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**Drug repurposing study based on *NDRG1* inhibition
in three-dimensional models derived from patients
with triple-negative breast cancer**

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Calahorra, J., Blaya-Cánovas, J. L., Castellini-Pérez, O., Aparicio-Puerta, E., Cives-Losada, C., Marin, J. J. G., Rementeria, M., Cara, F. E., **López-Tejada, A.**, Griñán-Lisón, C., Aulicino, F., Berger, I., Marchal, J. A., Delgado-Almenta, V., & Granados-Principal, S. (2024). Unlocking the effective alliance of β -lapachone and hydroxytyrosol against triple-negative breast cancer cells. *Biomedicine & pharmacotherapy*, 174, 116439. <https://doi.org/10.1016/j.biopha.2024.116439>

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Ávalos-Moreno, M., **López-Tejada, A.**, Blaya-Cánovas, J. L., Cara-Lupiañez, F. E., González-González, A., Lorente, J. A., Sánchez-Rovira, P., & Granados-Principal, S. (2020). Drug Repurposing for Triple-Negative Breast Cancer. *Journal of personalized medicine*, 10(4), 200. <https://doi.org/10.3390/jpm10040200>

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ABSTRACT

Triple-negative breast cancer (TNBC) is the most aggressive type of breast cancer exhibiting high rates of relapse, metastasis, and mortality. Despite recent therapeutic advancements for metastatic TNBC, there remains a critical need for novel biomarkers and targeted therapies to improve patient outcomes.

N-Myc downstream regulated gene 1 (NDRG1) exhibits pleiotropic activity across various cancer types, including TNBC. However, its intrinsic mechanism of action remains incompletely understood. Building upon our previous research, which demonstrated elevated NDRG1 expression following aggressive TGF- β stimulation, we hypothesized that this pathway could mediate NDRG1's tumorigenic role in TNBC.

Initially, we observed that enhanced levels of *NDRG1* gene expression correlated with poorer survival in TNBC patients through analyses of two public databases: the Kaplan-Meier plotter and GOBO database. We corroborated these findings through immunohistochemical assessment of NDRG1 protein expression in a cohort of 83 TNBC patients. Consequently, we concluded that high NDRG1 expression was indicative of poor prognosis compared to lower expression.

Subsequently, we investigated the modulation of TGF- β and NDRG1 activity. We elucidated that NDRG1 activity depends on TGF- β stimulation and the origin of tumor cells. In pleural-effusion-derived cells (MDA-MB-231, MDA-MB-436) *NDRG1* inhibition upon TGF- β stimulation reduced migration, invasion, and Vimentin expression, whereas these effects were not observed in primary-tumor-derived cell lines (SUM159, BT549). We also examined key oncogenic processes, including the maintenance of cancer stem cell (CSC) populations by flow cytometry. Notably, primary-tumor cells required extended TGF- β stimulation to enhance NDRG1-mediated aggressiveness. This suggests that post-metastasis, brief TGF- β exposure suffices to induce aggressive behavior. NDRG1 regulated ALDH1⁺ CSCs maintenance across all four cell lines, while CD44⁺/CD24^{low/-} and side population CSCs were maintained only in primary-tumor-derived cells.

Considering these findings and the potential tumor-suppressive role of NDRG1 in some TNBC patients, we pursued an alternative approach to direct NDRG1 inhibition. To identify drugs that mimic the antitumor transcriptomic signature resulting from inhibition of TGF- β -induced NDRG1, we performed a Connectivity Map study.

Drug repurposing has emerged as a promising, efficient approach to investigate new therapies, avoiding the long and costly process that *de novo* drugs imply. As we had observed that *NDRG1* inhibition following TGF- β stimulation produced an antitumor signature, we selected three candidates that might resemble this gene expression, which was studied via RNA sequencing technique. Our signature-based approach led to the selection of efavirenz, ouabain, and vinburnine as repurposed drug candidates. To validate their efficacy, we performed subsequent evaluations. These compounds significantly impaired tumor cell proliferation, CSC populations, self-renewal capacity, clonogenic properties, and migratory abilities in attached TNBC cell lines. Furthermore, given the absence of assessment of the combination of repurposed drugs in cancer research, we also study the effects of pair-combinations of our candidates. Notably, efavirenz + ouabain (EO) demonstrated superior efficacy in reducing CSC phenotypic properties. Furthermore, we studied their effects on epithelial-mesenchymal transition (EMT) markers by immunoblot and observed that they reduced Snail and Slug protein expressions.

Importantly, these repurposed drugs exerted their antitumor effects without directly inhibiting *NDRG1* protein expression, potentially avoiding adverse effects associated with suppressing the tumor-suppressive variant of *NDRG1*. Instead, their mechanism of action involved the blockade of AKT, as evidenced by reduced expression of its phosphorylated (Ser347) form in immunoblot analyses.

Given that chemoresistance is one of the main obstacles that TNBC tumors develop, we also assessed our repurposed drugs in a docetaxel-resistant cell line that we had previously established. Efavirenz and ouabain exhibited more sensitivity compared to their parental cell line. Conversely, vinburnine was able to re-sensitize TNBC cells when combined with docetaxel.

To enhance the translational potential of our findings, we attempted to assay our repurposed drugs in TNBC patient-derived organoids (PDOs). We could not successfully establish long-term PDOs cultures, probably due to the limited amount of biopsy tissue.

To overcome this limitation, we established three TNBC patient-derived xenograft (PDX) models, which were subsequently digested to produce PDX-derived organoids (PDXOs). One model (UGR01) successfully grew *in vitro*, demonstrating the absence of the murine population and recapitulating histopathological features of the TNBC patient origin. In this model, our three

repurposed drugs effectively reduced cell viability, both as monotherapies and in combination with docetaxel. Immunofluorescence staining corroborated the downregulation of phosphorylated AKT in treated PDxOs and the reduction of cell proliferation using Ki67 marker.

In conclusion, this project develops a comprehensive study that starts from unraveling the intrinsic aggressiveness of NDRG1 in TNBC to the validation of repurposed drugs in a patient-derived model. We present the rational choice of repurposing method to avoid malignant direct inhibition of NDRG1. Furthermore, we assess the effects on cell viability, apoptosis, CSC properties, migration, and EMT in adherent TNBC cell lines and the mechanism of action. To conclude, we validate our results in a PDxOs model that resembles the patient's characteristics. These findings support the importance and value of drug repurposing to enhance a new era of drug development against cancer.

RESUMEN

El cáncer de mama triple negativo (TNBC, por sus siglas en inglés) es el tipo más agresivo de cáncer de mama, presentando altas tasas de recaída, metástasis y mortalidad. A pesar de los recientes avances terapéuticos para el TNBC metastásico, persiste una necesidad crítica de nuevos biomarcadores y terapias dirigidas para mejorar los resultados clínicos de los pacientes.

NDRG1 (del inglés, N-Myc downstream regulated gene 1) exhibe una actividad pleiotrópica en varios tipos de cáncer, incluyendo el TNBC. Sin embargo, su mecanismo de acción intrínseco sigue sin comprenderse completamente. Basándonos en nuestra investigación previa, que demostró una expresión elevada de NDRG1 tras la estimulación agresiva con TGF- β , planteamos la hipótesis de que esta vía podría mediar el papel tumorigénico de NDRG1 en el TNBC.

Inicialmente, observamos que los niveles elevados de expresión génica de *NDRG1* se correlacionaban con una peor supervivencia en pacientes con TNBC mediante análisis de dos bases de datos públicas: Kaplan-Meier plotter y GOBO. Corroboramos estos hallazgos mediante la evaluación inmunohistoquímica de la expresión proteica de NDRG1 en una cohorte de 83 pacientes con TNBC. En consecuencia, concluimos que una alta expresión de NDRG1 era indicativa de un mal pronóstico en comparación con una expresión más baja.

Posteriormente, investigamos la modulación de la actividad de TGF- β y NDRG1. Elucidamos que la actividad de NDRG1 depende de la estimulación por TGF- β y del origen de las células tumorales. En células derivadas de efusión pleural (MDA-MB-231, MDA-MB-436), la inhibición de NDRG1 tras la estimulación con TGF- β redujo la migración, la invasión y la expresión de Vimentina, mientras que estos efectos no se observaron en líneas celulares derivadas de tumores primarios (SUM159, BT549). También examinamos procesos oncogénicos clave, incluyendo el mantenimiento de poblaciones de células madre cancerosas (CSC, por sus siglas en inglés) mediante citometría de flujo. Notablemente, las células de tumores primarios requirieron una estimulación prolongada con TGF- β para potenciar la agresividad mediada por NDRG1. Esto sugiere que, después de la metástasis, una breve exposición a TGF- β es suficiente para inducir un comportamiento agresivo. NDRG1 reguló el mantenimiento de CSCs ALDH1+ en las cuatro líneas celulares, mientras que las CSCs CD44+/CD24^{low/-} y la “side population” se mantuvieron solo en células derivadas de tumores primarios.

Considerando estos hallazgos y el potencial papel supresor tumoral de NDRG1 en algunos pacientes con TNBC, buscamos un enfoque alternativo a la inhibición directa de NDRG1. Para identificar fármacos que imiten la firma transcriptómica antitumoral resultante de la inhibición de *NDRG1* inducida por TGF- β , realizamos un estudio de Connectivity Map.

El reposicionamiento de fármacos ha surgido como un enfoque prometedor y eficiente para investigar nuevas terapias, evitando el largo y costoso proceso que implican los fármacos *de novo*. Como habíamos observado que la inhibición de *NDRG1* tras la estimulación con TGF- β producía una firma antitumoral, seleccionamos tres candidatos que podrían producir una expresión génica semejante, la cual se estudió mediante la técnica de secuenciación de ARN. Nuestro enfoque basado en firmas condujo a la selección de efavirenz, ouabaína y vinburnina como nuestros fármacos candidatos reposicionados. Para validar su eficacia, realizamos diferentes evaluaciones. Estos compuestos redujeron significativamente la proliferación de células tumorales, las poblaciones de CSC, la capacidad de autorrenovación, las propiedades clonogénicas y las capacidades migratorias en líneas celulares de TNBC adherentes. Además, dada la ausencia de estudios con combinaciones de fármacos reposicionados en la investigación del cáncer, también analizamos los efectos de las combinaciones por pares de nuestros candidatos. Notablemente, la combinación de efavirenz + ouabaína (EO) demostró una eficacia superior en la reducción de las propiedades fenotípicas de las CSC. Además, estudiamos sus efectos en los marcadores de transición epitelio-mesénquima (EMT, por sus siglas en inglés) mediante inmunoblot y observamos que reducían las expresiones proteicas de Snail y Slug.

Es importante destacar que estos fármacos reposicionados ejercieron sus efectos antitumorales sin inhibir directamente la expresión proteica de NDRG1, evitando potencialmente los efectos adversos asociados con la supresión de la variante supresora de tumores de NDRG1. En su lugar, su mecanismo de acción implicó el bloqueo de AKT, como se evidenció por la reducción de la expresión de su forma fosforilada (Ser347) mediante inmunoblot.

Dado que la quimiorresistencia es uno de los principales obstáculos que desarrollan los tumores TNBC, también evaluamos nuestros fármacos reposicionados en una línea celular resistente a docetaxel que habíamos establecido previamente. Efavirenz y ouabaína exhibieron mayor sensibilidad

en comparación con su línea celular parental. Por el contrario, la vinburnina fue capaz de resensibilizar las células TNBC cuando se combinó con docetaxel.

