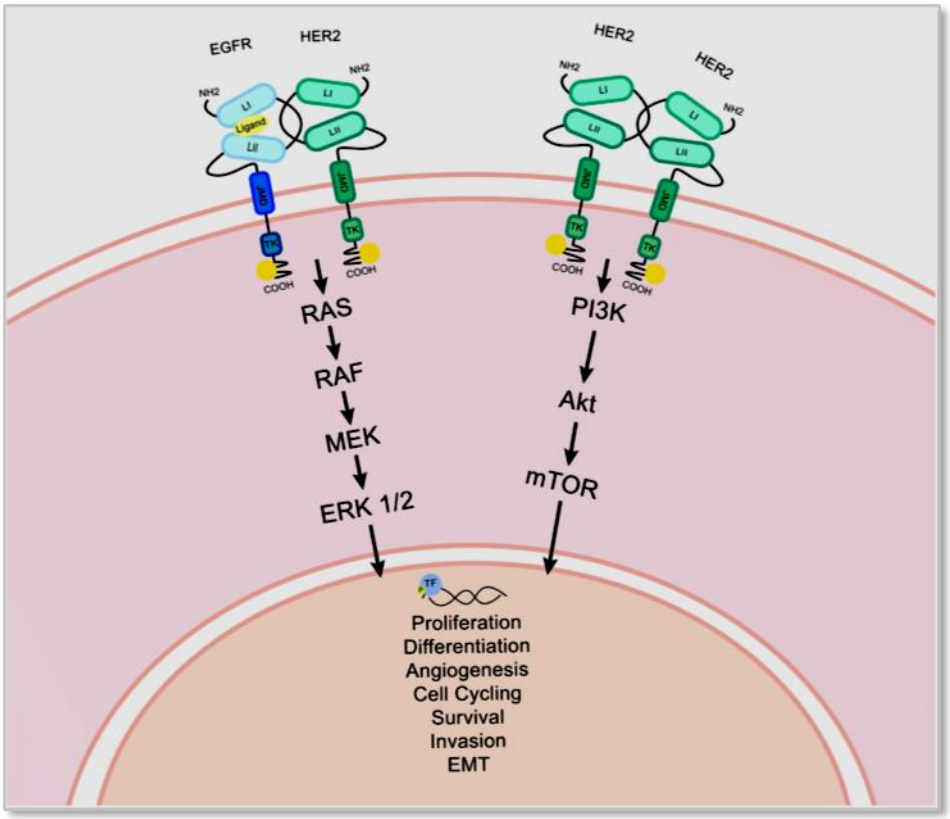

Figure 5. Example of HER2 signalling pathways. *The binding of a ligand to an Epidermal growth factor receptor (EGFR)/Epidermal Growth Factor Receptor 2 (HER2) heterodimer activates signalling pathways through the phosphorylation of the tyrosine kinase domain within the intracellular HER2 molecule. The HER2 homodimer is constitutively active, stimulating the Rat sarcoma (RAS)/Mitogen-activated protein kinases (MAPK) and Phosphatidylinositol-4,5-Bisphosphate 3-Kinase (PI3K)/Protein kinase B (Akt) pathways. This activation leads to the engagement of crucial downstream effectors and transcription factors, altering transcription and regulating gene expression. This signalling cascade influences various essential cellular functions associated with cancer hallmarks. Source: Adapted by Hart et al., Oncotarget, 2020[45]*

An aberrant truncated form of the HER2 receptor, known as p95, is also found in some HER2 BC [46]. Without the extracellular domain, p95 acquires constitutive activity and resistance to

antibodies that target the external domain [29]. However, membrane-bound HER2 can also translocate to the nucleus, binding to DNA at HER2-associated sequences (HASs) and acting as a coactivator of transcription factors [47]. Nuclear HER2 has revealed its role in BC growth, metastasis, and resistance to chemo- and radiotherapy [48,49]. Notably, nuclear HER2 has been associated with the induction of Cycloxygenase-2 (COX-2) expression as a gene promoter, stimulating its transcription [47]. Progestins induce the transcriptional complex composed of Activator protein 1 (AP-1), STAT3, PR, and HER2 at a region of the Cyclin D1 promoter [39,50]. Conversely, nuclear HER2 inhibits the transcription of the DEP-domain containing mTOR-interacting protein (DEPTOR), a direct inhibitor of mTOR. This inhibition suppresses its kinase activity, promoting cell proliferation and survival [43].

Furthermore, elevated ER and PR levels serve as favorable predictors of survival in HER2+ patients, highlighting the crosstalk between these signalling pathways. Particularly, a high PR level is associated with improved survival outcomes in HER2+ patients [51].

I.1.3.3 PI3K/AKT/mTOR pathway

The PI3K/AKT/mTOR pathway is involved in several tumourigenic processes, including cell proliferation, survival, invasion, migration, apoptosis, glucose metabolism, and DNA repair [52]. PI3K/AKT/mTOR pathway is activated in 70% of BC cases and is linked to numerous gene mutations, such as *PIK3CA* (a subunit of PI3K) and *AKT* [34].

In this pathway, ligand binding to a cell-membrane receptor triggers the activation of PI3K. This enzyme catalyzes the phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP2) into phosphatidylinositol 3,4,5-trisphosphate (PIP3), which is responsible for the recruitment of phosphoinositide-dependent kinase 1 (PDKI1) and AKT [52,53]. Once activated, AKT mediates the regulation of the cell cycle, growth, proliferation, and energy metabolism [54]. Additionally, activated AKT stimulates the mTOR complex 1 (mTORC1) complex, a key regulator of cellular growth and protein synthesis that includes mTOR [53]. AKT also phosphorylates Mouse double minute 2 homolog (MDM2), a protein that binds to p53, inhibiting its tumour suppressor activity [53] (Figure 6).

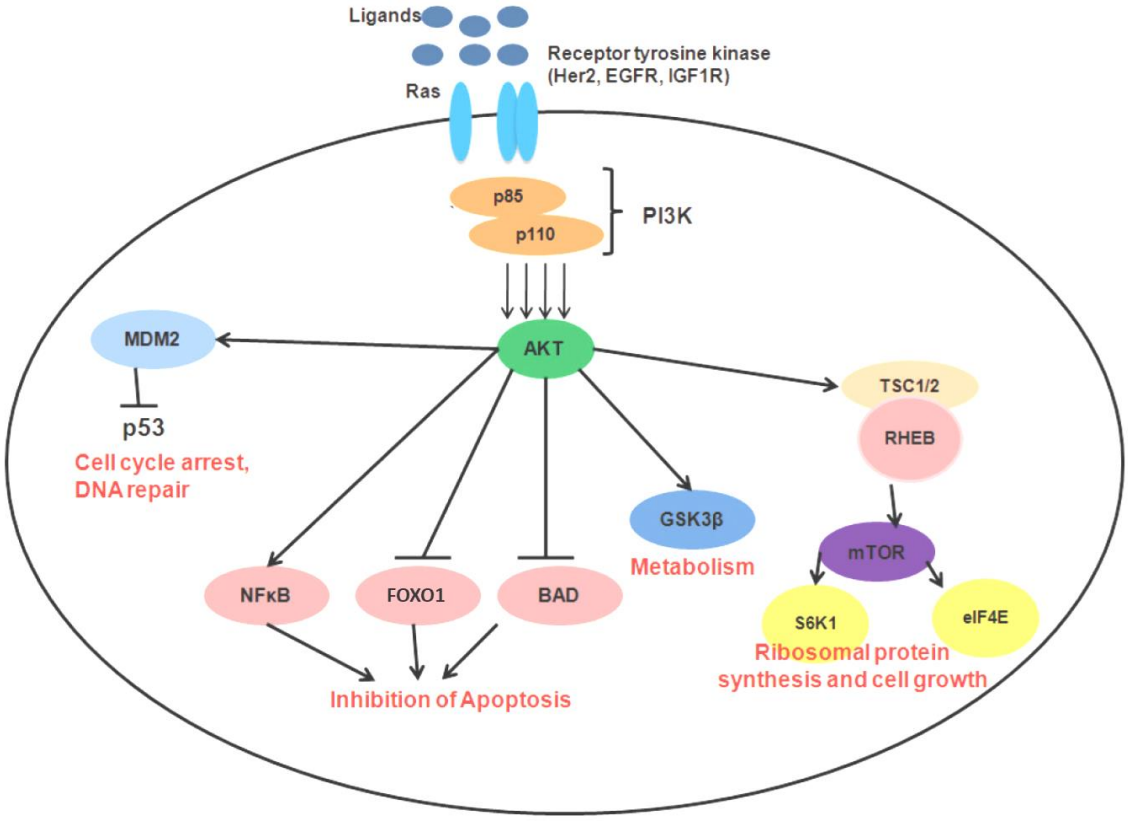

Figure 6. Example of PI3K/AKT/mTOR signalling pathways. *The binding of ligands to receptors tyrosine kinase, such as Epidermal growth factor receptor (EGFR), Insulin-like growth factor 1 receptor (IGF1R), Epidermal Growth Factor Receptor 2 (HER2), or Rat sarcoma (Ras) activates Phosphatidylinositol-4,5-Bisphosphate 3-Kinase (PI3K), a heterodimer with a regulatory subunit p85 and a catalytic subunit p110. PI_3K activates Protein kinase B (Akt) activation through phosphorylation. Once activated, Akt induces the activation of proteins involved in ribosomal protein synthesis and cell growth (mTOR, S6K1, eIF4E), metabolism (GSK3β), inhibition of apoptosis (NFkB, FOXO1, BAD), and cell-cycle arrest and DNA repair (MDM2) mediated by p53. BAD - BCL2 antagonist of cell death; eIF4E - Eukaryotic translation initiation factor 4E; FOXO1 - Forkhead transcription factor; GSK3β - Glycogen Synthase Kinase-3β; MAPK - Mitogen-activated protein kinases; MDM2 - Mouse double minute 2 homolog; mTOR - mammalian Target of Rapamycin; NFkB – Nuclear factor kappa B; p53 – Tumour protein 53; S6K1 - Ribosomal Protein S6 Kinase B1. Source: Adapted by Gaikwad and Ray, Am J Nucl Med Mol Imaging, 2012 [55]*

In BC, PI3K/AKT/mTOR pathway is the most frequently activated signalling pathway and interacts with the ER signalling pathway. On one way, the PI3K/AKT/mTOR pathway activates estrogen-independent ER transcriptional activity, on the other way, ER promotes the transcription of several genes encoding upstream effectors of PI3K/AKT/mTOR pathway [53]. Hyperactivation of this pathway can contribute to endocrine resistance through the downregulation of ER expression, promoting hormone-independent growth [53].

Additionally, the PI3K/AKT/mTOR pathway is activated through the overexpression or amplification of HER2 in BC. Furthermore, HER2 phosphorylation may lead to PI3K/AKT/mTOR pathway activation [52,56]. Constitutive activation of PI3K, associated with PIK3CA mutation and

loss of the tumour suppressor PTEN, is linked to resistance to therapies targeting HER2, as it sustains pathway signalling despite HER2 blockage [57].

I.1.3.4 Canonical Wnt/β-catenin pathway

Wingless-type MMTV integration site family (Wnt) proteins act as ligands for several receptors, initiating different downstream pathways [58]. In BC cells, the constitutive elements of Wnt signalling undergo alterations at DNA level (mutations, amplifications, and methylations), mRNA level (posttranscriptional modifications) and protein level (posttranslational modifications). These changes also encompass shifts in subcellular localizations, especially for β-catenin [59].

The canonical Wnt/β-catenin signalling pathway initiates with the binding of Wnt ligands to Frizzled receptors (FZD), which recruit the co-receptors low-density lipoproteins 5/6 (LPR5/6). This process inhibits β-catenin ubiquitination by β-transducin repeats-containing proteins (β-TrCP). The subsequent accumulation of β-catenin facilitates its translocation into the nucleus, initiating downstream transcriptional activation [60]. Conversely, Wnt signalling inhibitors such as Dickkopf 1 (DKK1), act as tumour suppressors by promoting the degradation of β-catenin. This impedes the transcription of oncogenes targeted by β-catenin, exerting a regulatory influence on Wnt-mediated cellular processes [26] (Figure 7).

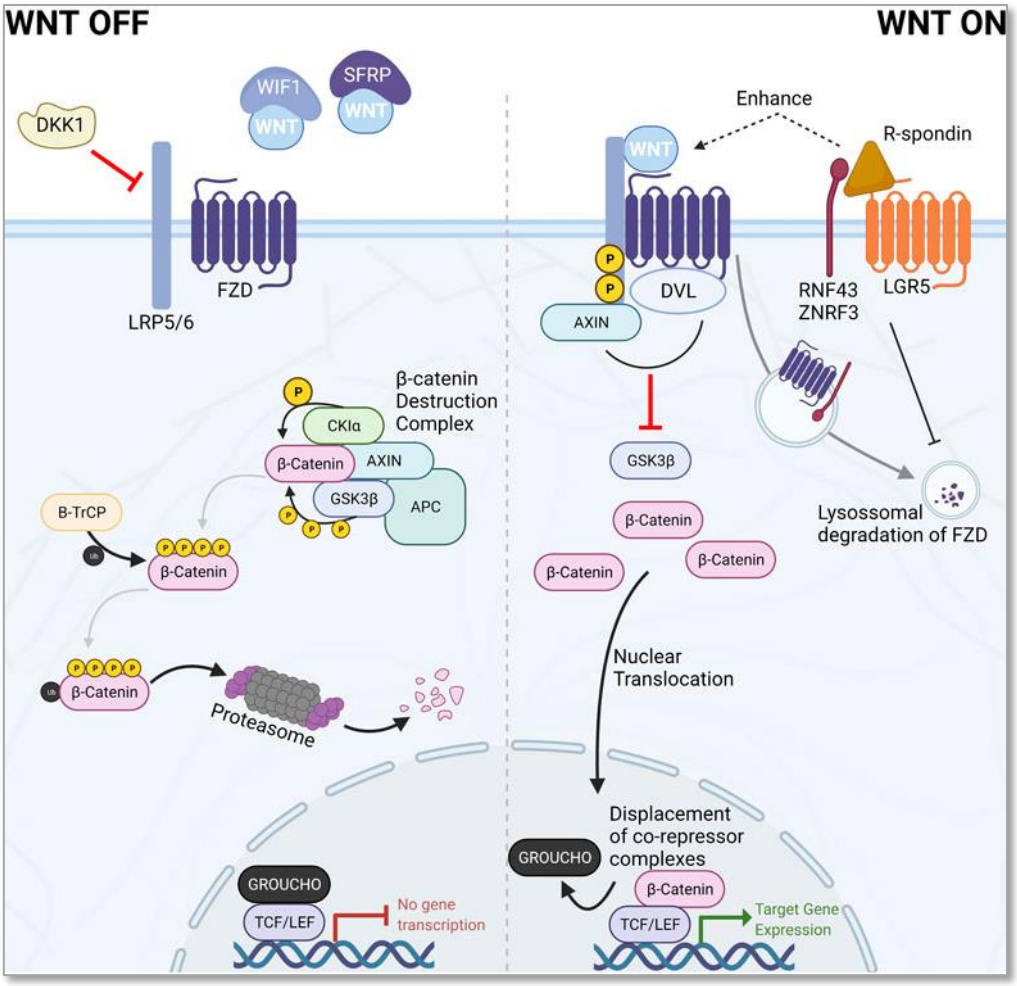

Figure 7. ***Example of Wnt/β-catenin signalling pathways.*** *In the absence of Wnt ligands or in the presence of antagonists like Dickkopf 1 (DKK1) (WNT OFF), Wnt inhibitory factor 1 (WIF1), or Secreted frizzled-related proteins (SFRPs), the Wnt signalling pathway remains inactive due to the degradation of β-catenin by the destruction complex, which includes Axis inhibition proteins 1/2 (AXIN)1/2, Adenomatous polyposis coli (APC), Casein kinase 1α (CK1α), and Glycogen Synthase Kinase-3β (GSK3β). This complex phosphorylates β-catenin, leading to its proteasomal degradation. When Wnt ligands bind to the Frizzled receptors (FZD)/Low-density lipoprotein receptor-related protein (LRP) co-receptor complex (WNT ON), the destruction complex disassembles, allowing β-catenin to accumulate, translocate to the nucleus, and activate Wnt target genes by displacing co-repressors from T-cell factor (TCF)/Lymphoid enhancer-binding factor (LEF) transcription factors. R-spondins further enhance this signalling by preventing the degradation of FZD receptors. Source: Oliveira et al., Front. Cell Dev. Biol., 2022* [61]

The constitutive activation of the Wnt/β-catenin pathway is particularly notable in TNBC, strongly contributing to its proliferation, migration, stemness, and chemoresistance [26,60,62]. Moreover, breast tumours driven by Wnt signalling are sustained by clones capable to re-activate Wnt overexpression after Wnt inhibition. This underscores the pivotal role of aberrant Wnt activation as a key driver in the recurrence and progression of BC [63].

I.1.4 Breast Cancer Microenvironment

Accumulated evidence highlights that a tumour comprises many more elements than just neoplastic cells. The microenvironment surrounding these cells plays a crucial role in tumour progression [64]. The Tumour microenvironment (TME) is implicated in inflammation and the suppression of antitumour immune responses, but also contributes to tumour heterogeneity [65]. BC cells are commonly surrounded by a dynamic microenvironment composed of blood vessels, fibroblasts, adipocytes, suppressive immune cells, stem cells, and an altered Extracellular matrix (ECM) (Figure 8). These components engage in intricate crosstalk with each other and with tumour cells, inhibiting effective antitumour immunity and contributing to tumour progression, propagation, and response to therapies [64–66].

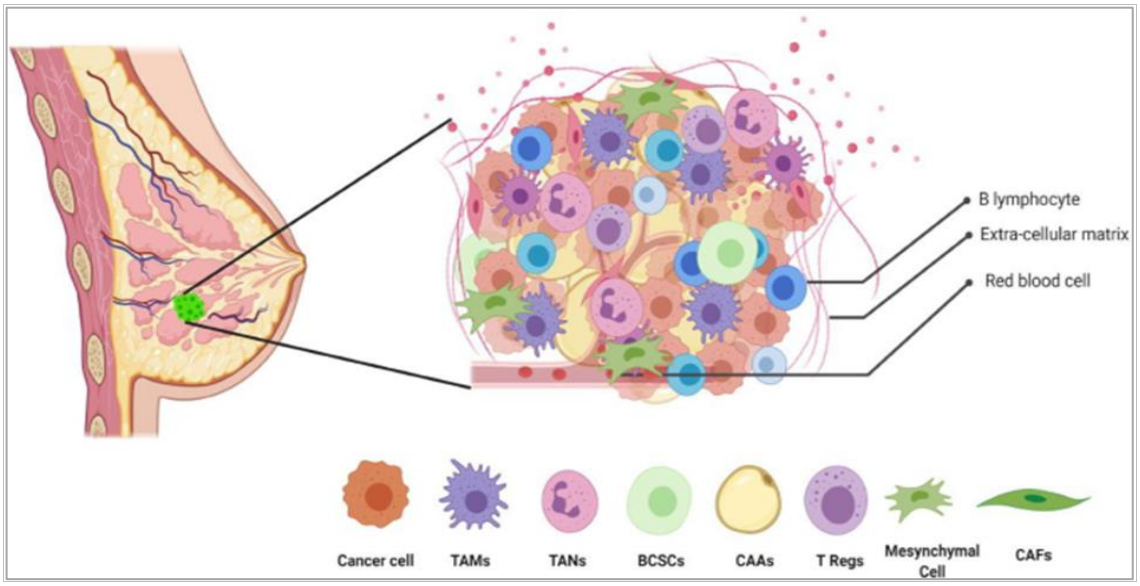

Figure 8. Tumour environment in breast cancer. *The tumour thrives within a permissive microenvironment defined by an extracellular matrix, which encompasses a variety of biomolecules (cytokines, chemokines, miRNAs, and growth factors) and various cell types such as Breast cancer stem cells (BCSCs), platelets, Tumour-associated macrophage (TAMs), mesenchymal stem cells, and Cancer-associated fibroblast (CAFs) contribute to creating favorable conditions for cancer progression. CAA – Cancer-associated adipocyte; TAN – Tumour-associated neutrophil. Source: Mehraj et al., Cell Oncol., 2021* [67]

Interaction between the tumour cells and the microenvironment results from direct cell-cell contact or by the release of autocrine or paracrine factors, activating pro-tumour signalling pathways and modulating tumour behavior [68]. In the primary site, stromal cells interact with tumour cells, changing gene expression of both cell types. For instance, the expression of several genes associated with hallmarks of cancer (metabolic reprogramming or tumour-promoting microenvironment) is dysregulated [69].

Stromal cells, for example, exhibit molecular alterations and abnormal signalling pathways [64]. Among these, cancer-associated fibroblasts (CAFs), the most abundant cell type in BC stroma, are responsible for producing cytokines, chemokines, growth factors, and ECM proteins, all of which are implicated in BC development and metastasis [70]. Inflammatory cytokines, such as interleukin-6 (IL-6) promote BC progression and metastasis by acting on BC stem cells [71]. Furthermore, the overexpression of the chemokine CXCL12 and its cell surface receptor C-X-C motif chemokine receptor 4 (CXCR4) axis induces migration and proliferation of stromal cells, but also the activation of Matrix metalloproteinases (MMP) including MMP-2, -11, and -14, which degrade matrix proteins, contributing to ECM remodeling and tumour invasion and metastasis [72–76].

CAFs form a highly heterogeneous group of cells, originating not only from fibroblasts, but also from mesenchymal stem cells, tumour stem cells, immune cells, and endothelial cells [77,78]. While most CAF subpopulations exert cancer-promoting effects, inducing proliferation, angiogenesis, metastasis, and drug resistance, some subsets play an inhibitory role in tumour growth, studied as a potential therapeutic migration inhibitory approach [77,79]. However, the gene expression profiles of CAFs differ depending on BC subtypes, influencing prognosis [80,81]. For instance, in HER2+ BC, CAFs may increase invasive behavior by dysregulating pathways associated with the cytoskeleton and integrin signalling, contributing to a poor prognosis in these patients [80]. In TNBC, CAF-S1 and CAF-S4 subsets are increased. CAF-S1 is associated with an immunosuppressive microenvironment by attracting and retaining CD4+CD25+ T cells. Additionally, CAF-S1 increases T lymphocyte survival and promotes their differentiation into CD25+Forkhead Box P3 (FOXP3+). Both mechanisms inhibit T effector proliferation [82]. Furthermore, CAF-S1 stimulates tumour cell migration and Epithelial-to-mesenchymal transition (EMT) through CXCL12 and Transforming growth factor-beta (TGF-β) pathways, while CSF-S4 induces tumour cell invasion via NOTCH signalling. Assessing CAF subsets can facilitate the choice of anti-TGF-β and/or NOTCH therapies in BC [83].

In the context of local invasion in BC, adipocytes undergo phenotypical changes, transforming into fibroblast-like cells known as Adipocyte-derived fibroblasts (ADF), a class of CAFs. These ADFs demonstrate altered secretion of fibronectin and collagen I, heightened migratory and invasive capabilities, and increased expression of the CAF marker Fibroblast specific protein-1 (FSP-1). Notably, the development of the ADF phenotype hinges on the reactivation of the Wnt/β-catenin pathway triggered by Wnt3a, a protein secreted by tumour cells [84]. These particular adipocytes can also overexpress proteases, such as MMP-11, and proinflammatory cytokines, such as IL-1β and IL-6, proteins associated with invasion promotion [85]. Upregulation

of IL-6 in BC adipocytes also promotes angiogenesis, proliferation, and tumour survival via Janus kinase (JAK)/STAT3 signalling pathway [86]. Additionally, adipocytes are associated with metabolic remodeling, providing pyruvate, lactate, ketone bodies, and fatty acids to BC cell proliferation [87].

Macrophages play a crucial role in the immune response and can be broadly classified into M1 and M2 types, each activated by distinct stimuli. M1 macrophages, stimulated by the type 1 T helper cell (Th1) cytokines, produce pro-inflammatory cytokines, such as Interferon-gamma (IFN-γ) and Tumour necrosis factor (TNF). They exert tumour-killing functions, recognizing and phagocytizing cancer cells. On the other hand, M2 macrophages, activated by Th2 cytokines, such as IL-4, IL-10, and IL-13, chemokines, and proteases act as tumour promoters. In the BC microenvironment, M2 macrophages tend to increase during progression and become the predominant type of Tumour-associated macrophages (TAMs) [64,88–90]. TAMs promote angiogenesis, cancer stemness, cell invasion, and metastasis, regulate energy metabolism, and modulate T-cell activity [88,90].

In normal conditions, the immune checkpoints, like Programmed death-1 (PD-1), cytotoxic T-lymphocyte antigen 4 (CTLA-4) and B- and T-lymphocyte attenuator, prevent excessive T-cell activation and inflammatory reactions. They maintain immune system stability through several cell-cell interactions [34,64,91]. Tumour cells often overexpress these immune checkpoints, inhibiting T-cell activity and successfully evading immune surveillance [34]. While high Tumour-infiltrating lymphocytes (TILs) are generally associated with a better prognosis, the composition of TILs varies with BC subtype and does not uniformly predict outcomes. In general, both CD4+ and CD8+ T cells are present at high levels and are involved in immune responses, but with distinct roles [92]. During BC progression, CD4+ T-cells undergo a shift from a Th1-dominant profile in the early stages that is linked to a favorable prognosis, to a prevalence of Regulatory T cells (Treg) and Th17 subsets in the later stages [92]. Treg cells are a subset of CD4+ T cells that significantly contribute to BC progression by suppressing antitumour responses. They inhibit the activity of CD8+ and CD4+ T-cells, as other immune effectors, such as Natural killer (NK) cells, B cells, and antigen-presenting cells. Furthermore, the abundance of Treg cells in the TME may hinder the anti-tumour immune response, allowing cancer cells to evade immune surveillance [71,93]. Conversely, CD8+ cytotoxic T-cells play a pivotal role in eliminating tumour cells, through the release of granzyme and perforin mediated by IFN-γ secretion, conducting an effective antitumour immune response, leading to improved patient outcomes [66,92]. However, the activation of CD8+ T-cells depends on Th1 cells, which enhance their interaction with APCs, rendering them more effective in presenting antigens to CD8 T cells [66].

Dendritic cells, crucial for initiating antitumour responses through antigen presentation and T-cell (TCD4, TCD8) stimulation, exhibit dysfunction in the BC microenvironment. This impairment hampers antitumour immune responses and supports tumour progression. In some instances, the tumour microenvironment can induce dendritic cells to acquire immunosuppressive properties, potentially promoting the generation of Treg cells or other immune-suppressive mechanisms, thus impeding effective anti-tumour responses [94,95].

Finally, the ECM is recognized as an important regulator of BC progression, primarily due to the alterations in the composition and organization of the tumour ECM. In general, the ECM encompasses structural and specialized components, such as fibronectin, collagen, laminins, glycoproteins, proteoglycans, matricellular proteins, and ECM remodeling proteins [96–98]. For instance, an increase in collagen I cross-linking induced by Lysyl oxidase (LOX) and LOX-like 2 (LOXL2) enzymes remodels the ECM and increases focal adhesions, thereby contributing to migration and invasion of BC cells [99,100]. In fact, these components play essential roles in BC progression, invasion, metastasis, and resistance to therapeutics [96,101]. Blocking LOX activity, for example, can decrease collagen cross-linking and fibrinogen assembly, enhancing drug penetration while downregulating Integrin subunit alpha 5 (ITGA5)/Fibronectin (FN1) expression. This cascade of events hampers the c-Src/Focal adhesion kinase (FAK) signalling pathway, leading to apoptosis and resensitization of cancer cells to chemotherapy [101].

Understanding the microenvironment components, the involved downstream signalling pathways, and the interplay between cells and molecules in the microenvironment and tumour cells is crucial for developing effective immunotherapy strategies for BC.

I.1.5 Breast Cancer Therapy

BC is a heterogeneous disease with distinct pathological attributes, clinical trajectories, and molecular subtypes. Defining treatment strategies to BC individual distinct characteristics and molecular subtypes involves a comprehensive approach comprising local therapies (surgery and radiation) and systemic therapies encompassing chemotherapy, endocrine therapy, targeted therapies, and immunotherapy (Figures 2 and 9). Complementary therapies, including bone stabilization agents, further enhance the treatment landscape [4].

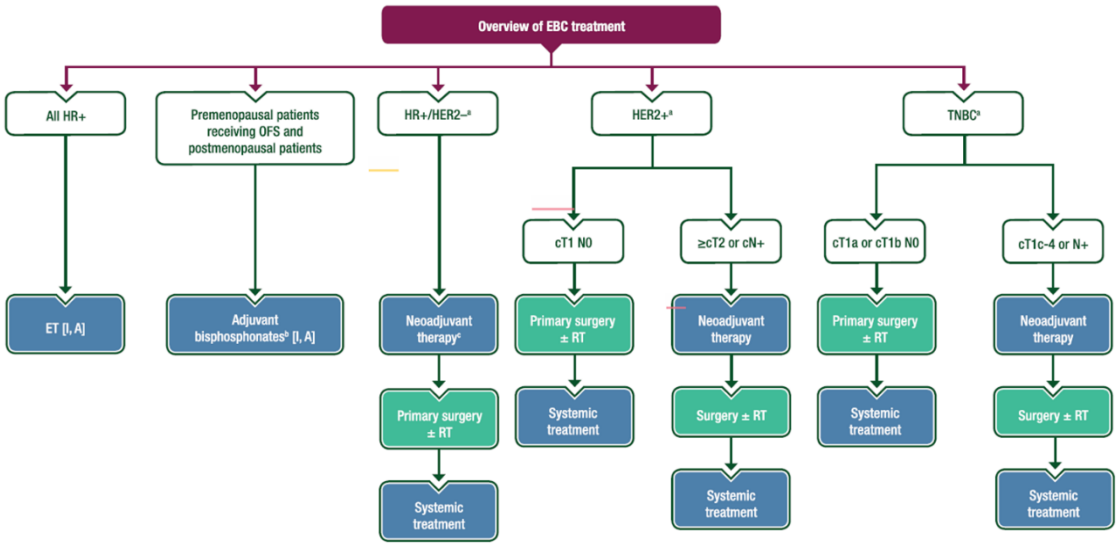

Figure 9. Example of breast cancer treatment. *Green boxes represent combination of treatments or other systemic anticancer therapy, blue boxes represent systemic treatments and white boxes show other aspects of management. ALN - Axillary lymph node; C - Clinical; ChT - Chemotherapy; CPG - Clinical Practice Guideline; DCIS - Ductal carcinoma in situ; EBC - Early breast cancer; ET - Endocrine therapy; HER2 - Human epidermal growth factor receptor 2; HR – Hormone receptor; N - node; OFS - Ovarian function suppression; T - tumour; TNBC- Triple-negative breast cancer; RT- radiotherapy*

a Need to evaluate ALN involvement and systemic therapy according to breast cancer subtype and special situations (elderly patients, male breast cancer and DCIS) are described in the CPG text.
b Bisphosphonates are approved for treating bone metastases and osteoporosis and not for prevention of relapse.
c If ChT is indicated it may be given in the neoadjuvant setting.

Source: Loibl et al., Ann of Oncol., 2023 [102]

While not all women with breast cancer require chemotherapy—such as those with small, early-stage tumours without lymph node involvement—chemotherapy remains a common therapeutic approach in both adjuvant and neoadjuvant settings [103].

The primary objective of chemotherapy is to target and destroy rapidly dividing tumour cells. Among the most frequently used chemotherapeutic agents are anthracyclines, such as doxorubicin and epirubicin, which inhibit topoisomerase II and intercalate into DNA strands, thereby disrupting essential processes like DNA replication and repair. Additionally, taxanes, including paclitaxel and docetaxel, prevent cell division by stabilizing microtubules, thereby inhibiting mitosis [104]. Other key agents include antimetabolites such as 5-fluorouracil and capecitabine, which disrupt DNA synthesis and thus inhibit cell replication [105]. Cyclophosphamide, an alkylating agent, adds alkyl groups to DNA, leading to cross-linking and strand breakage, which hampers DNA replication and triggers cell death [106]. Platinum compounds, like cisplatin and carboplatin, also form DNA cross-links, preventing DNA replication and inducing apoptosis [107]. The use of combinations of agents, each with a distinct mechanism

of action, enhances the overall efficacy of chemotherapy in targeting diverse pathways critical for tumour growth and survival.

In addition to conventional chemotherapy, advancements in our understanding of BC biology have created the foundations for personalized therapeutic strategies, resulting in improved responses based on the molecular subtype of each tumour. Endocrine therapy, for instance, is selectively administered to patients expressing ER and/or PR, notably the Luminal A and B subtypes [108]. This therapeutic approach aims to counteract the growth-promoting effects of estrogen through different ER-targeted mechanisms, including: i) selective ER modulators (e.g. tamoxifen), exhibiting agonistic or antagonistic effects on ER transcription, depending on the tissue; ii) selective ER down regulators (e.g. fulvestrant), which reduce ER expression; (iii) aromatase inhibitors (e.g. letrozole, anastrozole, exemestane), inhibiting estrogen biosynthesis in postmenopausal patients; and (iv) gonadotropin-releasing hormone analogs, inhibiting estrogen biosynthesis in premenopausal patients [53]. This therapeutic approach is effective in ER+ breast cancer; however, in advanced ER+ BC or upon the development of resistance, more aggressive treatments, such as chemotherapy, become necessary [53]. Drug resistance has prompted the development of new therapies, including Selective estrogen receptor degraders (SERDs), or combined therapies using inhibitors for cell cycle regulation or PI3K, for instance [18].

Following endocrine therapy for ER+ BC, some HER2- tumours can become HER2+. ER and HER2 can reciprocally regulate each other: ER downregulates HER2 and, conversely, HER2 downregulates ER expression. Consequently, blocking ER+ with endocrine therapy might upregulate HER2, and blocking HER2 might upregulate ER [29].

HER2+ tumours are biologically heterogeneous, depending on HER2 activation mechanisms, hormone receptor status, genome variations, tumour heterogeneity, and treatment resistance, impacting treatment benefits and patient outcomes [42]. Current therapeutic options for HER2+ patients include monoclonal antibodies (trastuzumab and pertuzumab, targeting different extracellular regions of the HER2 receptor, inhibiting dimerization), tyrosine kinase inhibitors (lapatinib, neratinib, and tucatinib, blocking HER2 receptors and disabling HER2 signalling), antibody-drug conjugates (such as Trastuzumab-emtansine (T-DM1) or trastuzumab-deruxtecan) which reduce systemic toxicity, enhance antitumour activity, and facilitate trastuzumab crossing the blood-brain barrier, or bispecific recombinant monoclonal antibodies (zanidatamab). These therapeutic strategies are employed in various combinations, including in neoadjuvant therapy [34,42,48,109].

The primary mechanism of action involves inhibiting downstream signals of HER2, particularly the PI3K/AKT and MAPK pathways [42]. However, several patients do not achieve an effective response or develop resistance. One of the key alterations related to resistance to trastuzumab and lapatinib is the overactivation of the PI3K/AKT pathway [41]. One possible reason for this resistance may be associated with nuclear HER2 and its participation in the STAT3/HER2/HER3 transcriptional complex, resulting in BC subtypes resistant to trastuzumab [48]. Despite these findings, the pathways involved in nuclear transport remain unclear, and there is currently no drug developed to block nuclear HER2 functions [41,48].

Due to the pivotal role of the PI3K/AKT/mTOR pathway in BC progression and its close interaction with ER, several clinical trials, predominantly involving PI3K inhibitors, have been conducted. These trials primarily focus on postmenopausal patients with ER+/HER2- metastatic BC, utilizing combinations like buparlisib with letrozole [110], or targeting ER+ metastatic BC refractory to aromatase inhibitors using pictilisib with fulvestrant [111]. Combining these inhibitors with endocrine therapy aims to prevent the occurrence of resistance [53]. The Mammalian Target of Rapamycin, as a downstream component of PI3K/AKT, is also an intriguing target for inhibitors. Everolimus, a potential mTOR inhibitor approved for renal cell carcinoma, is undergoing clinical trials for BC to potentially reduce resistance to endocrine therapy [52,112].

Other therapeutic approaches related to important BC progression pathways have also been adopted. For example, CDK4/6 (palbociclib, ribociclib, and abemaciclib) have been employed to overcome resistance to targeted and endocrine agents [24]. These CDK4/6 inhibitors are used for treating ER+, advanced HER2-, or metastatic BC, acting downstream of many mitogenic signalling pathways [33]. However, their effects are limited, and the development of resistance mechanisms is common, highlighting the need for new combinations [33]. Additionally, inhibitors targeting Porcupine, FZDs, Wnt/FZD/LRP, and β-catenin are undergoing various phases of clinical trials [59].

Despite advancements in the development of new therapies using different inhibitors, there is currently no specific therapy for TNBC. Standard treatments include chemotherapy and Poly(ADP-ribose) polymerase (PARP) inhibitors (veliparib, talazoparib, olaparib, and iniparib) due to their effectiveness in TNBC associated with *BRCA1/2* mutations, commonly observed in TNBC [7,34,108]. In fact, PARP, BRCA1 and BRCA2 are proteins with roles in DNA damage repair, with PARP acting in DNA single-strand repair and BRCA1 and BRCA2 in DNA double-strand repair. Inhibiting PARP promotes genome instability and tumour cell death [34].

Therapeutic outcomes for many patients are encouraging, but challenges such as metastasis and drug resistance persist. The inter-tumour and intratumour heterogeneity of BC, have distinct roles in tumour initiation and progression, and increase the difficulty of determining an appropriate therapeutic approach for each BC subtype [34].