Para mejorar el potencial traslacional de nuestros hallazgos, intentamos estudiar nuestros fármacos reposicionados en organoides derivados de pacientes (PDOs) con TNBC. No pudimos establecer con éxito cultivos de PDOs a largo plazo, probablemente debido a la cantidad limitada de tejido de biopsia.

Para superar esta limitación, establecimos tres modelos de xenoinjertos derivados de pacientes (PDX) con TNBC, que posteriormente se digirieron para producir organoides derivados de PDX (PDxOs). Un modelo (UGR01) creció con éxito *in vitro*, presentando ausencia de población murina y recapitulando las características histopatológicas del origen del paciente con TNBC. En este modelo, nuestros tres fármacos reposicionados redujeron eficazmente la viabilidad celular, tanto como monoterapias como en combinación con docetaxel. La tinción por inmunofluorescencia corroboró la disminución de la expresión de la forma fosforilada de AKT en los PDxOs tratados y la reducción de la proliferación celular utilizando el marcador Ki67.

En conclusión, este proyecto desarrolla un estudio integral que parte de desentrañar la agresividad intrínseca de NDRG1 en TNBC hasta la validación de fármacos reposicionados en un modelo derivado de pacientes. Presentamos la elección racional del método de reposicionamiento para evitar la inhibición directa de NDRG1. Además, evaluamos los efectos sobre la viabilidad celular, apoptosis, propiedades de las CSC, migración y EMT en líneas celulares de TNBC adherentes y el mecanismo de acción. Para concluir, validamos los resultados en un modelo de PDxOs que mantiene las características del paciente. Estos hallazgos respaldan la importancia y el valor del reposicionamiento de fármacos para potenciar una nueva era de desarrollo de fármacos contra el cáncer.

ABBREVIATIONS

3D	Three-dimensional
Ab	Antibiotic/antimitotic solution
ABC	ATP-binding cassette
ADCs	Antibody drugs conjugates
AdDMEM	Advanced dulbecco's modified eagle medium
AdDMEM/ F12+++	Advanced DMEM/F12 supplemented with 1% penicillin/streptomycin, 10 mM HEPES, and 1X Glutamax
AKT	Protein kinase B
ALDH	Aldehyde dehydrogenase
AR	Androgen receptor
ATCC	American Type Culture Collection
ATF4	Activating Transcription Factor 4
BAA	BODIPY-aminoacate
BL1	Basal-like 1
BL2	Basal-like 2
BLIA	Basal-like immune-activated
BLIS	Basal-like immune-suppressed
BME	Basement membrane extract
BSA	Bovine serum albumin
CLUE	CMap Linked User Environment
CMap	Connectivity Map
CSCs	Cancer stem cells
Ct	Comparative threshold cycle
D	Docetaxel
D+E	Docetaxel + Efavirenz
D+O	Docetaxel + Ouabain
D+V	Docetaxel + Vinburnine
DEAB	Diethylaminobenzaldehyde
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
E	Efavirenz
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMA	European Medicines Agency
EMT	Epithelial-mesenchymal transition
EO	Efavirenz + Ouabain
ER	Estrogen receptor
ESMO	European Society for Medical Oncology
EV	Efavirenz + Vinburnine

FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
FISH	Fluorescence <i>in situ</i> hybridization
GENyO	Centre for Genomics and Oncological Research
GFR	Growth factor-reduced
HER2	Human epidermal growth factor receptor 2
HR	Hazard ratio
IC50	Half-maximal inhibitory concentration
IF	Immunofluorescence
IHC	Immunohistochemistry
IL-6	Interleukin-6
IM	Immunomodulatory
iNOS	Inducible NOS
LAR	Luminal androgen receptor
LINE-1	Long interspersed nuclear element-1
LN	Lymph node
lncRNA	Long noncoding RNA
L-NMMA	NG-monomethyl-L-arginine
log₂ fold change	Logarithmic fold change
M	Mesenchymal
MAPK	Mitogen-activated protein kinase
ME	Mesenchymal-like
MES	Mesenchymal
MHC	Major histocompatibility complex
mRNA	Messenger RNA
MSFE	Mammosphere-efficiency forming
MSL	Mesenchymal stem-like
mTNBC	Metastatic TNBC
NDRG	N-myc downstream regulated gene
NO	Nitric oxide
NOS	Nitric oxide synthase
O	Ouabain
OV	Ouabain + Vinburnine
p-AKT	Phospho-AKT (Ser476)
PARP	Poly(ADP-ribose) polymerase
PBS	Phosphate buffer saline

PCLs	<i>Perturbagen</i> classes
PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
PDO	Patient-derived organoid
PDX	Patient-derived xenograft
PDxO	Patient-derived xenograft organoid
PFA	Paraformaldehyde
PI	Propidium iodide
PI3K	Phosphatidylinositol-3-kinase
p-NDRG1	Phospho-NDRG1 (Thr346)
PR	Progesterone receptor
PRISM	Protein Interactions by Structural Matching
RT-qPCR	Real-time quantitative PCR
ref	Reference
RFS	Relapse-free survival
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
RT	Room temperature
SCR	Scrambled siRNA
SD	Standard deviation
SDA-PAGE	Sodium dodecyl polyacrylamide gel
SGK1	Glucocorticoid-regulated kinase 1
STAT3	Signal Transducer and Activator of Transcription 3
STITCH	Search Tool for Interactions of Chemicals
TBS	Tris-buffer saline
TBST	Tris-buffer saline with tween-20
T-DXd	Trastuzumab deruxtecan
TG	Tris-glycine
TGF-β	Transforming Growth Factor β
TGS	Tris-glycine-SDS
TILs	Tumor-infiltrating lymphocytes
Trop-2	Trophoblast cell-surface antigen
V	Vinburnine
VEGF	Vascular endothelial growth factor
β-ADR	β -adrenergic receptor

INTRODUCTION

1. Breast cancer

Breast cancer is a heterogeneous disease that consists on the abnormal non-controlled growth of breast cells to form tumors[1]. According to the Global Cancer Observatory of the World Health Organization, in 2022 breast cancer was the second most frequently diagnosed cancer and the fourth cause of cancer death in absolute numbers in both sexes (Figure 1)[2].

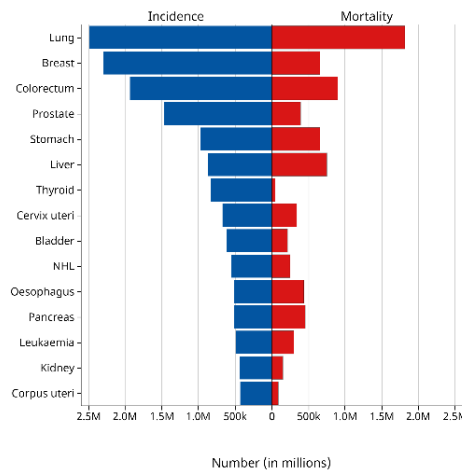


Figure 1. Bar chart of absolute cases of global incidence and mortality for 15 topmost types of cancer in 2022.

Rates are shown in descending order of absolute cases of incidence. [Source: GLOBOCAN 2022].

The main risk factor is the female gender as only 0.5-1% of cases occur in men[1]. Among women, it represents the type of cancer with the highest incidence (23.8%) and mortality (15.4%)[3] (Figure 2).

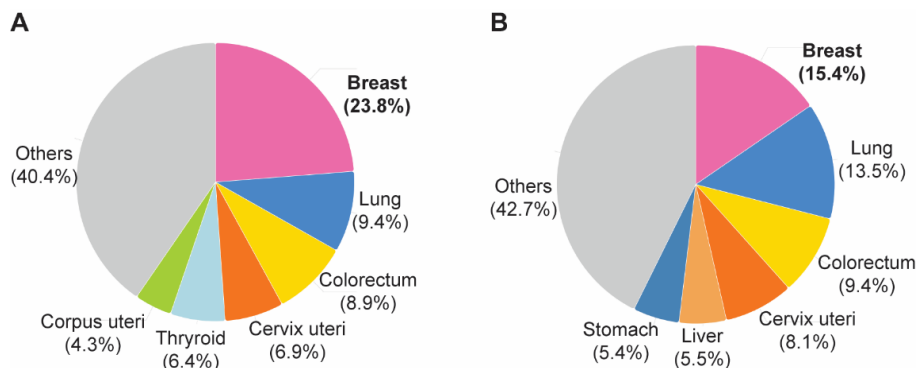


Figure 2. Pie charts representing the incidence (A) and mortality (B) of the 6 topmost types of cancer for female sex in 2022.

[Source: GLOBOCAN 2022].

1. Breast cancer

The most common molecular classification of breast cancer is based on the presence of hormone receptors and the amplification expression or overexpression of the human epidermal growth factor receptor 2 (HER2), which is easily diagnosed by immunohistochemistry (IHC). Four main groups are distinguished in Table 1[4].

Table 1. Classification of breast cancer by histochemistry.

ER, estrogen receptor; FISH, fluorescence in situ hybridization; HER2, human epidermal growth factor receptor 2; HR, hormone receptors; IHC, immunohistochemistry; PR, progesterone receptor.

Subtype	HR status by IHC	HER2 status by IHC/FISH	Ki67 expression
Luminal A	ER+/PR+	HER2-	Ki67<20%
Luminal B	ER+/PR<20%	HER2-	Ki67>20%
HER2+ B2	ER+/PR+	HER2 overexpression	
HER2+	ER-/PR-	HER2 overexpression	
Triple-negative	ER-/PR-	HER2-	

2. Triple-negative breast cancer

2.1. Definition and classifications

Triple-negative breast cancer (TNBC) is the **most aggressive** type of breast cancer, presenting 15-20% of cases, and is defined by the absence of hormone receptors and HER2 amplification. It is characterized by its earlier onset, typically affecting premenopausal women under 40 years old, as well as by its shorter survival time with a mortality rate of 40% within the first 5 years post-diagnosis. This subtype demonstrates a heightened propensity for metastasis, with approximately 46% of TNBC patients developing distant metastases, often to visceral organs and the brain. Furthermore, the treatment choice is systemic chemotherapy, with poor efficacy with postoperative adjuvant leading to tumor recurrence, hence there is an urgent need for more specific therapies[5].

To further understand the intrinsic characteristics of this type of breast cancer and propose different therapies, classifications based on gene expression profiling have been proposed[5]. In 2011, Lehmann et al. designed a classification system that divided TNBC into six subtypes[6] (Figure 3):

- **Basal-like 1 (BL1).** This subtype exhibits the elevated expression of cell cycle-regulating and DNA repair genes, including *MYC*, *PIK3CA*, *CDK6*, and *BRCA2*. BL1 tumors are particularly sensitive to platinum-based chemotherapy and poly(ADP-ribose) polymerase (PARP) inhibitors.
- **Basal-like 2 (BL2).** It is characterized by aberrant activation of growth factor signaling pathways, including epidermal growth factor receptor (EGFR), MET, and IGF-1R. Potential therapeutic targets for BL2 include mTOR inhibitors and growth factor receptor antagonists.
- **Mesenchymal (M) and Mesenchymal stem-like (MSL).** These subtypes display enhanced expression of genes involved in epithelial-mesenchymal transition (EMT) and stem cell-related pathways such as Transforming Growth Factor β (TGF- β) signaling. PI3K inhibitors and Src antagonists have shown promise in treating M and MSL subtypes.
- **Immunomodulatory (IM).** It exhibits enrichment of immune cell-associated genes and signaling pathways, resembling medullary breast carcinoma. Notably, approximately 20% of all TNBC cases display elevated expression of programmed death-ligand 1 (PD-L1). PD-L1 is expressed by both tumor and immune cells within the tumor microenvironment and interacts with programmed cell death protein 1 (PD-1), a transmembrane receptor found on adaptive immune cells, particularly T cells. This interaction initiates a complex signaling cascade that suppresses T-cell activity, promoting immune tolerance and enabling tumor immune evasion. Furthermore, activation of PD-L1/PD-1 axis disrupts the phosphatidylinositol-3-kinase/protein kinase B (PI3K/AKT) pathway, which is vital for T-cell proliferation and cytokine production[7]. Hence, IM subtype tumors show potential responsiveness to immune checkpoint inhibitors targeting PD-1 and PD-L1, offering promising therapeutic options.
- **Luminal androgen receptor (LAR).** It is defined by the high expression of androgen receptor (AR) and its downstream targets. Possible treatments would include AR antagonists and PI3K inhibitors[6].

Nonetheless, this classification was later refined to four subtypes (BL1, BL2, M, and LAR) after removing the IM and MSL subtypes due to their association with tumor-infiltrating lymphocytes (TILs) and tumor-associated stromal cells, respectively[8].

2. Triple-negative breast cancer

Conversely, in 2015, Burstein et al. proposed an alternative classification system based on mRNA expression and DNA genomic profiling, identifying four TNBC subtypes[9]:

- **LAR.** This subtype aligns with Lehmann's LAR cluster as it is described by AR signaling and luminal gene expression.
- **Mesenchymal (MES).** It exhibits elevated expression of genes involved in cell motility, extracellular matrix remodeling, and growth factor signaling. This subtype corresponds to Lehmann's M and MSL subtypes.
- **Basal-like immune-suppressed (BLIS).** It displays a basal-like gene expression signature with low expression of immune function genes and cytokine signaling. It presents the worst prognosis among the four.
- **Basal-like immune-activated (BLIA).** It is characterized by elevated expressions of immune-activating genes and signal transducer and activator of transcription (STAT) molecules. This subtype has the best prognosis among the four[9].

Furthermore, a third classification of TNBC subtypes has been done based on mRNA and lncRNA (long non-coding RNA) profiles by Liu et al[5,10]. Their proposal is composed of four clusters, whose objective is to facilitate individual treatment for this heterogeneous disease:

- **IM.** It is characterized by expression of immune-related genes and pathways such as *CXCL13*, *CXCL11*, *CXCL10*, and *CXCR2*.
- **LAR.** Similar to the previously described LAR group, it presents active hormonal regulation pathways and increased PPAR signaling. Possible therapies based on antiandrogen compounds.
- **Mesenchymal-like (ME).** This subtype is characterized by extracellular matrix-receptor interactions, gap junctions, and TGF- β signaling and is associated with growth factor pathways (ATP-binding cassette (ABC) transporters, adipokine signaling).
- **BLIS.** It is enriched for cell division, cell cycle, and DNA replication/repair genes and presents a high expression of proliferation-related genes (e.g., *CENPF*, *BUB*, *PRC1*). It is characterized by the downregulation of immune response genes and associated with worse prognosis and higher recurrence risk[10].

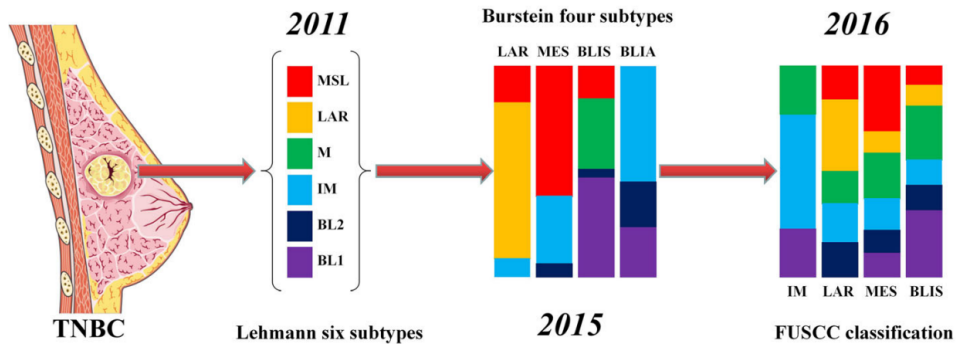


Figure 3. Classifications of TNBC molecular subtypes: Lehmann’s six subtypes, Burststein’s four subtypes, and FUSCC (Fudan University Shanghai Cancer Center) classification.

The size of rectangles depends on the proportion of the samples. BL1, basal-like 1; BL2, basal-like; BLIA, basal-like immune-activated; BLIS, basal-like immune-suppressed; IM, immunomodulatory; LAR, luminal androgen receptor; M; mesenchymal; MES, mesenchymal; MSL, mesenchymal stem-like. Source:[5].

2.2. Treatment

As previously introduced, the conventional therapy for TNBC is systemic cytotoxic chemotherapy due to the negative profile of ER, PR, and HER2. Even though TNBC is more chemosensitive than other breast cancer subtypes, it still presents a lower overall prognosis[7].

The recommended guidelines of the National Comprehensive Cancer Network include a variety of **systemic chemotherapies** (Figure 4) as the first line of TNBC treatment. Among the combinations, three main groups of cytotoxic drugs are distinguished[7]:

- **Taxanes** (e.g., docetaxel, paclitaxel). Their main mechanism of action is binding the microtubules, consequently, they disrupt mitosis producing cycle arrest and apoptosis[11]. They can be used in both monotherapy or combination and are a good option in case of resistance or contraindication of anthracyclines. The most important undesirable effect is neuropathy[12].
- **Anthracyclines** (e.g., doxorubicin or adriamycin, epirubicin). Originally studied as antibiotics, anthracyclines present different mechanisms of action responsible for their cytotoxicity such as DNA intercalation that follows damage in its replication, generation of reactive oxygen species (ROS) producing mitochondrial dysfunction, alteration of calcium

2. Triple-negative breast cancer

homeostasis and cell membranes, or induction of apoptosis, among others. The main undesirable effect is its high cardiotoxicity[13].

- **Cyclophosphamide.** This antineoplastic prodrug belongs to the group of nitrogen mustard derivatives and is enzymatically activated by the hepatic cytochrome P-450. Cytotoxicity is due to the production of strand DNA crosslink, hence impairing DNA replication. Resistance to cyclophosphamide is caused by high levels of aldehyde dehydrogenase (ALDH), which is the enzyme responsible for its detoxification[14].

Although the high efficacy of their combinations, other chemotherapeutic drugs were approved to expand the possibility of treatments:

- **Platinum-based compounds** (e.g., carboplatin). They also are alkylating agents, in other words, they bind DNA and interrupt its replication and transcription, leading to cell death. Patients carrying mutations in the *BRCA* gene are more sensitive to these drugs as they cannot successfully repair the DNA damage[15].
- **Antimetabolites.** Two compounds are approved for TNBC patients:
 - **Capecitabine.** It is a prodrug that, after activation by thymidine phosphorylase, produces the antimetabolite 5-fluorouracil. It is an analog of the nitrogen base uracil, hence, the drug blocks DNA synthesis[16].
 - **Gemcitabine.** This prodrug, after been phosphorylated, competes with deoxycytidine triphosphate and inhibits DNA replication[17].
- **Eribulin mesylate.** Its mechanism of action involves binding to the plus ends of microtubules, inhibiting their growth without affecting depolymerization. This leads to the formation of non-functional mitotic spindles, resulting in irreversible mitotic arrest and ultimately apoptosis. Additionally, eribulin has been shown to reverse the EMT in TNBC cells. It is approved for the treatment of metastatic TNBC (mTNBC)[18,19].
- **Vinorelbine.** It is a semi-synthetic vinca alkaloid derived from vinblastine. It exerts its antitumor activity primarily by binding to tubulin and inhibiting microtubule assembly. This leads to the formation of non-functional mitotic spindles, resulting in irreversible mitotic arrest and ultimately apoptosis. Vinorelbine also induces other complex molecular mechanisms, including the activation of tumor suppressor gene *p53* and modulation of key signaling pathways involved in cell survival and proliferation. It is used as a second-line treatment in mTNBC[20,21].

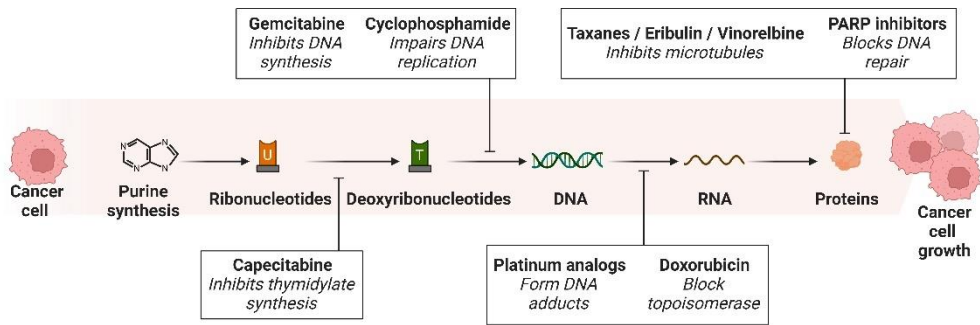


Figure 4. Main mechanisms of chemotherapy for TNBC.

PARP: Poly(ADP-ribose) polymerase. Created with Biorender.

More recently, advanced treatments have been approved but only for some cases of TNBC, depending on the expression of targets:

- **PARP inhibitors:** olaparib, talazoparib. PARP enzymes, particularly PARP1 and PARP2, are essential for the repair of single-strand DNA breaks. Inhibition of PARP activity results in the accumulation of unrepaired single-strand breaks, leading to the formation of double-strand breaks, which ultimately induces cell death. This effect is notably more pronounced in patients with *BRCA* mutations. The underlying reason is that *BRCA1/2* deficiencies impair homologous recombination, a key DNA repair mechanism, leaving PARP1 as the primary repair pathway. Thus, inhibiting PARP in these patients effectively blocks DNA repair, leading to cell death due to the accumulation of DNA damage[7,22].
- **Immunotherapy:** pembrolizumab, atezolizumab. As previously reported, the interaction of PD-L1 and PD-1 has been studied in TNBC and, consequently, targeted therapies have been approved for TNBC treatment[8]. Pembrolizumab (anti-PD-1) or atezolizumab (anti PD-L1), in combination with chemotherapy, are the first line of treatment in case of PD-L1+ mTNBC[23] (Figure 5).

2. Triple-negative breast cancer

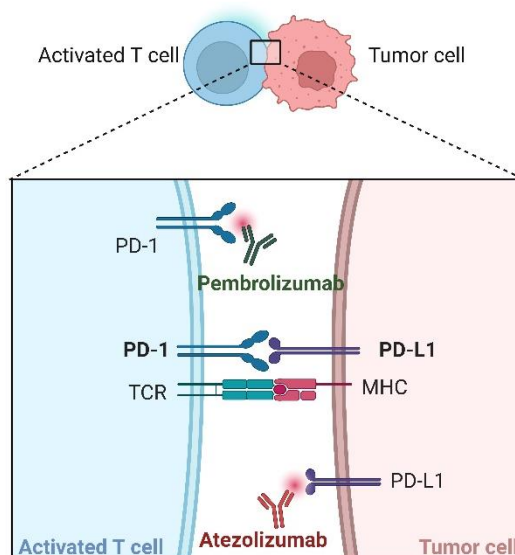


Figure 5. Representation of the mechanism of FDA-approved immunotherapy for TNBC.