I.2 Principles of cellular redox signalling

I.2.1 Reactive Oxygen Species

Reactive Oxygen Species (ROS) encompass a family of highly reactive chemical entities that can regulate signalling pathways and disrupt redox homeostasis depending on their concentration [113,114]. ROS include oxygen free radicals such as superoxide (O_2^{*-}), hydroxyl (HO^*), peroxyl (RO_2^*), and alkoxyl (RO^*), as well as non-radical species that can easily convert into radicals like hypochlorous acid (HOCl), ozone (O_3), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2) as the most representative nonradical molecules [113,115]. Physiological metabolic reactions in mitochondria, peroxisomes, ER, and responses to inflammation contribute to the production of these species [113,116]. Enzymatic reactions involving cyclooxygenases, membrane-bound NADPH oxidases (NOX1 to NOX5, Dual Oxidases [DUOX1 and DUOX2]), xanthine oxidases, and others also contribute to cellular ROS production [113,116]. Particularly, the NOX family is found in various organelles and can transport electrons across the plasma membrane, generating ROS [114,115,117]. External factors such as exposure to physical and chemical agents (e.g., ultraviolet rays, heat, or chemotherapy-related compounds) can also induce ROS generation [113].

During mitochondrial oxidative metabolism, most of the consumed oxygen is converted into water. However, a small percentage of molecular oxygen gives rise to ROS, primarily in the form of O_2^{*-} [118]. Mitochondria serve as the primary source of ROS in cells, resulting from redox reactions during cellular respiration and electron transport chain activity [116,117]. The generation of ROS by mitochondria is regulated by various metabolic and redox-sensitive pathways [119]. Specifically, O_2^{*-} generated in the inner mitochondrial membrane is controlled by antioxidant systems. At high levels, O_2^{*-} can escape into the intermembrane space and cytosol, participating in redox signalling or causing oxidative damage [120].

In addition to their role in energy production, mitochondria play vital roles in redox regulation, cell signalling, and cell death regulation [119,121]. ROS elevation affects not only the structure and function of mitochondria but also various biomolecules such as lipids, proteins, carbohydrates, and nucleic acids, resulting in potential damage, particularly at high levels [113,115,118,122]. ROS can induce oxidative stress through the peroxidation of fatty acids, affecting cell membrane function and permeability [113]. ROS can also interact with reactive cysteine groups on proteins, leading to their oxidation by H_2O_2 (but not by O^{2-}). These interactions, including protein thiol oxidation, can influence signalling pathways involved in cell

regulation, such as proliferation or apoptosis mechanisms [113,120,123]. The impact of high levels of ROS on cell regulation pathways can transmit signals from the cytoplasm to the nucleus, modifying the activity of transcription factors that control the expression of several genes. This alteration in gene expression can also contribute to an increased risk of mutations, ultimately leading to the transformation of normal cells into cancer cells [113,114].

I.2.2 Oxidative Stress and Redox Biology

In healthy organisms, the production of reactive species is balanced by antioxidant defense systems, which consist of enzymatic and non-enzymatic components. However, this balance is not perfect, and some damage caused by ROS occurs continuously, indicating that antioxidant defenses primarily control ROS levels rather than eliminate them [116,124]. Therefore, oxidative stress refers to a significant imbalance between oxidants and antioxidants, with a bias toward the oxidants, which have an impact on redox signalling and control, thereby potentially inducing molecular alterations [125,126]. This imbalance can result from an increase in ROS generation and/or a decrease in mechanisms that reduce ROS, such as antioxidant molecules [127]. Another form of imbalance, known as reductive stress, occurs when reducing agents exceed ROS levels, which may explain the limited success of antioxidant therapies in clinical studies [128].

In mitochondria, ROS are a byproduct of the respiratory chain [129,130]. Neutrophils and macrophages also produce ROS through the action of NOX on the plasma membrane in response to inflammation [118,131]. H_2O_2, which has a relatively long half-life, acts as a signalling messenger and can modulate various cellular processes by influencing the redox state of cysteine residues in proteins [117,132]. For instance, H_2O_2 can inactivate the tumour suppressor PTEN by oxidizing cysteine residues in its active site, forming a disulfide bond that impairs PTEN's ability to inhibit the PI3K/Akt pathway. This mitogenic pathway is crucial in regulating cell proliferation, cell cycle progression, and cell survival [117].

Both H_2O_2 and O^{2-} play central roles in redox signalling, and their concentrations are enzymatically controlled [129]. Mitochondrial O^{2-}, which cannot diffuse across membranes, is mainly dismutated to H_2O_2 and O^{2-} by Superoxide dismutase 2 (SOD2 (or Manganese superoxide dismutase [MnSOD]) in the mitochondrial matrix and SOD1 (or Copper-zinc-superoxide dismutase [CuZnSOD]) in the intermembrane space [129,133]. The generation of O^{2-} depends on metabolic and physiological states, which are integrated into the redox state and contribute to the generation of redox signals. This is achieved through a transitory increase in steady-state

H_2O_2 concentrations resulting from the conversion of O^{2-} by SOD2 in the matrix or SOD1 in the intermembrane space [134].

Following the conversion of H_2O_2 by SOD2 in the matrix, catalase (CAT) in peroxisomes and the oxidation of catalytic cysteine residues in peroxiredoxins (PRX) and thioredoxins (TXN), H_2O_2 is further reduced to water and O_2 [114,130,133]. PRX, a family of thiol-dependent peroxidases (PRX1-6), incorporate reactive cysteines and reduce hydroperoxides in cells and the extracellular matrix, depending on their isoform [135–137].

Oxidized PRX is then reduced by TXN, which possesses two adjacent thiol groups that convert into a disulfide unit upon oxidation. The process of reducing the disulfide unit back to its dithiol form and restoring oxidized TXN to its reduced form is catalyzed by thioredoxin reductase (TXNRD1), which utilizes NADPH as an electron donor (Figure 10a) [133,135,138]. H_2O_2 is also converted to water by the Glutathione peroxidase (GPX) family in the cytosol and mitochondria, with reduced glutathione (GSH) being converted to its oxidized form (GSSG). GSSG is then reconverted into its reduced form through the action of glutathione reductase (GSR), utilizing NADPH as an electron donor (Figure 10b) [114,118,135,136]. These enzymes are essential for maintaining cellular homeostasis by preventing the accumulation of ROS through the reduction of disulfide bonds in cytoplasmic proteins. Therefore, an increase in GSSG and a consequent decrease in GSH levels in cells and tissues may indicate oxidative stress, leading to alterations in signal transduction and cell cycle regulation [118,133].

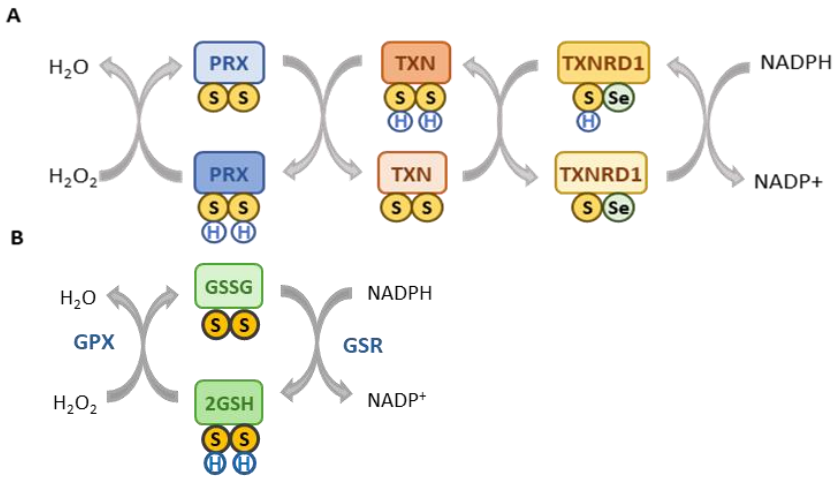

Figure 10. Reactions involving removal of ROS through the Thioredoxin and Glutathione systems*. A) Thioredoxin (TXN) catalyzes the reduction of peroxiredoxin (PRX), which in turn detoxifies hydrogen peroxide (H_2O_2). When TXN becomes oxidized, TXNRD1 reduces it, activating TXN, using NADPH as electron and hydrogen donor. B) In a process of converting H_2O_2, glutathione peroxidase (GPX) oxidizes glutathione (GSH/GSSG), which is then recycled by glutathione reductase (GSR), using NADPH as a cofactor. SS - disulfide bond (oxidize form); SH - thiol (reduced form); Se - selenium. Source: Adapted from AlOkda & Van Raamsdonk, Antioxidants, 2023* [139] *and Kageyama & Waditee-Sirisattha, Mar. Drugs, 2019* [140]

A key mechanism of redox regulation involves the formation of disulfide bonds through the oxidation of thiol groups in cysteine residues, which are sensitive to oxidation [141]. The network of thiols acts as a regulatory system involved in maintaining steady-state H_2O_2 levels, mitochondrial and cellular redox balance, and metabolic homeostasis [134,142]. This antioxidant system, based on thiol interactions, encompasses Glutaredoxin (GRX)/GSH/GSSD and TRX/TXNRD systems, which support the activities of glutathione peroxidases, peroxiredoxins, peroxidases, and methionine sulfoxide reductases. Alterations in this regulatory system can affect transcription, growth, and cell survival [114,142].

I.2.3 Role of Redox signalling in cancer progression

During tumourigenesis, mitochondrial enzymes and ROS play essential roles from tumour initiation to progression [119]. Mutations in the genes encoding these enzymes can generate oncometabolites ((R)-2-hydroxyglutarate, succinate and fumarate, for example), and alterations in mitochondrial signalling can contribute to tumour initiation [121]. The (im)balance of ROS levels in cancer is highly complex and tends to promote oxidative stress, which can disrupt transcriptional processes and signalling pathways. Generally, tumour cells exhibit higher levels of ROS due to increased cell growth, gene mutations, relative hypoxia, and metabolic rate [113,117]. ROS can modulate signalling pathways and dysregulate mechanisms related to tumour progression, such as proliferation, apoptosis, metabolic alterations, and angiogenesis, during the promotion step [117,138]. In fact, ROS exert a key role in affecting many of the hallmarks of cancer [143], as illustrated in Figure 11 and described below.

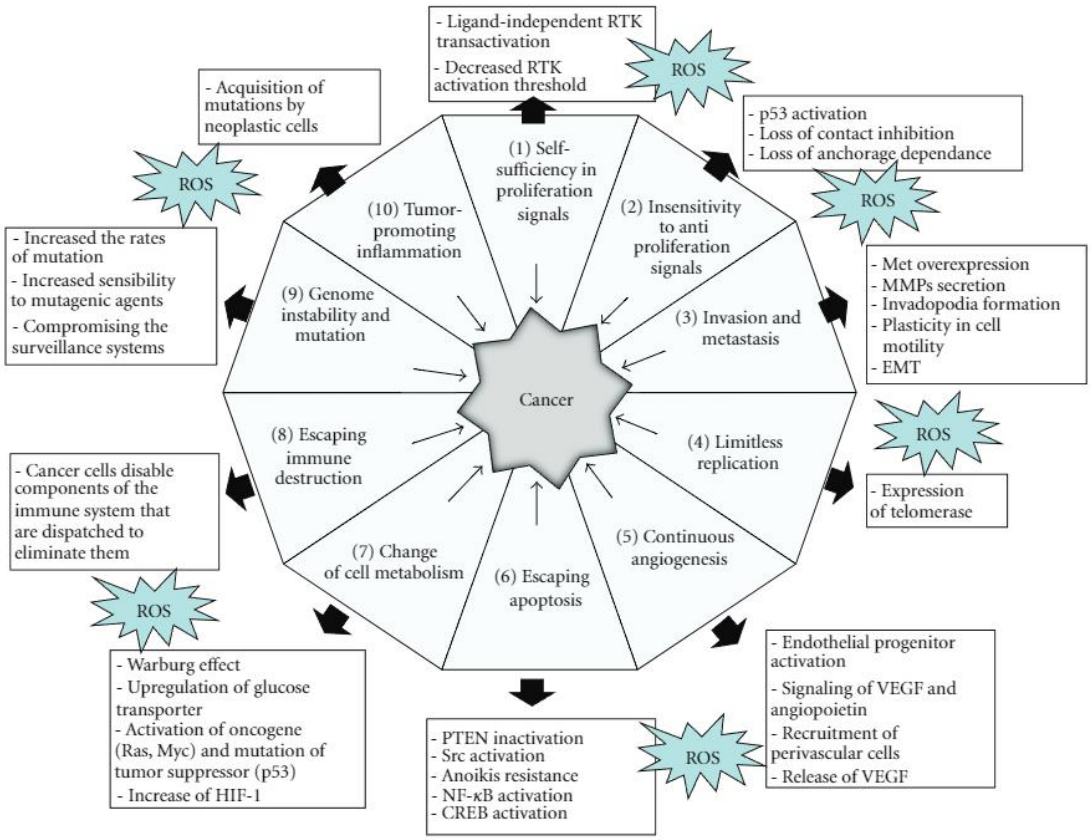

Figure 11. Roles of ROS in the hallmarks of cancer. *The contribution of ROS is highlighted for each hallmark. ROS plays a pivotal role in cell proliferation and growth, by promoting the loss of the anchorage dependence. At the same time, it facilitates the evasion of apoptosis and immune system detection, and the activation of continuous angiogenesis. Elevated intracellular ROS levels further contribute to increased genomic instability, disruptions in metabolic regulation and the promotion of migration and invasion. Source: Fiaschi and Chiarugi, Int J Cell Biol, 2012* [143]

The elevated level of ROS generates mutations upon DNA replication, thus increasing cell cycle perturbations and genomic instability, but also induces the activation of AKT/Cyclin D1 signalling, promoting proliferation [144]. Additionally, ROS contribute to the activation of tyrosine kinase receptors by growth factors, inducing cell proliferation, differentiation, migration, and survival [145].

Tumour cells often consume more glucose than normal cells to support their high proliferation rate. To sustain high energy levels and obtain the necessary metabolites, tumour cells can switch from mitochondrial phosphorylation to glycolysis. This phenomenon is known as the Warburg effect (Figure 12).

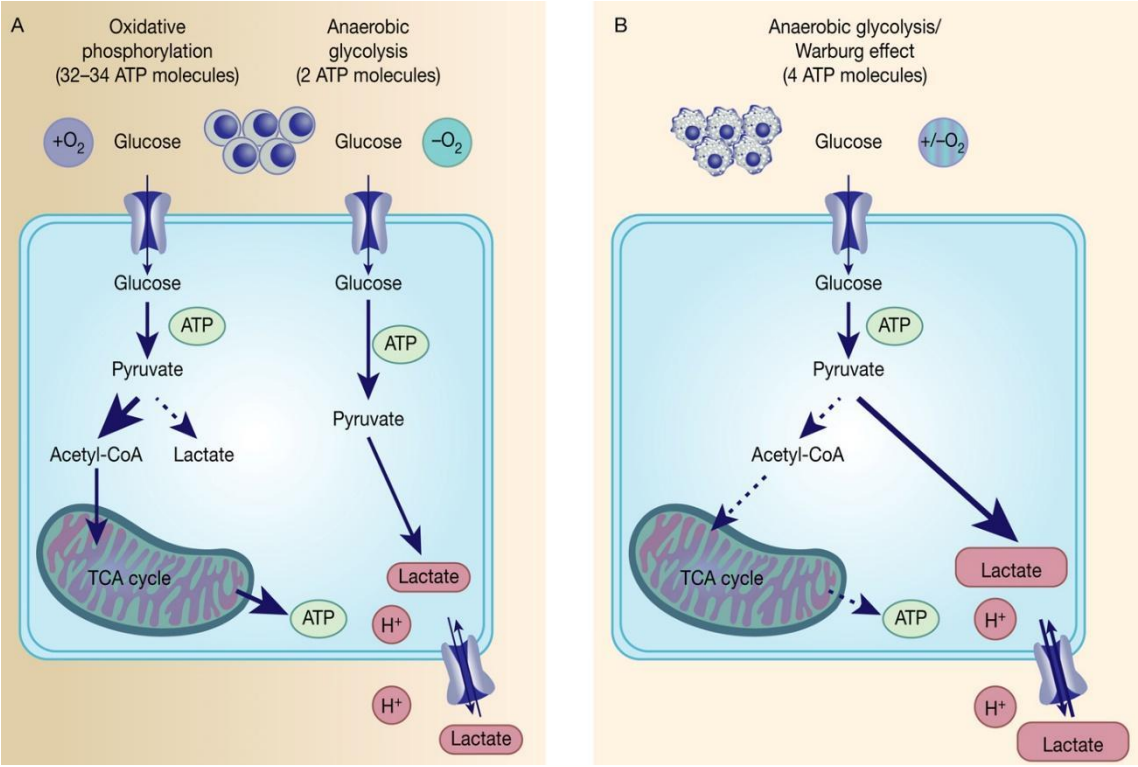

Figure 12. The Warburg effect in cancer*. A) In normal cells under normal oxygen levels, cells metabolize glucose through oxidative phosphorylation in the mitochondria, efficiently generating a large amount of ATP. When oxygen is scarce, cells switch to anaerobic glycolysis, converting glucose to lactate, which is excreted. This produces much less ATP but sustains minimal energy production for survival. B) Tumour cells predominantly convert glucose to lactate regardless of oxygen levels (Warburg effect). Despite being less efficient in ATP production compared to oxidative phosphorylation, this pathway allows cancer cells to quickly generate the energy and intermediates necessary for rapid growth and proliferation. Source: Unterlass & Curtin, Exp. Rev. Mol Med, 2019* [146]

This modification provides an additional advantage to tumour cells since the acidic environment generated by lactate production from glycolysis, coupled with low oxygen levels, promotes their proliferation compared to normal cells [119,147]. Additionally, tumour cells can adapt to nutrient depletion and hypoxic environments by modifying levels of mitochondrial redox proteins [121]. The role of mitochondrial redox proteins depends on cellular ROS levels: low ROS levels promote transcriptional activation and tumour cell proliferation, while increased ROS levels induce oxidative stress, genomic instability, DNA mutations, tumour invasiveness, and metastasis [114,117,119].

However, to counter the detrimental effects of increased oxidative stress, tumour cells activate the transcription and activation of antioxidant enzymes and mechanisms that upregulate various antioxidant systems. By buffering ROS levels, tumour cells can maintain ROS within a range that ensures survival in an unfavorable microenvironment compared to normal cells and promotes tumour progression (Figure 13) [113,114,148]. For instance, high ROS levels and low expression

of antioxidants downregulate the Sirtuin-3 (SIRT3) gene expression, a mitochondrial tumour suppressor gene that regulates Hypoxia-inducible factor 1-alpha (HIF1α) activity [119]. HIF-1α, a subunit of Hypoxia-inducible factor 1 (HIF-1), activates transcription factors that facilitate adaptation to a hypoxic environment and mediate the activation of vascular endothelial cells to promote angiogenesis, cell invasiveness, and metastasis in the tumour core [149,150]. Concurrently, the antioxidant system, including NADPH and GSH synthesis, is upregulated, maintaining a reductive phenotype. This adaptive response to increased ROS levels is crucial for acquiring and maintaining the cancer phenotype [119].

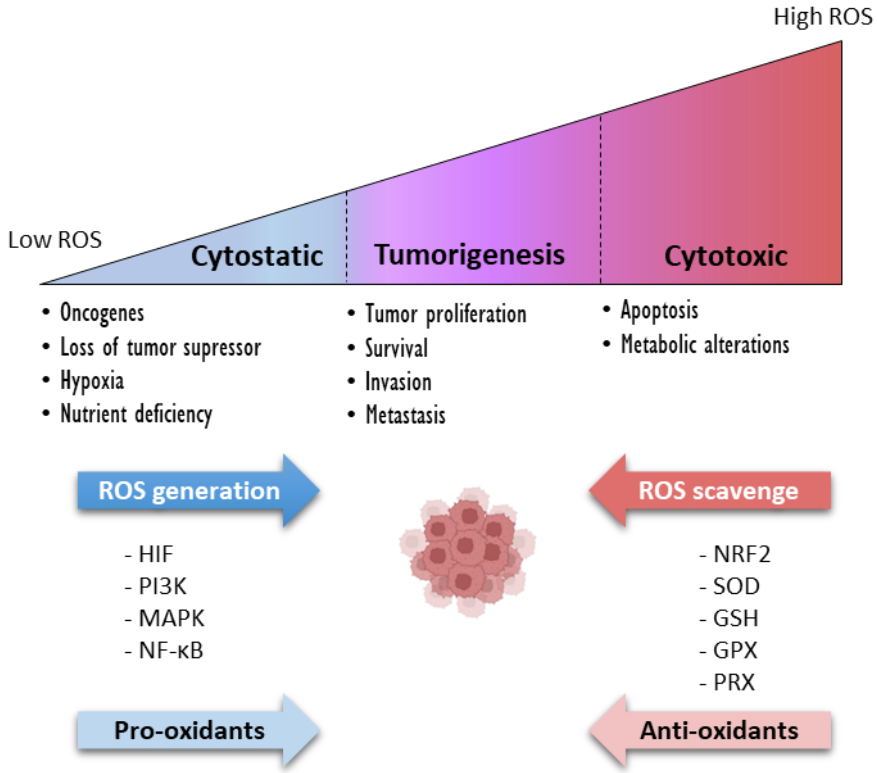

Figure 13. Contribution of ROS levels to tumourigenesis*. The equilibrium between ROS generation and ROS scavenging is crucial for tumour cells to sustain optimal ROS levels within the tumourigenic range. Oncogenes, loss of tumour suppressor, hypoxia, and nutrient deficiency activate mitochondrial ROS generation, leading to the upregulation of proteins associated with increased tumourigenicity. Concurrently, tumour cells elevate the expression of antioxidant proteins to prevent ROS from reaching cytotoxic levels that could hinder their growth. This orchestrated interplay between ROS generation and scavenging mechanisms highlights the adaptive strategies employed by tumour cells to establish a pro-tumourigenic redox environment. Source: Based on Schieber & Chandel, Curr Bio, 2014* [151] *and Sullivan & Chandel, Cancer & Metabol, 2014* [117]

It is important to highlight the role of Nuclear factor erythroid 2-related factor 2 (Nrf2) in tumour cells. Under normal physiological conditions, Kelch-like ECH-associated protein 1 (Keap1) inhibits Nrf2, a transcription factor that regulates the transcription of several antioxidant

proteins and detoxification response molecules. However, increased ROS levels promote the dissociation of Keap1 and Nrf2, allowing the translocation of Nrf2 into the nucleus to induce the translation of detoxification elements, such as SOD, GPX, PRX, CAT, GSH biosynthesis enzymes, NADPH production, and iron-sequestration agents, to prevent oxidative stress (Figure 14) [114,124,148,152]. NADPH plays a crucial role in cellular redox balance as a hydrogen donor in cancer cells. In specific cancer cell types, an inefficient redox cycling process leads to excessive NADPH consumption, resulting in a persistent state of oxidative stress, increased NOX levels, and impeding the maintenance of an adequate pool of NADPH [135,153].

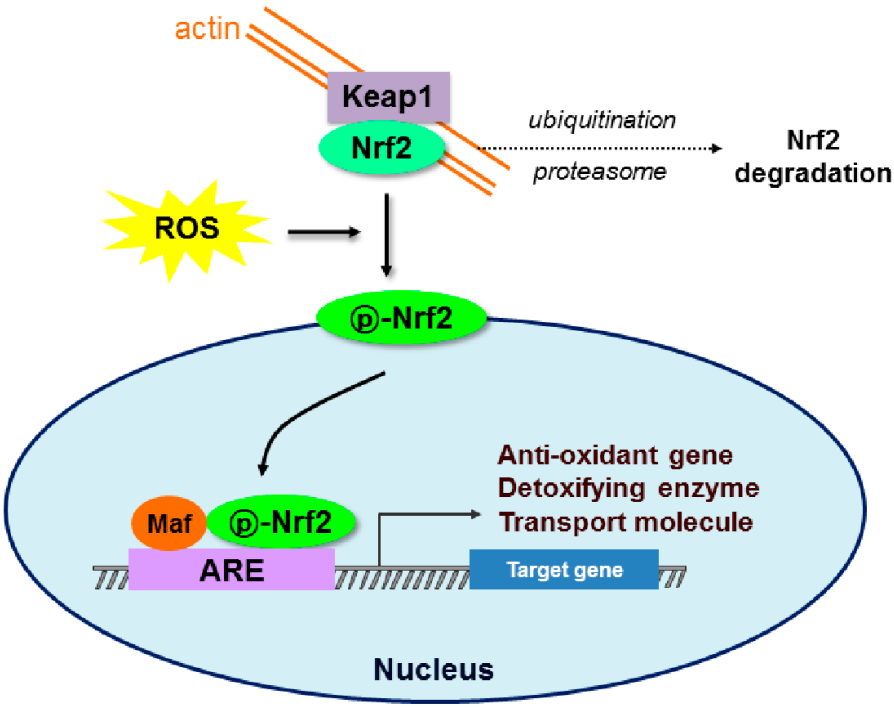

Figure 14. Kelch-like ECH-associated protein 1/Nuclear factor erythroid 2-related factor 2 (Keap1/Nrf2) signalling pathway. *Under normal conditions, KEAP1 binds to Nrf2 and targets it for degradation, keeping Nrf2 levels low. When ROS levels rise, KEAP1 undergoes oxidative modifications that prevent it from degrading Nrf2. This allows Nrf2 to accumulate, enter the nucleus, and activate the expression of genes that combat oxidative stress, enhancing cell survival and maintaining redox homeostasis. ARE - Antioxidant response element. Source: Oh & Jun, Int J. Mol Sciences, 2017* [154]

Notably, SODs have a dual role in cellular processes. They act as antioxidants by neutralizing superoxide anions but can also generate H_2O_2, acting as prooxidants. Consequently, H_2O_2 can impact protein tyrosine phosphatases and oxidize cysteine residues at their active sites, inhibiting and altering signalling pathways. Inhibition of SOD has significant consequences: it activates phosphatases, promotes apoptosis, and inhibits MAPKs in tumour cells. Inhibition of tyrosine phosphatases activates the tyrosine kinase cascade pathway, driving cell proliferation and promoting tumourigenesis [122].

In short, tumours need to maintain ROS levels within a specific range that promotes proliferation without causing cytotoxicity. To achieve this, a balance between ROS production and antioxidant expression is crucial for maintaining tumour proliferation through the induction of ROS levels [121]. The adaptive response associated with increased ROS levels can also be responsible for drug resistance, as tumour cells exhibit greater resistance to oxidant agents and oxidative stress when compared to normal cells [119]. It is worth noting that the roles of redox signalling can have both beneficial and detrimental consequences, depending on factors such as the type of cancer, metabolic state, tumour heterogeneity, tissue type, microenvironment, and tumour stage [119,121].

I.2.4 Redox signalling in breast cancer progression

In BC, ROS are not only generated by mitochondria and defense cells. Thymidine phosphorylase, an enzyme involved in thymidine catabolism, and lactoperoxidase, associated with estrogen metabolism, are also implicated in oxidative stress in BC [131]. In fact, estrogen-induced oxidative stress can lead to genome modifications in BC cells [116].

Breast carcinomas typically promote their growth by inducing angiogenesis, which results in cycles of hypoxia and reperfusion, leading to the generation of ROS and oxidative stress within the breast tumour. Hypoxia induces the accumulation of HIF-1 and activation of nuclear factor NF-kB, which stimulates the transcription of the angiogenic factor Vascular endothelial growth factor (VEGF). ROS frequently elevate the levels of these proteins, which play a crucial role in BC progression, contributing to mutations, tumour progression, activation of growth-promoting signalling pathways, resistance to therapy, and increased risk of metastasis [131].

In BC, ROS also affect signalling pathways such as MAPK/ERK1/2, related to the MAPK family, which includes p38α kinase, Extracellular-regulated kinase 1 (ERK1) and c-Jun N-terminal kinase (JNK), and is an intracellular signal transduction pathway involved in tumour growth, apoptosis and migration [131,135]. For instance, ROS inhibitors targeting ERK1/2 in metastatic BC MDA-MB-435 cells promote apoptosis and enhance cell adhesion [155].

Certain types of cancer rely on increased cysteine levels to enhance GSH biosynthesis. By promoting GSH production, cancer cells gain a survival advantage and enhance their ability to counteract the damaging effects of ROS [156]. In BC, GSH biosynthesis is linked to the oncogenic PI3K/Akt signalling pathway, which activates Nrf2 to upregulate GSH biosynthetic genes [157]. Nrf2 is constitutively activated in various tumour cells, including BC cells, facilitating increased

GSH biosynthesis required for PI3K/Akt pathway gain resistance to oxidative stress and stimulate angiogenesis, tumour growth, survival, and cell mobility [124,135,157,158]. However, inhibiting GSH biosynthesis does not contribute to controlling BC progression, likely due to the adoption of thioredoxin pathways [148].

While Nrf2 has a cancer-preventive function by inducting antioxidant enzymes, it plays a dual role in BC. Nrf2 activation can confer resistance to chemotherapy agents. In the case of BC treatments, like cisplatin, doxorubicin, and etoposide, which increase the production of ROS to cytotoxic levels, Nrf2 activation can lead to the activation of antioxidant response. This activation of antioxidant pathways enhances the survival of cancer cells and their resistance to chemotherapy [158,159].

Combination therapy targeting redox pathways shows promise as a potential treatment strategy. *In vitro* and *in vivo* BC models using combined inhibitors of the glutathione and thioredoxin pathways demonstrated synergistic cancer cell death. Glutathione is the most abundant antioxidant in the cell and is required for cancer initiation and thioredoxin may be required to buffer ROS levels. While individual pathway inhibitors have shown limited success, inhibiting both antioxidant pathways can induce cancer cell death [148].

ROS can also function as tumour-suppressor agents. Although high levels of ROS can promote tumourigenesis and contribute to resistance, the enhanced antioxidant defense observed in cancer cells suggests that reducing antioxidant defense and increasing ROS levels to a toxic threshold may suppress tumour development [160].

I.2.5 Impact of ROS production on the breast cancer Tumour Microenvironment

The TME plays a pivotal role in tumourigenesis and is particularly associated with metastatic spread in BC [68,161]. The TME encompasses not only tumour cells, but also a spectrum of components like normal fibroblasts, CAFs, endothelial cells, adipocytes, and immune cells [68,162]. Remarkably, ROS are produced by both tumour cells and different components of the TME, fostering a more tumourigenic and chemoresistant environment, thereby increasing angiogenesis and inflammation [163,164]. Hypoxia is common in the TME of BC and represents a significant driver of adaptations to evade host immunosurveillance [165].

Mitochondrial ROS, local hypoxia, and growth factors released by tumour cells and infiltrating immune cells, such as TNF-α and IL-1β, stimulate the differentiation of CAFs, an essential component of the TME [166]. In BC, the increase of ROS levels, combined with hypoxia,

contributes to HIF-1α and NF-kB activation in CAFs, resulting in autophagic degradation of Caveolin-1 (CAV-1) in stromal fibroblasts. The loss of CAV-1 shields adjacent tumour cells from apoptosis and is associated with tumour recurrence, lymph node metastasis, tamoxifen resistance, and poor clinical outcome [167,168].

These tumour progression mechanisms are facilitated by the upregulation of glycolytic enzymes, responsible for aerobic glycolysis and the release of lactate and pyruvate, also known as Reverse Warburg effect [168]. HIF-1 is a key factor in orchestrating the metabolic shift of tumour cells from oxidative phosphorylation to glycolysis [165].

Endothelial cells within the TME are phenotypically distinct from normal cells and are also known as Tumour-associated endothelial cells (TEC) [169,170]. These cells play an important role in BC development and progression, as TECs respond to hypoxic stress by increasing angiogenesis, potentiated by the interaction with tumour cells. This mechanism, essential for tumour spreading, is also responsible for resistance to angiogenic inhibitors [169].

Moreover, ROS exerts a significant influence on the expression of PD-1 and Programmed cell death-ligand 1 (PD-L1). Within tumour cells, PD-L1 attenuates the effector function in dendritic cells [171] and macrophages [172] by binding with PD-1, an inhibitory immune checkpoint receptor expressed on activated immune cells.

Immune cells, especially M2 macrophages, dendritic cells, and T cells, constitute the major components of the immunosuppressive TME induced by ROS-driven inflammation [173]. Elevated ROS levels induce M2 macrophages, and Myeloid-derived suppressor cells (MDSCs), both exhibiting immunosuppressive roles. These cells recruit Tregs to the TME, acting as immunosuppressors and hindering T cell activity [174–176]. Like other myeloid cells, M2 macrophages and MDSCs are also important sources of ROS production due to their role in inflammation.

ROS can influence the expression of FOXP3, a transcription factor crucial for Treg development and function [177]. Regulated by ROS within the TME, Treg cells suppress an exacerbated inflammatory response by secreting immunosuppressive cytokines and upregulating immune checkpoints. The potential to utilize lactic acid for a more efficient oxidative phosphorylation, allows them to avoid metabolic restrictions. The increased levels of Treg cells induce an immunosuppressive reprogramming in the TME, contributing to tumour growth and drug resistance [178,179].

Activation and differentiation of T cells require low levels of ROS, which activate T cell receptor (TCR) and Nuclear factor of activated T cells (NFAT), consequently promoting IL-2 expression [180,181]. Conversely, high levels of ROS in the TME, not only suppress NK viability and function but also inhibit T cell proliferation and anti-tumour function, by impeding the recognition between TCR and Major histocompatibility complex (MHC)-peptide complex, thereby suppressing cytotoxic T lymphocytes response [180,182]. Furthermore, elevated levels of ROS upregulate Fas expression and downregulate anti-apoptotic BCL2 expression, promoting T cell apoptosis. [183–185]. Both promote cancer progression by evading the immune response [182,186,187]. This was highlighted in a study that demonstrated an impaired MDSC-mediated T-cell proliferation when co-culturing MDSCs with T-cells in the presence of catalase [188].

Focusing solely on tumour cells may overlook the potential of the TME in therapeutic intervention, whether applied individually or in combination with other therapies. A comprehensive understanding of how the modulation of the redox balance within and between tumours and different immune cells infiltrating the TME holds promise for more efficient treatments [163].

I.2.6 New therapeutics for breast cancer using ROS approaches

A profound comprehension of ROS regulation mechanisms in BC can be a key to developing novel therapeutic approaches. Targeting the tumour through strategies such as enhancing ROS production to cytotoxic levels, inhibiting antioxidant defense mechanisms, or combining both approaches presents a distinct opportunity for intervention [160].

The therapeutic potential of exploiting increased intracellular ROS levels in tumour cells is evident. Celecoxib, a potent anticancer agent currently in phase III clinical trials for metastatic BC, rapidly elevates mitochondrial O^{2-} production, triggering cytotoxic effects [189,190]. By disrupting mitochondrial metabolism, Celecoxib leads to excessive ROS production directly from the mitochondria, ultimately activating the intrinsic apoptotic pathway in metastatic BC cells. Additionally, Celecoxib inhibits mitochondrial membrane potential, facilitating the collapse of the mitochondrial transmembrane potential, which further contributes to inducing intrinsic apoptosis in BC metastatic cells [190]. Similarly, 2-methoxyestradiol, a metabolite of 17-beta estradiol, induces apoptosis via ROS generation in both hormone-dependent and hormone-independent BC [191]. This compound inhibits mitochondrial respiration and promotes the formation of O^{2-}, leading to Cytochrome C release and activation of the Caspase-9 pathway [192].

These examples illustrate how redox knowledge is being harnessed in the discovery of new drugs for BC. However, numerous other studies involving different compounds are also underway, spanning various stages of development, from preclinical research to clinical trials. By deepening our understanding of how to exploit ROS to modulate cell-specific biological functions will not only pave the way for the possible creation of new anticancer therapies but may also enhance the efficacy of existing treatments.

I.3 Principles of cellular Ca^{2+} signalling

I.3.1 Calcium channels, pumps and exchangers

Calcium ions (Ca^{2+}) function as versatile second messengers essential to multiple intracellular signalling events. This second messenger plays a pivotal role in regulating various physiological processes such as fertilization, cell proliferation, gene transcription, contraction, secretion, learning and memory, synaptic transmission, and even cell death via necrosis or apoptosis [193,194]. These events are intricately regulated in multiple distinct ways, depending on cell type, cellular context, spatial and temporal dynamics of the Ca^{2+} signals [193,195]. For instance, Ca^{2+} prompts exocytosis within microseconds at synaptic junctions, while its involvement in controlling gene transcription and cell proliferation is associated with signals spanning hours [193].

The tight regulation of Ca^{2+} concentrations within cellular compartments ensures the sensitive regulation of cell signalling pathways, enabling accurate responses to various stimuli. Within cells, Ca^{2+} homeostasis is maintained by ensuring low cytosolic Ca^{2+} levels. Ca^{2+} concentration gradients are established between the cytosol and specific organelles, like the Endoplasmic reticulum (EndR), the Golgi apparatus, lysosomes, and mitochondria [196]. These organelles function as Ca^{2+} stores and house high-capacity Ca^{2+} binding proteins, like calsequestrin and calreticulin that play a crucial role in maintaining Ca^{2+} homeostasis within the EndR [197,198]. This enables the EndR to generate Ca^{2+} signals into the cytosol or mitochondria, thereby contributing to modulating the activity of Ca^{2+}-dependent proteins [198].

Calcium ion moves through different compartments, including the extracellular space, cytoplasm, EndR, Golgi apparatus, and mitochondria. This movement depends on specialized Ca^{2+}-handling and Ca^{2+}-sensing proteins unique to each compartment. Some proteins, such as pumps and exchangers, actively transport Ca^{2+} against concentration gradients, while others, like Ca^{2+}-permeable ion channels, allow Ca^{2+} to move passively. Certain proteins store Ca^{2+} within compartments using Ca^{2+}-binding proteins, while Ca^{2+}-dependent effectors trigger cellular processes [199] (Figure 15).