Activated T cells express PD-1 that interacts with PD-L1 protein on the surface of cancer cells. Pembrolizumab (green) and atezolizumab (red) maintain T-cell activity by blocking PD-1 or PD-L1, respectively. PD-1, programmed cell death protein 1, PD-L1, Programmed cell death ligand 1; MHC: major histocompatibility complex, TCR: T cell receptor. Created with Biorender.

- **Antibody drugs conjugates (ADCs):**
 - **Sacituzumab govitecan.** The combination of a monoclonal antibody that targets a protein of the cancer cell membrane connected to a cytotoxic molecule represents a new strategy to eradicate cancer cells more specifically. Particularly for TNBC, one approved ADC is composed of sacituzumab, which is the monoclonal antibody that targets trophoblast cell-surface antigen (Trop-2) and SN-38 or govitecan, which is a metabolite of irinotecan (topoisomerase I inhibitor). The principal advantage of this combination is that govitecan exerts its cytotoxic effect in TNBC cells as Trop-2, the receptor of sacituzumab, is overexpressed in mTNBC whereas it presents normal expression in normal cells[7].
 - **Trastuzumab deruxtecan (T-DXd).** It is composed of a monoclonal antibody that targets HER2 receptor linked to a

topoisomerase I inhibitor payload. In the DESTINY-Breast04 trial, T-DXd demonstrated significant improvements in progression-free and overall survival compared to standard chemotherapy in patients with HER2-low metastatic breast cancers, including TNBC. This landmark study has led to a paradigm shift in the classification and treatment of HER2-negative metastatic breast cancer, establishing T-DXd as the first HER2-targeted therapy to show clinically meaningful efficacy in HER2-low TNBC patients. [24,25]

- **Bevacizumab.** This monoclonal antibody targets vascular endothelial growth factor A (VEGF) and has demonstrated potential in normalizing tumor vasculature and enhancing the delivery of chemotherapy drugs, which may contribute to its anti-tumor effects[26]. It is approved for its use in mTNBC as it has shown promise in improving progression-free survival and response rates when combined with chemotherapy[27]. However, its efficacy in early-stage TNBC remains controversial[28]. Recent research has uncovered interesting immunomodulatory effects of bevacizumab in TNBC. A study found that bevacizumab treatment increased intratumor infiltration of stromal TILs, CD4+ and CD8+ T cells, and mature dendritic cells in primary TNBC tumors. These findings suggest that VEGF blockade could potentially enhance the efficacy of immunotherapy in TNBC by promoting a more favorable immune microenvironment[29].

The use of all these options depends on the individual patient, the intrinsic characteristics of the tumor, and the response to the previous treatment. To help the medical choice, the European Society for Medical Oncology (ESMO) published the Pan-Asian adapted **guidelines for diagnosis and treatment of patients with early and metastatic breast cancer**[23,30]. These guidelines summarize the choices of therapies that are available and the characteristics that patients need to accomplish to be considered. Briefly, the treatment choice is based on the type of breast cancer and multiple factors, such as the stage of disease, size of the tumor, or presence of lymph node involvement. However, the main common characteristic is that the management of TNBC is composed of surgery, radiotherapy if indicated, and chemotherapy. Figure 6 shows the organizational chart of management of early TNBC.

2. Triple-negative breast cancer

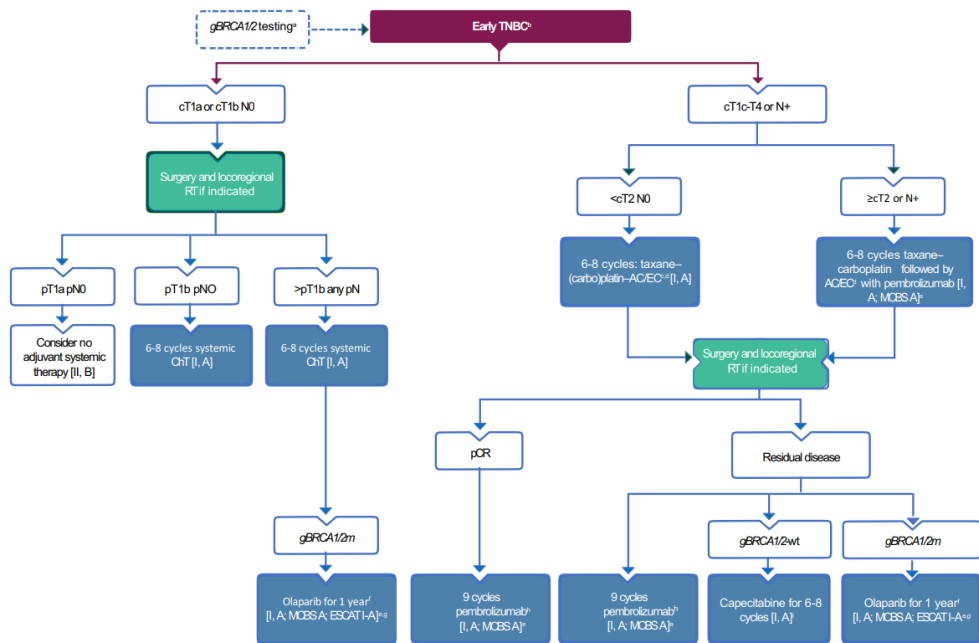


Figure 6. Organizational chart of management of early TNBC.

Burgundy box: general categories or stratification; blue box: systemic anticancer therapy; turquoise box: combination of treatments or other systemic treatments; white boxes: other aspects of management; dashed line: optional recommendation. AC, doxorubicin/cyclophosphamide; c, clinical; ChT, chemotherapy; EC, epirubicin/cyclophosphamide; ESCAT, ESMO Scale for Clinical Actionability of molecular Targets; ESMO, European Society for Molecular Oncology; gBRCA1/2, germline BRCA1/2; gBRCA1/2m, germline BRCA1/2; mutation; ICI, immune checkpoint inhibitor; m, mutation; MCBS, ESMO-Magnitude of Clinical Benefit Scale; N, node; p, pathological; pCR, pathological complete response; RT, radiotherapy; T, tumor; TNBC, triple-negative breast cancer; wt, wild type. Source:[30]

Regarding **mTNBC**, the study of **theragnostic markers** is mandatory because the presence or absence of PD-L1 and mutation in *BRCA* is the criteria of the treatment choice, which is explained in Figure 7.

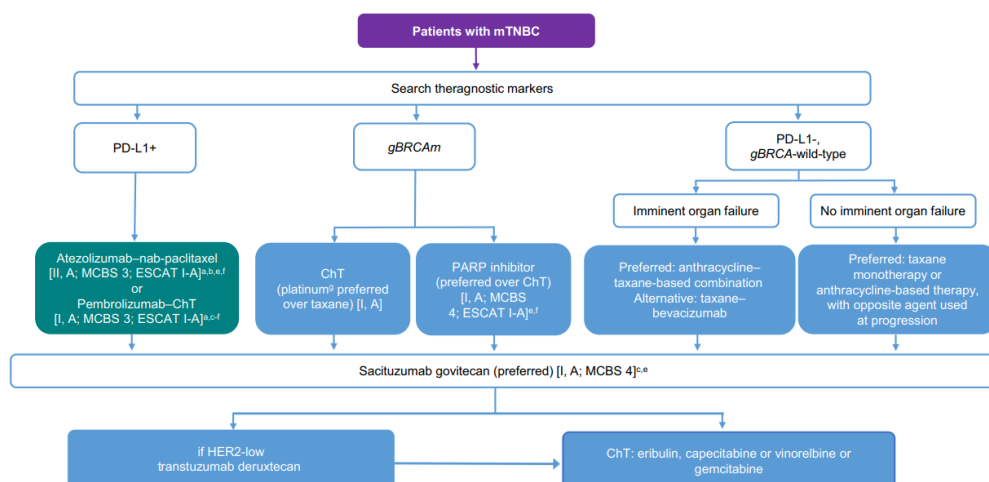


Figure 7. Organizational chart of management of metastatic TNBC.

Purple box: general categories or stratification; turquoise/green box: the combination of treatments or other systemic treatments; white boxes: other aspects of management; blue boxes: systemic anticancer therapy. ChT, chemotherapy; ESCAT, ESMO Scale for Clinical Actionability of Molecular Targets; *gBRCAm*, germline *BRCA1/2* mutation; MCBS, ESMO-Magnitude of Clinical Benefit Scale; mTNBC, metastatic triple-negative breast cancer; PARP, poly (ADP-ribose) polymerase; PD-L1, programmed death-ligand 1. Source:[23].

2.3. Cancer stem cells

Cancer stem cells (CSCs) are a subset of cells that exhibit characteristics analogous to those of normal stem cells, including unlimited self-renewal capacity, proliferative potential, and the ability to differentiate into diverse cellular lineages. These cells play a pivotal role in the pathogenesis and progression of cancer due to several key attributes[31].

CSCs can initiate and sustain tumor growth, even when present in small numbers, demonstrating their tumorigenicity and the possibility of establishing metastatic lesions. Among its characteristics, they often display enhanced resistance to conventional chemotherapeutic agents and ionizing radiation, potentially due to increased expression of drug efflux pumps, anti-apoptotic proteins, and enhanced DNA repair mechanisms[32].

Compared to other breast cancer subtypes, TNBC is particularly enriched with CSCs, which contribute to the aggressive nature and poor clinical outcomes associated with this subtype[33–35]. The two most well-established and studied CSC populations in TNBC are those expressing ALDH1 and those with a CD44⁺/CD24^{low/-} phenotype[34]. These CSC populations are associated with

2. Triple-negative breast cancer

chemotherapy resistance, tumor recurrence, and metastasis in TNBC patients[33]. Moreover, the presence of these populations in tumor tissues and circulating tumor cells has been correlated with poor prognosis, shorter disease-free survival, and reduced overall survival in TNBC[34,36].

On one hand, **ALDH1** is a detoxifying enzyme that belongs to the aldehyde dehydrogenase superfamily and is responsible for the oxidation of intracellular aldehydes[36]. The ALDH1+ cell population demonstrates enhanced invasive and metastatic capabilities, contributing to TNBC progression[34]. The resistance of ALDH1+ cells to chemotherapy is attributed to several factors, such as a high power of chemotherapeutics detoxification, particularly cyclophosphamide and other alkylating agents; stem cell properties including slow cell cycling[37] and enhanced DNA repair mechanisms; and higher expression of ABC transporters, which can actively pump out chemotherapeutic agents from the cells[38]. This CSC phenotype is characteristic of epithelial-like cells and usually are localized in the center of the tumor[39].

The **CD44⁺/CD24^{low/-}** phenotype is another important CSC marker in TNBC. CD44 is a cell surface glycoprotein involved in cell-cell interactions, cell adhesion, and migration, while CD24 is a cell adhesion molecule. Cells with the CD44⁺/CD24^{low/-} phenotype exhibit increased proliferative and tumorigenic potential[34]. They are predominantly in mesenchymal-like cells and localized in the periphery of the tumor[39]. These cells are also associated with chemoresistance, tumor recurrence, and metastasis in TNBC patients[34].

Interestingly, while both ALDH1+ and CD44⁺/CD24^{low/-} populations represent CSCs in TNBC, the overlap between these phenotypes is low, estimated at approximately 1% in primary tumors. However, cells expressing both markers are more tumorigenic than other cell populations[36].

Furthermore, another subpopulation of cells with stem cell-like properties identified in various cancers, including TNBC is the “**side population**”. This subset of cells is characterized by the ability to efflux compounds such as Hoechst 33342 dye due to the enhanced expression of ABC transporter[32,40,41]. In TNBC, the side population exhibits resistance to chemotherapy and self-renewal capacity, conferring the pivotal characteristics that contribute to the aggressive nature and treatment resistance of this breast cancer subtype[41].

2.4. Epithelial-mesenchymal transition

EMT is a biological process whereby **epithelial cells lose their cell-cell adhesion and polarity**, gaining migratory and invasive properties to become mesenchymal cells[42,43]. In cancer, including TNBC, this process contributes to therapy resistance and metastasis, as it enables tumor cells to detach from the primary site and invade surrounding tissues, establishing distant focus[42]. EMT is also associated with the acquisition of CSC characteristics as it induces stem cell-like properties in cancer cells. This connection is particularly relevant in TNBC, where the enrichment of therapy-resistant CSCs is more pronounced compared to non-TNBC subtypes[33].

Moreover, recent studies have revealed that TNBC tumors often contain a significant **population of hybrid epithelial/mesenchymal cells**, which exhibit both traits. These hybrid cells are thought to be highly plastic and possess enhanced metastatic potential compared to cells at either end of the EMT spectrum[42].

EMT is a complex process regulated by multiple signaling pathways also related to CSC features. Among the different signaling pathways, **TGF- β and Wnt** signaling play crucial roles in the initiation and progression of EMT (Figure 8).

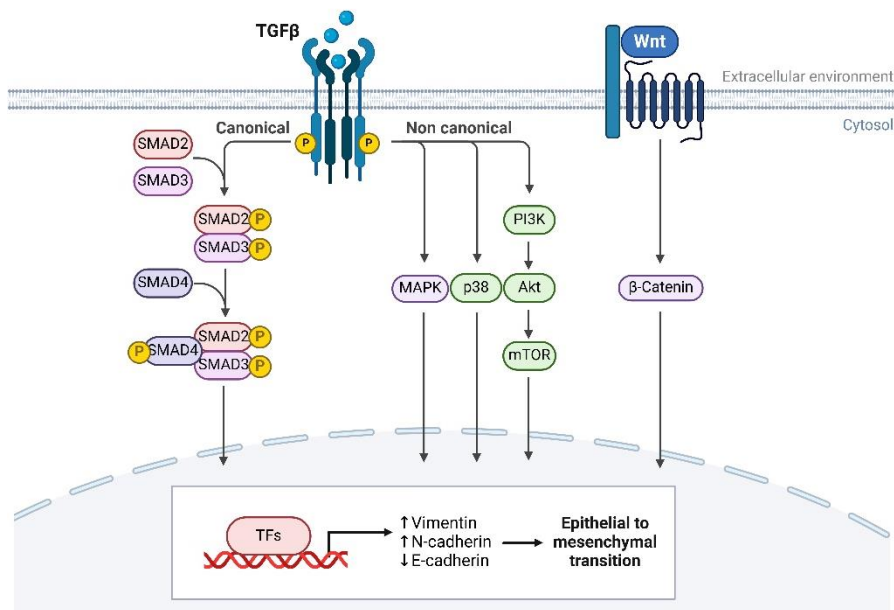


Figure 8. Schematic representation of TGF- β and Wnt pathways.

TFs: Transcription factors. Created with Biorender.

2. Triple-negative breast cancer

2.4.1. TGF- β signaling

TGF- β signaling induces EMT through multiple molecular mechanisms and its major isoform is TGF- β 1[44]. TGF- β elicits its effects through specific cell surface receptors, T β RI and T β RII, which form a heteromeric complex upon ligand binding. The canonical pathway involves the phosphorylation of SMAD2 and SMAD3, which form complexes with SMAD4 to regulate the expression of specific target genes[44,45]. These SMAD complexes activate the expression of key EMT transcription factors, including *SNAI1* (whose corresponding protein is Snail), *SNAI2* (whose protein is known as Slug), *ZEB1/2*, and *Twist*[45,46]. These factors orchestrate the downregulation of epithelial markers like E-cadherin and upregulation of mesenchymal markers such as N-cadherin and Vimentin, leading to enhanced cell motility and invasive capacity[45]. Additionally, TGF- β signaling can induce EMT through non-canonical pathways, including the activation of RAS-mitogen-activated protein kinase (MAPK), p38 MAPK, and PI3K signaling cascades[45].

2.4.2. Wnt signaling

The Wnt signaling pathway is a key regulator of EMT in TNBC[47]. In TNBC, dysregulation of both canonical and non-canonical Wnt signaling has been observed, contributing to tumor progression, metastasis, and poor patient outcomes[48].

Canonical Wnt signaling involves the stabilization and nuclear translocation of β -catenin, which acts as a transcription co-factor with TCF/LEF family proteins [47,48]. This activation results in the upregulation of key EMT-associated transcription factors, which in turn repress epithelial markers and induce mesenchymal markers[47,49]. Additionally, Wnt signaling enhances the expression of matrix metalloproteinases, promoting extracellular matrix degradation and facilitating tumor cell invasion[47].

The Wnt/ β -catenin pathway can synergize with other signaling cascades, such as TGF- β and EGFR, to enhance EMT and stemness in TNBC cells[49]. Moreover, the Wnt/ β -catenin/EMT axis has been implicated in promoting therapy resistance in TNBC[47].

3. N-myc downstream regulated gene 1

The N-myc downstream regulated gene (NDRG) family comprises four known proteins (NDRG1, NDRG2, NDRG3, and NDRG4) belonging to the α/β hydrolase superfamily. Initially identified as tumor suppressors, subsequent research has revealed their potential role in promoting tumorigenesis, depending on the cancer type[50].

Particularly, **NDRG1** has attracted considerable attention in TNBC research. This protein is encoded by the *NDRG1* gene, located on human chromosome 8q24.2, and consists of 394 amino acids[50,51]. NDRG1 is subject to various **post-translational modifications**, with **phosphorylation** being the most prominent. Several phosphorylation sites have been identified, including Ser330, Thr346, Thr356, and Thr366, which are targeted by the serum and glucocorticoid-regulated kinase 1 (SGK1)[52,53].

NDRG1 exhibits a **complex subcellular distribution** pattern, contributing to its diverse functions. While predominantly cytosolic (p-Thr346), it is also localized in the nucleus (p-Ser330), plasma membrane, mitochondria, perinuclear region, vesicles, and centrosome. This varied localization suggests that NDRG1 performs multiple functions with organelle specificity[52]. The protein is ubiquitously expressed in tissues, and its expression is induced in response to cellular stress signals, such as DNA damage or hypoxia. Various response mechanisms involved in cell differentiation, proliferation, growth arrest, neoplasia, tumor progression, and metastasis have been proposed and demonstrated[54].

NDRG1 has been extensively characterized as a **tumor suppressor** in multiple cancer types, including prostate[55,56], pancreatic[57,58], colon[56,59], and breast cancers[60]. In breast cancer research, numerous studies support NDRG1's role as a tumor metastasis suppressor and associate this protein with multiple pathways, including PTEN[60], SGK1[61,62], and Wnt/ β -catenin[63]. However, paradoxically, several recent studies have described a **pro-oncogenic role** for NDRG1 in liver[64,65], gastric[66,67], and bladder cancers[68]. These pleiotropic functions and their underlying mechanisms remain poorly understood. For instance, Park et al. observed differences in NDRG1 phosphorylation at Thr346 in prostate cancer and hepatocellular carcinoma after *PTEN* silencing, suggesting that differences in interactions with other molecules could be pivotal for the pleiotropic properties of NDRG1[52].

3. *N-myc downstream regulated gene 1*

In the context of TNBC, Nagai et al. investigated NDRG1 expression in patient samples using IHC and reported that its overexpression correlated with poorer prognosis[69]. Furthermore, in previous research by our team examining the **role of Activating Transcription Factor 4 (ATF4) downstream of TGF- β /SMAD** and mTOR pathways, we observed **hyperphosphorylation of NDRG1** (Thr346) following exposure of TNBC cells to TGF- β 1. Consequently, we postulated that in this TGF- β -mediated context of malignancy, overexpression of NDRG1 in this breast cancer subtype might exert a pro-oncogenic function[70].

4. Drug repurposing

4.1. Definition

Drug repurposing, also known as drug repositioning or reprofiling, has emerged as a promising strategy to accelerate the development of novel cancer therapies. This approach represents a tactic for **identifying new therapeutic applications for existing drugs** that were originally developed and approved for other indications by regulatory agencies such as the FDA (Food and Drug Administration) or EMA (European Medicines Agency)[71,72]

Reduced development timelines and costs are among the most highlighted advantages over *de novo* drug discovery. While the *de novo* discovery is currently estimated with a timeline of 10-17 years and associated with costs ranging from US\$1-2 billions[72], drug repurposing is characterized by a time development of 3-12 years and a significant reduction of economic investment estimated in US\$0.4-0.8 billions[73] (Figure 9). Moreover, drug repurposing offers **lower risks of failure** due to established safety profiles, whereas *de novo* strategy is characterized by substantial attrition rates during clinical trials, with approximately 90% of candidate compounds failing to progress due to unforeseen pharmacological properties or adverse effects[74]. While repurposed drugs are not immune to failure in clinical trials, they generally exhibit a lower probability of attrition due to toxicity-related issues as these candidates have already undergone extensive safety evaluations in their original indications. However, it is important to note that safety profiles may differ in new therapeutic contexts, necessitating continued vigilance throughout the clinical development process[71].

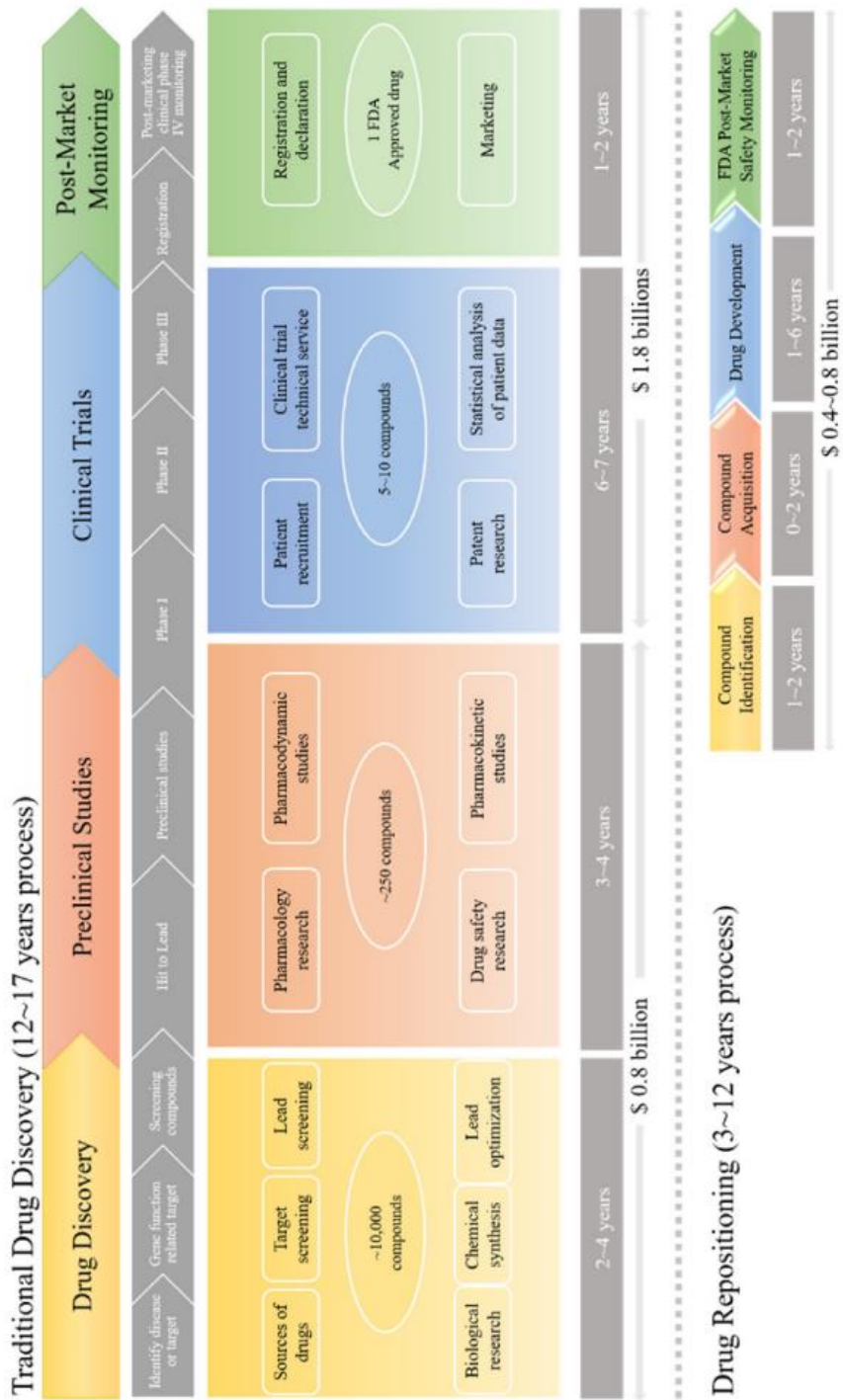


Figure 9. Schematic comparison between *de novo* drug development and drug repurposing.