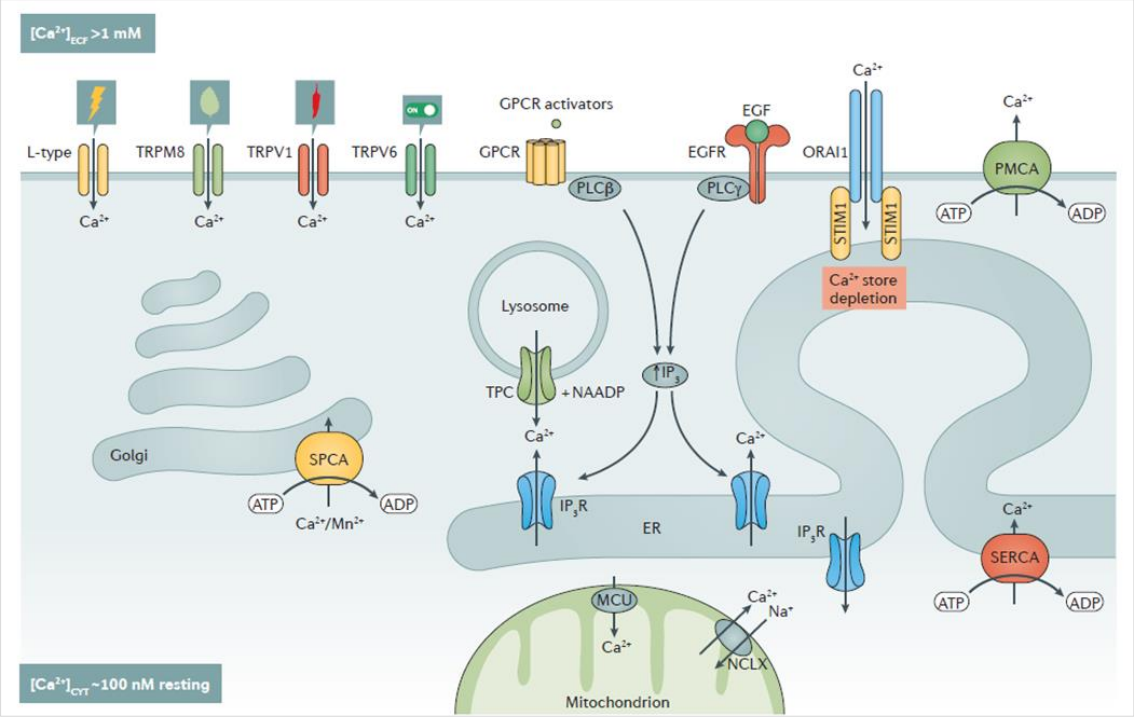

Figure 15. Calcium ion cellular homeostasis regulation by Ca^{2+}-permeable channels, pumps, and exchangers located at the plasma membrane and intracellular organelles. *The concentration of free Ca^{2+} in the extracellular fluid ($[Ca^{2+}]_{ECF}$ >1 mM) is significantly higher than the resting free Ca^{2+} level in the cytosol ($[Ca^{2+}]_{CYT}$ ~100 nM). The maintenance of this gradient is regulated by Plasma membrane Ca^{2+}-transporting ATPase (PMCA) transporting Ca^{2+} across the plasma membrane. Several channels also contribute to the increase of $[Ca^{2+}]_{CYT}$, such as voltage-gated channels (L-type, illustrated, but also T-type, P/Q-type, R-type, and N-type), and members of the transient receptor potential (TRP) family (like TRPM8 activated by cool and menthol, TRPV1 activated by heat and capsaicin, and TRPV6, a highly Ca^{2+} selective channel with constitutive activity in certain cells). $[Ca^{2+}]_{CYT}$ levels can also be elevated through the release of Ca^{2+} from stores such as the Endoplasmic reticulum (ER), the Golgi complex, the lysosome or the mitochondria. For instance, an increase in inositol 1,4,5-trisphosphate (IP_3) activates IP_3 receptors (IP_3Rs) located in the ER, releasing Ca^{2+} into the cytoplasm and mitochondria. Mitochondria located near to ER, capture Ca^{2+} through the Mitochondrial calcium uniporter (MCU) complex. The depletion of these Ca^{2+} stores is detected by the Ca^{2+} sensor stromal interaction molecule 1 (STIM1), which activates the calcium channel protein 1 (ORAI1)-dependent Ca^{2+} influx pathway, releasing Ca^{2+} into the cytosol. This leads to the sarcoplasmic/ER Ca^{2+}-ATPase (SERCA) pump transports Ca^{2+} back into the ER. Ca^{2+} is also sequestered into the Golgi apparatus using the secretory pathway Ca^{2+} ATPase (SPCA) pump, which transports Mn^{2+} as well. Channels such as the mitochondrial Na+/Ca^{2+} exchanger (NCLX), and lysosomal two-pore channels (TPCs) also contribute to Ca^{2+} transport. Source: Monteith et al., Nature Reviews, 2017* [196]

I.3.2 Extracellular and intracellular Ca^{2+}-signalling

In resting cells, cytoplasmic Ca^{2+} levels remain low. The intracellular Ca^{2+} level is determined by a balance between "on/off" reactions, ie., Ca^{2+} introduction into the cytoplasm and Ca^{2+} removal mechanisms, orchestrated by a network of buffers, pumps, and exchangers, as illustrated in Figure 15 [193,194]. External channels responding to stimuli or internal stores, primarily the EndR (and also the nuclear envelope, Golgi apparatus, lysosomes, and mitochondria), initiate these reactions based on Ca^{2+} gradients or messengers [195]. In "on" reactions, external stimuli

trigger Ca^{2+} entry and second messenger formation, releasing stored internal Ca^{2+} from the EndR. Ca^{2+} is mainly bound to buffers, but a small portion activates effectors that trigger Ca^{2+}-dependent cellular processes. These released channels are sensitive to factors within the cytosol and EndR lumen. During "off" reactions, Ca^{2+} dissociates from effectors and buffers, being released from the cytoplasm and storing organelles, such as EndR and Golgi apparatus, through various exchangers and pumps [193,196]. In greater detail, Ca^{2+} signalling is activated by the influx from extracellular space or released from intracellular stores (Figure 16).

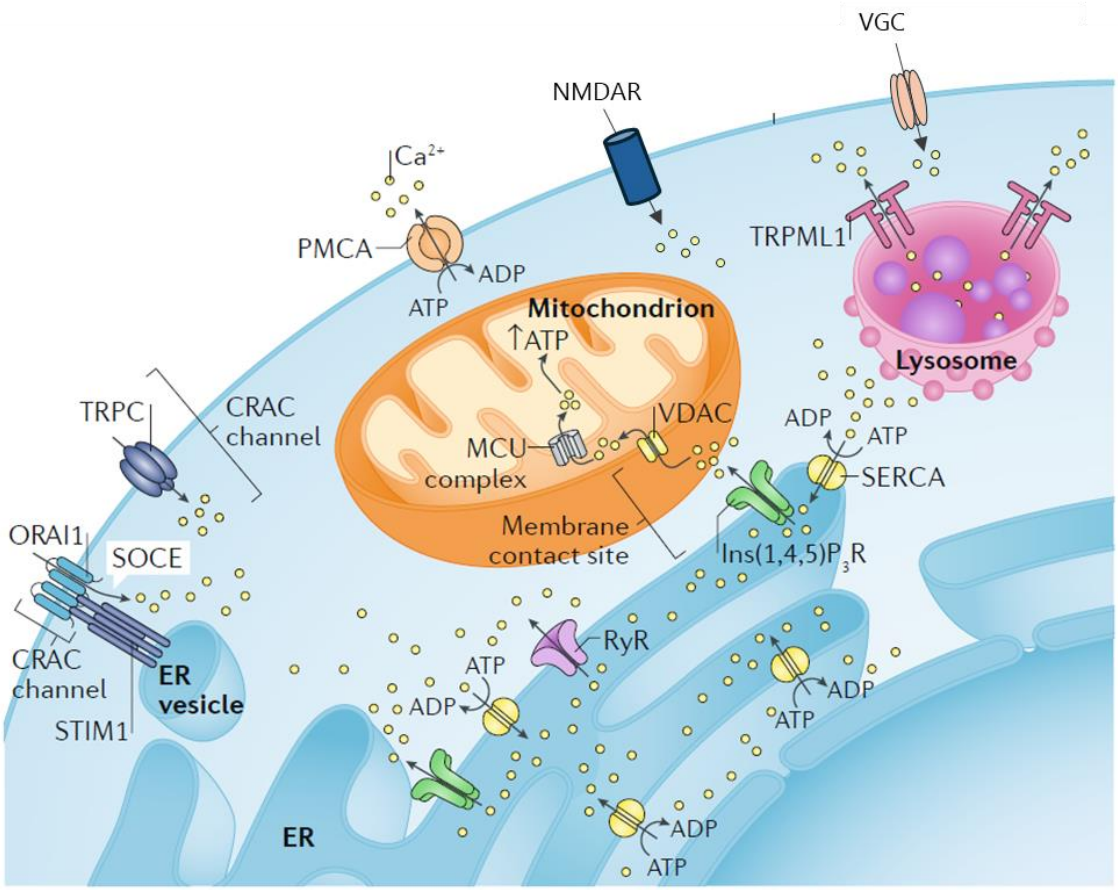

Figure 16. Calcium ion influx occurs from both external and internal sources*. External stimulation can generate Ca^{2+} signals by inducing Ca^{2+} entry through channels in the plasma membrane. Various stimuli, such as glutamate, activate N-methyl-D-aspartate receptors (NMDARs), while electrical fields open voltage-gated channels (VGCs). Transient receptor potential canonical (TRPC) channels and ORAI calcium release-activated calcium modulators (ORAI) respond to internal stimuli. Internal Ca^{2+} stores are regulated by inositol 1,4,5-trisphosphate (Ins(1,4,5)P3) receptors (InsP3R) and Ryanodine receptors (RYRs). Source: Adapted from Giorgi et al., Nat Rev Mol Cell Biol, 2018* [200]

The influx of Ca^{2+} from the extracellular space serves as Ca^{2+} signalling during the “on” reaction, driven by an electrochemical gradient across the plasma membrane [193]. Distinct entry channels play crucial roles in responding to different external signals. Receptor-operated channels (ROCs) respond to specific ligands binding to cell surface receptors, while the

neurotransmitter glutamate activates N-methyl-D-aspartate receptors (NMDARs) (Figure 16). Second-messenger-operated channels (SMOCs) are controlled by internal messengers and Store-operated channels (SOCs) are activated in response to decreased EndR Ca^{2+} concentration [193,194]. In response to this decline, Stromal Interaction Molecule (STIM) proteins, engage ORAI Calcium release-activated calcium modulator (ORAI) channels located in the plasma membrane, allowing external Ca^{2+} entry via Store-operated calcium entry (SOCE) and restoring Ca^{2+} levels (Figure 16) [201,202]. Other important entries are Voltage-gated channels (VGCs), which rapidly generate Ca^{2+} fluxes upon activation (Figure 16) and are critical for processes like muscular contraction [193,194].

The Transient receptor potential ion-channels (TRP) family, comprising 28 channel proteins and divided into seven subfamilies - ankyrin (TRPA), canonical (TRPC), melastatin (TRPM), mucolipin (TRPML), NO-mechanopotential (TRPN), polycystin (TRPP), and vanilloid (TRPV) - modulate various signalling pathways and slower cellular processes. In fact, TRP channels tend to have low conductance, enabling them to operate over longer time scales without overwhelming the cell with excessive Ca^{2+}, allowing them to control slower cellular processes, including cell proliferation, and also modulate different signalling pathways, such as MAPK, TGF-β, NF-kB and AMP-activated protein kinase (AMPK). [193,203].

The “on” reactions are also associated with internal stores of Ca^{2+}, which are regulated by two crucial players: Inositol 1,4,5-trisphosphate (IP_3) receptors (IP_3Rs) and Ryanodine receptors (RYRs) (Figure 16). These receptors unleash Ca^{2+} from intracellular stores in response to various stimuli, leading to swift spikes in Ca^{2+} cytosol concentration levels during "on" reactions and the emergence of regenerative Ca^{2+} waves [193].

The triggered release of Ca^{2+} from the EndR takes place by intracellular stimuli or when extracellular stimuli bind to G-coupled protein receptors, activating Phospholipase C (PLC) to generate IP_3 [198]. IP_3Rs are modulated by the binding of IP_3, enhancing the receptor's sensitivity to Ca^{2+}. Interestingly, IP_3 presents a biphasic behavior – activating the receptor at low concentrations but exerting inhibitory effects at higher concentrations upon Ca^{2+} release [193,194]. This regulation is influenced by direct Ca^{2+} action but is also indirectly modulated by Calmodulin (CAM), a protein that plays both activating and inhibitory roles. CAM undergoes conformational changes upon binding to Ca^{2+}, allowing it to interact with specific targets [198]. Furthermore, Ca^{2+} can augment the sensitivity of IP_3Rs via EndR luminal lectin chaperones, such as calreticulin and calnexin, which are Ca^{2+}-binding proteins that sensitize the receptors through interaction with the receptor. Several signalling pathways further modulate IP_3Rs, including

phosphorylation by Ca^{2+}/CAM-dependent kinase (CaMKII), GMP-dependent protein kinase (PKG), protein kinase C (PKC), and cAMP-dependent protein kinase (protein kinase A; PKA) [193,194].

RYRs are also located in the EndR and are equally important channels for Ca^{2+} release. These channels are subject to regulation by diverse signals [204]. Notably, the activity of RYR2 varies with Ca^{2+} concentrations, remaining inactive at low nM levels, becoming active at low µM levels, and being inactivated by high mM concentrations [193].

Several mechanisms contribute to the "off" reaction, each characterized by varying roles, namely: Plasma-membrane Ca^{2+}-ATPase (PMCA), Na^{+}/Ca^{2+} exchanger (NCX), Sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA), Secretory pathway Ca^{2+} ATPase (SPCA), and Mitochondrial calcium uniporter (MCU). While PMCA, SPCA, and SERCA pumps exhibit modest transport rates yet high affinities, suitable for responding to slight Ca^{2+} level increases and establishing basal Ca^{2+} levels, NCX and the MCU boast significantly higher transport rates that can limit Ca^{2+} transients over a wider dynamic range. This range of activities allows cells to select the combination of "off" reactions required based on their Ca^{2+} demands. NCX and PMCA drive Ca^{2+} expulsion across the plasma membrane, whereas SERCA and SPCA pump direct Ca^{2+} back into the EndR and the Golgi apparatus, respectively. Mitochondria also play a pivotal role in recovery, capturing Ca^{2+} through the MCU and gradually releasing it back into the cytosol, where SERCA and PMCA manage Ca^{2+} levels. Cellular survival relies on Ca^{2+} homeostasis, ensuring that Ca^{2+} fluxes during "off" reactions align with those during "on" reactions [193,202,205,206].

Beyond directly activating cell responses, Ca^{2+} transients also participate in feedback mechanisms that regulate transcriptional events. This Ca^{2+}-dependent transcriptional regulation is adaptive, enabling cells to respond to changes in their Ca^{2+}-signalling systems through compensatory measures [193]. The size, kinetics, and spatial distribution of cytoplasmic Ca^{2+} signals play critical roles in determining the specific Ca^{2+}-dependent response triggered, as well as the timing and duration of its activation [207]. Moreover, Ca^{2+} signalling exhibits mutual modulation with other cellular signal transduction pathways, fostering the crosstalk between these pathways [195].

The regulatory function of Ca^{2+} within signalling pathways offers valuable insights into the remodeling mechanisms the system deploys to adapt to internal or external stimuli [193]. Therefore, acquiring a comprehensive understanding of the regulation and the consequences of alterations in Ca^{2+} homeostasis presents an intriguing avenue for comparing dysregulation arising from pathological conditions.

I.3.3 Role of Calcium signalling in cancer progression

Calcium ion signalling is pivotal for maintaining cellular homeostasis, required to control cell proliferation, programmed cell death, cell motility, response to oxygen levels and nutrient supply. Extensive evidence has demonstrated that during tumourigenesis, intracellular Ca^{2+} homeostasis undergoes intricate remodeling, which modifies the regulation of these pivotal events. This remodeling can contribute to heightened cell proliferation, evading programmed cell death, promoting angiogenesis, adapting to hypoxia and nutrient scarcity, modifications of cellular metabolism, as well as enabling cell invasion and metastasis, all of which are included in the hallmarks of cancer [199,202,204,208,209].

The remodeling of intracellular Ca^{2+} homeostasis occurs due to the dysregulation of expression and/or activity of various Ca^{2+} channels, exchangers, and pumps [197,204,210]. Alterations in activation and/or expression of plasma membrane Ca^{2+} channels, especially SOCE stores and TRP channels, contribute to mechanisms linked with tumourigenesis. Therefore, elevated expression and/or activation of these stores and channels result in increased Ca^{2+} influx, fostering cell growth, proliferation, invasion, and migration [209,211]. Recent work reveals that various TRPC channels play a role in cell cycle progression, thus contributing to the control of tumour cell growth [212]. The impact of SOCE is extensively explained in the final paragraph of this section, highlighting its crucial role in various tumourigenic processes.

Calcium ions can also exert a regulatory function over gene expression for various cancer-related factors. For instance, upon Ca^{2+} binding to CAM, it activates calcineurin, a CAM-dependent phosphatase that in turn activates the Nuclear Factor of Activated T-cell (NFAT) [213]. Activated NFAT subsequently phosphorylates Cyclin D, contributing to tumour cell proliferation (Figure 17) [214]. A notable subset of Ca^{2+} transporters, including PMCA, SERCA, and SPCA, and Ca^{2+} channels like VGCs and TRPs, commonly undergo altered expression levels in tumour cells. For that, accurate identification of the altered site of expression is crucial to discern the associated functional changes [197,208].

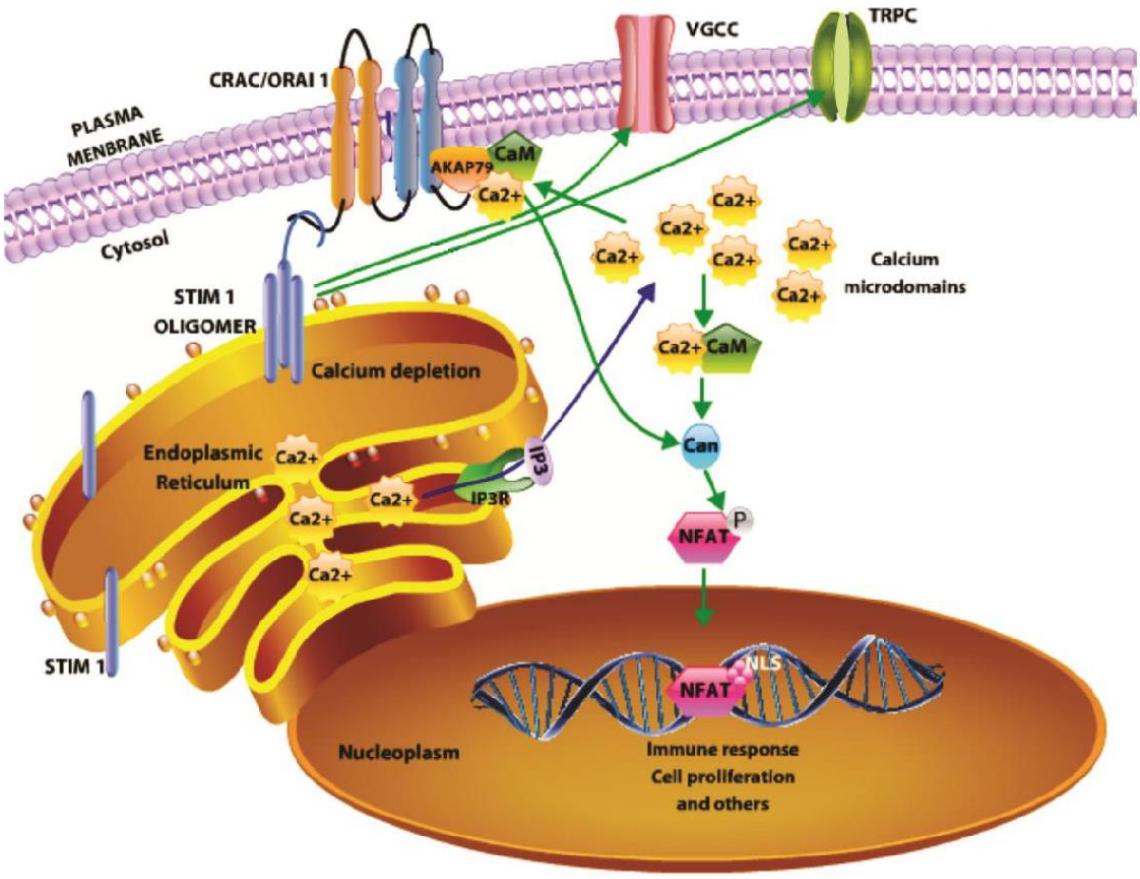

Figure 17. Example of Calcium ion role in cancer proliferation. *The elevation of Ca^{2+} levels in the Endoplasmic reticulum triggers Ca^{2+} release via Inositol 1,4,5-trisphosphate receptor (IP_3R) and enhances external Ca^{2+} entry through Store-operated calcium entry (SOCE), mediated by Stromal interaction molecule (STIM) and ORAI Calcium Release-Activated Calcium Modulator (ORAI) proteins. Additionally, STIM can activate Transient receptor potential ion-channels canonical (TRPC) and Voltage-gated channel (VCG, also known as Voltage-gated calcium channels or VGCC) channels, ensuring a sustained Ca^{2+} influx. This influx activates the Ca^{2+}/calmodulin (CAM) and calcineurin pathways, which in turn activate the Nuclear factor of activated T cells (NFAT). The activated NFAT translocates to the nucleus, facilitating cell cycle progression and activation of transcription factors. Adapted from Pinto et al., Cell signalling, 2015* [215]

Concerning cell proliferation, a process heavily influenced by Ca^{2+} dysregulation, significant links were identified with cell cycle progression control, particularly through the regulation of Ras activity [197]. In response to mitogenic growth signals, cytoplasmic Ca^{2+} levels increase, initiating interactions between Ca^{2+}-handling molecules that activate the cell cycle. An illustrative pathway involves Ca^{2+} binding to CAM, triggering the activation of CAMKII, which in turn regulates various cell cycle checkpoints [216,217].

SERCA, IP_3R, and VDAC are responsible for transferring Ca^{2+} from the EndR to the mitochondria, playing essential roles in cellular responses to apoptotic signals. SERCA pumps Ca^{2+} back into the EndR from the cytosol, maintaining low cytosolic Ca^{2+} levels [218]. Impairment of SERCA function

by ROS leads to the depletion of EndR Ca^{2+} stores, resulting in an increase in cytosolic Ca^{2+} levels, which can activate pro-apoptotic pathways, through p53 non-canonical actions [219] or via the Bax and Bak pathway [220]. IP_3R regulates Ca^{2+} release from the EndR and can be influenced by ROS [221]. During early apoptosis, Cytochrome C binds to IP_3R, increasing Ca^{2+} release and causing cytoplasmic and mitochondrial Ca^{2+} overload, extensive Cytochrome C release, and maximal caspase activation [222]. This binding prevents Ca^{2+}-dependent inhibition of IP_3R, leading to heightened Ca^{2+} release from internal stores, increasing cytoplasmic Ca^{2+} concentration and mitochondrial Ca^{2+} overload, triggering apoptosis [223].

Among the three VDAC isoforms, VDAC1 plays a prominent role in regulating the release of apoptotic proteins like Cytochrome C [224]. VDAC1 oligomerization leads to the formation of the mitochondrial permeability transition pore (mPTP) and consequently the release of pro-apoptotic proteins, such as Translocator Protein (TSPO), Bcl-2, and hexokinase, thereby activating mitochondrial apoptosis pathways [224,225]. Studies suggest that inhibiting VDAC1 can prevent apoptosis in different cell types, including macrophages, by preventing Ca^{2+}-mediated ROS generation, mitochondrial membrane permeabilization, and Cytochrome C release [226].

The Bcl-2 protein family, comprising both pro- and anti-apoptotic members, is located at the junctions between mitochondria and the EndR. These proteins regulate intracellular Ca^{2+} movement by interacting with channels such as VDAC [227] and IP3R [228] on both mitochondria and the EndR. This interaction is crucial for mitochondrial Ca^{2+}-dependent processes. Additionally, Bcl-2 proteins influence cellular ATP production and ROS generation, thereby determining cell survival or apoptosis [229,230]. Some Bcl-2 proteins inhibit Ca^{2+} release from the EndR to the mitochondria, preventing Ca^{2+}-induced cell death [231]. Conversely, others modulate mitochondrial Ca^{2+} uptake based on the energy requirements of the cells. Proteins like Bax and Bak induce Ca^{2+} release from the EndR, leading to apoptosis by sensitizing mitochondria to Cytochrome C [232–234].

The impact of Ca^{2+} on apoptosis hinges on the sustained elevation of intracellular Ca^{2+} and a prolonged decline in EndR Ca^{2+}. These factors are cell type dependent. Tumour cells evade apoptosis by reducing Ca^{2+} influx via the downregulation of Ca^{2+}-permeable channels expression or by dampening the activation of associated signalling pathways. This adaptation enables cells to cope with the prolonged EndR Ca^{2+} depletion [199,216]. This reduced influx restricts excessive Ca^{2+} release triggered by pro-apoptotic signals, thereby preventing activation of both mitochondrial and cytoplasmic apoptotic pathways [235,236]. The reduction in cytoplasmic Ca^{2+}

influx inhibits the initiation of the mitochondrial Permeability Transition Pore (PTP) formation, thus preventing the release of apoptosis-inducing factors (Figure 18) [199].

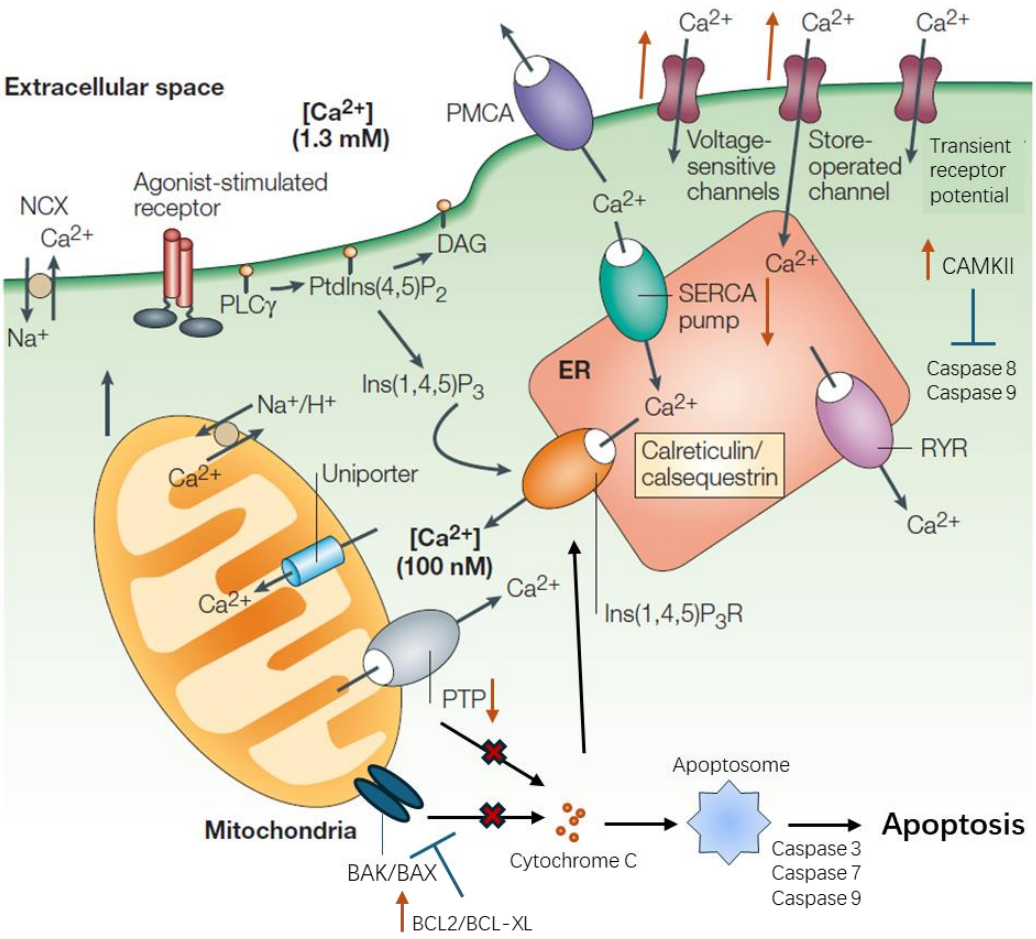

***Figure 18. Calcium-dependent apoptosis inhibition**. Increased Ca^{2+} influx through upregulation of Voltage-sensitive, Store-operated and Transient receptor potential channels activates Ca^{2+}/CaM-dependent protein kinase II (CAMKII), inhibiting caspases 8 and 9. Furthermore, downregulation of Sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) decreases Ca^{2+} levels in the Endoplasmic reticulum (ER), while downregulation of Inositol-1,4,5-trisphosphate receptor ($Ins(1,4,5)P_3R$) and mitochondrial PTP reduces mitochondrial Ca^{2+} levels. Mitochondria-associated anti-apoptotic proteins like B-cell lymphoma 2 (BCL2) and B-cell lymphoma-extra large (BCL-XL) are often overexpressed in cancer cells, inhibiting the release of cytochrome c and other pro-apoptotic factors from the mitochondria, preventing apoptosis. Source: Adapted from Orrenius et al., Nature Rev Mol Cell Biol, 2003* [237]

Moreover, tumour cells adapt to the decreased Ca^{2+} levels within the EndR, leading to a decline in the activation of EndR stress-dependent reactions [216]. Notably, alterations in Ca^{2+} transporters, such as the NCX and the PMCA, are closely linked to processes like proliferation and apoptosis resistance [209]. Other intracellular transporters, such as SERCA, also play a role in determining the fate of tumour cells (Figure 18) [209].

Normal cells typically cease division after a certain number of cycles due to telomere shortening and other control mechanisms to avoid propagation of damaged cells, known as replicative senescence. This process can induce apoptosis if DNA damage persists [238]. Cancer cells can evade senescence and apoptosis by upregulating telomerase to maintain telomeres [239]. However, the Ca^{2+}-binding protein S100A8 in the human epidermal keratinocyte line HaCaT, which contains EF-hand domains, inhibits telomerase activity, accelerating cellular senescence [240]. This suggests that modifications in Ca^{2+} signalling, particularly reduced signalling, may contribute to cancer cell immortality [239] via telomerase activity control.

Calcium ion also plays a vital role in modulating cellular bioenergetics, acting as an allosteric activator of metabolic enzymes and modulating metabolic pathways. This interplay between Ca^{2+} and metabolism is essential for maintaining cellular homeostasis [241]. However, due to their heightened proliferation rate, tumour cells require increased biomass and energy, leading to metabolic adaptations. Specific Ca^{2+} transporters, such as MCU and VDAC, play a role in maintaining these cancer-related metabolic features by limiting mitochondrial Ca^{2+} influx and preserving glycolysis as a protective measure against oxidative stress [242–244].

Mitochondrial Ca^{2+} levels are pivotal in regulating the metabolic transition from oxidative phosphorylation to aerobic glycolysis observed in cancer cells, known as the Warburg effect, which fuels their proliferation [244]. Specifically, decreased levels of mitochondrial Ca^{2+} activate pyruvate dehydrogenase phosphatase, leading to the phosphorylation and subsequent inactivation of pyruvate dehydrogenase. Consequently, pyruvate is diverted away from entering the Krebs cycle as acetyl-CoA, and is instead utilized for lactate production. This metabolic shift contributes to the high glycolytic rate characteristic of cancer cells [200,245]. Furthermore, metastatic cells exhibit elevated mitochondrial Ca^{2+} uptake capacity and increased ROS generation. While these features enhance cell migration and invasiveness, they also render cancer cells more susceptible to Ca^{2+}-mediated apoptosis [200]. So, alterations in mitochondrial Ca^{2+} homeostasis are a hallmark of tumourigenesis contributing to survival and increased proliferative and migratory activity of cancer cells [200].

As a second messenger, Ca^{2+} is implicated in various steps of cell motility, including cytoskeleton organization, extracellular matrix proteolysis, focal adhesion, traction force generation, and directional sensing, mechanisms related to cell migration [209]. In tumour cells, augmented intracellular Ca^{2+} levels facilitate rear detachment through a transient front-to-rear gradient [246,247]. Cytoskeleton dynamics, regulated by Ca^{2+}/CAM, activates Myosin Light Chain Kinase (MLCK), driving actomyosin contraction (Figure 19) [248]. Concurrently, calpain, a Ca^{2+}-sensitive

protease, cleaves Focal Adhesion Kinase (FAK), integrin, talin and mucin, controlling cell adhesion and motility [249]. Spatially confined Ca^{2+} signals stimulate both directed actin polymerization and the growth of focal adhesions at the leading edge of migrating cells, facilitating membrane extension, and contributing to lamellipodia retraction and adhesion cycles (Figure 19) [250]. This gradient significantly influences focal adhesion and actin cytoskeleton activation through the activation of calpain, CAMKII, or Protein tyrosine kinase 2 (PYK2), which phosphorylate FAK [251–253]. This migration process involves components like TRPs and VGCs channels and the STIM1/ORAI1 complex that regulates Ca^{2+} influx pathways, but also the PLC/ IP_3R pathway, which releases Ca^{2+} from EndR stores [254,255].

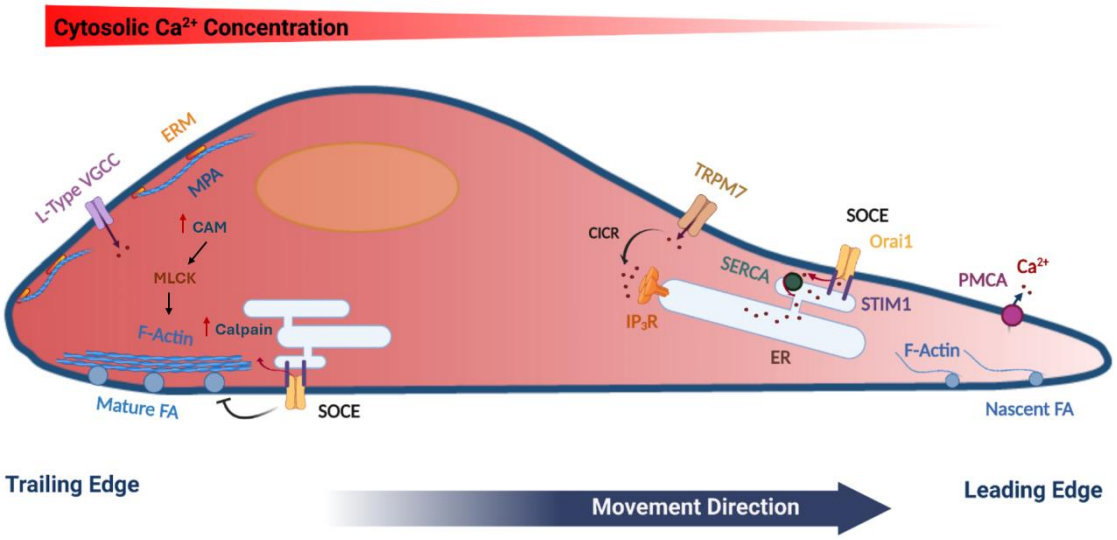

Figure 19. Calcium ion participation in tumour cell migration. *Upregulation of Transient receptor potential ion channel melastatin 7 (TRPM7) and Plasma membrane Ca^2-ATPase (PMCA) enhances Ca^{2+} influx. Store-operated Ca^{2+} entry (SOCE), composed of STIM1 and ORAI1, maintains Ca^{2+} homeostasis by refilling Ca^{2+} stores and is involved in focal adhesion disassembly. The increased Ca^{2+} influx activates signalling pathways that regulate cytoskeletal dynamics through the elevated expression of Calmodulin (CAM) and Myosin light chain kinase (MLCK). MLCK phosphorylates myosin light chains, thereby increasing myosin ATPase activity and promoting the formation of focal adhesions, which enhances the contractile forces within tumour cells. Source: Adapted from Hammad & Machaca, Cells, 2021* [255]

Another Ca^{2+} flux with important roles in cancer progression is the SOCE, which primarily relies on ORAI channels on the plasma membrane and STIM proteins functioning as EndR Ca^{2+} sensors. This pathway is a major Ca^{2+} influx mechanism, with substantial implications for tumourigenesis. STIM and ORAI constitute the core components of the Ca^{2+} release-activated Ca^{2+} (CRAC) channel [201,256]. When EndR Ca^{2+} levels drop, activated STIM proteins interact with ORAI, triggering SOCE. This influx provides Ca^{2+} for EndR stores and fuels the transduction of Ca^{2+}

signalling pathways [201]. Overexpression of STIM and ORAI proteins, along with the subsequent increase in SOCE activity, is a recurrent phenomenon across various cancer types, such as esophagus squamous cell carcinoma [257], non-small cell lung carcinoma [258] and gastroesophageal cancer [259]. This SOCE dysregulation is often linked with poorer patient outcomes [260]. Their involvement in cancer progression-related events spans cell cycle regulation, proliferation, apoptosis resistance, drug resistance, cell migration, invasiveness, and angiogenesis [201]. While STIM and ORAI encompass other isoforms (STIM2, ORAI2, ORAI3), STIM1 and ORAI1 stand as the most extensively studied, revealing functional disparities between normal and tumour tissues [260–262].

I.3.4 Role of Calcium ion signalling in breast cancer progression

The intricate and multifaceted nature of Ca^{2+} signalling complicates the identification of the specific mechanisms dysregulated in BC. However, Ca^{2+} holds a profound connection with breast biology, owing to its significance in milk development through neonatal development [263]. In fact, some of the Ca^{2+} channels and pumps implicated in milk transport, such as SPCA1, SPCA2, PMCA2, SERCA2 [264] and ORAI1 [265] display altered expression patterns in BC.