Source:[73]

4. Drug repurposing

Even though the expectations are promising, drug repurposing still faces several **challenges** that must be addressed. Despite the established safety profiles of repurposed drugs, they still are at risk of **failure in later stages** of clinical trials. Drugs initially developed for specific indications may require different dosages or administration routes to achieve therapeutic efficacy in new applications[72]. Furthermore, **intellectual property considerations** also pose significant challenges. Securing patent protection for repurposed drugs can be difficult, especially if the new use is already known in scientific literature. Off-patent drugs offer a limited return on investment, discouraging industry funding. The regulatory landscape for drug repurposing is often complex and lacks clear guidance, consequently, insufficient regulatory incentives and the absence of formal guidelines on the repurposing process can discourage companies from investing in this area[71,72].

4.2. Classification of methods for drug repurposing

Drug repurposing in oncology has traditionally relied on understanding disease pathways or serendipitous discoveries. However, innovative strategies combining computational and experimental methods are now enhancing the success of drug repurposing efforts[71,72]. **Experimental approaches** utilize tools such as induced pluripotent stem cell models[72] and phenotypic screenings to perform high-throughput screening to study the potential of multiple compounds[71,72]. On the other hand, **computational or in-silico methods** are predominantly data-driven, involving the systematic analysis of diverse data types including gene expression, chemical structure, genotype, proteomic data, and electronic health records. The methodologies follow various techniques to identify potential new indications for existing drugs and facilitate their repurposing[71]. Actually, computational and experimental are often used synergistically to potentially accelerate the identification of promising repurposed drugs for cancer treatment, offering new avenues for therapeutic intervention[72].

4.2.1. Computational approaches

Molecular docking

Molecular docking is a strategy that intends to predict the binding affinity between drugs and target proteins. This approach is particularly useful for

identifying drugs that may interact with novel targets implicated in cancer pathogenesis[75]. Researchers can efficiently screen large libraries of existing drugs for potential anticancer activity by leveraging high-resolution protein structures and advanced docking algorithms. There are multiple docking programs like AutoDock (<http://autodock.scripps.edu/>) or GOLD (<https://www.ccdc.cam.ac.uk/solutions/software/gold/>). The virtual high-throughput screening programs need access to different databases of chemical compounds such as PubChem (<https://pubchem.ncbi.nlm.nih.gov/>) or DrugBank (<https://www.drugbank.com/>)[75,76].

Network-based methods

Network-based methods in drug repurposing integrate diverse biological data, including protein-protein interactions, drug-target interactions, and disease-gene associations, to construct comprehensive molecular networks. In these networks, nodes typically represent components such as genes, proteins, or drugs, while edges denote their associations. Protein-protein interaction networks can facilitate the discovery of novel therapeutic targets, with notable tools including PRISM (Protein Interactions by Structural Matching; <http://gordion.hpc.eng.ku.edu.tr/prism>) and OmicsNet (<https://www.omicsnet.ca/>). For drug-target networks, where nodes represent both drugs and their targets, platforms like DT-web (<https://alpha.dmi.unict.it/dtweb/>) and STITCH (Search Tool for Interactions of Chemicals; <http://stitch.embl.de/>) offer valuable resources. These computational approaches enable researchers to identify potential new indications for existing drugs by leveraging complex biological relationships[75,76].

Knowledge-based methods

Knowledge-based drug-repositioning methods leverage diverse information sources, including drug-target networks, signaling pathways, and clinical trial data, to enhance prediction accuracy in identifying new uses for existing drugs. These approaches utilize various computational techniques, such as network analysis, similarity inference, and binding site comparisons. These methods collectively demonstrate the power of integrating multiple data sources and computational techniques to uncover novel therapeutic applications for existing drugs[75]. For instance, meta-analysis of clinical trial data represents a valuable

4. Drug repurposing

approach for drug repurposing[71]. Retrospective analyses of clinical data, such as the method developed by Su and Sanger[77], enable the discovery of potential drug candidates for indications beyond their original testing conditions.

Signature-based strategies

Gene signatures, defined as characteristic gene expression patterns resulting from normal or pathological conditions, represent one of the resources for computational drug repurposing. This strategy compares the gene expression of diseases with those induced by drugs and provides potential new indications of therapeutic candidates. One of the main platforms for this type of drug repurposing is Connectivity Map[75].

4.2.1.1. Connectivity Map

The Connectivity Map (CMap) is a comprehensive resource that **leverages gene expression alterations between pathological states and therapeutic interventions**. This platform applies comparative analysis of gene expression signatures induced by disease states or *perturbagen* treatments, to identify similarities and further discover novel drug repurposed opportunities. To note, *perturbagens* are chemical or genetic agents that induce measurable changes in gene expression[78,79].

The CMap database contains information on over 27,000 genetic *perturbagens*, facilitating the creation of more than 470,000 unique expression signatures[78]. It incorporates 12,328 distinct genes, with 978 designated landmark genes due to their direct expression quantification[80]. This curated gene subset represents a **highly informative selection**, as these genes exhibit widespread expression patterns and accurately predict the expression of remaining genes through computational inference[81]. Of the 11,350 inferred genes, 9,196 are subjected to high-fidelity algorithmic analysis[80]. The set of these 10,174 highest-quality genes forms the core dataset utilized by the CMap tool for querying similar gene expression profiles within the database. Noteworthy, this focused approach may result in excluding certain genes from the database, emphasizing the importance of considering this limitation during analysis[82].

This platform includes the tool **CLUE** (CMap Linked User Environment), which **performs access to the data and generates a hierarchical clustering**

heatmap of *perturbagens*, ranked by their transcriptional similarity to the input gene signature query. A positive connectivity score indicates that the *perturbagen* induces a concordant gene expression profile, while a negative score denotes an inverse transcriptional response. The quantitative measure of this similarity is expressed as the "**connectivity score**" or ***tau***, a normalized metric ranging from -100 to +100. For each *perturbagen*, *tau* is defined as "the proportion of reference gene sets exhibiting greater similarity to the *perturbagen* than the query signature". Conventionally, *tau* values $\geq +90$ or ≤ -90 are considered statistically significant and merit further investigation[83]. The CMap database categorizes *perturbagens* into distinct classes, including small molecule compounds, gene knockdowns, and gene overexpression constructs[84].

In addition to individual *perturbagen* lists, CLUE generates heatmaps of ***Perturbagen Classes (PCLs)***, which are clusters of compounds sharing similar mechanisms of action or biological functions based on literature-derived annotations. PCL analysis and corresponding *tau* values provide a comprehensive perspective on the molecular mechanisms underlying specific gene signatures[85].

Furthermore, the CMap database incorporates **eight cancer-derived lines**: A375 (human malignant melanoma), A549 (human non-small cell lung carcinoma), HCC515 (human non-small cell lung adenocarcinoma), HEPG2 (human hepatocellular carcinoma), MCF-7 (human breast adenocarcinoma), PC3 (human prostate adenocarcinoma), VCAP (human metastatic prostate cancer), and HT29 (human colorectal adenocarcinoma); **and one non-cancerous line**, HA1E (immortalized human liver epithelial cells)[86].

CLUE search results present *perturbagen* lists with respective *tau* values for each cell line, along with two categories that summarize these values: **General** analysis' connectivity score is based on the median of *tau* scores for each cell line, and **Summary's *tau*** is derived from maximum quantile statistic. The latter provides a more sensitive measure for a subset of cell lines compared to mean or median values. These summary statistics are particularly useful when the optimal cell line is uncertain or when investigating conserved transcriptional responses across multiple cell types[87].

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4.3. Drug repurposing for TNBC

In the specific case of TNBC, numerous drug repurposing studies have been conducted to identify targeted therapies. There is **not any predominant methodology** among these approaches and, in some instances, serendipitous discoveries have initiated investigations. Some mechanisms of action explored in TNBC drug repurposing efforts are shown in Figure 10.

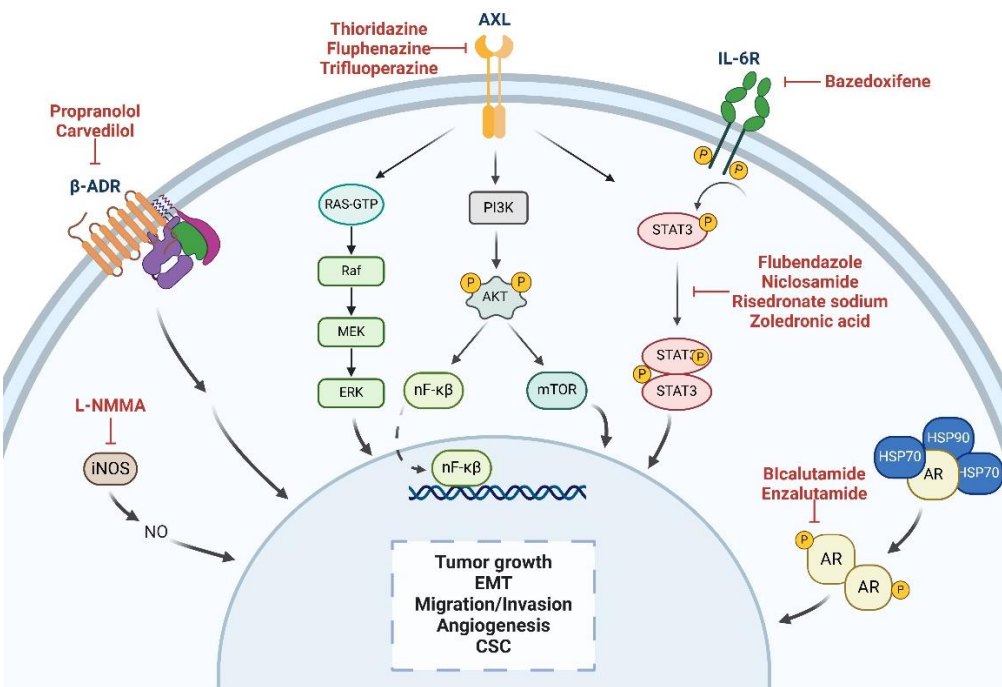


Figure 10. Representation of some pathways that are being studied for drug repurposing in TNBC.