Beyond the link between Ca^{2+} signalling and lactation, numerous Ca^{2+} pumps and channels have been uncovered in the context of BC [263]. The remodeling of Ca^{2+} fluxes and signals differs across different BC subtypes, [210,263]. For instance, SPCA1 exhibits high expression in the MDA-MB-231 basal-like breast cell line, and its silencing results in reduced cell proliferation [266]; TRPV4 is overexpressed in basal-like subtypes and presents a differential expression pattern when compared with other subtypes [267]; ORAI1 overexpression is observed in basal-like BC cell lines, while elevated ORAI3 levels are common in luminal BC cell lines, coinciding with ER+ subtypes [268]. Moreover, hypoxia induces ORAI3 expression in basal BC cell lines via HIF-1α, while silencing ORAI3 mitigates hypoxia-induced phosphorylation of EGFR and downregulates genes associated with cell migration and immune responses [268]. In MCF-7 luminal BC cells, down-regulation of ORAI3 halts cell-cycle progression and triggers apoptosis [269]. Additionally, elevated ORAI3 levels are associated with poorer clinical outcomes in basal, ER-, and TNBC when coupled with low ORAI1 expression, indicating a potential regulatory role for ORAI3 in basal BC progression [268].

In BC, the key contributors to intracellular Ca^{2+} increase include the TRP channels and the ORAI family - these elements notably impact several cancer hallmarks, such as cell proliferation, invasion, and migration (Figure 20) [209,270]. Some TRP channels possess kinase activity,

including TRPC1, TRPC6, TRPV6, TRPM7, and TRPM8, which are implicated in BC cell proliferation. However, the specific activated channels differ among various BC subtypes [209,271,272]. For instance, MCF7 cell line proliferation is modulated by a Ca^{2+} entry reliant on TRPC6/TRPC1 channels, while MDA-MB-231 cell line proliferation, dependent on Ca^{2+}, is regulated by TRPC6/TRPC3 channels [209,272,273]. Similarly, TRPM7 and TRPV6 overexpression significantly influences BC cell proliferation and migration, with their roles modulated by Ca^{2+} levels [271,274]. High expression of TRPM7 predicts poor survival in BC patients, due to its role in tumour cell migration [270]. By regulating cellular tension linked to myosin II, TRPM7 significantly contributes to the alteration of focal adhesion, cell-cell adhesion, and polarized cell mobility in MDA-MB-231 BC cells [270]. In MCF-7 BC cells TRPV6 maintains optimal cytoplasmic Ca^{2+} levels and facilitates cell proliferation. This role is intricately linked to its association with a recently identified binding partner protein, NUMB Endocytic adaptor protein (Numb1), which exhibits a negative modulatory effect on TRPV6 activity [275]. This highlights the vital influence of TRP channel regulators in shaping oncogenesis.

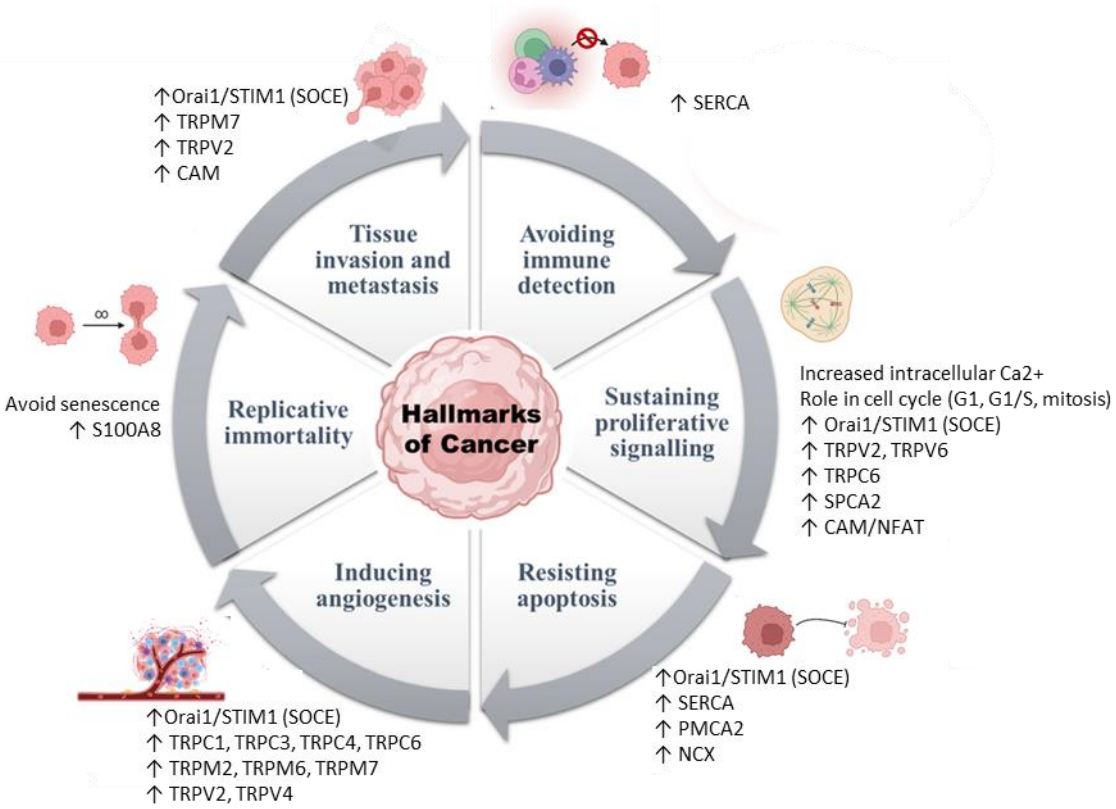

Figure 20. Impact of Calcium ion remodeling on various hallmarks of breast cancer*. Elevated intracellular Ca^{2+} levels stimulate proliferative signalling, inducing a sustained cell cycle. Moreover, Ca^{2+} contributes to the evasion of senescence, enabling continuous cell replication. Adaptive alterations in key Ca^{2+} channels and pumps further support angiogenesis, resistance to apoptosis, enhanced cell invasion and migration, and avoid the immune system. Source: Own, adapted from Mishra et al., Cancer Reports, 2023* [276]*; Bruce & James, Cancers, 2020* [239]

Another crucial mechanism in BC progression is angiogenesis, the formation of new blood vessels. Notably, TRPV4 overexpression in breast tumour-derived endothelial cells suggests that it can promote angiogenesis and thus support tumour growth (Figure 20) [277]. ORAI1 also plays a role in BC progression by contributing to Ca^{2+} influx, which triggers the release of pro-angiogenic factors, particularly in MDA-MB-231 and BT-549 triple-negative BC cells [278].

In fact, ORAI1 channels are involved in other critical mechanisms related to BC progression. ORAI1 facilitates a constitutive Ca^{2+} entry through interaction with SPCA2 pumps (Figures 20 and 21A) particularly in HER2+ and luminal BC cell lines [279]. This influx promotes proliferation via the ERK pathway and cell cycle progression via Cyclin D1, both of which are controlled by ERK activation (Figure 21A), often mediated through the Ca^{2+}/CAM/CAMK pathway [209]. Conversely, SPCA2 knockdown diminishes ERKI1/2 pathway activity, potentially explaining the decrease in proliferation [279].

The EndR IP_3-activated Ca^{2+} release channels, IP_3Rs, also regulate BC cell proliferation, through the activation of the PLC/IP_3/IP_3R/MAPK pathway induced by G protein-coupled receptor (Figure 21B) [263]. Interestingly, silencing both IP_3R1 and IP_3R3 is detrimental to luminal A MCF-7, luminal A T47D[1], and triple-negative HS578T BC cells, as it induces autophagy related to the silencing of these IP_3Rs [280].

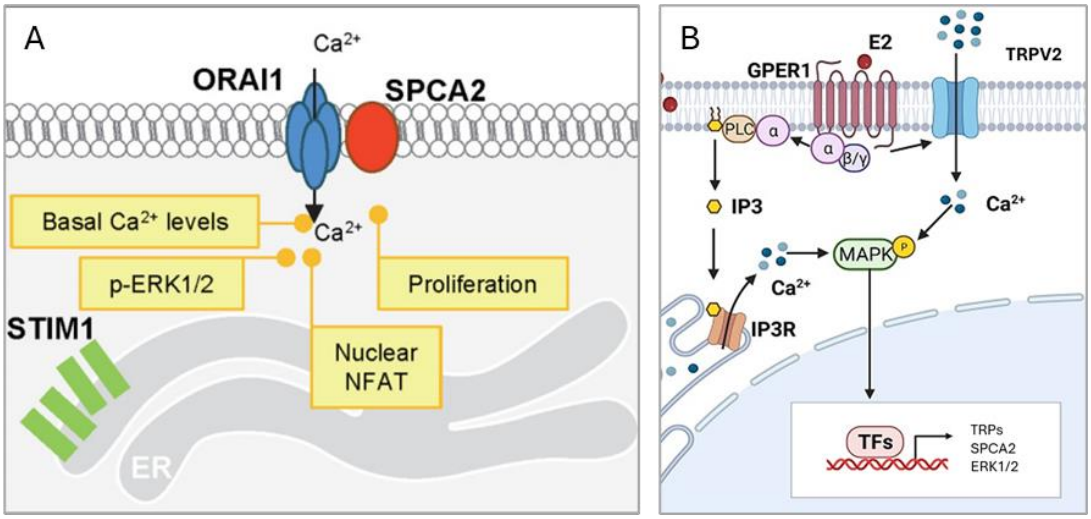

Figure 21. Impact of Calcium ion in breast cancer proliferation*. A) Constitutive Ca^{2+} entry promoted by the interaction between ORAI Calcium Release-Activated Calcium Modulator 1 (ORAI1) and Secretory pathway Ca^{2+} ATPase (SPCA2). This Ca^{2+} influx contributes to the phosphorylation of Extracellular-regulated kinase 1/2 (ERK1/2) and Nuclear factor-activated T cells (NFAT) which promotes cell cycle progression and the transcription of proliferative proteins. B) The PLC interacts with G protein-coupled receptors and activates the IP_3/IP_3R/MAPK pathway promoting cell cycle progression. Source: Adapted from Monteith al., J Biol Chemistry, 2012* [281] *and Huang et al., Int J Biol Sci, 2022* [282]

[1] MCF-7 and T47D are both luminal A cell lines, but T47D is more susceptible to progesterone.

ORAI1 channels also contribute to the metastasis mechanism in BC since the overexpression of both ORAI1 and STIM1 induces Ca^{2+} influx through SOCE entry, resulting in tumour invasion. The precise mechanism may be attributed to the increased Ca^{2+} levels impacting FAK activity, consequently influencing focal adhesion turnover [201,283]. High levels of ORAI3 are also associated with cell growth and invasiveness in ER+ BC cell lines, with a pronounced dependence on SOCE entry. This influx of Ca^{2+} leads to the activation of ERK1/2 and FAK, contributing to the loss of cell adhesion [284]. In contrast, the downregulation of ORAI3 inhibits proliferation by arresting the cell cycle at the G1 phase and inducing apoptosis due to excessive intracellular Ca^{2+} levels [269].

Furthermore, Ca^{2+} signalling also plays a crucial role in EMT contributing to BC cells migration. CAMK2β, activated by Ca^{2+} signals, promotes EMT in MCF-7 BC lines, inducing changes in gene expression, cytoskeleton organization, and protein transport. Inhibiting CAMK2β reverses this state rapidly compared to inhibiting TGF-β, another EMT inducer, suggesting a distinct role for Ca^{2+}-driven EMT in enhancing cellular plasticity, with important implications for cancer therapy [285]. Elevated intracellular Ca^{2+} also regulates key EMT-inducing transcription factors, such as Snail. SOCE, through ORAI3 and STIM1, is crucial for TGF-β-dependent Snail family transcriptional repressor 1 (Snai1) transcription. Blocking SOCE in MDA-MB-231 BC cells reduces migration but increases Snai1 gene activation via the AKT pathway and NF-κB binding at the Snai1 promoter in response to TGF-β. Increased Snail stability results in decreased E-cadherin expression and loss of epithelial cell-cell connections, leading to increased EMT. This suggests a dual role for Ca^{2+} channels in regulating cell migration and Snai1 transcription during EMT [286]. Furthermore, STIM2 drives BC metastasis by modulating the expression of NFAT1, facilitating its activation of the TGF-β1 promoter. This process increases the expression and secretion of TGF-β1, promoting EMT, while NFAT1 knockdown reduces TGF-β1 secretion. These findings underscore the significance of STIM2 and NFAT1 in BC progression and metastasis.

Resisting apoptosis is another hallmark of BC in which Ca^{2+} plays a pivotal role (Figure 20). For instance, upregulation of PMCA2 expression leads to changes in the magnitude and recovery of Ca^{2+} transients in T47D BC cells. This augmented capacity to remove Ca^{2+} from the cytoplasm results in reduced apoptosis when exposed to Ca^{2+}-related stressors [208,287]. Consequently, PMCA2 overexpression could contribute to the development of apoptosis-resistant characteristics, potentially fostering cancer progression and unfavorable patient outcomes [287].

I.3.5 Impact of Calcium ion homeostasis on breast cancer Tumour Microenvironment

The impact of Ca^{2+} signalling in different cell types within the stroma surrounding the primary tumour, pre-metastatic niches, and metastatic sites remains poorly understood [196]. However, alterations in Ca^{2+} signalling within cancer cells triggered by immune cells, alongside the regulatory role of Ca^{2+} signalling in key immune cell functions, demonstrate this interplay [196,239].

The low extracellular pH in the TME hampers the ability of immune cells like CTLs, NK cells, and antigen-presenting DCs to eliminate tumour cells, while simultaneously bolstering the immunosuppressive properties of Tregs, myeloid cells, and TAMs [289]. This is particularly relevant because every Ca^{2+} channel and transporter is highly sensitive to pH, especially CRAC channels [290] and acid-sensing ion channel 1 (ASIC1), a proton-gated cation channel [291]

MCF-7, T47D, and MDA-MB-231 BC cells express ASIC1, crucial for ROS generation and NF-κB and Akt activation induced by acidosis, which are critical for tumourigenesis. Mechanistically, ASIC1 facilitates acidosis-mediated signalling through Ca^{2+} influx. Furthermore, ASIC1, located in the cytoplasmic membrane, is associated with mitochondria, suggesting a potential role in regulating mitochondrial Ca^{2+} influx [291].

Calcium ion also influence the regulation of immune cells. C-C motif chemokine 18 (CCL18) often released from breast TAMs promotes MDA-MB-231 BC cells invasiveness and metastasis. These effects occur through its chemokine receptor, membrane-associated Phosphatidylinositol transfer protein 3 (PITPNM3), activated by the increased intracellular Ca^{2+} levels. Subsequently, this activation triggers downstream Ca^{2+} signalling pathways [292]. Moreover, under tumour conditions, CD4+ T cells exhibit up-regulation of SERCA3 expression, due to an imbalance in Ca^{2+} homeostasis, leading to ER stress and subsequent apoptosis of CD4+ T cells. This process contributes to immune suppression in MCF-7 BC cells [293].

I.3.6 New therapeutics for breast cancer using Calcium ion remodelling approaches

Given their involvement in various tumourigenic mechanisms, Ca^{2+} channels and pumps have become attractive therapeutic targets. Two primary therapeutic approaches have been pursued: (i) altering the activity of the channels or pumps to reduce activation of Ca^{2+}-dependent pathways associated with tumourigenesis, and (ii) utilizing inhibitors or activators of dysregulated channels or pumps to either decrease or increase Ca^{2+} concentrations [197]. However, selecting an anti-cancer therapeutical approach based on the remodeling of the Ca^{2+}

fluxes requires a clear understanding of which channels or pumps are dysregulated in each type of BC and the consequence of that specific dysregulation for cancer progression.

The therapeutic potential of targeting Ca^{2+} permeable channels and pumps has led to the development of clinical trials [294]. SOR-C13, a TRPV6 Ca^{2+} channel inhibitor, is currently in phase I clinical trials for advanced solid tumours, including BC, aiming to reduce proliferation and/or invasion pathways in tumour cells by modulating cytosolic Ca^{2+} signalling [295].

Additionally, drugs like leflunomide and teriflunomide, originally used for other pathologies, have been identified *in silico* as potential suppressors of SOCE [296]. The effects of both drugs, but also CM4620 and BTP2, selective SOCE inhibitors, were evaluated on different BC subtypes, MDA-MB-231 (mimic TNBC) and MCF-7 (mimic Luminal A). The findings showed that CM4620 and BTP2 effectively inhibited SOCE in both BC cell lines. Additionally, Leflunomide and Teriflunomide disrupted Ca^{2+} signalling specifically in TNBC subtype cells, without affecting Ca^{2+} entry in the Luminal A subtype [297]. In another investigation involving two TNBC cell lines, MDA-MB-231 and MDA-MB-468, BTP2 and another SOCE inhibitor, Synta66, were found to reduce serum-activated migration. However, they exhibited differential effects on proliferation and EGF-activated migration, indicating the need for further exploration of the mechanisms of action of different SOCE inhibitors [298]. Furthermore, the significance of inhibiting SOCE was highlighted in a study on angiogenesis, demonstrating that in vitro inhibition of SOCE with BTP2 prevented endothelial colony-forming cell proliferation and tubulogenesis by down-regulating EndR Ca^{2+} levels [299].

In anthracycline-resistant metastatic BC, verapamil, a potent Ca^{2+} channel blocker used for hypertension, has shown promise in improving patient survival rates. Its mechanism likely involves potentiating the effects of alternative chemotherapeutic agents like 5-fluorouracil, although the exact mechanism remains unclear [300].

Clinical trials have explored the use of different Ca^{2+} strategies in cancer treatment. In melanoma, supraphysiological Ca^{2+} doses combined with electroporation have shown promise as an effective and safe anticancer treatment [301]. Due to the promising outcomes, this approach is being investigated for its potential application in BC. For example, in BC cell lines sensitive (MCF-7/WT) and resistant to doxorubicin (MCF-7/DOX), Ca^{2+} combined with electroporation demonstrated effectiveness in treating BC [302]. Additionally, a case study involving cutaneous metastases in HER2+ BC patients showed that Ca^{2+} combined with electroporation, along with trastuzumab over 5 years, preserved skin integrity, was well-tolerated, and controlled cutaneous metastases without recurrence, possibly indicating local

immunity [303]. Molecularly, Ca^{2+} electroporation induces acute ATP depletion and mitochondrial dysfunction, leading to cell death without causing DNA damage. These findings suggest the therapeutic potential of Ca^{2+} modulation in BC treatment, particularly in combination with electroporation [304,305].

The widespread presence of Ca^{2+} handling machinery and its multifunctional roles in normal cells pose challenges in the targeted manipulation of its components in tumour cells. While some studies targeting Ca^{2+} transport proteins show promising anti-neoplastic effects in cell lines, the lack of specificity, significant side effects, and difficulties in targeted delivery underscore the importance of ongoing research into new targets and delivery strategies. It remains imperative to identify Ca^{2+} channels, pumps, and exchangers with features suitable for use as targets or potential biomarkers for cancer diagnosis, prognosis, and treatment.

I.4. ROS and Calcium ion inter-regulation in carcinogenesis

The dynamic interplay between Ca^{2+}-mediated ROS production and ROS-mediated Ca^{2+} signalling highlights the concept of ROS and Ca^{2+} crosstalk. In addition to the role of Ca^{2+} in regulating ROS generation/elimination, the cellular overall redox state and the ROS also influence the activity of various Ca^{2+} channels, pumps, and exchangers [306].

Maintaining redox balance is essential for regulating various receptors, proteins, and signalling molecules that directly or indirectly affect Ca^{2+} signalling pathways, thereby influencing local and global Ca^{2+} homeostasis [306]. Disruption of redox equilibrium, caused by ROS accumulation or clearance, affects cellular signalling pathways, leading to their dysfunction and eventually to diseases such as cancer. Consequently, mitochondrial ROS and Ca^{2+} signalling work synergistically to maintain cellular homeostasis, highlighting their interdependent roles in maintaining cellular health [307].

Numerous studies demonstrate that intracellular Ca^{2+} levels regulate ROS production and ROS scavenging mechanisms, altering the cellular redox state [130,306]. The best-documented function of Ca^{2+} in this context is to enhance ATP synthesis and consequently ROS production during mitochondrial respiration, as well as to modulate ROS generation from NOX and nitric oxide synthase (NOS) [308,309]. Conversely, antioxidant enzymes regulate Ca^{2+} levels by mitigating oxidative stress-induced Ca^{2+} influx and maintaining Ca^{2+} homeostasis [310]. This interaction between antioxidant enzymes and Ca^{2+} channels can also influence gene expression. For instance, extracellular glutathione modulates the function of Ca^{2+}-sensing receptors [311]. Similarly, catalase affects oxidative stress-induced Ca^{2+} signalling by regulating TRP cation channel activation in human neuroglial cells, highlighting its role in Ca^{2+} regulation [312].

The redox regulation of Ca^{2+} homeostasis is evident in various contexts [313]. Under oxidative conditions, phosphatases like PP2B and PP2A, which are responsible for dephosphorylating and regulating CaMKII, become inactivated. This oxidative inactivation results in elevated CaMKII phosphorylation, thereby impacting downstream Ca^{2+} signalling pathways and emphasizing the critical role of redox regulation in Ca^{2+}/CaMKII signalling [314]. Calcium channels such as VDCC, SOCE, TRP channels, and IP3R are regulated by ROS levels due to the presence of redox-sensitive cysteine residues in their domains [315–319]. Notably, cysteine residues at position 195 in Orai1 and Orai2 channels are critical for redox sensitivity. The absence of this cysteine residue in Orai3 reduces its sensitivity to H_2O_2 [320]. Consequently, the ratio of Orai isoforms may modulate Ca^{2+} signals during oxidative stress in pathological conditions such as cancer [320]. STIM1, another element of the SOCE mechanism, undergoes S-glutathionylation at cysteine 56 in response to

oxidative stress, resulting in constitutive Ca^{2+} entry regardless of intracellular Ca^{2+} store status. This stress-induced Ca^{2+} entry disrupts mitochondrial Ca^{2+} handling and bioenergetics, leading to cell death [321]. Additionally, the EndR oxidoreductase ERp57 modulates the EndR luminal domain of STIM1 through interactions with conserved cysteine residues, C49 and C56. ERp57 binding influences STIM1 oligomerization, thereby inhibiting SOCE. In the absence of ERp57, SOCE is stimulated, suggesting that ERp57 normally functions to decelerate this process. Mutations in the C49 and C56 residues of STIM1, which disrupt its interaction with ERp57, result in reduced SOCE, illustrating the ERp57 modulatory role in Ca^{2+} entry and homeostasis [322].

In tumour cells, SOCE can be regulated by mitochondrial Ca^{2+} as well as mitochondria-derived redox signalling [323–325]. However, the precise mechanisms underlying the redox regulation of this complex in cancer are not completely understood [313]. For instance, Hempel and Trebak [313] suggested that the location of cysteine residues and the surrounding environment can determine susceptibility to ROS-mediated changes. Furthermore, hypoxia increases ROS levels, inducing the translocation of STIM1 to the plasma membrane, which activates Ca^{2+} channels and increases intracellular Ca^{2+} levels. Increased Ca^{2+} levels subsequently activate CaMKKβ, which activates AMPK independently of AMP/ATP levels. ROS and increased Ca^{2+} also influence the MAPK pathways, contributing to cellular responses to stress [326]. Additionally, the STIM1 expression can be positively regulated by HIF-1α during hypoxic tumour growth, mediating SOCE opening and increasing Ca^{2+} influx [327]. Conversely, another study showed that in HEK293 kidney cells, hypoxia and acidification-induced uncoupling of the STIM-Orai complex resulted in decreased SOCE, which prevents excessive Ca^{2+} overload [328]. These conflicting findings highlight the importance of comprehensively understanding the role of different hypoxia states in SOCE regulation in tumour cells, as this can be a key mechanism for maintaining tumour survival and progression.

Certain tumour cells have adapted mechanisms to manage ROS and Ca^{2+} signalling to avoid cell death by altering SOCE composition, increasing the Orai1/Orai3 ratio to maintain homeostasis under oxidative stress. In prostate cancer cell lines, this inactivation of SOCE is induced by H_2O_2, which prevents excessive Ca^{2+} influx and the initiation of apoptosis [329].

In addition to SOCE, several members of the TRP channel family exhibit redox-dependent activation [330]. Examples of such channels include TRPM7, TRPC3, and TRPC5, which can be activated through oxidative stress in various cell lines [331–334].

Another example of the ROS-Ca2+ interplay is the activation of NOX5 regulated by Ca^{2+} levels that plays a crucial role in various physiological and pathological processes, such as cancer. NOX5

generates superoxide using NADPH as a substrate and is involved in functions related to ROS regulation under different conditions [335]. NOX5 activity is primarily regulated by Ca^{2+} flux through its Ca^{2+}-binding domain or by calmodulin, affecting its conformation and superoxide-generating capability [335–337]. Alterations in this regulation can influence cellular responses. For example, moderate expression of NOX5 in several cell lines induces proliferation, but excessive levels of NOX5 caused by an increase in Ca^{2+} release results in the production of toxic levels of superoxide, leading to ROS-mediated apoptosis [338].

All the accumulated evidence, reviewed by Hempel and Trebak [313] highlights the evidence of this interplay in metabolic alterations, apoptosis resistance, cell proliferation, and metastasis mechanisms. The subsequent sections will analyze these processes and others related to the impact of Ca^{2+}/ROS crosstalk in tumourigenesis.

I.4.1 ROS and Calcium ion interplay in cancer metabolic processes

Mitochondria play a pivotal role in cellular Ca^{2+} dynamics, sequestering and releasing Ca^{2+} to integrate various cellular functions [200,339]. Mitochondrial Ca^{2+} regulates aerobic metabolism by stimulating key enzymes in the Krebs cycle, such as pyruvate dehydrogenase, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase. This regulatory mechanism facilitates efficient pyruvate oxidation, acetyl CoA production, and subsequent ATP generation through the electron transport chain. This highlights the critical role of mitochondrial Ca^{2+} in cellular energy metabolism and redox homeostasis, as the mitochondrial respiratory chain promotes ROS production by conversing O_2- to H_2O_2 [306,340]. The metabolic state of mitochondria significantly influences how Ca^{2+} affects mitochondrial ROS levels. When the mitochondrial membrane potential is high, indicating no ATP synthesis, Ca^{2+} uptake reduces ROS generation [306] (Figure 22).

During cancer progression, Ca^{2+} can promote metabolic activity, leading to higher oxygen consumption, and increasing electron leakage in the respiratory chain, resulting in increased ROS production. On the other hand, several Ca^{2+} channels, such as IP3R and RyR, are regulated by ROS levels, facilitating their transfer to mitochondria through VDAC and MCU. These EndR and mitochondria close contact regions, known as Mitochondria-associated EndR membranes (MAMs), enable Ca^{2+} flux between the EndR and mitochondria and are activated in response to changes in redox conditions [341,342] (Figure 22).

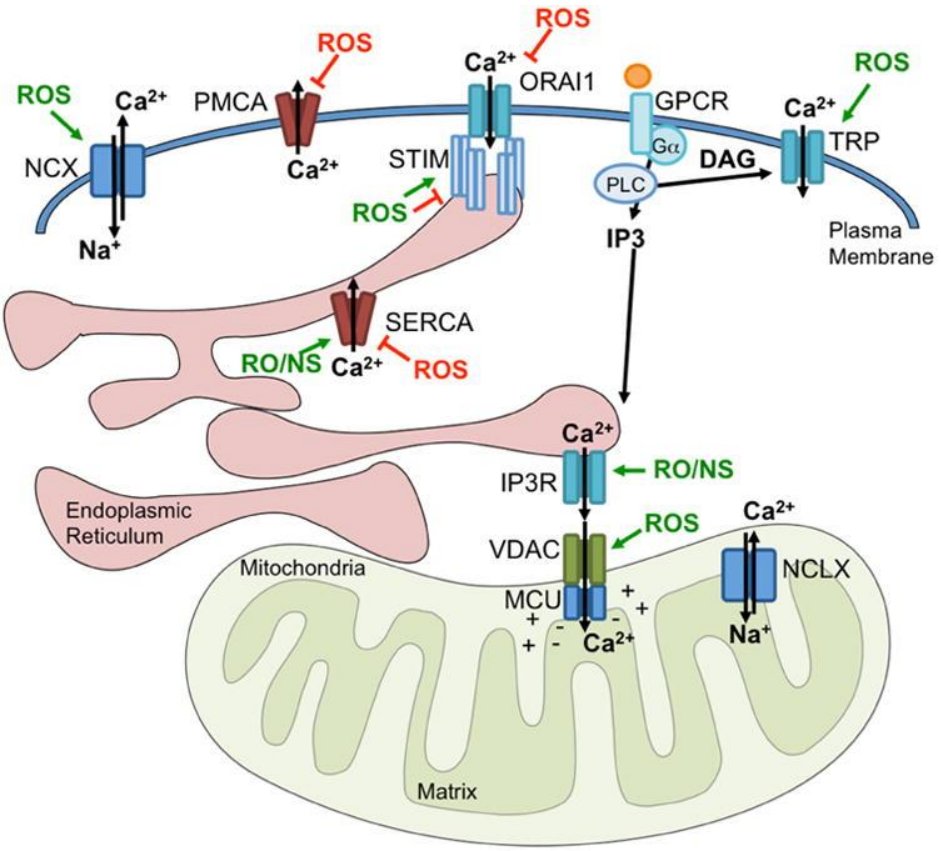

Figure 22. Calcium and ROS interaction between the endoplasmic reticulum (EndR) and mitochondria in cancer metabolism. *The increase of ROS enhances the release of Ca^{2+} from the EndR to the mitochondria via Ca^{2+} channels such as Ryanodine Receptors (RyR) and Inositol 1,4,5-trisphosphate Receptors (IP3R). Additionally, Ca^{2+} released into the cytoplasm can enter mitochondria through Voltage-Dependent Anion Channels (VDAC) or the Mitochondrial Calcium Uniporter (MCU). Elevated Ca^{2+} levels within mitochondria boost respiratory chain activity and increase ROS production. Source: Hempel and Trebak, Cell Calcium, 2017* [313]

However, the role of MCU varies between different cancer types. Dysregulation of MCU can increase mitochondrial Ca^{2+} uptake, promoting mitochondrial biogenesis and cell growth through ROS-induced NF-kB signalling in colorectal cancer [343]. In hepatocellular cancer, upregulation of MCU increases mitochondrial Ca^{2+} uptake, promoting ROS production by downregulating the ratio of nicotinamide adenine dinucleotide (NAD+)/reduced form of nicotinamide adenine dinucleotide (NADH) and sirtuin 3, thereby inhibiting SOD2 activity [344].

Another crucial player in cancer metabolism is VDAC1, which interacts with an enzyme called hexokinase (HK-II) that is critical for glucose metabolism. In cancer cells, elevated levels of HKII bind to VDAC1, promoting increased glycolysis and cell growth [345]. This interaction highlights the importance of metabolic adaptations in supporting proliferative and survival adaptations of cancer cells. In BC, HKII is induced by HIF-1α and plays a critical role in tumour progression by

increasing glycolytic activity [346]. Moreover, HKII overexpression has also been detected in brain metastasis tissues of BC and is associated with poor prognosis [347].

I.4.2 ROS and Calcium ion interplay in cancer avoiding apoptosis

Mitochondrial-related apoptosis may result from increased ROS production, decreased ATP production, and disruption in mitochondrial Ca^{2+} homeostasis [348]. For instance, oxidants like H_2O_2 inhibit PMCA through mitochondrial depolarization and mPTP opening, independent of ATP depletion, potentially leading to a Ca^{2+} overload response [349]. This mechanism suggests that increased oxidative stress enhances cytosolic Ca^{2+} levels. Furthermore, oxidative stress leads to rapid inactivation, conformational changes, aggregation, internalization, and proteolytic degradation of PMCAs, mediated by calpains and caspases [350]. Specifically, the PMCA 4b subtype is a substrate of caspase 3 in early apoptosis [351].

To ensure their survival, tumour cells adopt antioxidant defenses to maintain ROS optimal levels and sustain pro-tumourigenic signalling [352]. This regulation of ROS generation is mediated by mitochondrial Ca^{2+} signalling, which promotes proliferation and invasion while avoiding the sustained Ca^{2+} fluxes associated with apoptosis triggered by oxidative stress [313].

In tumour cells, mitochondrial ROS accumulation enhances ROS production through positive feedback, promoting mitochondrial Ca^{2+} uptake by modifying VDAC and MCU [353,354] (Figure 23). Despite high ROS levels, tumour cells activate antioxidant pathways to ensure their survival and prevent excessive mitochondrial Ca^{2+}, thus avoiding apoptosis mechanisms [313,355]. Moreover, apoptosis can be triggered in response to EndR stress, but tumour cells modify Ca^{2+} signalling and the response to EndR stress to support cell survival [356]. The regulation of Ca^{2+} homeostasis by ROS can be demonstrated through the modulation of key proteins involved in Ca^{2+} transport and storage.

Increased ROS levels modify the cellular redox state, inducing the release of cytosolic Ca^{2+} from the EndR via IP_3Rs. Subsequently, a reduction in the activity of the SERCA enables the recapture of this Ca^{2+} [357] (Figure 23). Moreover, tumour cells desensitize mPTP regulation and reduce pore opening, limiting Ca^{2+} buildup in mitochondria and activating apoptotic mechanisms (Figure 23) [358]. This adaptation enables tumour cells to survive and evade apoptosis through oxidative stress tolerance.

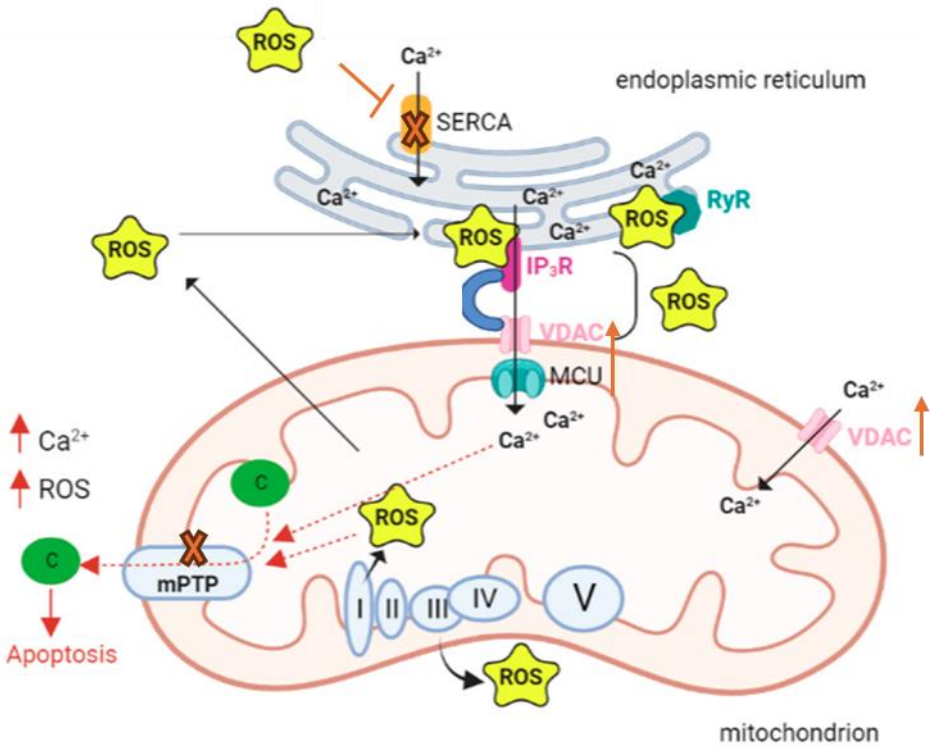

Figure 23. Calcium and ROS interaction between the endoplasmic reticulum (EndR) in cancer cells to avoid apoptosis*. Elevated ROS levels can inhibit sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) activity, accumulating Ca^{2+} in the cytosol. ROS enhances the sensitivity of voltage-dependent anion channel (VDAC) and mitochondrial calcium uniporter (MCU) to Ca^{2+}, increasing mitochondrial Ca^{2+} uptake in levels that support metabolism without triggering the mitochondrial permeability transition pore (mPTP) and apoptosis. Cancer cells often express anti-apoptotic proteins (e.g., Bcl-2) that help stabilize the mPTP and prevent its prolonged opening. Source: Adapted from De Nicolo, et al., Antioxidants, 2023* [359]

Research demonstrates that tumour cells, including those from leukemia, lung, and ovarian cancers, modify the expression of anti-apoptotic proteins like Bcl-2 and Bcl-XL. These proteins block IP3Rs, RyRs, and VDACs, thereby hindering Ca^{2+} transfer from the EndR to the mitochondria and suppressing Ca^{2+}-ROS-dependent apoptosis [360–364]. Additionally, overexpression of HK-I and HK-II increases their binding to VDAC1, inhibiting Cytochrome C release and the activation of the oxidative stress-induced apoptotic cascade [365,366]. To highlight this mechanism, inhibition of survival and proliferation of colon cancer cells has been observed with the HK-II inhibitor 3-bromopyruvate acid [367].

PMCA isoforms, specifically PMCA2 and PMCA3, play crucial roles in modulating ROS levels in cancer cells. Suppression of PMCA2 results in higher glutathione (GSH) levels, indicating efficient protection against oxidative stress [368]. Conversely, a decrease in PMCA3 levels increases total glutathione S-transferase (GST) activity, suggesting an adaptive mechanism against cytotoxicity

mediated by elevated Ca^{2+} levels, thereby influencing the susceptibility of the cells to apoptosis [368]. In BC cell lines (T47D, MDA-MB-231), PMCA2 overexpression evades high Ca^{2+} levels, protecting tumour cells from apoptosis. Consequently, high PMCA2 expression in BC is associated with poor prognosis due to increased resistance to apoptosis [287,369]. Silencing PMCA2 in MDA-MB-231 cells enhances cell death initiated by the Bcl-2 inhibitor ABT-263 [370]. Furthermore, estrogen receptor-negative and basal-like BC exhibit higher levels of MCU, which may confer survival protection by inhibiting caspase-independent cell death pathways [371].

A notable example of ROS-regulated Ca^{2+} channels is TRPM2, which is activated by ROS and plays a significant role in cell survival by adapting cells to oxidative stress. In normal cells, high oxidative stress activates TRPM2 and the increase of Ca^{2+} influx, inducing apoptosis; however, cancer cells evade this by relocalizing TRPM2 to the nucleus and expressing TRPM2-antisense mRNA, as observed in prostate cancer cell lines [372]. This adaptive mechanism protects the cells from TRPM2-mediated apoptosis, and when TRPM2 antisense mRNA expression decreases, apoptosis is initiated, inhibiting tumour growth in prostate cell lines [372]. Another protective mechanism against TRPM2-mediated apoptosis is the upregulation of TRPM2-L (full-length isoform) in response to hypoxia and elevated ROS levels, inducing an antioxidant response mediated by proteins such as Prx1, Prx3, and Gpx4, and cofactors like GSH, NADPH, and NADH regulated by Nrf2 [373,374]. In neuroblastoma xenografts, TRPM2-L expression is associated with tumour growth, increased HIF-1/2α expression, and chemoresistance to doxorubicin [375]. Under moderate oxidative stress, TRPM2-L, unlike the TRPM2-S (short) isoform, promotes cell survival by facilitating pro-survival Ca^{2+} signalling [376]. This Ca^{2+} channel is highly expressed in various cancers, such as pancreatic, prostate, melanoma, leukemia, neuroblastoma, and BC, contributing to tumour cell survival [377]. In non-small cell lung cancer cells, TRPM2 inhibition leads to elevated intracellular levels of ROS and RNS, resulting in DNA damage and activation of the JNK pathway. This activation leads to G2/M arrest and ultimately triggers cell death [378]. In MCF-7 and MDA-MB-231 BC cell lines, TRPM2 plays a nuclear role in minimizing DNA damage, thereby maintaining cell proliferation and inhibiting apoptosis mechanisms [379]. TRPM2 inhibition increases DNA damage and cell death via pathways other than the typical apoptotic, autophagic, or caspase-independent mechanisms, although the precise pathways involved are not well-understood [380].

In cancer cells, HIF-1α induces the expression of several glycolytic proteins, contributing to the inhibition of apoptosis [381]. Stress conditions such as hypoxia, nutrient deprivation, and cell detachment prompt tumour cells to develop adaptive mechanisms for survival. One such mechanism involves AMPK activation upon matrix detachment, regulated by a combination of

intracellular Ca^{2+} and ROS signalling. Under cell detachment conditions, SOCE is activated in response to oxidative stress, triggering the LKB1, Ca^{2+}/calmodulin-dependent protein kinase kinase β (CaMKKβ), and AMPK pathways leading to resistance against anoikis and promoting survival [382].

While mitochondrial Ca^{2+} promotes ROS generation, which modifies the expression of Ca^{2+} channels, certain antioxidant enzymes and transcription factors also control Ca^{2+} channel expression. For instance, TRPA1 is upregulated by the antioxidant transcription factor Nrf2, promoting oxidative stress tolerance in various tumour spheroid models, including of BC cells [383]. Activation of Nrf2 under oxidative stress triggers the expression of antioxidants, such as enzymes related to GSH production, glutathione peroxidases, and peroxiredoxins [152]. Additionally, activated TRPA1 increases intracellular Ca^{2+}, activating Ca^{2+}-dependent anti-apoptotic pathways such as the PI3K/AKT/mTOR/RAS/ERK signalling pathways [383].

I.4.3 Redox and Calcium ion inter-regulation: implications for cell migration in tumourigenesis

The TME significantly impacts metastasis and is modulated by the crosstalk between ROS and Ca^{2+} signalling, although few studies analyze this inter-regulation. For instance, in T lymphocytes H_2O_2 can inhibit ORAI1 channel activity but not ORAI3. Moreover, as naïve human T helper lymphocytes differentiate into effector T helper lymphocytes, their redox sensitivity diminishes, correlating with elevated mRNA levels encoding the less responsive ORAI3 protein. Alterations in the composition of ORAI channels, affecting their sensitivity to ROS, may enable T lymphocytes to regulate cellular responses in oxidizing environments, such as those encountered during inflammation [320]. As effector T cells migrate to inflamed sites, including the TME where ROS levels are elevated, upregulating the ORAI3 to ORAI1 ratio may serve as an adaptive mechanism to sustain Ca^{2+} signals essential for cell proliferation and cytokine production [384]. Additionally, adopting this less redox-sensitive mechanism could help maintain the influx of Ca^{2+} into cells through SOCE and prevent cell death pathways triggered by ROS [384]. Tumour cells also modulate the ORAI3/ORAI1 ratio, although the findings are not unanimous. For example, ER+ BC cell lines are mediated by STIM1 or STIM2 along with ORAI3, whereas ER- BC cell lines prefer the STIM1/ORAI1 pathway [385]. Moreover, silencing ORAI3 reduces hypoxia-associated phosphorylation of the epidermal growth factor receptor (EGFR) and the expression of genes associated with cell migration and immune response in MDA-MB-468 basal BC cells [268].

Cytokines such as interferon-gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α), and interleukin-1 (IL-1) significantly drive ROS production by tumour cells [133] and macrophages [386] and neutrophils [387], which produce high levels of O_2-, promoting H_2O_2 accumulation in the TME. To control ROS levels at non-toxic levels, tumour cells induce the expression of antioxidant enzymes, maintaining low to moderate ROS levels in cells[2] [152].

In cancer cells, Transmembrane B cell lymphoma 2-associated X protein inhibitor motif-containing 4 (TMBIM4) (also known as human Golgi anti-apoptotic protein [hGAAP]), plays a significant role in promoting cell migration and invasion by regulating the flux of Ca^{2+} from the Golgi apparatus to the mitochondria [388]. This Ca^{2+} influx into mitochondria enhances mitochondrial respiration, increasing ROS production, particularly H_2O_2 [340]. The accumulation of H_2O_2 driven by TMBIM4 overexpression, acts as a second messenger, oxidizing proteins associated with cell motility and invasion mechanisms. Despite these findings, the precise molecular mechanism through which TMBIM4 regulates these processes remains unclear [388].

Another member of the TMBIM family, the Transmembrane B cell lymphoma 2-associated X protein inhibitor motif-containing 6 (TMBIM6) (also known as Bax Inhibitor-1), an EndR protein with Ca^{2+} channel-like properties, plays a pivotal role in promoting cancer cell metabolism and migration [389]. TMBIM6 modulates intracellular Ca^{2+} release, facilitating the assembly of mTORC2, and subsequently enhancing AKT signalling. This process is intricately linked to the regulation of oxidative stress, as TMBIM6 influences GSH synthesis, thereby modulating ROS levels. By maintaining ROS within a moderate range, TMBIM6 ensures the activation of pro-migratory signalling pathways [390]. Notably, TMBIM6 is frequently upregulated in various cancers, including BC [391]. Targeting TMBIM6, either by inhibiting its function or disrupting its interaction with mTORC2, has the potential to inhibit AKT activation and reduce cancer cell migration [390].

In BC cells, increased ROS resulting from mitochondrial dysfunction induces overexpression of CXCL14, promoting cell motility via the activator protein 1 (AP-1) pathway. Furthermore, CXCL14 induces cytosolic Ca^{2+} elevation through interaction with IP3R [392].

In addition to alterations in TME, in TNBC, MCU expression correlates with tumour size and lymph node infiltration, suggesting that mitochondrial Ca^{2+} uptake might contribute to metastasis formation. This cell migration process, promoted by alterations in Ca^{2+} homeostasis, is regulated by SOCE [393]. Additionally, MCU prompts sustained mitochondrial ROS generation,

[2] Discussed in Chapter 2

fueling the activation of the HIF-1α signalling pathway, which promotes tumour growth and metastasis [394]. Particularly, in MDA-MB-231 BC cells, deletion of MCU reduces mitochondrial ROS production and downregulates HIF-1α expression, underscoring the effector role of ROS in MCU/Ca^{2+} regulation of tumour progression and metastasis [394].

Considering these previous findings and the intricate and poorly explored interplay, identifying genes that are co-dysregulated in both redox and Ca^{2+} signalling pathways could provide valuable prognostic information and therapeutic insights in BC research. This approach may lead to a more comprehensive understanding of cancer biology and potentially improve patient outcomes through better prognostic tools and targeted therapies.

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Chapter II – Hypothesis and Objectives

II.1 Hypothesis

Can the individual or combined expression levels of redox- and Ca^{2+} signalling-related genes be used as biomarkers and/or therapeutic targets for breast cancer (BC) progression?

II.2 Main objective

Using available gene expression data from The Cancer Genome Atlas (TCGA) project and bioinformatic tools, this thesis aims to explore the contribution of individual or combined expression profiles of cellular redox- and Ca^{2+} signalling-related genes that impact BC progression as a strategy to identify novel druggable targets or prognostic biomarkers.

II.2.1 Specific objectives

- To compare the usability and available resources of the most common online platforms used to analyze TCGA gene expression and patient survival data. This approach allows the selection of the most adequate platforms for the subsequent analyses, according to the main thesis objective.

- To evaluate the association between the expression co-dysregulation of Ca^{2+} and ROS-associated genes and BC prognosis, and to elucidate the potential biological processes involved.

- To explore the association between the expression dysregulation of LOX, LOXL1, LOXL2, LOXL3 and LOXL4 genes on BC patient survival and their association with the prevalence of tumour cell infiltrates. Ultimately, this analysis aims to guide the development of novel LOXs inhibitors with specificity profiles tailored to target specific BC cancer subtypes more effectively.

- To analyze the association between the altered expression of redox-related genes in HER2+ BC and their relation with the prevalence of tumour cellular infiltrates in the tumour microenvironment and with patient survival. This approach aims to elucidate the role of redox-related genes in the progression of HER2+ BC and to identify potential prognosis biomarkers.

Chapter III – Gene expression and survival analysis in cancer research using online open access platforms: a comparative analysis

The work presented in this chapter was partially published in:

Ramos S, Fernandes AS, Saraiva, N. Gene expression and survival analysis in cancer research using online open access platforms: a comparative analysis. Biomedical and Biopharmaceutical Research, 2020;17(2):222-240. https://doi.org/10.19277/bbr.17.2.247

III.1 Introduction

The prevalence of cancer has prompted extensive research efforts and the accumulation of enormous amounts of data, resulting in vast databases encompassing genomic and transcriptomic data alongside clinical information. The Cancer Genome Atlas (TCGA) is one of the most used cancer databases, housing data from over 11,000 cancer patients across 33 different cancer types over a decade [1,2]. This extensive database provides insights into various facets of carcinogenesis, incorporating information relative to DNA copy numbers, somatic mutations, gene expression, microRNA analysis, survival data, DNA methylations, histological features, clinical information, and more, enabling a comprehensive multi-omics approach to cancer research [2].

For transcriptome profiling of tumour and normal tissue samples, the TCGA database utilized RNA sequencing (RNA-seq) with the Illumina system, along with microarrays analysis using Agilent G4502A_07, Affymetrix HT_U133 array, and Exo1.0 platforms, the latter of which was released before November 2015 [3]. There was a significant overlap in samples analyzed by both technologies. For example, 1,662 samples were profiled using both Agilent arrays and RNA-Seq [3]. RNA-seq stands out as a high-precision technique capable of identifying and quantifying transcripts, including rare ones, isoforms, and non-coding RNAs, across a wide range of samples [4,5]. Microarray analysis protocols are standardized to detect more significantly expressed genes and exons than RNA-seq [6]. For microarray data, gene level normalization was performed using the Robust Multi-array Average (RMA) algorithm on GenePattern. Agilent expression values were gene-centered [7]. The outcomes of these techniques enable the analysis of gene expression and simplify comparisons between tumour and normal tissues, providing the foundation for the compilation of current studies.

Moreover, with TCGA researchers collecting 1,098 BC samples, the significant number of tumour samples enhances statistical power, strengthening the reliability of molecular alteration-related outcomes in cancer research [5].

To facilitate the analysis of these complex and vast datasets, various TCGA-associated platforms and tools for multi-omics analysis and visualization have been developed [2]. Specialized platforms focus on specific data types, such as microRNA profiles and gene expression. Others, provide genomic and epigenomic data, or patient survival data and gene expression. Emphasizing gene expression analysis, this study evaluates the platforms cBioPortal (https://www.cbioportal.org/) [8], UCSC Xena (https://xena.ucsc.edu/) [9], GEPIA (http://gepia2.cancer-pku.cn/#index) [10,11], UALCAN (https://ualcan.path.uab.edu/) [12], and

OncoLnc (http://www.oncolnc.org/) [13], using Thioredoxin-1 (TXN) expression in BC as a case study. TXN expression is associated with aggressive tumour behavior and poor patient outcomes, highlighting its potential as a therapeutic target and biomarker.

III.2 Objective

By comparing the selected TCG data analysis free-access platforms, this study aims to aid users in choosing the most adequate and navigating the diverse transcriptomic (gene expression), and patient survival resources available.

III.3 Methodology

The methodology involved the selection of platforms capable of analyzing gene expression and patient survival using TCGA datasets. Criteria for platform selection included accessibility, user-friendliness, coverage of various cancer types and stages, and inclusion of patient survival information. Initial platform identification was conducted through keyword searches, such as “gene expression database” or “gene expression platform”, and a review of the relevant literature.

Four platforms meet the inclusion criteria: cBioPortal, UCSC Xena, GEPIA, and UALCAN. Additionally, OncoLnc was included for its ability to provide gene expression and survival data for individual TCGA patients in an easy-to-use format. The utility of each platform was assessed using the TCGA Breast Invasive Carcinoma (TCGA-BRCA) dataset, with the TXN gene serving as a model for testing. Evaluation criteria for each platform encompassed general platform features, gene expression analysis capabilities, and differential survival analysis functionalities. Each criterion was rated on a scale of 1 to 3 based on availability and limitations, as described in Table 1.

The quality of general features of the platforms was evaluated by a 3-point scale (1,2 or 3) based on three criteria: user-friendliness, quality of graphics (both online and in downloaded versions), and data attribute extraction capabilities (Table 1). For gene expression analysis and differential survival analysis, a rating of 1 indicates the analysis is unavailable, 2 signifies availability with limitations, and 3 denotes full availability (Table 1).

Table 1: *Score system for the criteria used to classify the platforms.*

General features of the platform	Gene expression analysis	Differential survival analysis
User-friendly 1. Information provided by tutorials and articles is required but is not sufficiently clear or not easily available 2. Use of tutorials and/or articles of the authors is required 3. Resources are easy to find, the platform use is intuitive and without the need for tutorials	Normal vs Cancer comparison 1. Non-available 2. Selection process is not so clear 3. Available and clear	Kaplan-Meier parameters 1. Non-available 2. Only partially possible 3. Customization of Kaplan-Meier parameters
Graphical quality 1. Two or the three parameters were lacking 2. If one of the previous parameters is not good 3. The graphs are easy to generate, are nicely presented with a clean layout, have good definition, contrast colors, and x and y axis labelled with easy-to-identify units.	Cancer stage analysis Same criteria for Normal vs Cancer Tissue	Logrank and p-value 1. Absence of Logrank test 2. Only the p-value is shown 3. The test is available, and its p-value is calculated
Data Extraction 1. Raw data was completely unavailable. 2. Raw data is partially available 3. Access (downloadable) to all the raw data used to build the graphs	Gene expression output options 1. Non-available. 2. If one or more features are unavailable 3. Possibility to generate different types of graphs, descriptive analysis, and statistical analysis available	Hazard ratio and p value Same criteria as the Logrank test

III.4 Results and Discussion

Analyzing gene expression across tumour stages and its influence on patient survival is crucial for the process of identification of potential biomarkers or therapeutical targets in cancer. Given the dimension, format, and complexity of the currently available data, accessible online analysis platforms play a vital role, especially for specialists lacking bioinformatics or programming expertise. Our study assessed five open-access platforms, comparing their ability to perform

gene expression quantification and analysis across cancer stages and to analyze the survival of patients via Kaplan-Meir curves and Hazard ratio calculation.

III.4.1 Comparison of gene expression analysis provided by the different platforms

The gene expression analysis highlights the importance of its quantification in understanding signalling pathways in oncological pathology. The four platforms selected using the criteria mentioned in the methods (cBioPortal, UCSC Xena, GEPIA, and UALCAN) offer varying capabilities for gene expression analysis.

The cBioPortal allows access to TCGA and International Cancer Genome Consortium (ICGC) data for various cancer studies, offering a graphical representation of high and low gene expression percentages using a Z-score (Figure 1A) and clinical information. However, it lacks a direct comparison between cancer and normal tissues. UCSC Xena provides diverse genomic analysis tools, including survival analysis and statistical tests, with data from various studies, namely Caldas (2007) [14], Yau (2010) [15], and TCGA. It enables filtering and comparing across different tumour genomic contexts and subgroups and supports analysis of tumour *versus* normal tissues (Figure 1B). GEPIA offers access to TCGA and Genotype-Tissue Expression (GTEx) databases for gene expression comparison across cancer types. It supports various analyses, including top differential gene expression in each cancer type and gene expression analysis between tumour and normal tissues (Figures 1C and 1D) in a graphic format. Still, a direct collection of expression values is not available. UALCAN and GEPIA provide detailed gene expression analysis from TCGA data, facilitating the identification of key genes across different cancer types and comparing tumour and normal tissues (Figure 1E). UALCAN supports analysis across various subpopulations and offers a unique "gene scan" function for identifying differentially expressed genes.

While all platforms employ different visualization methods, cBioPortal stands out for its easy study identification, while UCSC Xena and UALCAN provide detailed statistical comparisons. GEPIA is very friendly, and the graphs are clear. However, it only estimates expression levels without providing exact values and statistical significance values.

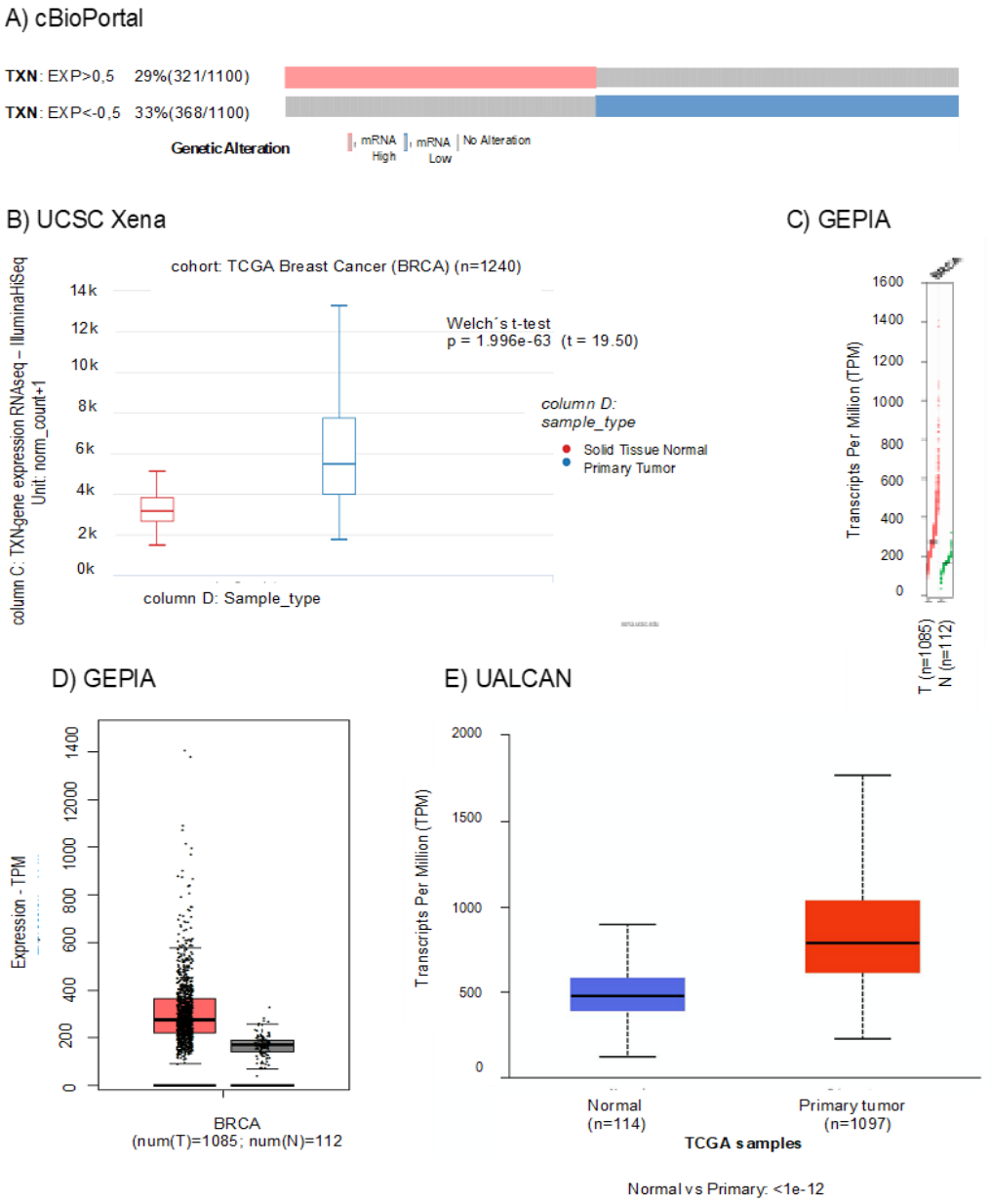

Figure 24. Comparative analysis between the graphical output of TXN mRNA expression *– The expression levels of the TXN gene in the TCGA Breast Cancer (BRCA) dataset were analysed by the different platforms. (A) cBioportal, using ± 0.5 z-score threshold. By default, the platform selects only the samples where the mRNA expression was analyzed. (B) Boxplot of TXN expression in UCSC Xena using sample type, which compares normal and tumour tissues, using Welch´s t-test. (C) Dot plot of TXN expression in normal and tumoural cells in GEPIA using one-way ANOVA test. Boxplot with a comparison between normal and tumoural cells presented in GEPIA (D) and UALCAN (E), using one-way ANOVA test and t-test, respectively. While cBioPortal uses log2(norm_count+1) and USCS Xena uses norm_count+1, GEPIA and UALCAN uses transcript per million as gene expression unit.*

III.4.2 Gene expression analysis by cancer stage: platforms comparison

Tumour staging plays a critical role in prognosis definition and treatment selection by categorizing the tumour type at diagnosis and the progression of the malignant disease over time post-diagnosis. It is essential for identifying appropriate cancer therapies and is often utilized in clinical research to define study populations and reduce bias in treatment group comparisons.

In cBioPortal, tumour stage-associated expression is shown with boxplots and includes an option for raw data exploration. Multiple classification systems are available, like Converted Stage and Tumour Stage or Neoplasm Disease Stage American Joint Committee on Cancer Code. Users can select mRNA profiles based on different expression types, such as microarray of mRNA expression (Figure 2A). UCSC Xena offers various classification systems depending on the study. It provides statistical analysis including maximum, minimum, quartiles, median, and ANOVA test for each stage (Figure 2B). GEPIA presents pathological stages or "sub-stages" based on the American Joint Committee on Cancer Code, along with one-way ANOVA and p-value comparisons between stages (Figure 2C). UALCAN analyzes gene expression across pathological stages, providing statistical comparisons using t-test and visualization (Figure 2D).

Not all platforms use the same pathological stage classifications. Some use the TNM system while others use the Neoplasm Disease Stage American Joint Committee on Cancer Code. This discrepancy makes direct comparisons challenging. While platforms like GEPIA show consistent median expression levels across stages, the number of cases included in each stage is often unavailable, hindering direct comparison. Despite this, platforms like UCSC Xena and UALCAN display similar trends in mean expression levels and well-defined quartiles (Figures 2B and 2D).

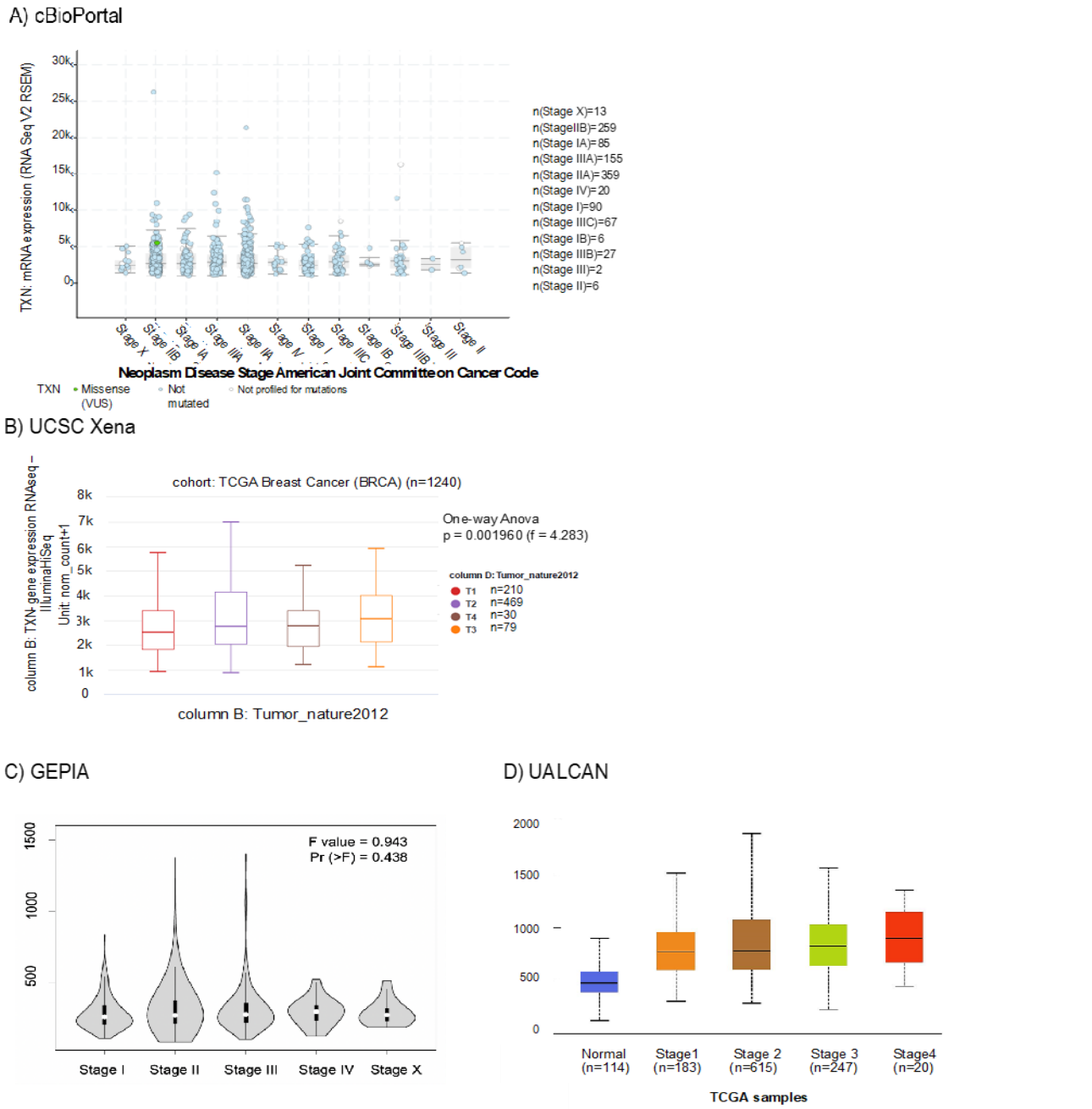

Figure 25. Comparative overview of TXN mRNA expression level analysis according to tumour stage – *The levels of TXN expression by tumour stage were explored with cBioportal, using Neoplasm Disease Stage American Joint Committee on Cancer Code and Chi-squared test(A), UCSC Xena, using classification defined as Tumour_nature2012 and one-way ANOVA test (B), GEPIA (C) and UALCAN (D), using pathological major stages and one-way ANOVA and t-test, respectively. While cBioPortal uses log2(norm_count+1) and USCS Xena uses norm_count+1, GEPIA and UALCAN uses transcript per million as gene expression unit.*

III.4.3 Differential survival analysis based on the expression level of a specific gene: platforms comparison

Identifying cancer biomarkers through gene expression levels is crucial for estimating patient outcomes and guiding therapeutic decisions. Survival and hazard functions, derived from specific survival data, assess patient outcomes based on the expression of a gene. The survival function predicts the likelihood of patient survival beyond a given time, while the hazard function measures event rates at specific time points for surviving individuals. Kaplan-Meier curves visualize survival trends over time, assuming events (patient death) occur independently. Statistical models like the Cox proportional hazards model assess survival differences between groups, yielding hazard ratios (HR) and corresponding p-values for each variable in the model.

The cBioPortal allows for comparison of gene expression levels, categorizing cases as under or overexpressed based on defined z-score thresholds. It provides overall survival data, including Logrank test p-values and median survival months for each group (Figure 3A). UCSC Xena generates Kaplan-Meier plots for two, three, or quartile-based groupings, with Logrank test statistics for survival analysis (Figure 3B). GEPIA offers survival analysis using Logrank and Mantel-Cox tests and includes hazard ratio values, allowing for overall or disease-free survival assessment, based on gene expression levels (Figure 3C). UALCAN enables survival analysis based on gene expression levels or clinical-pathological characteristics, with Logrank test statistics (Figure 3D). OncoLnc complements other platforms by exploring survival correlations and providing clinical data associated with gene expression from TCGA (Figure 3E). Users can select a gene and conduct Cox regression across various cancer types, generating Kaplan-Meier plots with customizable percentiles.

cBioPortal has less intuitive parameter definitions for survival analysis compared to other platforms. UALCAN and UCSC Xena lack customization options, although UCSC Xena allows quartile selection. Survival analysis across all platforms showed no significant differences between groups when using quartiles. However, GEPIA analysis yielded a p-value close to significance (Figure 3C), suggesting a potential difference in patient survival based on TXN gene expression levels. Unlike GEPIA, the UCSC Xena and cBioPortal platforms allow timeline customization and both yielded consistent results ($p<0.05$).

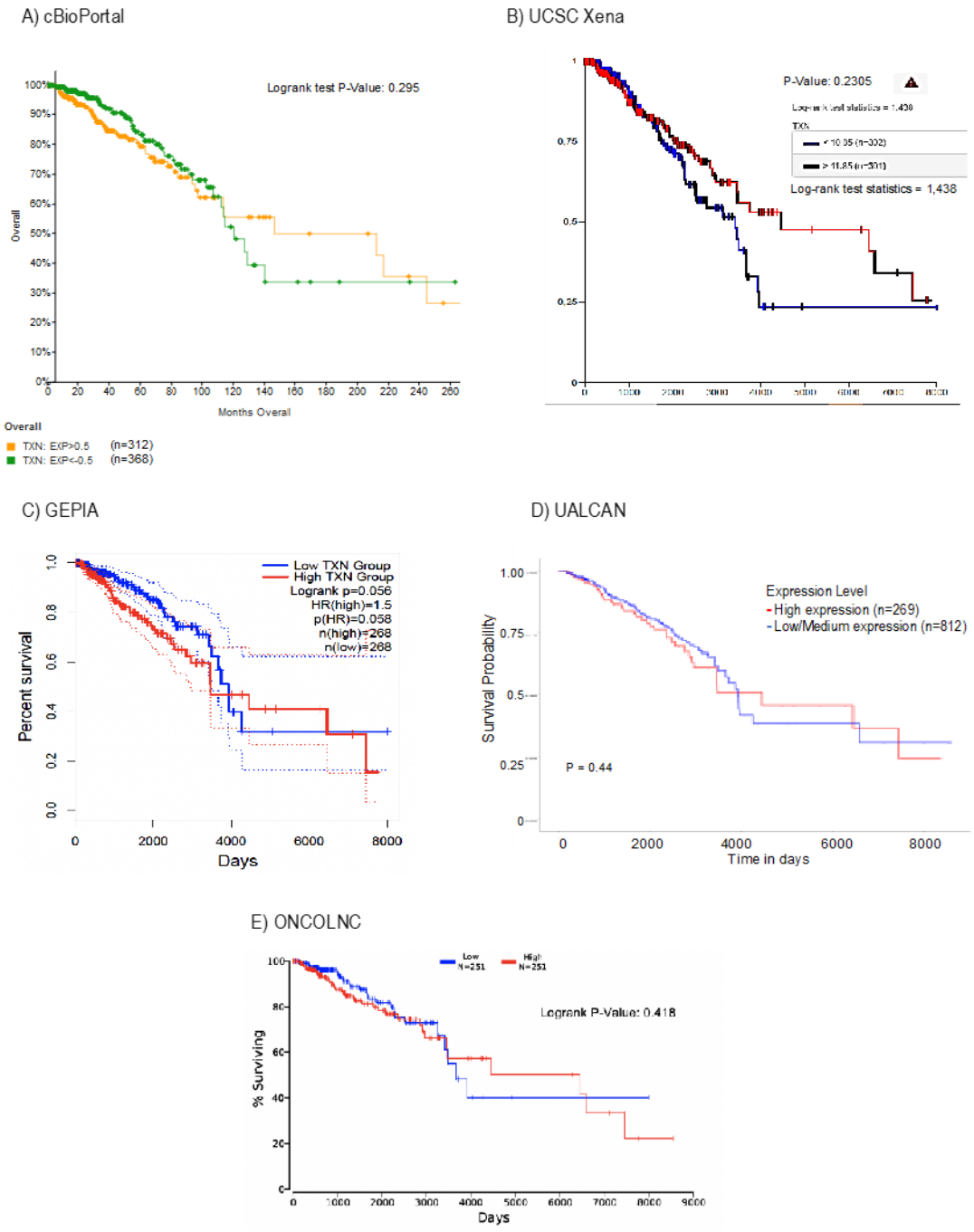

Figure 26. Comparison between the typical survival analysis output from different platforms *– Kaplan-Meier survival curves for high and low TXN expression levels were generated with (A) cBioPortal, using expression thresholds between EXP<-0.5 and EXP>-0.5 (standard deviations from the mean). (B) UCSC Xena Kaplan-Meier using quartiles as cut-offs. (C) GEPIA with Logrank test and hazard ratio customized using quartiles as stratification. (D) UALCAN with upper quartile and below upper quartile division and (E) OncoLnc with Logrank p-value and quartiles cut-off customization.*

III.4.4 Platform comparative analysis

Figure 4 illustrates a comparative analysis using color gradients to represent different scores, with darker colors indicating a better performance. A blue gradient was chosen for general platform features, while a green gradient was used for gene expression and differential survival analysis.

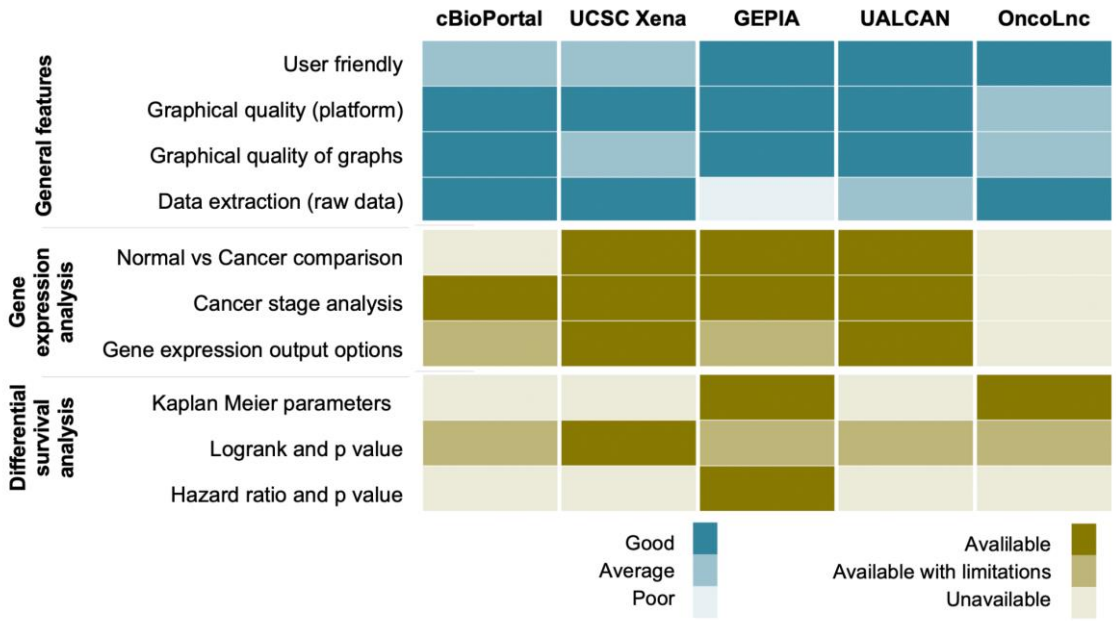

Figure 27. Global comparative analysis of the platforms. *Three general features of the platforms that are related to gene expression and differential survival analysis were considered. A colour gradient was used to classify platforms using three three-point scale. More intense colour reflects better classifications. General features (blue) were classified as "good", "average" or "poor" in terms of user-friendliness, quality of the graphs (presented online and downloaded versions), and data extraction availability/flexibility. Gene expression and differential survival analysis (green) were classified as "available", "available with limitations" or "non-available". For gene expression analysis, comparison between normal and cancer tissue, availability of cancer stage analysis, and different types of output (types of graphical outputs, descriptive and statistical analysis) were considered. For survival analysis, the customization of Kaplan-Meier parameters, test and the p-value for Logrank and hazard ratio were analysed.*

All platforms reviewed are user-friendly with helpful tutorials, and each has distinct advantages and limitations summarized in Table 2. CBioPortal lacks normal tissue gene expression data, limiting differential expression analysis to tumour origin, stage, or type comparisons. While it offers diverse cancer attributes for analysis, its survival curve presentation lacks customization options and only allows it to perform the Logrank test. UCSC Xena is less intuitive but includes a wider range of studies, data sources, and clinical tracks. Its ability to examine multiple hubs and genomic contexts simultaneously, allows for comprehensive analysis, although some studies may lack certain information. GEPIA does not provide gene expression level values and lacks

certain clinical information. However, it offers an easy selection of genes of interest and comprehensive survival analysis, including hazard ratio and customizable cut-offs. UALCAN offers detailed quantification of gene expression and comprehensive survival analysis. It provides correlations between the expression of two genes and various survival curves but has fixed cut-offs and only presents the Logrank p-value for statistics. OncoLnc simplifies querying TCGA data, allowing customization of gene expression cut-offs and providing patient data for survival analysis. However, it focuses on survival analysis and does not offer a gene expression analysis presentation.