Repurposed drugs are shown in red. AR, androgen receptor, β -ADR, beta-adrenergic receptor; CSC, cancer stem cell; EMT, epithelial-mesenchymal transition; IL-6R; interleukin-6 receptor, iNOS, inducible isoform of nitric oxide synthase; L-NMMA; NG-monomethyl-L-arginine; NO, nitric oxide. Adapted from[76]. Created with Biorender.

Androgen Receptor (AR). As previously mentioned, AR signaling plays a crucial role in the LAR subtype of TNBC, characterized by AR overexpression and enrichment in hormonally regulated pathways[88,89]. In TNBC, AR activation has been implicated in altering the tumor microenvironment, promoting tumor growth, and activating EMT pathways, thereby enhancing metastatic potential and CSC maintenance[90,91]. Given the significance of AR signaling in TNBC, several AR-targeted therapies, initially FDA-approved for prostate cancer, are

being investigated for drug repurposing. Notable among these are bicalutamide and enzalutamide, which are currently under clinical evaluation[92].

Bicalutamide, a first-generation, non-steroidal antiandrogen, functions as a competitive AR inhibitor[89,91]. *In vitro* studies have demonstrated its efficacy in reducing cellular proliferation, inhibiting colony formation, and inducing apoptosis in TNBC cell lines, which was mediated through the inhibition of the Wnt/ β -catenin signaling pathway. Furthermore, *in vivo* studies using TNBC xenograft models have corroborated these findings, showing decreased cellular viability and enhanced apoptosis[93]. The NCT00468715 phase II clinical trial in AR-positive TNBC patients yielded promising results in terms of clinical benefit rate[89]. Additional clinical trials combined with other therapies (NCT06365788, NCT03090165) are ongoing[89].

Enzalutamide, a second-generation, non-steroidal antiandrogen with higher AR binding affinity than bicalutamide[88,89,91], has also shown promising results in preclinical studies. Both *in vitro* and *in vivo* xenograft experiments have demonstrated its efficacy showcasing a reduction of proliferation or tumor growth due to the induction of apoptosis[90,94]. The NCT01889238 clinical trial resulted in improvements in progression-free survival and overall survival[89]. Additional clinical trials investigating enzalutamide combined with chemotherapy are underway, including a phase II trial (NCT02689427) and a phase Ib/II study (NCT02457910).

Beta-adrenergic receptor antagonists (β -blockers) are pharmacological agents primarily utilized for the management of cardiovascular pathologies, including arrhythmias and hypertension. These compounds are categorized based on their selectivity for β -adrenergic receptor subtypes (β_1 , β_2 , and β_3)[95]. β -adrenergic receptor (β -ADR) signaling in β -ADR-expressing tumor cells activates metastatic-associated genes involved in inflammation, angiogenesis, and EMT processes, downregulating antitumor response genes[96].

In the context of TNBC, *in vitro* studies have demonstrated increased expression of β_2 receptors, suggesting a potential therapeutic target to study drug repurposing[97]. Extensive *in vitro* and *in vivo* investigations have explored the antineoplastic properties of various β -blockers, with **propranolol** emerging as a particularly promising candidate. This non-selective β -blocker has demonstrated efficacy in reducing proliferation, migration, and invasion in TNBC *in vitro*, as well as inhibiting metastasis, angiogenesis, and tumor growth *in*

4. Drug repurposing

vivo[98,99]. Additionally, animal models have shown enhanced survival when propranolol is combined with conventional chemotherapeutic agents such as paclitaxel or fluorouracil. **Carvedilol**, a non-selective β -blocker with additional α -blocking properties, has also exhibited significant inhibitory effects on migration and invasion in TNBC cell lines[98]. While some researchers posit that non-selective β -blockers may offer superior antineoplastic properties in breast cancer[100], β 1-selective agents like atenolol have also demonstrated antiproliferative effects in TNBC *in vitro*[98].

Signal Transducer and Activator of Transcription 3 (STAT3). This pathway is a critical mediator of breast cancer pathogenesis, serving as both an early diagnostic marker and a contributor to tumor aggressiveness. STAT3 activation is implicated in cancer progression, proliferation, apoptosis evasion, metastasis, and chemoresistance. The STAT3 signaling cascade is initiated by various cytokines, including interleukin-6 (IL-6), and growth factors that activate a complex pathway culminating in STAT3 phosphorylation, which is responsible for transcriptional activation. Consequently, STAT3 activation induces the transcription of genes involved in CSC maintenance, EMT promotion, apoptosis suppression, and chemoresistance enhancement in breast cancer[101]. Several compounds targeting different mechanisms within this pathway are currently under investigation.

Bazedoxifene, a selective ER modulator FDA-approved for osteoporosis treatment in postmenopausal women[102], was identified as a novel IL-6 receptor inhibitor through structure-based drug repurposing[103]. In TNBC models, both *in vitro* and *in vivo* studies have demonstrated its ability to decrease cell viability, migration, and colony formation while increasing apoptosis and resensitizing cells to paclitaxel when used in combination[102,104].

Flubendazole, an FDA-approved anthelmintic agent that disrupts tubulin polymerization[105], has been shown to reduce cell proliferation *in vitro* and tumor growth *in vivo* at clinically relevant doses by inducing G2/M phase cell cycle arrest. Additionally, it has demonstrated efficacy in reducing the CD44^{high}/CD24^{low} CSC population, mammosphere-forming ability, and the expression of stemness genes in TNBC, as well as attenuating chemoresistance when used in combination[106].

Niclosamide, another FDA-approved anthelmintic agent, inhibits cell growth *in vitro* and tumor growth *in vivo* in TNBC studies[107]. It was identified as a breast

CSC inhibitor through high-throughput drug screening[108]. This drug reverses EMT and inhibits the stem-like phenotype in cancer cells, suggesting the potential for reversing cisplatin resistance[107]. Furthermore, it has been demonstrated that niclosamide acts as a radiosensitizer in both *in vitro* and *in vivo* TNBC models by reversing radioresistance generated through STAT3 and Bcl-2 activation and reduction of ROS[109].

Risedronate sodium and zoledronic acid, oral bisphosphonates approved for osteoporosis treatment, were identified as potential STAT3 inhibitors through comparative *in silico* docking studies. Their toxicity in TNBC cells *in vitro* was further demonstrated[110]. Zoledronic acid, in particular, has shown promising results for TNBC repurposing, reducing cell viability by inducing cell cycle arrest, inhibiting self-renewal capability, and decreasing EMT markers' expression[111]. When combined with doxorubicin *in vivo*, zoledronic acid shows promising results[112]. Additionally, a randomized phase II clinical trial (UMIN000003261) investigating zoledronic acid in combination with neoadjuvant chemotherapy in TNBC patients demonstrated an improved pathologic complete response rate in the combination group compared to chemotherapy alone that was later translated into higher disease-free survival in the combination group versus the chemotherapy group[113].

AXL, a member of the receptor tyrosine kinase family, plays a pivotal role in tumor progression through the activation of diverse signaling cascades. Upon ligand binding, AXL undergoes autophosphorylation at its cytoplasmic domain, triggering context-dependent downstream signaling pathways, including PI3K/AKT/mTOR, MAPK, and STAT cascades. These pathways orchestrate various tumorigenic processes, such as cell motility, invasion, and proliferation[114]. In TNBC, elevated AXL expression correlates with poor clinical outcomes, primarily due to its promotion of EMT through upregulation of mesenchymal markers (Vimentin, Twist, Snail, and Slug). AXL overexpression is also strongly associated with chemoresistance, enhanced tumorigenesis, metastatic potential, and CSC maintenance[114,115]. The inhibition of AXL signaling has demonstrated attenuation of these oncogenic processes, leading to the identification of potential therapeutic agents for TNBC through gene signature analysis of AXL blockade. CMap analysis revealed **phenothiazines**, a class of antipsychotic compounds traditionally used as dopamine receptor antagonists, as potential candidates for TNBC treatment. *In vitro* and *in vivo* studies of three phenothiazine derivatives (thioridazine, fluphenazine, and

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trifluoperazine) demonstrated significant antineoplastic properties. *In vitro* experiments revealed decreased cell invasion, proliferation, and viability, coupled with increased apoptosis in TNBC cell lines. Notably, these compounds enhanced sensitivity to standard chemotherapy when combined with paclitaxel. *In vivo* studies demonstrated significant reductions in tumor growth and metastasis. Intriguingly, the mechanism of action for these compounds in cancer cells does not involve dopamine receptor antagonism or direct inhibition of AXL activity. Instead, they suppress the PI3K/AKT/mTOR pathway, aligning with the gene signature profile observed upon AXL inhibition. This mechanistic insight provides a compelling rationale for repurposing phenothiazines as potential therapeutic agents in TNBC treatment[115].

Nitric Oxide Synthase (NOS) is the enzymatic catalyst responsible for the biosynthesis of nitric oxide (NO), a pleiotropic signaling molecule with diverse physiological functions. NO, characterized by its short half-life, interacts with various cellular targets, resulting in the formation of nitrites, nitrates, S-nitrosothiols, and nitrosamines. These nitrogenous compounds have been implicated in DNA damage and subsequent mutagenesis[116]. The inducible isoform of NOS (iNOS) has been correlated with poor prognosis in breast cancer patients, attributed to its role in promoting tumor aggressiveness by us[117]. Dave and Granados-Principal et al. elucidated a mechanistic pathway wherein overexpression of *RPL39* and *MLF2* genes, known to enhance CSC properties and metastatic potential, induces iNOS expression[118]. Consequently, this discovery prompted our investigations into iNOS inhibition as a targeted therapeutic strategy for TNBC. Among the evaluated NOS inhibitors, the pan-NOS inhibitor **L-NMMA** (NG-monomethyl-L-arginine) demonstrated superior efficacy in both *in vitro* and *in vivo* assays[117,119]. L-NMMA exhibited antiproliferative, anti-migratory, and anti-CSC self-renewal properties *in vitro*, while it suppressed tumor growth, CSC self-renewal, and tumor initiation in TNBC xenograft models. Furthermore, the combination of L-NMMA with docetaxel significantly prolonged survival in murine models[117] and enhanced chemotherapeutic response in patient-derived xenograft (PDX) models of TNBC[119]. The clinical translation of this therapeutic approach is currently under investigation in a phase Ib/II study involving patients with refractory locally advanced or metastatic TNBC (NCT02834403).

5. Three-dimensional study models

The development of novel therapies for TNBC is significantly constrained by the scarcity of models that accurately recapitulate human disease. These models are crucial for researchers to **elucidate the complex biology of tumors**, as well as to serve as tools in the drug development pipeline, enabling preclinical evaluation of potential therapeutic agents[120].

Even though traditional two-dimensional cell culture is still extensively used for drug research, three-dimensional (3D) models have emerged as powerful tools for studying cancer, offering a more accurate representation of tumor biology[120,121]. Among these models, PDXs, patient-derived organoids (PDOs), and PDX-derived organoids (PDxOs) have gained significant attention in cancer research and drug discovery (Figure 11)[121–123].