Table 2: Main advantages and disadvantages of each platform.

Platform	Advantages	Disadvantages
cBioPortal FOR CANCER GENOMICS	Includes clinical tracks, diagnosis age, different stage classifications, patients' data Several data sources	Only cancer tissue samples Need to select patients/cases to analyze overall survival related to under and overexpression
UCSC Xena	Several hubs and genomic contexts simultaneously Various data sources and clinical tracks User friedly and good graphical quality Detailed results	Absence of statistical significance in downloaded graphs Requires some tutorials to understand
GEPIA	Overall survival with under and overexpression, Hazard ratio, median, quartile and custom cut-off	Gene expression and statistical related not quantified Unable to visualize patient/case data
UALCAN	Data from boxplot detailed (min, max, quartiles) Statistical comparisons (TxN and NxS)	Cut-off in survival analysis is fixed Unable to obtain patient/case data
OncoLnc	Patient data (gene expression, status, survival days)	Gene expression by stage not presented

III.5 Conclusion

All platforms analyzed provide easy access to genomic, transcriptomic, and clinical data and facilitate the exploration of individual and multiple gene expression patterns and their association with patient survival. They offer features to relate gene expression to disease stages and analyze survival associated with gene expression levels. Users can filter data by selecting specific attributes like cancer type, molecular alterations, recurrence, and histological aspects.

Data visualization through graphs enables the distribution of gene expression and the establishment of statistical relationships.

The diversity of available information contributes significantly to studying the complex mechanisms involved in cancer pathogenesis, allowing for multiple investigative approaches. The choice of platform depends on the specific information sought for each purpose. If one platform lacks certain information or resources, combining data analysis from multiple platforms is possible, provided they use the same database as the source.

III.6 Additional bioinformatic tools and analysis

Given the pivotal influence of immune cells within the microenvironment on BC progression [16–18], the association between genes expression and immune cell infiltrates was explored in BC (Chapter V studies 3 and 4). Furthermore, gene enrichment analysis emerged as a fundamental component in elucidating the biological significance and functional roles of a set of genes [19–21] (Chapter IV and paper under preparation) By identifying overrepresented biological terms, particularly through gene ontology (GO) terms analysis, valuable insights were gained into the underlying mechanisms.

III.6.1 Correlation between gene expression and immune cell infiltrates

Tumour progression is strongly influenced by the composition and abundance of immune cells within the tumour microenvironment [22]. By analyzing the tumour immune infiltrates, insights can improve the understanding of escape mechanisms utilized by tumour cells to evade the immune response, as well as potential avenues for enhancing the efficiency of therapeutic responses [23,24].

To estimate the immune cell composition and the interaction with gene expression, a set of computational algorithms based on marker gene and deconvolution approaches can be used [22,25]. Marker gene-based algorithms, such as xCell [26] and MCP-counter [27], quantify the presence and abundance of immune cell types using specific gene expression profiles. These algorithms depend on predefined gene sets to assess cell abundance in heterogeneous samples [25]. Deconvolution methods, like TIMER [28], quanTIseq [29], EPIC [30], and CIBERSORT [31], estimate cell type fractions within mixed populations from the tumour sample by analyzing gene expression profiles as linear combinations of predefined immune genes signatures. These

algorithms infer cell type abundance without relying on predefined marker genes, instead using reference expression profiles [29].

The Tumour Immune Estimation Resource 2.0 (TIMER2.0, available at http://timer.cistrome.org/) was utilized to estimate the immune cell composition and its interaction with gene expression profiles. This platform is a comprehensive resource for systematic analysis of immune infiltration across 32 different cancer types, including gene expression, clinical outcomes, somatic mutations, somatic copy number alterations, and the characterization of the tumour-immune infiltration based on TCGA cohorts [22,32]. To explore the correlation between tumour-infiltrating immune cells and gene expression, appropriate algorithms were employed, as suggested by Sturm et al. (algorithms described in [25]). In all cases, a Spearman correlation, adjusted for tumour purity, was used to determine the statistical significance ($p<0.05$) between the expression levels of each gene and the abundance of immune infiltrate [22].

III.6.2 Gene Ontology Enrichment Analysis

Functional enrichment analysis involves identifying the biological terms, such as GO terms or pathways, that are significantly over-represented in a list of genes compared to a background gene set [20,33]. This analysis helps to understand the underlying biological processes or functions associated with a set of genes, providing insights into the more relevant molecular mechanisms and cellular processes [33]. GO enrichment analysis classified mechanisms into biological processes, molecular functions, and cellular components [34]. In particular, biological processes were analyzed, because they provide a framework for understanding how genes contribute to broader biological phenomena, interpreting gene significance, enabling comparative analysis, and elucidating specific molecular processes underlying larger biological goals [35].

ShinyGo V0.76 (http://bioinformatics.sdstate.edu/go/) [36] was used as the tool for GO enrichment analysis of genes identified during data mining analyses (Chapters IV and V). The False Discovery rate (FDR) was applied as an adjustment method for the p-values of enrichment terms, allowing the determination of the likelihood of enrichment occurring by chance. Moreover, fold enrichment was used to indicate the degree to which genes of a certain pathway were overrepresented. To enhance the robustness of the analysis, the method adopted involved

selecting by FDR and sorting by fold enrichment, thereby prioritizing the top pathways based on their significance, as described on the website.

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Chapter IV – Association between Dysregulated Expression of Ca^{2+} and ROS-Related Gene Pairs and Breast Cancer Patient Survival

The work presented in this chapter was submitted for publication.

Ramos S, Gregório J, Fernandes AS, Saraiva N. Association between Dysregulated Expression of Ca2+ and ROS-Related Gene Pairs and Breast Cancer Patient Survival

IV.1 Introduction

Breast cancer (BC) is the most common cancer in women, with around 2.3 million new cases each year, representing 11.6% of all cancer cases, and causing 666,000 deaths globally [1]. This significant prevalence has led to the creation of guidelines for various diagnostic and therapeutic strategies [2,3]. Despite extensive research, the exact molecular mechanisms behind BC initiation and progression remain unclear.

Breast tumour tissues are highly susceptible to ROS, particularly those induced by estrogen, and surrounding adipose tissue exacerbates this pro-oxidative environment, contributing to cancer progression [4–8]. While ROS play a dual role, supporting normal cell functions (growth, proliferation, apoptosis, differentiation, migration, invasion, and angiogenesis) at low levels [5,9], in tumour cells, elevated ROS levels activate signalling pathways like HIF1 and PI3K/Akt, promoting tumour growth, survival, and resistance to therapy [9–11]. Tumour cells counterbalance high ROS through upregulation of antioxidant enzymes, such as superoxide dismutases (SOD), glutathione peroxidases (GPX), glutathione reductases (GR), glutaredoxins (GRX), thioredoxins (TXN), peroxiredoxins (PRDX), and catalase, are essential for regulating ROS and promoting tumour growth [12–15]. However, excessive ROS can lead to senescence and cell death, highlighting the complex role of ROS in BC [16,17].

Another key system in BC development is Ca^{2+} signalling pathways, which influence processes like cell growth, apoptosis resistance, angiogenesis, and metastasis [18–21]. Ca^{2+} signalling, driven by various channels, pumps, and internal Ca^{2+} stores such as the EndR and Golgi apparatus, is altered in cancer cells [19,22–24]. These alterations can either increase Ca^{2+} influx, promoting tumour progression [20,25–27], or reduce Ca^{2+} influx, inhibiting apoptosis pathways [22,28–30]. Key players in Ca^{2+} signalling in BC include TRP and ORAI channel families, which are linked to cell proliferation, angiogenesis, and migration [20,31–35].

Research shows that intracellular Ca^{2+} levels can impact the production of ROS, which, in turn, affects Ca^{2+} signalling. In particular, Ca^{2+} can activate mitochondrial enzymes in the Krebs cycle [36–38] and control antioxidant enzymes [66], while ROS can also influence Ca^{2+} signalling-regulated pathways [36]. This complex interaction benefits cancer cells by inhibiting apoptosis pathways while promoting tumour growth, inflammation, and invasion [37].

Despite significant research, the exact mechanisms associated with Ca^{2+} dysregulation and the progression of specific cancer types, including BC, remain unclear. Key aspects of BC biology, particularly the roles and interactions of redox and Ca^{2+} signalling in BC progression, are not well

understood. Therefore, gaining a deeper understanding of these mechanisms is crucial for finding new therapeutic targets and developing better BC treatments.

IV.2 Objective

Using a bioinformatic analysis of TCGA data, this study aims to explore the association between the co-dysregulation of genes involved in redox and Ca^{2+} homeostasis and BC patient survival. Additionally, associations between selected genes and biological processes related to tumour progression were identified.

IV.3 Methodology

IV.3.1 Gene selection criteria

The gene selection was conducted through a comprehensive PubMed search to identify redox- and calcium-related genes associated with BC progression and patient survival. Specific keywords and combinations were used, such as “Breast cancer AND redox”, “Breast cancer AND calcium”, “Breast cancer AND survival AND redox genes”, “Breast cancer AND survival AND calcium genes”, “Breast cancer genes AND redox AND progression”, “Breast cancer genes AND calcium AND progression”, “Breast cancer genes AND patients survival”, “Breast cancer AND redox regulation”, “Breast cancer AND calcium regulation”, “Breast cancer AND redox AND migration”, “Breast cancer AND calcium AND migration”, “Breast cancer AND redox AND proliferation”, “Breast cancer AND calcium AND proliferation” and “Breast cancer AND redox genes AND calcium genes”. Only genes supported by evidence from human BC models, including cell lines, xenografts, or human tissues, were considered.

This process yielded two sets of genes: 39 redox-related and 47 Ca^{2+}-related genes, all of associated with BC progression and/or patient survival according to published studies (Supplementary Table 1)

The Cancer Genome Atlas (TCGA) database, specifically the Breast Invasive Carcinoma (BRCA) dataset comprising 1,098 patients, was used to source gene expression data, at mRNA level and patient survival [39]. For the analysis, the UALCAN (http://ualcan.path.uab.edu/) and Gene Expression Profiling Interactive Analysis 2 (GEPIA2; http://gepia2.cancer-pku.cn/) platforms facilitated access to TCGA datasets.

The association between gene expression levels and overall survival in BC patients was analyzed using median and quartile cutoffs in GEPIA2 [40]. Statistical differences between Kaplan-Meier survival curves were assessed by the Log-rank test and Hazard ratios (HR). Genes showing significant differences ($p<0.05$) between survival curves (using median or quartile cutoffs) and with hazard ratios (HR) less than 0.8 or greater than 1.2 were selected for further analysis. UALCAN [41] was then used to determine differential gene expression between normal breast and BC tissues, with only statistically significant genes ($p<0.05$) being selected.

Additionally, a set of redox and Ca^{2+} signalling-related genes, such as *LOXL2* [42–44], *NOX4* [45,46], *SOD2* [47–49], *ORAI1* [50–52], and *STIM1* [53–55], were included despite not showing significant expression dysregulation. These genes were included because previous studies strongly suggested these genes to impact BC progression.

IV.3.2 Association between expression levels of gene pairs and BC patient survival

To analyze the relationship between the differential expression of gene pairs and patient survival, data from each patient was sourced from the TCGA database via OncoLnc (www.oncolnc.org) [56], focusing on survival days, patient status (alive/dead), and gene expression levels (RSEM RNA-SeqV2) of selected genes.

Gene expression levels were categorized into quartiles (Q1-Q4) (Supplementary data 1). Kaplan-Meier survival analysis and the Log-rank test were employed to evaluate differences in patient survival curves based on the expression levels of each gene pair. For each pair, one gene was used as a factor (Gene 1) and the other as strata (Gene 2), and this setup was then reversed. The analysis focused on a five-year prognosis time point, commonly recognized in the scientific and medical communities for assessing cancer survival rates [57]. Cumulative survival proportion and survival time were used to calculate risk, defining death as the event of interest.

Statistical significance was set at a 95% confidence level ($p<0.05$), and analyses were conducted using Statistical Package for Social Science for Windows (SPSS; v.27). Graphics depicting cumulative survival proportions over five years were generated based on the quartiles of the gene pairs. Gene pairs with consistent trends in survival outcomes associated with the first (Q1) or last quartile (Q4) were selected for further study, totaling 52 pairs that exhibited these consistent tendencies.

IV.3.3 Gene enrichment analysis

Gene enrichment analysis was conducted to identify significant biological processes associated with gene pairs linked to BC progression and patient survival. Using the UALCAN platform, Pearson correlation coefficients were calculated to find genes with strong positive or negative correlations with the selected gene pairs. Only genes showing a Pearson correlation coefficient lower than -0.3 or higher than 0.3 were considered. Venn diagrams were then used to identify genes correlated with both genes within each pair.

To understand the functional implications of the overlapped genes between each combination, the ShinyGO tool (http://bioinformatics.sdstate.edu/go/) [58] was utilized to perform Gene Ontology (GO) analysis, focusing on biological processes (BP). Pathways with a minimum of ten genes and a False Discovery Rate (FDR) less than 0.05 were considered significant, and the top ten pathways were selected based on FDR and sorted by fold enrichment. This process helped prioritize the most relevant pathways associated with gene dysregulation. Furthermore, genes with similar BP were grouped, and the DiVenn tool (https://divenn.tch.harvard.edu) [59] was employed to detect common subsets of upregulated and downregulated genes across at least three gene pairs. Figure 1 schematizes the research workflow detailing the main methodology steps used.

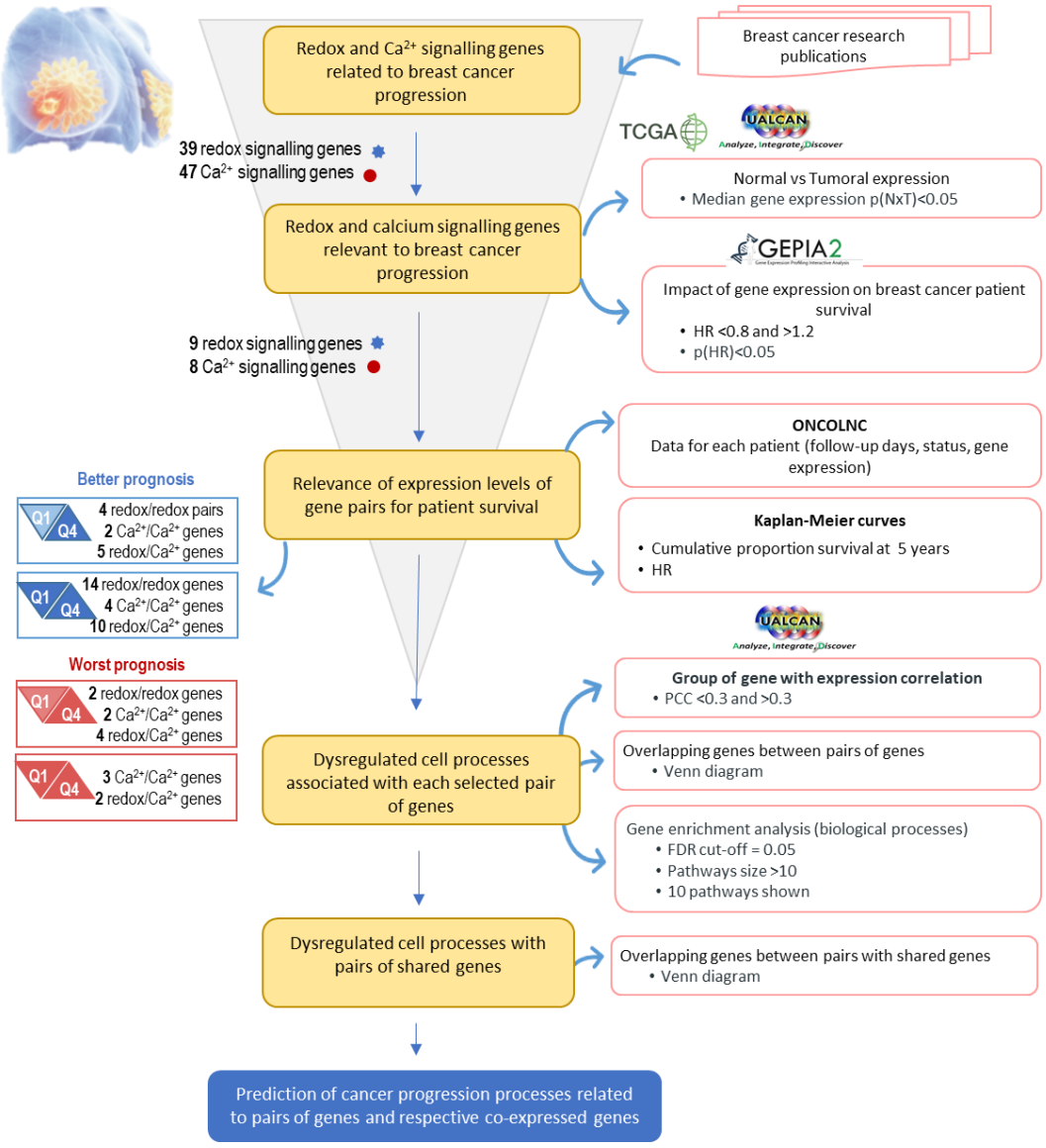

Figure 28. Research workflow. *Flowchart showing the data sources, methodology, and criteria for gene selection. The main methodology key points are described on the right.*

IV.4 Results and Discussion

IV.4.1 Identification of redox and Ca^{2+}-related genes potentially relevant in BC

The comprehensive literature review followed by a gene expression analysis, identified 39 genes related to redox signalling and 47 associated with Ca^{2+} regulation with an impact on BC progression or patient survival. A summary of their expression values and HR is provided in Supplementary Table 1. Among these genes, six redox-related genes—*GLRX2*, *GLRX3*, *LOXL3*, *PRDX4*, *TXN*, and *TXNRD1*—and five Ca^{2+}-related genes—*ATP2C2*, *CALM2*, *CAMK2G*, *PLCD1*, and *TRPM8*—fulfilled three key criteria: i) significant differential expression between normal and

cancerous tissues, ii) significant impact on overall survival (indicated by median or quartile cutoffs) and iii) HR lower than 0.8 or higher than 1.2. Although *LOXL2*, *NOX4*, *SOD2*, *ORAI1*, and *STIM1* only met one of these criteria, they were included in the study due to their well-documented roles in BC progression (Figure 2 and Supplementary Table 1).

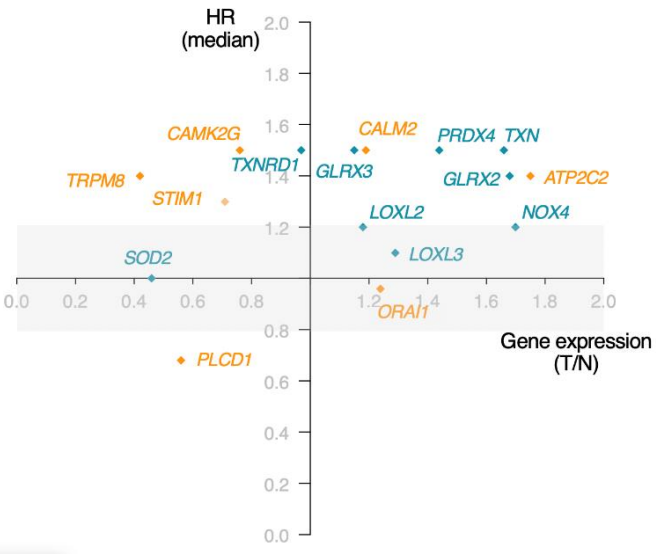

Figure 29. Identification of dysregulated redox and Ca^{2+}-related genes associated with BC patient survival. *Gene selection was represented based on the gene expression ratio between Tumour and Normal tissues (T/N), and its impact on patient survival using hazard ratio (HR) applying median as cutoff. Redox-related genes are represented in green and Ca^{2+}-related genes in orange. Genes that meet only one of the selection criteria (statistically differential gene expression between tumour and normal tissues, statistically significant impact on patient survival based on median cutoff and HR <0.8 and >1.2) are included in the grey area. Exceptionally, LOXL3 selection was based on the quartile cutoff for HR calculation. Significance level: p<0.05.*

Studies providing evidence for the impact of selected genes on BC progression are summarized in Supplementary Table 2. Our selection method highlighted several genes encoding thiol proteins, such as Glrx2, Glrx3, Prdx4, Txn, and TrxR1. These proteins feature a sulfur atom in their active site, often within a cysteine residue, making them highly reactive and crucial in redox reactions [60]. Although not all redox homeostasis proteins are thiol-dependent, many use thiol groups to regulate cellular redox balance [61–63]. Thiol-disulfide balance, due to its role in BC pathogenesis, can be a valuable biomarker in BC patients [62]. In addition to thiol-related genes, our selection includes *LOXL2*, *LOXL3*, *NOX4*, and *SOD2*. Loxl2 and Loxl3 are involved in extracellular matrix (ECM) remodeling, facilitating the cross-linking of elastin and collagen, which is linked to tumour cell invasion and migration [44]. NOX4, like other Nox proteins, generates oxidants that contribute to tumourigenesis [64,65].

All these genes are directly involved in H_2O_2 metabolism, a key redox signalling molecule as shown in Figure 3A. H_2O_2 mediates the reversible oxidation of cysteine residues in enzymes and

transcription factors, affecting major aspects of cancer cell behavior, including apoptosis, cell cycle progression, proliferation, energy metabolism, and angiogenesis [66]. The present findings underscore the crucial role of H_2O_2 in BC. Despite its recognized importance, the precise mechanisms and effects of H_2O_2 in cancer cell fate remain unclear. Emerging evidence suggests that the impact of H_2O_2 is shaped not just by its concentration but also by its spatial and temporal variations [66]. Our results reveal genes associated with both H_2O_2 generation and detoxification, indicating that altered expression of some genes might correlate with either better or worse prognosis depending on the context. Furthermore, the identified redox-related genes encode proteins localized in various organelles, emphasizing the importance of H_2O_2 distribution. H_2O_2 gradients and selective accumulation in specific cellular regions promote localized thiol oxidation, leading to targeted H_2O_2 signalling events [96].

The selected genes related to Ca^{2+} homeostasis exhibit a variety of molecular functions (Figure 3B). Four of these Ca^{2+}-related genes (*ORAI1*, *STIM1*, *PLCD1*, and *ATP2C2*) encode proteins that regulate Ca^{2+} levels in key intracellular stores: the EndR and the Golgi apparatus. *ORAI1* and *STIM1* facilitate Ca^{2+} uptake by the EndR, while *ATP2C2* supports Ca^{2+} uptake by the Golgi. TRPM8 and *ATP2C2* enable Ca^{2+} influx from the extracellular space into the cytosol, and *PLCD1* controls the activation of IP_3R, influencing cytosolic Ca^{2+} levels sensed by various Ca^{2+}-responsive proteins, including *CALM2* and *CAMK2G*. The dysregulation of these genes is frequently linked to cell survival, migration, and tumour growth [50,67–70]. Additionally, *CALM2* plays a role in BC cell cycle regulation [71].

While there is substantial evidence of the individual contributions of these genes to BC, their combined effects on the mechanisms driving BC progression and patient outcomes remain poorly understood.

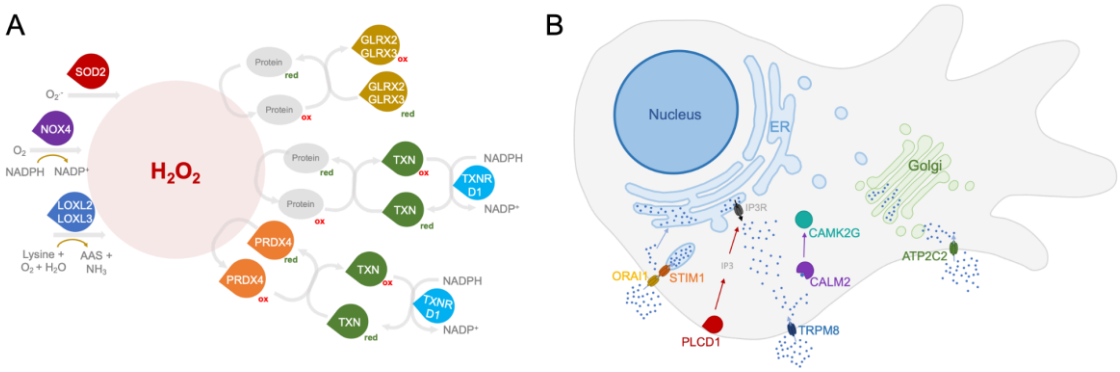

Figure 30. Simplified schematic representation of the (A) involvement of the redox-related genes identified in this work to H_2O_2 metabolism; and of the (B) function and subcellular localization of the proteins encoded by genes identified in this work that are related to Ca^{2+} homeostasis. *Information regarding protein function was obtained from The Protein Atlas.*

IV.4.2 Combined dysregulation of redox- and Ca^{2+}-related genes and BC patient survival

To investigate the relation between BC patient prognosis and the combined expression of gene pairs, cumulative survival proportions were calculated. Focusing on the first (Q1) and last quartiles (Q4) of gene expression, four scenarios were considered: (i) reduced patient survival associated with low expression of one gene and high expression of the other (Figure 4A); (ii) reduced survival linked to high expression of both genes (Figure 4B); (iii) improved patient survival associated with low expression of one gene and high expression of the other (Figure 4C); and (iv) improved survival linked to high expression of both genes (Figure 4D). Using cumulative survival proportions, the patient outcomes for various gene pair combinations were determined (Figure 5).

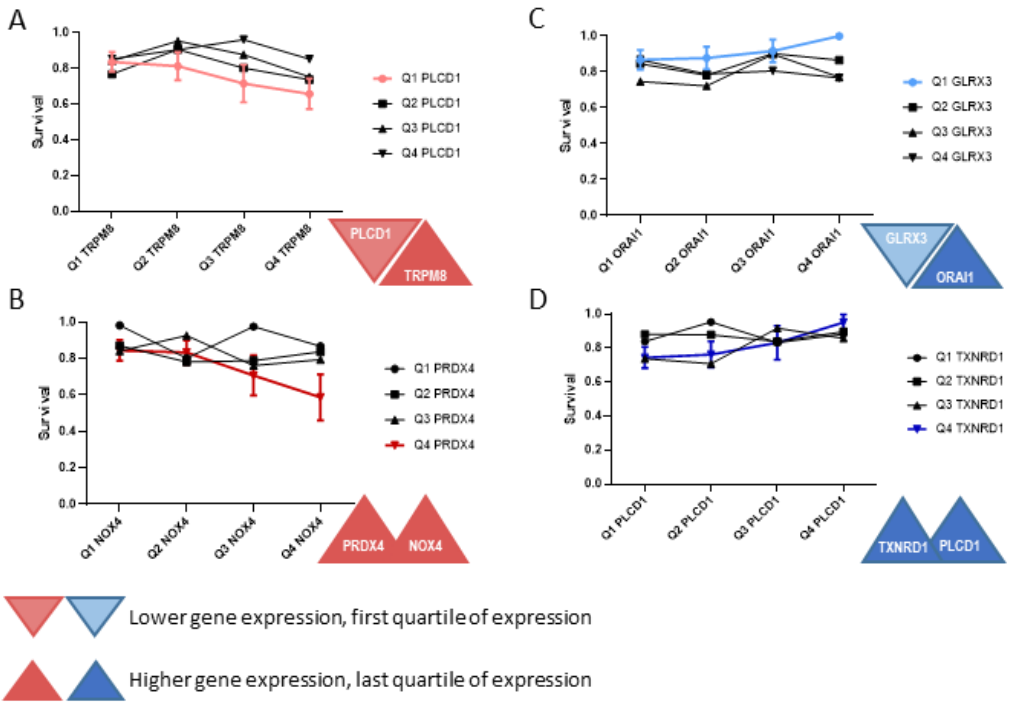

Figure 31. Possible scenarios concerning the expression of pairs of redox and Ca^{2+} signalling genes and the survival of BC patients. *Graphs (A-D) represent the cumulative overall survival proportion of patients related to the quartiles of expression of one gene associated with the quartiles of expression of the second gene. Only when all four points showed an increased/decreased tendency were considered as a significant trend. A-D provides examples of the four possible combinations. As an example: (A) Patients with lower expression of PLCD1 and higher expression of TRPM8 have a worse prognosis than patients with lower expression of both genes; (B) Increased expression of PRDX4 and NOX4 is associated with decreased survival. In the opposite direction, (C) Patients with lower expression of GLRX3 and higher expression of ORAI1 have a better prognosis than those with low expression of both genes, and (D) the increased expression of TXNRD1 and PLCD1 is associated with increased survival.*

The approach illustrated in Figure 4 was utilized for all possible combinations of the selected genes (Supplementary File 1). Gene pairs with expression changes associated with significant differences in patient survival outcomes are represented in Figure 5.

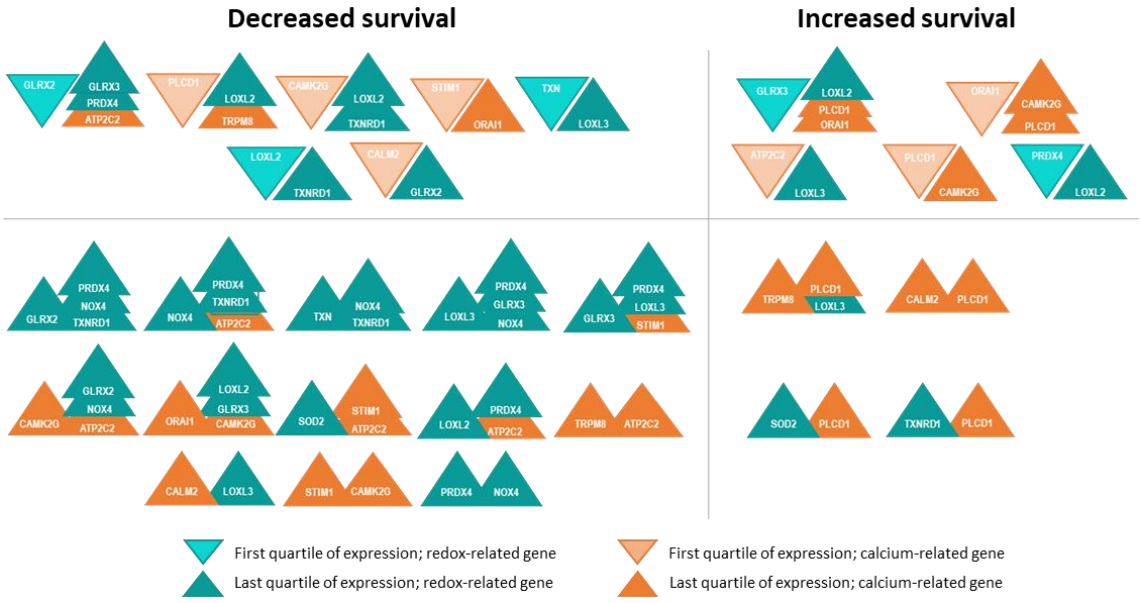

Figure 32. Schematic representation of the association between expression of gene pairs and BC patient survival. *The diagram summarizes the cases where an association was found between the combined expression dysregulation of pairs of genes involved either in Ca^{2+} (orange) or redox (green) homeostasis and the overall survival of BC patients.*

This analysis revealed 52 gene expression combinations linked to changes in BC patient survival. Among these, 39 combinations were linked to decreased survival, while 13 were associated with improved survival. The genes *PLCD1*, *GLRX3*, and *PRDX4* frequently showed significant correlations with patient survival when paired with other genes, highlighting their potential significance in BC progression.

PLCD1, encoding the enzyme Phospholipase Cδ1, is involved in energy metabolism, Ca^{2+} homeostasis, and intracellular signalling pathways [69,72]. This phospholipase maintains Ca^{2+} homeostasis by converting phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) into the signalling molecules inositol-1,4,5-trisphosphate (IP3) and 1,2-diacylglycerol (DAG). These molecules regulate intracellular Ca^{2+} levels and activate protein kinase (PKC) signalling pathways [72]. In BC cells, PLCD1 acts as a potential tumour suppressor, inducing G2/M cell cycle arrest and inhibiting cell migration and invasion through the ERK1/2/β-catenin/MMP7 signalling pathway [69,72]. Similar to its role in other types of cancer, such as renal cell carcinoma [73], pancreatic cancer

[74], and esophageal squamous cell carcinoma [75], *PLCD1* is frequently silenced in BC cells through promotor methylation, leading to reduced expression and its tumour-suppressing potential [69,76]. Our findings revealed that patients with lower *PLCD1* expression combined with higher expression of *LOXL2* and *TRPM8* showed a poor prognosis. Conversely, higher *PLCD1* expression combined with lower expression of *GLRX3* and *ORAI1*, or with higher expression of *TRPM8*, *CALM2*, *SOD2*, and TXNRD1, were associated with improved patient survival. Additionally, lower PLCD1 expression alongside higher *CAMK2G* expression is associated with better survival outcomes. Further research is needed to elucidate the mechanisms behind these associations.

GLRX3, encodes Grx3, an iron-sulfur protein vital for cellular response to oxidative stress. This may be triggered by the translocation of Grx3 to the nucleus under oxidative stress conditions [77]. Glrx32 also contributes to maintaining genome stability by regulating DNA-damage response and repair mechanism, activating ATR, a kinase that in turn activates Chk1 and Chk2, crucial for these processes [78]. In BC, the overexpression of *GLRX3* suggests a potential role in the regulation of NF-kB signalling, impacting tumour cell growth and metastasis [79]. *GLRX3* knockout has been shown to downregulate the IKKα/IKKβ/IKKγ complex, a regulator of NF-κB, inhibiting cell proliferation, survival, and invasion *in vitro* [79]. Additionally, the oxidation of NF-κB decreases its DNA binding activity [80]. These findings suggest that *GLRX3* may play a regulatory role in NF-κB activity, supporting its involvement in redox signalling mechanisms. Our findings showed that higher expression of *GLRX3*, combined with lower expression of GLRX2, or with higher expression of *LOXL3*, *PRDX4*, *STIM1*, and *ORAI1* are associated with poor patients prognosis. Conversely, lower expression of *GLRX3* combined with higher expression of *LOXL2*, *PLCD1*, and *ORAI1* are associated with better patients survival.

Some of these findings are supported by other authors. The *GLRX2* gene encodes Grx2, an antioxidant enzyme that contributes to maintaining mitochondrial redox homeostasis and apoptosis induction in response to elevated ROS levels [81]. The combination of low expression of *GLRX2*, which impairs apoptosis, coupled with high expression of *GLRX3*, which modulates NF-κB activity, may synergistically contribute to a poor prognosis in BC patients. The reduced expression of *GLRX2* impairs the apoptosis process, essential for the elimination of cancer cells, while the elevated expression of *GLRX3* modulates NF-κB activity, a key transcription factor involved in cancer progression.

The poor survival of BC patients associated with elevated *GLRX3*, *STIM1*, and *ORAI1* expression remains not fully elucidated. However, it is plausible that the constitutive Ca^{2+} entry induced by

store-operated calcium entry (SOCE)—a complex involving the STIM sensor and ORAI channel [82]—is triggered by ROS elevation. This could explain the necessity for heightened protein expression to sustain tumour cell survival and proliferation.

The synergistic effect between the overexpression of *GLRX3* and *PRDX4* may contribute to enhanced proliferation and tumour cell survival, facilitated by *GLRX3* upregulation, alongside increased angiogenesis driven by *PRDX4* upregulation. *PRDX4* is highly expressed in BC cells and released into the extracellular matrix (ECM), where it mediates cell-cell interactions [83]. Additionally, higher expression of *PRDX4* is associated with lower expression of *GLRX2* and higher expression of *GLRX2*, *NOX4*, *LOXL3*, and *LOXL2*, resulting in poor patient survival. Conversely, lower expression of *PRDX4* combined with higher expression of *LOXL2* showed a better prognosis for BC patients.

Prx4 primarily functions as an antioxidant, which can activate NF-κB, a transcription factor that stimulates the production of the angiogenic factor VEGF in BC cells [84,85]. The varied prognosis associated with the upregulation and downregulation of *GLRX2* may be influenced by different breast cancer subtypes, highlighting the need to investigate apoptosis-related mechanisms regulated by *GLRX2*. Furthermore, elevated levels of *NOX4* increase cellular ROS, activating the transcription factors NF-κB and Nrf2. These transcription factors are crucial for cancer cell antioxidative defense, leading to the upregulation of antioxidant enzymes and contributing to proliferation, angiogenesis, apoptosis suppression, and tumour cell metastasis [86–89].

As described, the impact of many of these combinations of gene expression dysregulation in BC must be experimentally verified, as their mechanisms remain undefined. Nonetheless, leveraging existing mechanistic data on individual proteins allows for speculation about potential mechanisms that might explain the observed effects on patient outcomes when both genes exhibit dysregulation.

IV.4.3 Gene Ontology Analysis

Genes either positively or negatively correlated with the sets of gene combinations were identified using UALCAN. From these, genes commonly correlated with both members of each gene pair shown in Figure 5 were determined. An enrichment analysis was conducted to identify the biological processes associated with these correlated genes. This analysis identified 11 gene pairs with more than 10 correlated genes each (Figure 6 Venn diagrams for details: *GLRX3.PRDX4*, n=257; *GLRX2.GLRX3*, n=111; *GLRX2.PRDX4*, n=85; *CALM2.GLRX2*, n=60; *GLRX2.TXNRD1*, n=74; *TXN.TXNRD1*, n=72; *GLRX3.ORAI1,* n=27; *NOX4.TXNRD1*, n=88;

NOX4.CAMK2G, n=59; *GLRX3.LOXL2*, n=13; *CAMK2G.TXNRD1*, n=810). The functional enrichment analysis, focusing on GO-Biological processes, was performed on the correlated genes (a complete list is available in Supplementary File 2). For several pairs, significant enrichment was observed in two primary biological processes: i) cell cycle regulation (Figure 6A) and ii) cell adhesion and projection (Figure 7A). The GO analysis of genes co-dysregulated with *GLRX2.GLRX3*, *GLRX2.PRDX4*, *GLRX3.PRDX4*, *GLRX2.TXNRD1*, *GLRX3.ORAI1*, *CALM2.GLRX2,* or *TXN.TXNRD1* strongly indicates their involvement in cell cycle regulation (Figure 6A). The overlap of genes in these enriched combinations (Figure 6B) shows that most are upregulated in BC, with some genes appearing in multiple gene pairs.

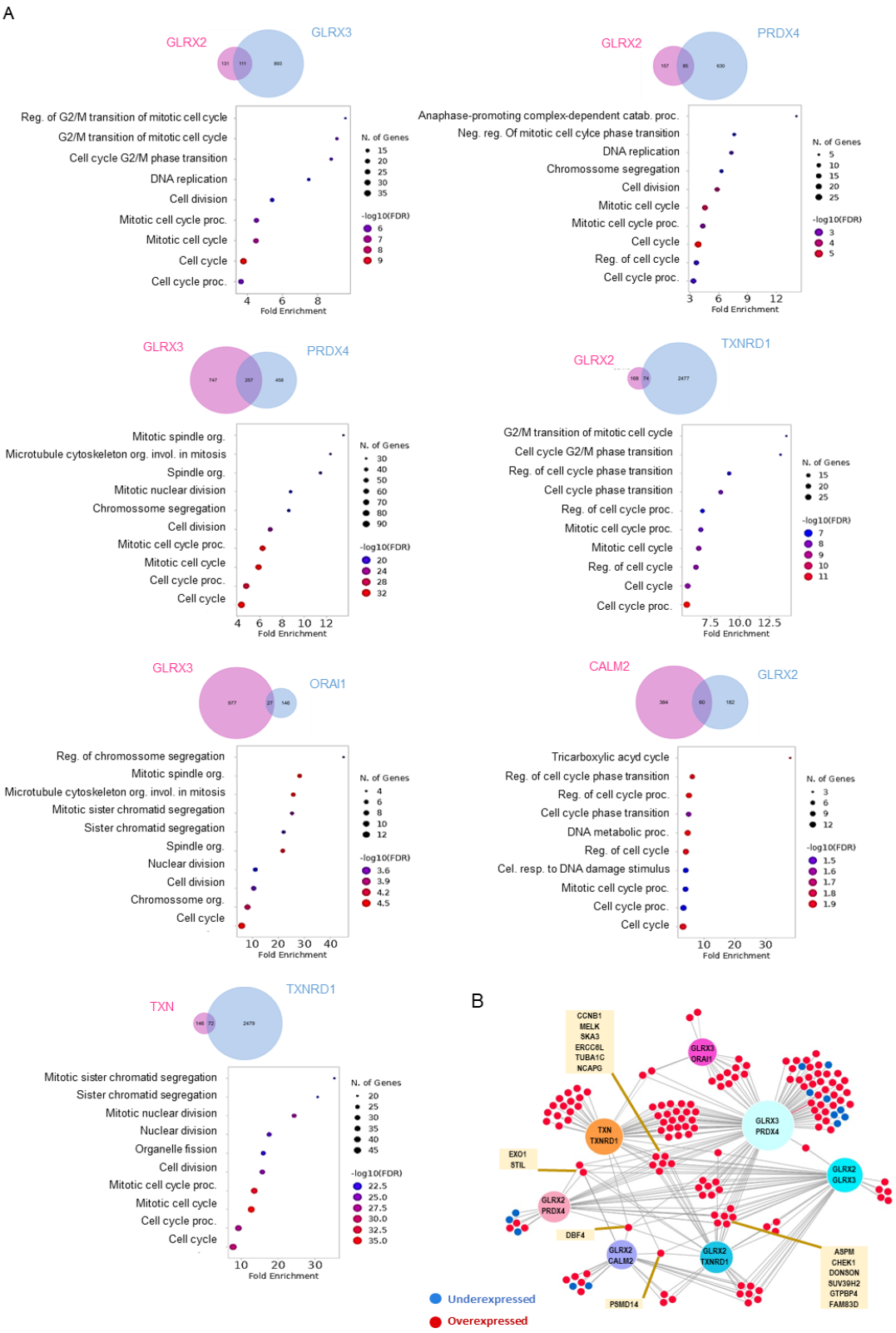

Figure 33. Cell cycle regulation as a biological process associated with co-dysregulated genes. *A) Genes with expression correlated to both elements of the pair were identified using Venn´s diagrams and used for an enrichment analysis. B) Co-expressed genes associated with cell cycle regulation were combined to identify overlapped genes between groups. Overexpressed genes are colored red while under-expressed genes are colored blue. Genes overlapping between more than three pairs are highlighted.*

The second cluster identified comprises the enrichment of the NOX4.TXNRD1, CAMK2G.NOX4, and GLRX3.LOXL2 gene pairs. The findings indicate a possible link between these genes and processes related to cell adhesion and projection, including the regulation of GTPase activity, cellular component biogenesis, and adhesion to substrates. Analysis of the overlapping enriched genes (Figure 7B) showed that the majority are downregulated in BC patients.

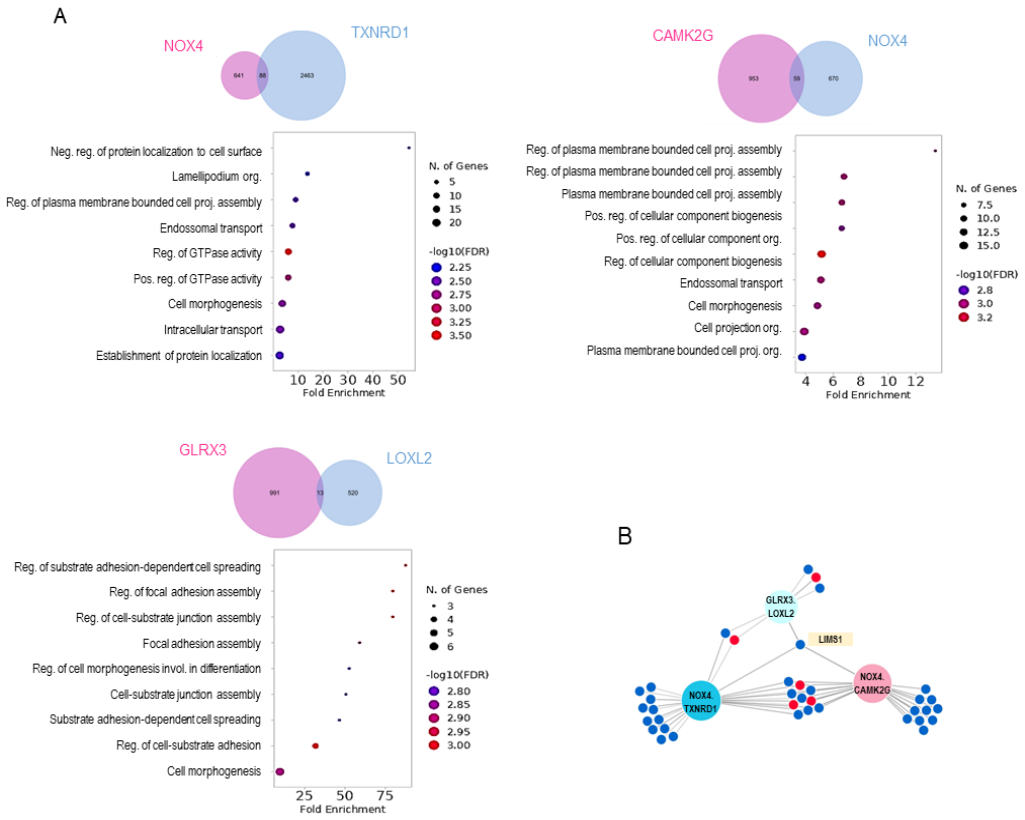

Figure 34. Adhesion and cell projection mechanisms as biological processes associated with co-dysregulated genes. *A) Co-expressed genes were combined using Venn´s diagrams and those which are overlapped were used for an enrichment analysis. B) Co-expressed genes associated with adhesion and cell projection mechanisms were combined to identify overlapped genes between selected gene pairs. Genes in red are overexpressed, and genes in blue are under-expressed in BC patients.*

Dysregulation of the cell cycle and adhesive properties of cancer cells significantly influence key cancer progression-associated hallmarks [90,91]. The associations observed between the gene pairs identified and these processes underscore their potential as biomarkers for personalized medicine or as targets for pharmacological intervention.

Further investigation into the biological mechanisms driving these correlations is crucial. Although this approach is promising, it requires experimental validation to assess the practical utility of these gene pairs as therapeutic targets or biomarkers. It is important to note that the

bioinformatic analysis performed relied on mRNA expression data, whereas protein expression and activity may not always correlate directly with mRNA levels.

IV.5 Conclusion

Several Ca^{2+} and redox-related genes have been previously identified as potential biomarkers or drug targets in BC. Our approach sheds light on the potential significance of expression combined dysregulation of gene pairs involved in Ca^{2+} and redox homeostasis and signalling, offering a more refined understanding of the BC disease mechanisms. Analyzing gene pairs revealed interactions and co-regulatory mechanisms that may have been overlooked when considering isolated dysregulated genes. This approach helps to identify synergistic effects and compensatory pathways, which can enhance biomarker discovery and support the development of combination therapies. By focusing on Ca^{2+} and redox-related gene pairs, we can uncover new functional relationships and network dynamics, providing deeper insights into BC molecular complexity. Ultimately, this approach may promote more effective and personalized treatment strategies, advancing the field of precision oncology.

IV.6 References

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Chapter V – Lysyl oxidases inhibitors for breast cancer treatment: bioinformatic clues on the importance of selectivity

Part of the work described in this chapter was conducted in collaboration with another PhD student and resulted in the publication of the following article:

Ramos S*, Ferreira S*, Fernandes AS, Saraiva N. Lysyl Oxidases Expression and Breast Cancer Progression: A Bioinformatic Analysis. Front. Pharmacol. 2022;13:883998. https://doi.org/10.3389/fphar.2022.883998 (* shared first authorship)

V.1 Introduction

Breast cancer is the most prevalent cancer among women, with around 2.6 million new cases diagnosed each year [1]. While early-stage, non-metastatic BC can be effectively treated, resulting in cure rates of 70-80%, advanced BC with distant metastases remains difficult to treat, leading to significantly lower patient survival rates [2]. This disparity highlights the importance of considering the molecular heterogeneity of BC in treatment decisions, which has been shown to improve patient outcomes. Within this context, the development of new therapeutic strategies should also take into account the diverse molecular characteristics of BC [3].

The human Lysyl oxidase (LOXs) family, which includes LOX and its related LOX-Like enzymes LOXL1-LOXL4, plays a crucial role in catalyzing the cross-linking of elastin and collagen in the extracellular matrix (ECM) [4]. Alterations in the expression of these genes can disrupt ECM remodeling, leading to changes in tissue stiffness and subsequently affecting cancer cell proliferation, survival, invasion, migration, and epithelial-to-mesenchymal transition, all of which are critical to tumour progression [5]. Evidence has linked the dysregulation of LOXs enzymes to metastasis and survival rates in various cancers [6–10], suggesting that these enzymes could be targeted to prevent BC metastasis [8,11].

Several inhibitors have been developed to target enzymes from the LOXs family, with varying specificity for LOXL2, LOX/LOXL2, LOXL2/LOXL3, or pan-LOX inhibition [8]. However, it remains unclear how selectively inhibiting each of these enzymes might impact different BC subtypes.

V.2 Objective

Using a bioinformatics approach using data from The Cancer Genome Atlas (TCGA) Breast Invasive Carcinoma (BRCA) project, this study aims to examine the association between the mRNA expression levels of each LOX gene and BC patient survival, as well as the co-expression of genes associated with cancer progression and the correlation with tumour infiltrates across BC subtypes. Ultimately, this analysis aims to guide the development of novel LOXs inhibitors with specificity profiles tailored to target specific BC cancer subtypes more effectively.

V.3 Methodology

V.3.1 Impact of LOXs family genes expression on Breast cancer patient survival

To assess the significance of each LOXs gene in BC and in their subtypes (Basal-like, HER2+, Luminal A, and Luminal B), a bioinformatics analysis was adopted utilizing the mRNA expression data from the TCGA BRCA dataset. The expression patterns of LOXs family enzymes in BRCA and its subtypes, as well as their association with patient survival, were analyzed using GEPIA2 (http://gepia2.cancer-pku.cn/#analysis) [12,13].

To determine the differential expression between normal and tumour tissues the one-way ANOVA test was used. The analysis of the association of each LOXs expression level on the prognosis of BRCA patients was performed using Kaplan-Meier survival analysis and the Cox Proportional Hazard (PH) Model, with median cut-offs and both overall survival (OS) and disease-free survival (DFS). Heatmaps were developed to present Cox proportional hazard ratios (HR) for high expression, and differences between Kaplan-Meier survival curves were evaluated using the Log-rank test.

V.3.2 Analysis of functions of correlated genes

To investigate the association between LOXs expression and other genes, the top one hundred genes with the highest Pearson correlation for each LOXs were identified from the TCGA BRCA dataset, utilizing GEPIA2 and UALCAN – a web tool for analysis of TCGA gene expression data (http://ualcan.path.uab.edu) [14]. Non-protein-coding mRNAs were excluded from the analysis, and only genes that appeared consistently on both platforms were considered. Pearson correlation values were obtained from GEPIA2. Each selected gene was functionally categorized using data from GeneCards and UniProt, and then grouped into four categories: i) cell migration, adhesion, and ECM regulation; ii) cell survival and proliferation; iii) angiogenesis and tumour progression; and iv) others (other functions).

Additionally, the ShinyGO platform (http://bioinformatics.sdstate.edu/go/) [15] was used in selected genes to conduct biological processes from Gene Ontology (GO) enrichment analysis. Pathways with at least ten genes and a False Discovery Rate (FDR) below 0.05 were defined as significant. The top ten pathways were selected, based on FDR and sorted by fold enrichment. This approach allowed for the identification of the most pertinent pathways linked to LOXs-correlated genes.

V.3.3 Association between LOXs gene expression and immune infiltrates in Breast cancer

The relation between LOXs gene expression and the abundance of immune infiltrates in BRCA and its subtypes was assessed using TIMER2.0, a TCGA-based data platform (http://timer.cistrome.org/) [16]. Spearman's correlations were calculated utilizing the deconvolution method Estimate the Proportion of Immune and Cancer Cells (EPIC) [17] and the marker gene-based Microenvironment Cell Populations-counter (MCP-counter) [18]. EPIC predicts the frequency of each cell type, comparing the level of expression of genes in a tumour with a library of the gene expression profiles from specific cell types found in tumours [17]. MCP-counter selected a set of genes characteristic for a cell type and quantified each independently, aggregating them into an abundance score of multiple immune and non-immune stromal populations [18]. The Spearman correlations were plotted in a heatmap, considering $p<0.05$ as significant.

V.4 Results and Discussion

V.4.1 LOXs family gene expression and association with Breast cancer patient survival

To assess the expression levels of *LOX* and *LOX-like* (*LOXL1-4*) genes in normal and BC tissues, mRNA data from the TCGA BRCA dataset were analyzed. The results show that while LOX expression remained relatively unchanged, *LOXL1*, *LOXL2*, and *LOXL3* were significantly elevated in BC tissues compared to normal tissues, particularly in Luminal A and Luminal B subtypes (Figure 1A). Among these, only *LOXL1* showed statistically significant differences between expression in normal and cancerous tissues across BRCA, Luminal A, and Luminal B subtypes. Previous research has highlighted the upregulation of *LOXL2* and *LOXL3* in invasive or metastatic BC cells compared to non-invasive or non-metastatic counterparts, supporting their critical roles in BC progression [6,19]. Clinical and preclinical data further indicate that elevated *LOXL2* expression correlates with the invasiveness of basal-like BC cells [9].

In contrast, *LOXL4* mRNA levels are significantly lower in BC tissues and in subtypes compared to normal tissues (Figure 1A). Given the involvement of LOXs enzymes in cancer-related cellular processes, their impact on patient survival was examined. As expected, the association between *LOX* expression and DFS was more significant than between OS (Figure 1B). The analysis revealed that the upregulation of *LOXL2* and also *LOXL1* generally correlated with poorer patient outcomes, indicated by an increased hazard ratio (HR), while *LOXL4* upregulation appears to have a protective effect associated with lower HR. Specifically, higher levels of *LOXL1*, *LOXL2*,

and *LOXL3* are associated with an increased HR, with LOXL3 upregulation showing a significant association with Luminal A, while *LOXL2* more strongly associates with outcomes in the Basal subtype (Figure 1B). A previous study showed that elevated levels of *LOXL2* in basal-like BC are associated with poor prognosis and distant metastasis [9].

Conversely, *LOXL4* downregulation can positively influence both OS and DFS, particularly offering potential protective effects in the HER2+ subtype (Figure 1B). The role of LOXL4 in cancer biology is complex, with its upregulation or downregulation leading to either progressive or repressive effects depending on the cancer type, context, and tumour stage [20,21]. Studies in various cancer models suggest that *LOXL4* downregulation is generally linked with tumour progression, enhanced growth, and metastasis [20,22], in particular in BC [23,24]. Despite the unclear role of LOXL4, lower expression is linked to ECM remodeling, including collagen synthesis, deposition, and structural alterations, which in turn promote tumour growth and metastasis, correlating with poor clinical outcomes in TNBC [24].

The analysis of LOX impact across different BC subtypes is somewhat limited by sample size, particularly in subgroups like HER2+ where only 66 samples are available, hence caution should be taken when interpreting these results.

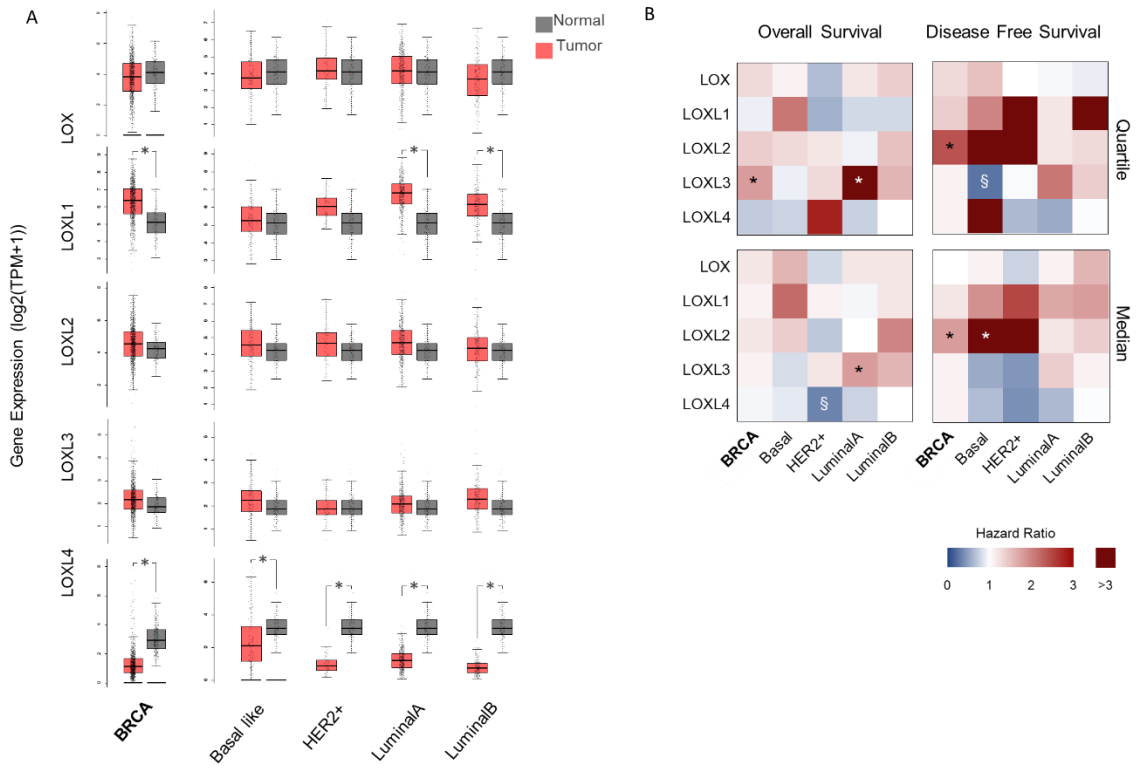

Figure 35. LOXs family gene expression and impact on breast cancer patient survival. *(A) LOXs mRNA expression profiling comparative analysis between BRCA (n=1085) or its subtypes (Basal-like n=135; HER2+ n=66; Luminal A n=415; and Luminal B n=194) and normal (n=112) tissue samples. *p < 0.05 (one-way ANOVA). (B) Hazard ratio of LOXs increased expression in patient overall and disease-free survival calculated from Kaplain-Meier curves using the Cox PH Model. Hazard ratios using LOXs expression median or quartiles were calculated for BRCA and its subtypes (median cut-off: nBRCA=535, nBasal-like=67, nHer2+=31; nLuminal A=206, nLuminal B=95; quartile cut-off: nBRCA=268; nBasal-like=34; nHer2+=15; nLuminal A=103; nLuminal B=48). *p < 0.05, §p ≤ 0.055 (Logrank test)*

V.4.2 Correlation between LOXs expression and other genes in Breast cancer: LOX, LOXL1-2 follow a distinct trend from LOXL3-4

To assess the correlation between altered LOXs enzymes expression and the mRNA levels of other genes in BC, the genes most closely correlated with LOXs expression were identified using their correlation coefficients (Figure 2A). These genes were categorized based on their involvement in processes related to cancer progression, particularly in cell migration, adhesion, ECM regulation, and angiogenesis (Figure 2B). Given the role of LOXs enzymes in ECM remodeling, it was expected that changes in their expression would significantly influence the expression of genes linked to these pathways.

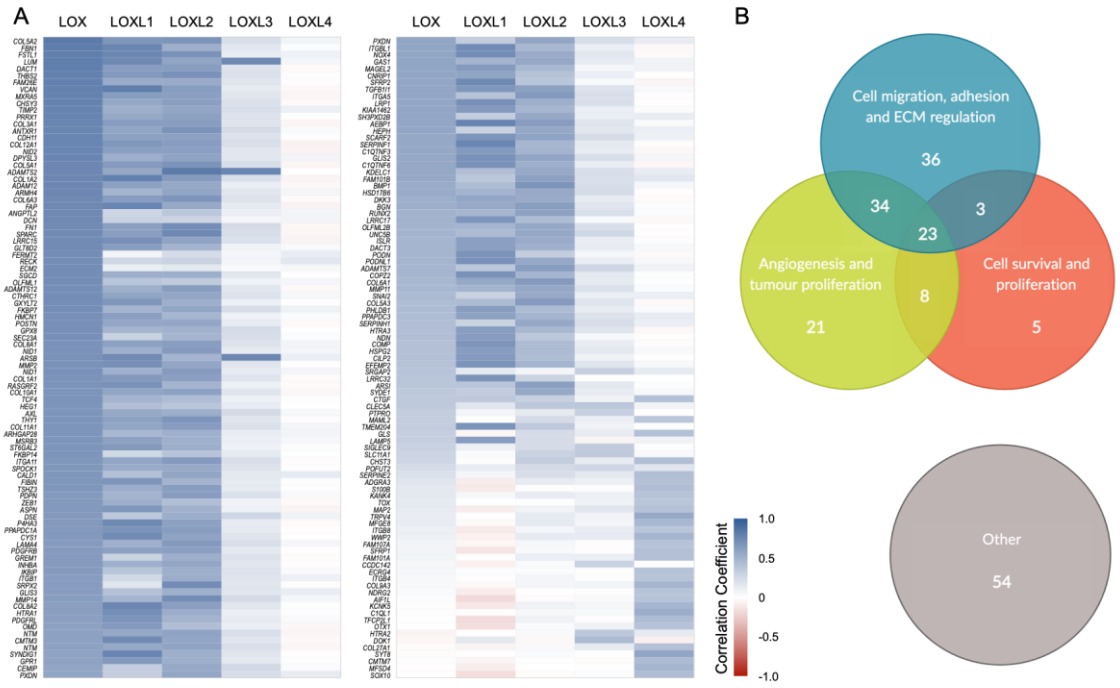

Figure 36. Correlation between the expression of LOXs and other genes in breast cancer. *(A) Heat map showing Pearson´s correlation coefficients between mRNA expression of LOXs family genes and other genes. For each LOXs, the one hundred most strongly correlated genes in BC were collected from GEPIA and UALCAN, and only those common to both platforms were selected. The blue gradient represents a positive Pearson´s correlation and the red gradient a negative correlation. (B) Genes were clustered according to their described functions, highlighting specific groups relevant to BC progression-related events.*

Interestingly, *LOX*, *LOXL1*, and *LOXL2* generally showed similar correlation patterns, suggesting they may have a more similar effect on cancer cells. Furthermore, a moderate and strong positive correlation was found between these LOXs genes and ECM-related genes, such as Platelet-derived growth factor receptor beta (*PDGFR-β*), Matrix metalloproteinases 2 and 14 (*MMP-2*, *MMP-14*), Tissue inhibitor of metalloproteinases 2 (*TIMP2*), Fibronectin 1 (*FN1*), Integrin Subunit Alpha 5 (*ITGA5*) and Zinc Finger E-Box Binding Homeobox 1 (*ZEB1*). MMP-14 is widely expressed in cancer cells and drives tumour progression by remodeling the basement membrane, promoting invasion, angiogenesis, and fibrous tissue expansion [25]. MMP-14 overexpression in TNBC correlates with tumour progression and metastasis [25]. MMP-2 strongly participates in BC metastatic cascade due to its ability to cleave a large repertoire of matrix and non-matrix substrates [26]. Its inhibitor, TIMP-2, has contradictory roles. On one hand, it acts as a suppressor of growth and metastasis through modulation of the EMT in TNBC cancer [27], but on the other hand, it increases MMP-2 and decreases TIMP-2 levels playing an important role in lymph node invasion [28].

In TNBC, a hypoxic environment also promotes the expression of *ITGA5* and its ligand, *FN1*, induced by *LOX* overexpression [29]. This dysregulation induces collagen crosslinking and fibronectin fibril assembly which reduce drug penetration into tumour cells [29]. Also regulated by hypoxia, high levels of *ZEB1* expression, downregulate endogenous E-cadherin and induce EMT in BC cell lines, promoting the conversion of tumour cells to invasive and metastatic phenotypes [30,31]. This evidence suggests that ZEB1 has the potential to directly inhibit E-cadherin, which correlates with poor prognosis and survival of cancer patients [31].

Conversely, *LOXL4* often exhibited an opposing correlation trend, indicating it might play a distinct or even opposing role in BC. *LOXL3*, however, did not show strong correlations with any specific gene groups within this context. These observations are consistent with the observations from *LOX* dysregulation, where *LOXL4* upregulation tends to be opposite to those seen with *LOX*, *LOXL1*, and *LOXL2* upregulation, and *LOXL3* has a relatively minor impact.

These differences among *LOXs* genes were further validated by functional enrichment analysis (Figure 3). The analysis revealed that *LOX*, *LOXL1*, and *LOXL2* are primarily correlated with genes involved in ECM organization, extracellular structure organization, and external encapsulating structure organization. These findings align with the known roles of these LOX family members. LOX, LOXL1, and LOXL2 are well-established in their involvement in ECM organization and structural integrity, which is reflected in their strong correlation with genes related to these processes. Conversely, *LOXL4* demonstrated a strong correlation with genes associated with the central nervous system, though it exhibited lower fold enrichment scores and moderate FDR values, suggesting a more specialized or context-dependent function. The limited number of genes correlated with *LOXL3* did not allow to conduct a functional enrichment analysis.

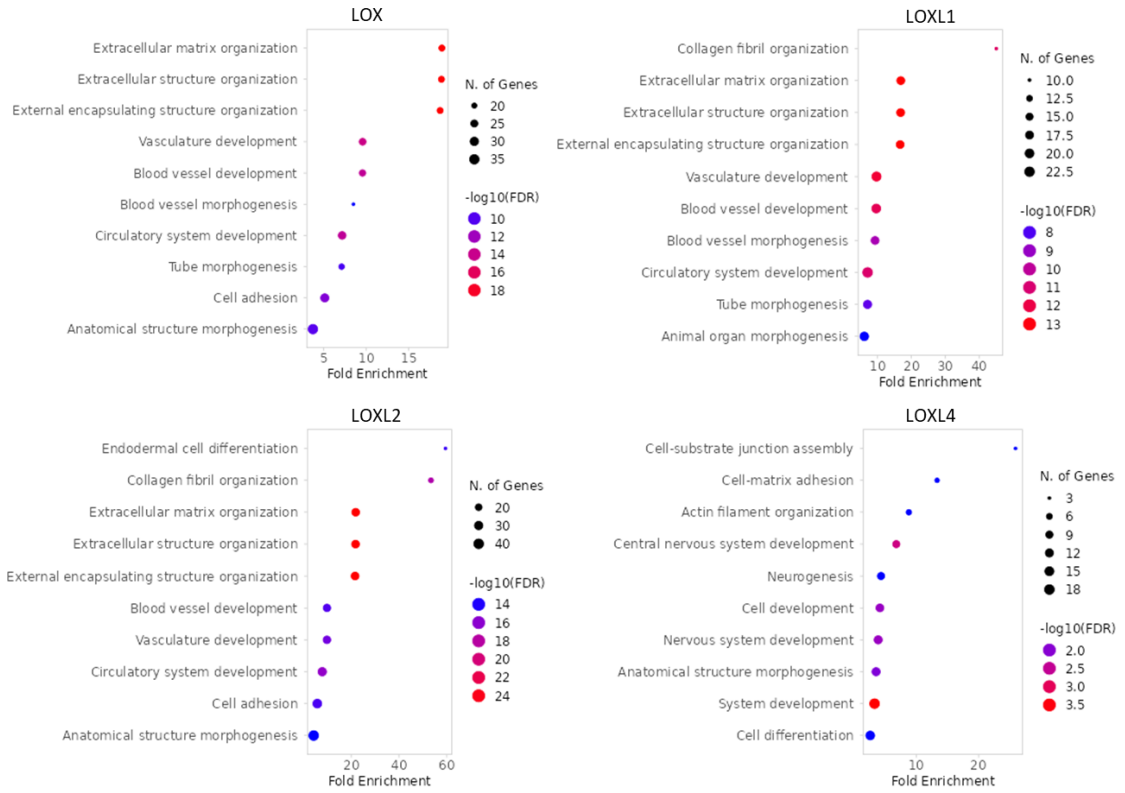

Figure 37. Functional enrichment analysis of LOXs family genes*. Genes whose expression correlated with each LOXs gene (LOX, LOXL1, LOXL2, and LOXL4) were identified and analyzed using an enrichment analysis based on biological processes from Gene ontology. The enrichment analysis was conducted by selecting pathways by FDR and sorting by fold enrichment.*

V.4.3 Influence of LOXs family gene expression in Breast tumour infiltrates

The tumour microenvironment is crucial in cancer progression and given the role of the LOXs family in ECM remodeling, it is likely that these enzymes influence the tumour infiltrates landscape. Research has demonstrated that LOX released in the hypoxic environment of invasive BC can attract inflammatory cells to distant locations, facilitating the creation of premetastatic niches [32].

To investigate how LOXs affect the BC microenvironment, immune deconvolution of gene expression-based methods were used to assess tumour-infiltrating immune cells (Figure 4). Data showed that higher expression of LOXs enzymes correlates with increased cancer-associated fibroblasts (CAFs) across all BC subtypes, particularly with *LOX*, *LOXL1*, and *LOXL2* overexpression, compared to *LOXL3* and *LOXL4*.

In Luminal A and Luminal B, a positive association was found between *LOX*, *LOXL3*, and *LOXL4* expression and the increase of macrophages and monocytes. Conversely, in general, *LOX*, *LOXL1*, and *LOXL2* expression were negatively associated with B, TCD4+, and T CD8+ cell infiltration.

This is supported by earlier studies showing that elevated levels of tumour-infiltrating lymphocytes are linked to a poor prognosis in BC [33].

LOXL3 and *LOXL4* had a less pronounced effect on immune infiltrates, particularly on B and T cells. Nevertheless, a positive correlation was observed between *LOXL4* expression and macrophage infiltration [23]. In general, the upregulation of *LOX*, *LOXL1*, and *LOXL2* led to a distinct tumour cell infiltrate profile compared to *LOXL3* and *LOXL4*. The patterns of tumour infiltration associated with *LOX*, *LOXL1*, and *LOXL2* were closely aligned and differed significantly from those linked to *LOXL3* and *LOXL4*.

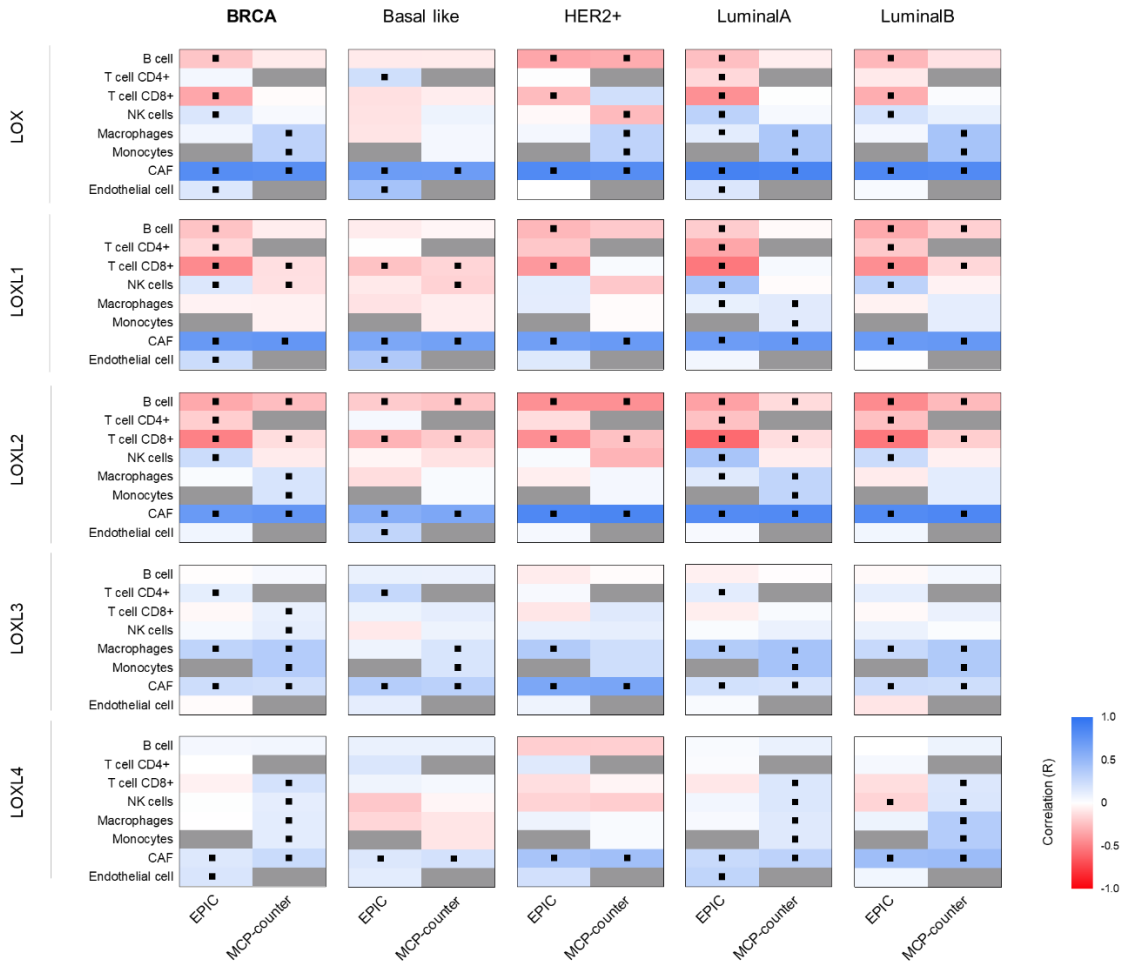

Figure 38. Impact of LOXs expression in breast cancer tumour infiltrates. *The correlations between the expression of LOX family members and the abundance of immune infiltrates in BC and its subtypes were calculated based on immune deconvolution methods. The Estimate of the Proportion of Immune and Cancer Cells (EPIC) and the marker gene-based Microenvironment Cell Populations-counter (MCP-counter) algorithms were used. Blue gradient represents a positive Spearman´s correlation and red gradient a negative correlation, ■ p<0.05 (Spearman). [nBRCA=1100; nBasal-like=191; nHer2+=82; nLuminal A=568; nLuminal B=219].*

V.5 Conclusion

Several molecular mechanisms driving BC invasion and metastasis remain unclear. Given the significant impact of metastasis on patient outcomes, there is a strong need for therapies that take into account the high molecular heterogeneity of tumours. In this context, LOX and LOXL1-4 emerge as promising targets for modulating BC progression. The data presented emphasize the importance of the dysregulation of these enzymes and their association with patient survival and relapse. In particular, elevated expression of *LOX*, *LOXL1*, and *LOXL2* are associated with similar patterns in patient survival, tumour infiltration, and interactions with genes involved in cancer processes. In contrast, *LOXL3* shows minimal impact, and *LOXL4* exhibits contrary effects. Research has shown that, unlike *LOXL2*, the overexpression of *LOXL4* can inhibit cancer growth and progression in various models [20,22,24]. Thus, our findings align and further support the known roles of LOX family enzymes in cancer.

Current therapeutic strategies mainly focus on inhibiting the enzymatic activity of LOX and LOXL2 members with small molecule inhibitors of varying selectivity. However, a well-defined approach for creating selective inhibitors developed for specific therapeutic needs is still lacking. This study helps to address this gap for BC. We hypothesize that while targeting LOXL3 might have variable effects depending on the BC subtype, focusing on the therapeutic inhibition of LOXL1 and LOXL2, rather than LOXL4, could be more advantageous and effective. These differences should guide the development of new LOX inhibitors for BC treatment.

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Chapter VI – Association between redox regulation genes and immune infiltrates in HER2+ breast cancer

The work described in this chapter was conducted in collaboration with another PhD student and will be submitted for publication:

Luz P*, Ramos S*, Oliveira MJ, Saraiva N, Costa JG, Fernandes AS. Interaction between redox regulation, immune activation and response to treatment in HER2+ breast cancer. (* shared first authorship)

VI.1 Introduction

HER2 is overexpressed in 40-60% of ductal carcinomas *in situ* and 15-30% of BC, mostly due to gene amplification, with rare cases linked to mutations or other chromosomal changes [1,2]. Monoclonal antibodies targeting the ligand-binding receptor domain of this plasma membrane receptor are part of the standard treatment, but not all patients respond, highlighting the need for new therapeutic approaches [3,4].

The role of TILs in BC is gaining attention, but the relationship between TILs and ROS still requires further research. Tumours often show a redox imbalance due to increased production and accumulation of ROS attributable to heightened metabolic activity of cancer cells. This oxidative stress significantly impacts the TME, affecting the survival and function of endothelial cells, fibroblasts, and immune cells [5].

ROS can act as a chemoattractant, drawing various immune cells to the tumour site [6–8]. However, depending on their concentration, ROS can either promote or inhibit an effective inflammatory response [9,10]. The balance between ROS production and consumption is a key factor in regulating T cell apoptosis, activation, differentiation, proliferation, and function, although the exact mechanisms remain unclear [11].

At low to moderate levels, ROS are essential for T-cell activation, growth, and function. They activate pathways like MAPK/mTOR/AMPK, which support T-cell survival and activation [12]. ROS also increases Ca^{2+} levels, triggering the NFAT, a transcription factor that boosts IL-2 production, leading to T-cell proliferation [13]. Mitochondrial ROS regulate IL-2 and IL-4 expression, further controlling T-cell activity [14]. When ROS are suppressed by antioxidants or NOX inhibitors, Treg cells decrease, allowing CD8+ T cells to become more active [15]. Reduced ROS levels also decrease phosphorylation of JNK and NF-κB, impacting CD8+ T cell function [16].

On the other hand, high levels of ROS can disrupt the immune response by interfering with T cell receptor recognition of proteins in the main histocompatibility complex. They also increase the expression of immune checkpoint proteins like PD-1 on T cells and its ligand PD-L1 on cancer cells, which hampers T cell activity [17,18].

ROS impact different T cell subpopulations in distinct ways. At high levels, ROS can activate Tregs, which can suppress the harmful overactivity of CD8+ T cells. Low ROS levels are linked to a more protective inflammatory response, while high ROS levels promote an anti-inflammatory immune environment. This suggests that targeting ROS could help reduce Treg activity and alter the immunosuppressive environment [19].

High ROS levels are also linked to increased tumour diversity, bringing in pro-inflammatory cells like M1 macrophages, CD4+ T cells, CD8+ T cells, and B cells, as well as anti-inflammatory cells like M2 macrophages and Tregs. Notably, a higher ROS score, determined based on the ROS-related gene expression in BC patients, tends to correlate with poorer overall survival in patients with ER+/HER2- BC, but not in other BC subtypes [20]. Significantly, research on the impact of oxidative stress on the immune system and the prognosis of HER2+ BC patients is limited, highlighting a crucial gap in the field.

VI.2 Objective

This study aims to identify redox-related genes that are differentially expressed in HER2+ BC and explore their relationship with patient survival and the abundance of immune cells in the TME.

VI.3 Methodology

VI.3.1 Impact of redox-regulated genes expression on HER2+ Breast cancer patients survival

A list of 727 genes was extracted from Gene Ontology (GO) using the keywords “oxidation,” “redox,” and “oxidase.” The TCGA HER2+ Breast Invasive Carcinoma (BRCA) dataset was employed to examine the expression levels of these genes and their association with patient survival. Gene expression data from both tumour and normal tissues, as well as the association between the expression of each gene and overall survival, were assessed using GEPIA2 (http://gepia2.cancer-pku.cn/#index) [21,22]. Genes were categorized into low and high-expression groups based on the median gene expression level for Kaplan-Meier survival analysis. Statistical significance was evaluated using a Log-rank test ($p<0.05$), and hazard ratios (HR) were determined through a Cox proportional hazards model.

For the 87 genes that significantly impacted HR, differential expression between HER2+ tumour and normal tissues was analyzed (Figure 1). Welch's t-test was used for this analysis, performed through the TCGA-UALCAN platform (http://ualcan.path.uab.edu/index.html) [23]. Genes with ratios between tumour and normal tissue expression lower than 0.8 or above 1.2 and with statistically significant differences ($p<0.05$) identified by Welch's t-test, were selected.

VI.3.2 Association between the expression of redox-regulated genes and immune infiltrates in HER2+ Breast cancer

The TCGA-based TIMER2.0 platform (http://timer.cistrome.org/) [24] was employed to assess the relationship between the expression of the 55 selected genes and the levels of immune cell infiltration in HER2+ BC patients. The TIMER algorithm, adjusted for tumour purity, was used to estimate the abundance of tumour-infiltrating cells. This algorithm calculates the abundance of six types of immune cells based on a scoring system using linear least squares regression [25]. Statistical significance was determined with a p-value of <0.05 using the Spearman correlation coefficient (Figure 1).

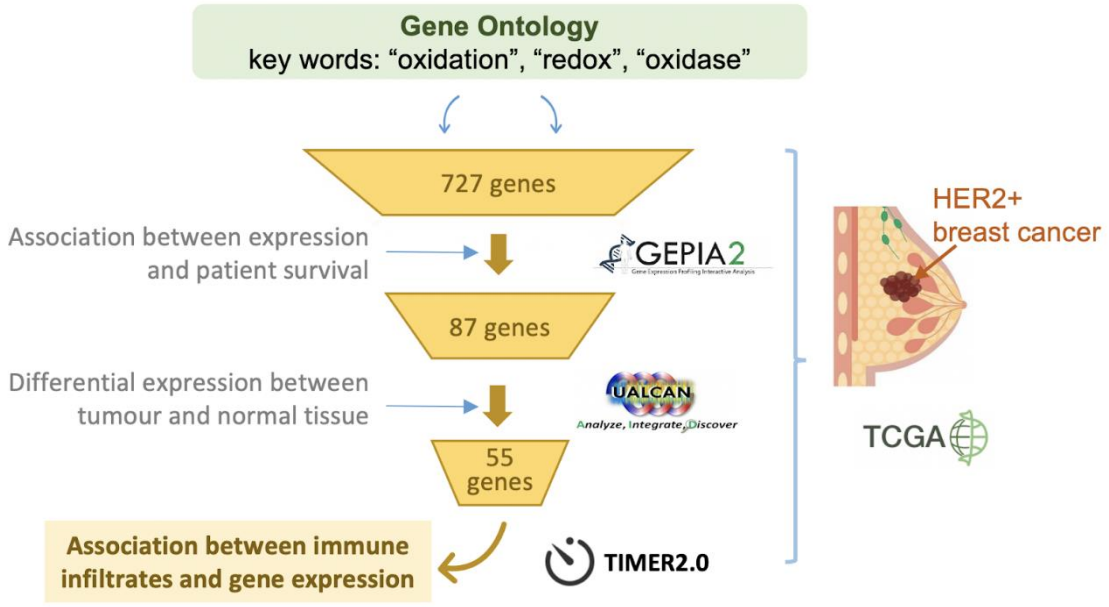

Figure 39. Methodological approach used to explore the associations between the expression of redox-related genes with HER2+ BC patient survival and with tumour infiltrates composition.

VI.4 Results and Discussion

The precise mechanisms and proteins involved in redox regulation that affect HER2+ BC progression are not well understood. To contribute to fill this gap, the TCGA BRCA HER2+ mRNA expression dataset was used to identify redox-related genes that are dysregulated in these tumours and associated with patient survival (Figure 2). Although the HER2+ subset of the TCGA BRCA dataset had a limited number of samples, 53 redox-related genes were identified. Among these, 43 genes with higher expression were associated with increased HR, while only 10 genes were linked to a lower HR.

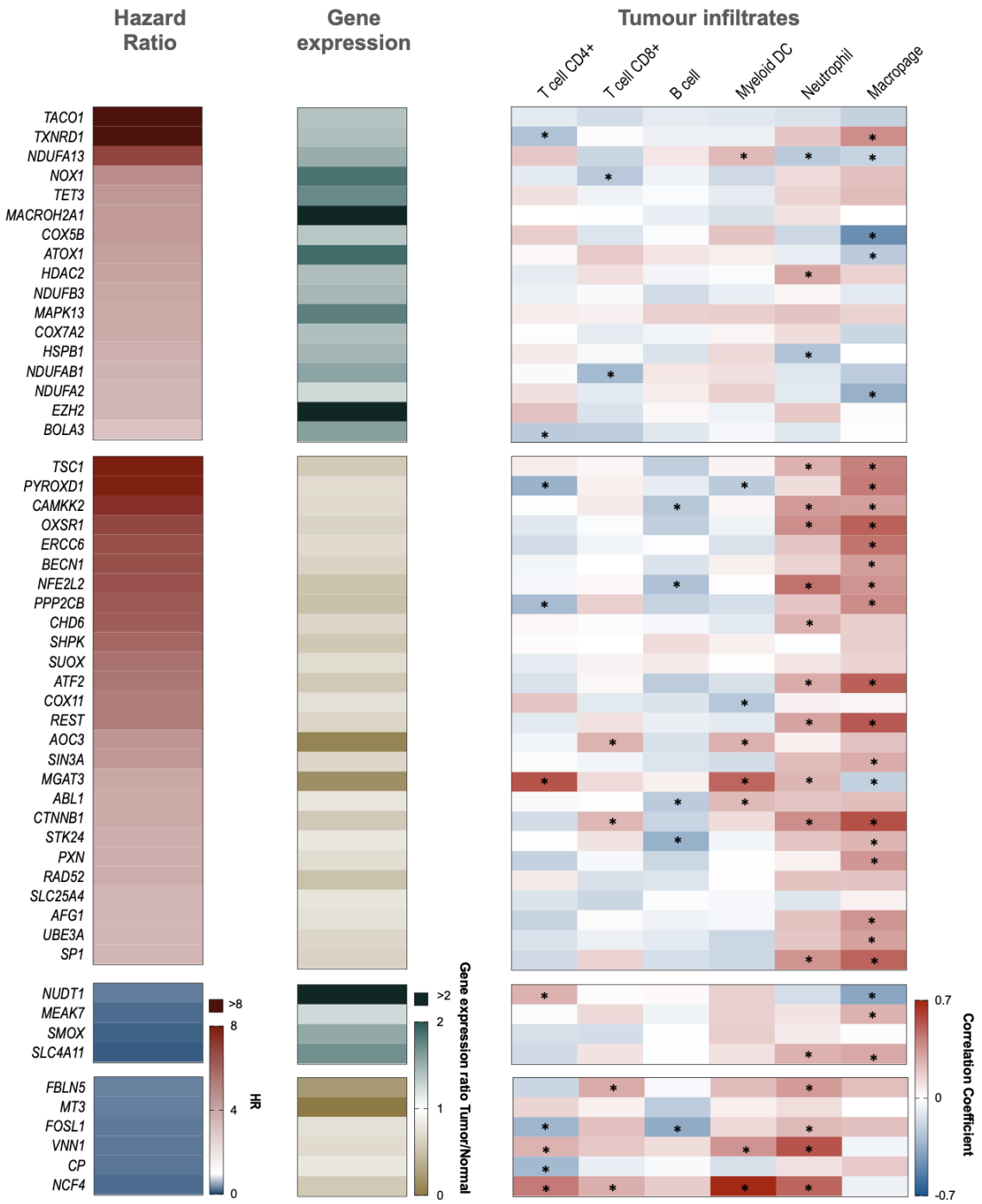

Figure 40. Identification of genes related to redox homeostasis that are relevant for HER2+ BC. *The list of 55 genes with expression dysregulation that significantly impact HER2+ BC patient survival is depicted. For each gene, the values corresponding to the hazard ratio, gene expression dysregulation, and impact on the estimated abundance of different tumour infiltrate cell populations are represented in heatmaps. *p<0.05 from Spearman correlation*

Among the 53 genes identified, only a few have been experimentally validated for their impact on HER2+ BC. For example, Enhancer of Zeste 2 Polycomb Repressive Complex 2 Subunit (*EZH2*) is notably upregulated in HER2+ BC and is associated with poorer patient survival. EZH2 overexpression correlates with a worse prognosis and more aggressive tumour behavior, as it

drives the activation of ERα, promotes tumour growth, metastasis, and resistance to HER2-targeted therapies [26]. Similarly, *MAPK13* is elevated in HER2+ BC and is linked to increased invasiveness, metastasis, and reduced survival rates [27]. NFE2-Like BZIP Transcription Factor 2 (*NFE2L2*) is downregulated in these tumours and is associated with poorer outcomes. This transcription factor is crucial for cellular antioxidant responses and survival signalling, and its dysregulation can disrupt redox balance and lead to higher oxidative stress [28,29]. Histone Deacetylase 2 (*HDAC2*) is upregulated in HER2+ BC and may be a potential therapeutic target due to its link to higher HR and the relation between its inhibition and the improvement of the effects of HER2-targeted therapies [30]. Macrohistone 2A1 (*MACROH2A1*) promotes HER2 expression in cancer cells [31], while loss of growth inhibitory protein tuberin (*TSC2*) function is associated with resistance to anti-HER2 therapies [32].

Several redox-related genes were found to be significantly dysregulated and associated with altered HR, though many lack specific studies on their role in HER2+ BC. Examples of such genes include: *TACO1*, *PYROXD1*, *CAMKK2*, *AOC1*, *MGAT3*, *NUDT1*, *SLC4A11*, *TXNRD1*, *MT3*, and *NCF4*.

Notably, 17 redox-related genes associated with high HR are expressed at higher levels in HER2+ tumours when compared to normal breast tissue, without a clear association with a particular TME signature to immune cell subpopulations. These genes are *TACO1*, *TXNRD1*, *NDUFA13*, *NOX1*, *TET3*, *MACROH2A1*, *COX5B*, *ATOX1*, *HDAC2*, *NDUFB3*, *MAPK13*, *COX7A2*, *HSPB1*, *NDUFAB1*, *NDUFA2*, *EZH2*, and *BOLA3*. Conversely, 26 high HR-associated genes are downregulated in HER2+ tumours compared to normal tissue and generally show strong correlations with increased macrophage and neutrophil infiltration. These are *TSC1*, *PYROXD1*, *CAMKK2*, *OXSR1*, *ERCC6*, *BECN1*, *NFE2L2*, *PPP2CB*, *CHD6*, *SHPK*, *SUOX*, *ATF2*, *COX11*, *REST*, *AOC3*, *SIN3A*, *MGAT3*, *ABL1*, *CTNNB1*, *STK24*, *PXN*, *RAD52*, *SLC25A4*, *AFG1*, *UBE3A*, and *SP1*.

Among the 10 redox-related genes associated with low HR, four are highly expressed in HER2+ tumours compared to normal tissue (*NUDT1*, *MEAK7*, *SMOX*, *SLC4A11*), while six are downregulated (*FBLN5*, *MT3*, *FOSL1*, *VNN1*, *CP*, *NCF4*). These low HR-associated genes are associated with increased infiltration of macrophages, neutrophils, myeloid dendritic cells, and cytotoxic CD8+ T cells (Figure 2).

Given the potential impact of redox regulation on HER2+ BC progression and therapy resistance, these genes could serve as valuable targets or biomarkers and warrant further investigation. Many of these are already described to be linked to cancer progression in other models, emphasizing their potential relevance for HER2+ BC.

VI.5 Conclusion

The study of tumour-infiltrating immune cells is becoming increasingly important due to their potential to predict patient prognosis and response to therapy. Moreover, moderate levels of ROS play a role in shaping the TME by influencing the recruitment, proliferation, and activation of various immune cells, contributing to a pro-inflammatory environment. This bioinformatic analysis has shown that the altered expression of several redox-related genes may be associated with the presence of specific immune cell populations in the TME and with patient survival. These genes could serve as potential prognostic biomarkers or therapeutic targets, though further experimental research is needed to validate their significance.

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Conclusion remarks

Breast cancer (BC) remains the most prevalent cancer among women, underscoring the urgent need for innovative therapeutic strategies to improve patient survival rates. This research explored the role of ROS and Ca^{2+}-related genes—key players in maintaining cellular homeostasis—as significant contributors to BC progression. Leveraging advanced bioinformatics tools and comprehensive data from TCGA-BRCA, this study aimed to identify individual and combined expression profiles of redox and Ca^{2+} signalling genes with the potential to be used as therapeutic targets or prognosis biomarkers. To achieve these objectives, the study employed a multi-faceted approach, beginning with the identification of suitable bioinformatic tools to analyze TCGA data (Chapter III). Three distinct investigative strategies were then pursued: analyze the interplay between co-dysregulation of redox- and Ca^{2+}-related genes and their roles in BC prognosis (Chapter IV); selective analysis of LOXs family genes in different BC subtypes (Chapter V); and identification of dysregulated redox-related genes in HER2+ BC and their relationship with tumour cellular infiltrates (Chapter VI).

The first phase of the research involved optimizing the parameters and filters of the selected bioinformatics tools, including patient survival analysis settings in GEPIA2, algorithms in TIMER2.0, Gene Ontology filters, and ShinyGO biostatistical methods. This careful optimization ensured that the analyses were both accurate and meaningful.

Recognizing the impact of redox- and Ca^{2+} homeostasis on BC hallmarks, the research hypothesized that the co-dysregulation of genes encoding proteins involved in these regulatory pathways might significantly affect BC patient survival (Chapter IV). By adopting an innovative bioinformatic approach that filtered gene expression combinations based on high and low quartiles, the study identified several gene pairs with potential synergistic effects on patient survival. This approach highlighted the importance of analyzing the combined expression of genes involved in both regulatory mechanisms, which are crucial for cancer progression. Notably, the expression of many of these gene pairs was correlated with genes involved in cancer progression-related processes such as cell cycle regulation, adhesion, and cellular projection. These findings suggest that the interplay between ROS and Ca^{2+} regulatory mechanisms is a promising avenue for identifying biomarkers that could predict disease prognosis and guide tailored therapeutic strategies.

The research also delved into the LOXs family enzymes, analyzing their differential expression in BC and in BC subtypes and their association with disease outcomes (Chapter V). The overexpression of LOXL1, LOXL2, and LOXL3 in BC and their correlation with poorer survival rates, especially in Basal-like, HER2+, and Luminal B subtypes, underscores the therapeutic

relevance of these enzymes. Conversely, the downregulation of LOXL4 and its association with poor outcomes in HER2+ patients further emphasize the complexity of redox-related pathways in BC. These findings emphasize the need to consider the molecular heterogeneity of BC subtypes when developing targeted therapies. They also highlight the therapeutic potential of inhibiting LOXL1 and LOXL2 but not LOXL4, which could lead to more effective interventions.

In its final approach, the research focused on identifying redox-related genes that are differentially expressed in HER2+ BC, with a particular emphasis on their correlation with tumour cell infiltration (Chapter VI). This analysis led to the identification of 55 redox-related genes, several of which were significantly dysregulated, revealing their potential as new therapeutic targets or biomarkers for HER2+ BC. Some of these genes, such as TXNRD1 and EZH2 were overexpressed, while NFE2L2 and TSC1 were downregulated. The interaction between these genes and tumour cellular infiltrates adds another layer of complexity to BC pathogenesis, as redox enzymes have the potential to recruit and activate immune cells, thereby contributing to the pro-inflammatory environment that may foster cancer progression and metastasis. These findings support the relevance of further experimental validation to evaluate the potential of these genes as HER2+ prognostic biomarkers.

In conclusion, this research has significantly advanced our understanding of the role of redox and Ca^{2+} signalling in BC, identifying several promising biomarkers and therapeutic targets. It has also shed light on the intricate interactions between redox-related genes, their interplay with Ca^{2+}-related genes, other molecular pathways, and the BC tumour microenvironment. These findings underscore the potential of targeting redox balance in cancer progression and highlight new avenues for therapeutic intervention.

However, this research has some limitations that must be acknowledged. First, the study was entirely supported by bioinformatic analyses, representing the initial phase of several investigative pathways that require experimental validation. While bioinformatics offers powerful insights, the predictive nature of these tools necessitates further laboratory-based experiments to confirm the findings. Additionally, the research focused on gene expression data, without considering post-transcriptional and post-translational modifications. These modifications can significantly impact protein expression and activity, potentially leading to biases that could alter the conclusions of the study. Therefore, future research should include both gene expression, protein level, and protein activity analyses to provide a more comprehensive understanding of the mechanisms involved.

Specific limitations also apply to the investigation of co-dysregulation of redox- and Ca^{2+}-related genes. The study identified these genes based on existing literature, which primarily focuses on well-characterized genes from patient samples, cell lines, and xenografts. This approach may exclude less-studied genes that could play crucial roles in these regulatory mechanisms. Expanding the scope to include a broader array of genes might enhance our understanding of the interplay between redox and Ca^{2+} signalling pathways in BC. Moreover, the study was confined to BC, without differentiating between its various subtypes due to the insufficient number of available samples in each subtype. The analysis of the LOX family (Chapter V) demonstrated the importance of considering the molecular differences between BC subtypes. Each subtype may have distinct regulatory mechanisms and responses to redox signalling, necessitating a more nuanced approach in future studies.

These findings highlight the importance of redox balance in cancer progression and suggest that targeted interventions that take these into account could be beneficial. However, further experimental validation is essential to translate these insights into effective clinical applications. Overall, this study contributes to the ongoing efforts to improve BC treatment and patient outcomes by integrating bioinformatics with molecular cancer research.

Looking forward, these limitations can be seen as opportunities for future research or novel approaches to understanding (dys)regulatory mechanisms in BC and other cancer types. Beyond experimental validation, additional bioinformatic analyses could be pursued, such as using chemoinformatics approaches to identify potential inhibitors for therapeutic targets described or predicting mechanisms of downregulation for tumour suppressor genes identified.

While experimental validation remains crucial, the findings of this study lay the groundwork for developing more precise and effective therapeutic strategies, ultimately contributing to improved outcomes for BC patients.

Supplementary data

Supplementary Table 1 – Redox signalling genes

Gene	Gene Expression (Tpm)								Overall Survival (Median)		Overall Survival (Quartil)	
	Normal			Tumoral			T/N	p(NxT)	HR(high)	p(HR)	HR(high)	p(HR)
	Q1	Med	Q3	Q1	Med	Q3	(Med)					
LOX	14358	25862	41660	9976	20246	35135	0,78	2,78E-01	1,20	0,200	1,30	0,230
LOXL1	25410	40784	58432	54412	91240	143813	2,24	1,00E-12	1,10	0,680	0,91	0,690
LOXL2	16021	23071	29787	16813	27233	43309	1,18	9,99E-16	1,20	0,210	1,40	0,170
LOXL3	**2194**	**2768**	**3906**	**2441**	**3579**	**5095**	**1,29**	**6,70E-11**	**1,10**	**0,540**	**1,80**	**0,009**
LOLX4	7957	10187	15207	692	1337	2404	0,13	1,62E-12	0,95	0,760	0,72	0,180
UCP2	83626	105074	142355	93239	145968	222239	1,39	1,62E-12	1,20	0,220	1,20	0,350
MAP3K1	15049	18663	24306	5462	8450	12087	0,45	1,62E-12	1,10	0,640	0,92	0,750
PRDX1	41239	462701	545518	635081	820458	1116 35	1,77	1,00E-12	1,30	0,160	1,40	0,100
PRDX2	241258	271424	323250	319135	402806	511942	1,48	1,00E-12	1,20	0,310	1,50	0,110
PRDX3	179225	198523	228501	145190	196503	253349	0,99	2,51E-01	1,40	0,563	1,50	0,100
PRDX4	**98011**	**117045**	**134269**	**128935**	**168163**	**222397**	**1,44**	**1,62E-12**	**1,50**	**0,009**	**1,70**	**0,019**
PRDX5	184064	218623	245283	221585	283402	360186	1,30	1,00E-12	1,10	0,570	1,40	0,150
PRDX6	216753	272348	378685	190540	238652	296428	0,88	2,19E-07	1,40	0,062	1,40	0,140
TXN	**391749**	**475845**	**577699**	**616945**	**789196**	**1033313**	**1,66**	**1,00E-12**	**1,50**	**0,021**	**1,50**	**0,059**
TXNRD1	**43139**	**50475**	**58010**	**33767**	**49123**	**71812**	**0,97**	**2,27E-05**	**1,50**	**0,023**	**1,70**	**0,015**
TXN2	97198	105652	114878	76310	94842	114856	0,90	4,62E-07	1,30	0,170	1,10	0,600
TXNRD2	11246	15240	17325	11894	16453	22604	1,08	1,66E-12	1,30	0,072	1,30	0,320
TXNIP	939658	1243701	1642085	18241	276418	424679	0,22	1,00E-12	0,98	0,880	0,95	0,840
GLRX	27031	34522	46920	25661	39554	61328	1,15	1,29E-07	0,99	0,960	0,90	0,650
GLRX2	**12257**	**14275**	**16743**	**18440**	**23986**	**31331**	**1,68**	**1,62E-12**	**1,40**	**0,035**	**1,40**	**0,130**
GLRX3	**46992**	**50768**	**55222**	**48921**	**58328**	**71276**	**1,15**	**1,62E-12**	**1,50**	**0,019**	**2,60**	**1,60E-04**
GLRX5	656582	60115	65893	61043	74144	90059	1,23	1,00E-12	1,10	0,410	1,10	0,760
GSR	16921	21407	26494	17411	29513	47238	1,38	1,62E-12	1,20	0,270	1,40	0,140
GPX1	230273	315742	412515	255733	327972	418808	1,04	9,89E-01	0,96	0,780	0,75	0,230
GPX3	199548	571757	1158976	21391	37299	68213	0,07	4,77E-15	0,99	0,970	1,00	0,840
GPX4	292989	415664	659066	275789	371489	480788	0,89	1,48E-03	0,92	0,630	0,77	0,250
GPX7	13952	16648	20111	9479	16267	24303	0,98	2,47E-07	0,78	0,140	1,20	0,460
GPX8	12042	15650	20270	11603	19288	28368	1,23	1,11E-16	1,30	0,150	1,40	0,120
MPO	128	254	357	27	61	107	0,24	5,60E-15	0,98	0,900	1,20	0,520
CAT	131996	179536	281433	46266	62203	85727	0,35	1,62E-12	0,88	0,450	0,91	0,670
SOD1	**300608**	**336732**	**385995**	**332818**	**413668**	**512191**	**1,23**	**1,62E-12**	**1,40**	**0,025**	**1,10**	**0,680**
SOD2	370650	455919	620360	147069	210030	324541	0,46	1,62E-12	1,00	0,910	0,78	0,300
SOD3	50448	92925	147110	4868	9472	18891	0,10	1,62E-12	0,88	0,440	0,72	0,170
NOX1	0	14	31	11	3	65	0,21	1,00E-12	1,00	0,990	0,84	0,530
CYBB	13077	21907	29575	9600	17538	30197	0,80	9,23E-01	1,00	0,800	1,10	0,820
NOX4	710	1901	5094	1758	3226	5179	1,70	5,99E-02	1,20	0,300	1,30	0,220
NOX5	91	149	224	57	157	393	1,05	7,56E-11	1,20	0,240	1,20	0,350
DUOX1	1393	2865	4365	539	1192	2613	0,42	5,21E-02	1,10	0,390	1,40	0,110
DUOX2	162	302	470	3	65	138	0,22	2,47E-08	0,92	0,600	1,00	0,880

Supplementary Table 1 – Calcium signalling genes

Gene	Gene Expression (Tpm)								Overall Survival (Median)		Overall Survival (Quartil)	
	Normal			Tumoral			T/N	p(NxT)	HR(high)	p(HR)	HR(high)	p(HR)
	Q1	Med	Q3	Q1	Med	Q3	(Med)					
TRPV1	3154	4162	5433	2341	3465	5132	0,83	0,125	0,96	0,800	0,96	0,800
TRPV2	4236	6397	9588	4214	6451	9827	1,01	0,649	0,97	0,870	0,80	0,370
TRPV4	2677	4742	6809	1483	2619	3977	0,55	0,091	0,87	0,410	0,98	0,930
TRPV6	3802	7531	11352	562	2073	6153	0,28	0,930	0,98	0,920	1,20	0,460
TRPA1	12	26	41	29	73	208	2,81	0,000	0,98	0,880	0,94	0,770
TRPC1	3966	5110	6158	953	1699	2695	0,33	0,000	1,10	0,500	1,10	0,550
TRPC3	68	123	183	56	106	176	0,86	0,003	0,94	0,690	0,80	0,390
TRPC6	2550	3593	4447	621	997	1585	0,28	0,000	1,20	0,370	1,00	0,900
TRPM7	20924	25538	30410	14951	20539	26694	0,80	0,000	1,20	0,270	1,40	0,200
TRPM8	**23**	**67**	**123**	**9**	**28**	**62**	**0,42**	**0,000**	**1,40**	**0,038**	**1,50**	**0,065**
ORAI1	16115	19758	23120	18887	24481	32225	1,24	0,000	0,96	0,780	1,30	0,270
ORAI2	3202	4396	5278	5537	7516	9727	1,71	0,000	1,00	0,910	0,91	0,650
ORAI3	17727	20593	22543	16350	23637	32609	1,15	0,000	1,00	0,900	1,10	0,720
STIM1	41579	47098	52490	25102	33289	43191	0,71	0,000	1,30	0,130	1,20	0,380
STIM2	13799	17532	20916	8771	11623	14592	0,66	0,000	1,00	0,990	0,97	0,900
SARAF (SOCE)	271149	319484	363630	178182	246665	348529	0,77	0,001	1,20	0,380	0,96	0,840
ATP2B1 (PMCA1)	16327	22225	27611	10017	14389	19053	0,65	0,000	0,93	0,640	1,40	0,140
ATP2B2 (PMCA2)	119	173	232	25	52	103	0,30	0,727	0,92	0,620	1,00	0,880
PMCA4	54077	75796	109030	26753	40121	56093	0,53	0,000	0,83	0,250	1,10	0,820
ATP2A3 (SERCA3)	12687	16250	21303	17979	32532	58117	2,00	0,000	1,30	0,110	1,10	0,750
CALM1 (CAM)	118722	158565	183047	99194	124711	154815	0,79	0,000	1,30	0,076	1,40	0,170
CALM2 (CAM)	**616842**	**707061**	**806536**	**685901**	**840372**	**1017677**	**1,19**	**0,000**	**1,50**	**0,023**	**1,60**	**0,031**
CALM3 (CAM)	138388	154589	171119	151077	185510	227464	1,20	0,000	1,10	0,490	1,30	0,240
CAMKIA	16298	26839	41388	9134	12539	16533	0,47	0,000	1,30	0,100	0,92	0,730
CAMKID	2541	3273	4325	2179	3632	5906	1,11	0,000	1,20	0,290	1,50	0,120
CAMK2B	509	1127	2407	255	765	1971	0,68	0,539	0,78	0,140	0,67	0,098
CAMK2D	16424	18360	20631	5801	9864	14530	0,54	0,000	1,20	0,220	1,40	0,140
CAMK2G	**18598**	**21459**	**23774**	**12826**	**16391**	**20401**	**0,76**	**0,000**	**1,50**	**0,024**	**1,80**	**0,011**
CAMKK1	4462	6175	7355	3467	4927	7131	0,80	0,856	1,40	0,064	1,60	0,051
CAMKK2	33277	36973	40805	23958	30963	37575	0,84	0,000	0,99	0,960	0,94	0,780
ATP2C2 (SPCA2)	**2170**	**4422**	**7401**	**4267**	**7752**	**13289**	**1,75**	**0,000**	**1,40**	**0,038**	**1,40**	**0,130**
ITPR1 (IP3R1)	33242	51434	71372	10247	19081	33096	0,37	0,000	1,30	0,150	1,30	0,260
ITPR2 (IP3R2)	16249	23623	32550	6023	10797	18229	0,46	0,000	0,88	0,440	0,86	0,520
ITPR3 (IP3R3)	9923	14708	19137	14897	21973	29633	1,49	0,000	1,10	0,630	1,10	0,680
FAM38A (PIEZO1)	39669	48170	60729	34522	48039	67265	1,00	0,002	1,00	0,960	0,96	0,880
FAM38B (PIEZO2)	2938	5737	8841	1781	4268	8798	0,74	0,000	1,10	0,600	1,00	0,850
TPCN1 (TPC1)	31915	35567	40969	17376	24741	33427	0,70	0,000	1,20	0,180	1,50	0,081
TPCN2 (TPC2)	5577	6553	7563	3848	5115	6759	0,78	0,474	1,00	0,940	1,00	0,840
CAPN1	73037	101480	116306	96634	121296	154653	1,20	0,000	1,20	0,230	1,50	0,083
CAPN2	116136	140962	160238	84923	124747	181214	0,88	0,454	1,20	0,230	1,40	0,130
CAST	136079	165625	201340	82317	111466	144311	0,67	0,000	1,10	0,440	1,00	0,920
CCDC109A (MCU)	12035	13856	15405	11524	14814	18916	1,07	0,001	1,30	0,140	1,10	0,640
UCP2	83626	105074	142355	93239	145968	222239	1,39	0,000	1,20	0,220	1,20	0,350
UCP3	625	872	1142	471	737	1045	0,85	0,330	0,78	0,130	0,78	0,310
PLCD1	**14102**	**16795**	**18813**	**6473**	**9387**	**13175**	**0,56**	**0,000**	**0,68**	**0,019**	**0,63**	**0,053**
NTSR1	9	19	57	7	23	56	1,21	0,000	1,20	0,360	1,20	0,470
TMBIM4	6442	76318	84314	59224	82102	110009	1,08	0,004	1,00	0,820	1,10	0,750

Supplementary Table 2 - Summary of studies related to the impact of the selected redox- and Ca^{2+}-related genes in BC outcome

Gene	Main gene function	Effect in BC *
Glutaredoxin-2 **(*GLRX2*)**	Glutathione-dependent oxidoreductase enzymes	10.1096/fasebj.23.1_supplement.LB253 10.1074/jbc.M408011200
Glutaredoxin-3 **(*GLRX3*)**		10.1172/JCI43144
Lysyl oxidase like 2 **(*LOXL2*)**	Copper-dependent amine oxidases that catalyse the formation of crosslinks in collagens and elastin in the extracellular matrix.	PMID: 12154058 10.1007/s10549-013-2662-3 10.1002/emmm.201100156
Lysyl oxidase like 3 **(*LOXL3*)**		10.1038/s41388-022-02258-1
NADPH Oxidase 4 **(*NOX4*)**	Catalytic subunit the NADPH oxidase complex. Acts as an oxygen sensor and catalyzes the reduction of molecular oxygen to various reactive oxygen species	10.4161/cbt.10.3.12207 10.1016/j.bcp.2013.05.011
Peroxiredoxin-4 **(*PRDX4*)**	Thiol-specific peroxidase that catalyzes the reduction of hydrogen peroxide and organic hydroperoxides to water and alcohols, respectively	10.1016/j.canlet.2015.03.012 10.23937/2378-3419/3/2/1053
Superoxide dismutase 2 **(*SOD2*)**	Oxidoreductase that converts superoxide anion to hydrogen peroxide and diatomic oxygen	10.1016/j.freeradbiomed.2014.08.026 10.1016/j.freeradbiomed.2008.09.005
Thioredoxin **(*TXN*)**	Catalyzes dithiol-disulfide exchange reactions. Plays a role in the reversible S-nitrosylation of cysteine residues in target proteins	10.1016/j.redox.2015.12.004 10.1186/bcr2599
Thioredoxin reductase 1 **(*TXNRD1*)**	Reduces disulfideprotein thioredoxin to its dithiol-containing form	10.1186/bcr2599 10.1038/srep36860
ATPase Secretory Pathway Ca^{2+} Transporting 2 (***ATP2C2***)	ATP-driven pump that supplies the Golgi apparatus with Ca^{2+} and Mn^{2+} ions. Interacts with *ORAI1*	10.1016/j.cell.2010.08.040 10.1016/j.redox.2022.102240
Calmodulin 2 (**CALM2**)	Mediates the control of enzymes, ion channels, aquaporins and other proteins through calcium - binding.	10.1007/s10549-008-0097-z 10.3390/proceedings2251550
Ca^{2+}/Calmodulin-stimulated protein kinase II gama (***CAMK2G***)	Ca^{2+}/calmodulin-dependent protein kinase.	10.1038/srep33132
Ca^{2+} release-activated Ca^{2+} modulator 1 (***ORAI1***)	Ca^{2+} release-activated Ca^{2+} channel which mediates SOCE activation by the Ca^{2+} sensor STIM1	10.1016/j.ccr.2008.12.019 10.1016/j.bbrc.2011.07.025
Stromal interaction molecule 1 (***STIM1***)	Ca^{2+} sensor in the ER and upon Ca^{2+} depletion, translocate from the ER to the PM where it activates ORAI1	
1-Phosphatidylinositol-4,5-bisphosphate phosphodiesterase delta-1 (***PLCD1***)	Catalyzes the hydrolysis of PIP2 into the second messengers DAG and IP3	10.18632/oncotarget.16072 10.4161/cbt.10.5.12726
Transient receptor potential melastatin 8 (***TRPM8***)	Receptor-activated non-selective cation channel.	10.1007/s13277-014-2077-8 10.1186/1471-2407-10-212

Supplementary Data 1

SPSS file available in https://doi.org/10.5281/zenodo.12701468

Supplementary File 1 and File 2

Supplementary File 1 containing three Excel files with cumulative proportion survival from redox-related genes, calcium-related genes, and redox and calcium-related genes.

Supplementary File 2 including an Excel file with a functional enrichment analysis using redox and calcium correlated genes. Cell cycle regulation (sheet 1) and Cell adhesion and projection (sheet 2) were the biological processes more enriched.

Available at https://doi.org/10.5281/zenodo.12168552