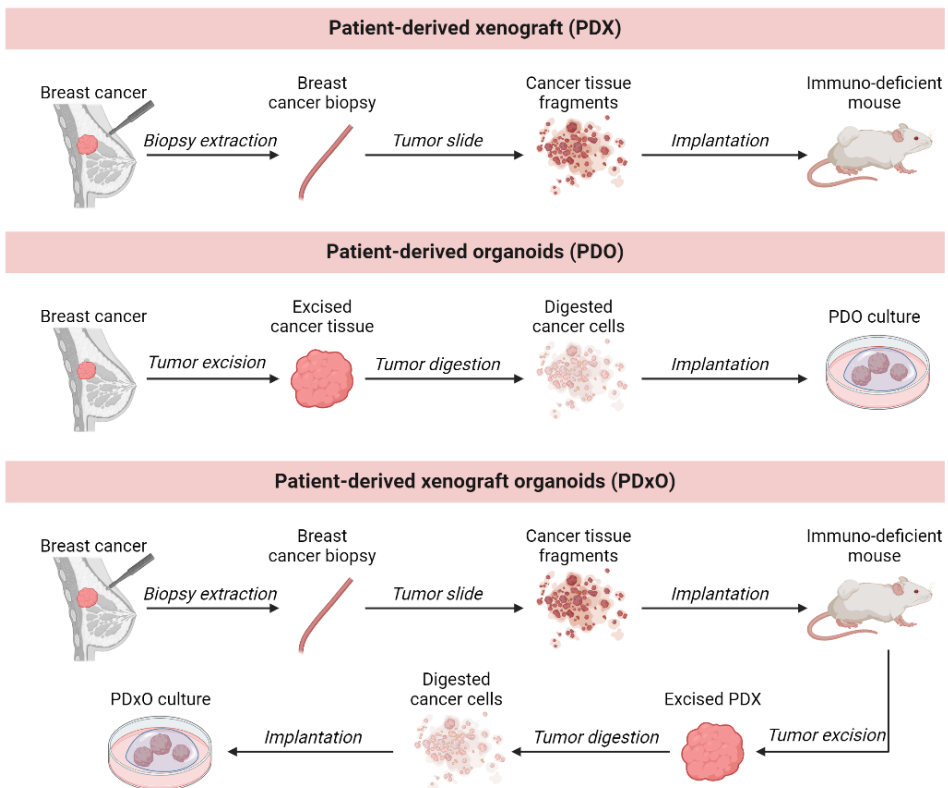


Figure 11. Summary of three-dimensional models for breast cancer research.

PDOs, patient-derived organoids; PDXs, patient-derived xenografts, PDxOs, patient-derived xenograft organoids. Created with Biorender.

5. Three-dimensional study models

5.1. Patient-derived xenografts (PDXs)

PDXs have emerged as a powerful tool in breast cancer research, offering significant advantages over traditional cell line-based approaches. PDXs are established by **implanting fresh tumor tissue** from patients **directly into immunodeficient mice**, more specifically, in the case of breast cancer, tumor grafts localized orthotopically in the mouse breast[120].

PDX model preserves the heterogeneity, histopathology, and genetic complexity of the original tumor. For instance, observed variations are not due to the murine environment but to the spontaneous evolution of solid tumors[121,124]. Hence, this model is particularly valuable in breast cancer research:

- **Tumor biology studies.** The high fidelity to the original tumor enables the research of tumor progression, metastasis, tumor microenvironment, and identification of biomarkers for diagnosis, prognosis, and treatment[121,124].
- **Drug development and preclinical testing.** PDX models can recapitulate patient responses to various therapies, including chemotherapy, and targeted agents[121,125].

Despite these advantages, it is important to note some **limitations** of PDXs. These include the loss of human stromal components[121,124,125]; the lack of a fully functional immune system, as immunocompromised mice have to be used; and the potential selection bias during engraftment[121,125], as most breast cancer PDX collections predominantly represent more aggressive tumors, typically characterized by low ER positivity, high Ki67 expression, and lymph node involvement[125]. Moreover, this 3D model is not preferred for high throughput drug screening and implies high costs[123].

5.2. Patient-derived organoids (PDOs)

PDOs are 3D cultures derived from patient tumor samples, **grown in a specialized extracellular matrix**, that closely recapitulate the histological and molecular characteristics of the original tumor. This model which has been successfully established for several tumors including breast cancer, has emerged as a powerful tool for studying cancer, offering several advantages over traditional two dimensional cell cultures and PDXs[126]. PDOs present remarkable potential for studying tumor heterogeneity and personalized drug responses.

First, as they **retain the cellular diversity and genetic profile** of the original tumor, it is more feasible to study the intrinsic mechanisms of tumorigenicity, intratumor heterogeneity, and potential biomarkers. Furthermore, PDOs can **be genetically modified** more easily compared to PDXs[126].

Regarding treatment responses, the use of PDOs allows a double perspective. They can be used for **drug high-throughput screening** as a preclinical model and also represent a **real-time platform for personalized medicine** as they can predict patient responses to various therapies[127]. For instance, the landmark study performed by Sachs et al. successfully cultured more than 100 breast cancer organoids from surgical breast cancer tissues, establishing a pioneering breast cancer organoid repository. They observed that the histopathology and hormone receptor status of breast cancer organoids were similar to those of the original tumor. Moreover, they noted that breast cancer organoids can be used for *in vitro* drug screening and respond similarly to *in vivo* xenografts and patients[128].

Furthermore, another advantage of PDOs is that it is possible to establish **culture from healthy and tumor tissue**. Hence, it provides benefits to testing drugs in both kinds of tissue and improving targeted therapy studies[126].

Despite their promise, PDOs face some limitations, and consequently, this model presents advantages and inconveniences compared to PDXs. On one hand, PDOs require less time and costs, they can be maintained in culture for **long periods of time**[126]. Moreover, as they avoid the use of animals, **no alterations between species** are possible. However, PDOs are characterized by the **absence of vasculature**, lower heterogeneity, **limitations of metastatic** models, and lower clinical validation and they also lack a complete tumor microenvironment[126]. This last issue is trying to be overcome by developing co-culture systems with immune cells[129] (Table 2).

5. Three-dimensional study models

Table 2. Comparison between PDXs and PDOs model characteristics.

Respective features were judged as best (+++), suitable (++), possible (+), not very suitable (±) or unsuitable (-). ^a: the immune system could be implemented by co-culturing PDOs with hematopoietic cells. Source:[126]

Features	PDXs	PDOs
Success rate of initiation	++	+++
Ease of maintenance	±	++
Resource consumption	High	Medium
Expansion	+	++
Retention of phenotypic features <i>in vitro</i>	+++	++
Retention of genetic features <i>in vitro</i>	++	++
Representation of cancer spectrum	++	++
Amenable to genetic modification	-	+++
Matched normal controls	-	+++
Tumor-stroma interactions	++	-
Incorporation of an immune system	-	± ^a
Genetic cancer modeling (initiation and progression)	-	+++
Low-throughput drug screens	+	+++
High-throughput drug screens	-	++
Biobanking	-	+++

5.3. PDX-derived organoids (PDxOs)

PDxOs represent a **bridge between PDXs and PDOs** models. They are created by establishing organoid cultures from existing PDX tumors. PDxOs have been shown to **maintain high similarity** to their parental PDXs in terms of genomics, histopathology, and drug response profiles, suggesting a biological equivalence between the two models[123].

PDxOs represent an innovative approach to breast cancer research, combining the advantages of PDX and organoid cultures. This model confers a new platform suitable for studying new therapies based on genomic alterations as they maintain high fidelity with the original tumor while providing a **more accessible and high-throughput platform** for experimentation[123,130].

Similar to PDOs cultures, they present several advantages such as **long-term maintenance**, preservation of **tumor heterogeneity**, and the possibility to enable rapid and cost-effective drug screening[123]. Nevertheless, they also share the main limitation which is the **absence of tumor microenvironment and immune system**. Consequently, it is not possible to study these conditions

nor to test drugs involving them. Furthermore, candidates cannot be required to be activated by the liver[130].

Contrary to PDOs, the necessary time to establish the PDXs tumors avoids the possibility of a real-time co-clinical assessment[130]. However, the chance to generate **both PDXs and PDxOs** models from the same tumor sample provides a powerful tool for **validating *in vitro* findings in an *in vivo*** setting that is not offered with the establishment of PDOs[123].

To date, PDxOs have been successfully established from various types of cancer including prostate[131], breast[123], colorectal cancer[132], salivary gland carcinoma[133], medulloblastoma[134], and esophageal squamous cell carcinoma[135].

Concerning breast cancer, the landmark study of Guillen et al.[123] established a large collection of breast cancer PDX models and matched PDxOs, showcasing their potential for drug screening and therapy decision-making. The study reported an 85% success rate in generating PDxOs lines from PDX tumors, with cultures maintained for over 200 days.

HYPOTHESIS AND JUSTIFICATION

The primary challenge in TNBC lies in identifying specific molecular targets for the development of targeted therapies. Currently, the standard of care remains chemotherapy, which is associated with a significant burden of adverse effects.

In previous investigations conducted by our research group, which focused on elucidating the role of ATF4 downstream of the TGF- β signaling pathway, we observed a notable upregulation of NDRG1 following TGF- β 1 stimulation in TNBC cells. Given the pleiotropic activity of NDRG1 in cancer, we hypothesize that this protein exhibits pro-oncogenic characteristics when the TGF- β pathway is stimulated. Consequently, the aim of this doctoral thesis is to elucidate this subject.

Furthermore, this finding suggests a potential therapeutic target in the TGF- β -NDRG1 axis for TNBC treatment. However, given the pleiotropic and unpredictable nature of NDRG1, direct or indirect inhibition of this protein may pose significant risks. To address this therapeutic challenge, we propose a novel strategy utilizing RNA sequencing in TNBC cells following *NDRG1* gene inhibition and TGF- β 1 stimulation.

This approach is expected to yield valuable transcriptomic data that will facilitate the identification of repurposable drugs capable of modulating gene expression in a manner analogous to the silencing of TGF- β -activated pro-oncogenic *NDRG1*. By doing so, we aim to standardize the unique NDRG1-associated condition observed in specific patients, effectively "democratizing" this therapeutic approach.

To enhance the validation of these candidate drugs and expedite translation into clinical practice, we propose the use of PDOs or PDxOs from TNBC patients. The utilization of these 3D models, which recapitulate the heterogeneity of tumors, will allow the identification of patients who are likely to benefit from this novel therapeutic strategy and the evaluation of drug efficacy in patient-specific microtumors grown *in vitro*.

This hypothesis, if validated, has the potential to significantly impact TNBC treatment strategies, offering a more personalized and effective approach to managing this aggressive form of breast cancer. By leveraging advanced genomic technologies and patient-derived models, we aim to bridge the gap between bench research and bedside application, potentially revolutionizing TNBC treatment paradigms.

OBJECTIVES

The **primary aim** of this study is to elucidate the pro-oncogenic role of NDRG1 in TNBC cell lines and subsequently design and validate targeted therapeutic approaches using TNBC patient-derived organoid cultures.

The **specific objectives** are:

- I. To study the role of NDRG1 after TGF- β stimulation and perform a drug repurposing study by transcriptomic analysis.
- II. To validate the efficacy of repurposed candidates in TNBC cell lines, analyze their effect against the phenotypic properties of CSCs, and unravel their mechanisms of action.
- III. To generate organoid cultures derived from patients with TNBC (PDOs).
- IV. To assess the efficacy of repurposed drugs on organoids.

MATERIALS AND METHODS

1. *NDRG1* analysis in breast cancer patients in public datasets

To study if the expression of *NDRG1* could be related to malignancy in breast cancer patients, we analyzed the association between *NDRG1* mRNA expression and relapse-free survival (RFS) with KM plotter and GOBO. We performed this study in all cases of breast cancer, luminal A, luminal B, HER2+, basal-like, and TNBC. Specifically, we split patients by median expression of *NDRG1* in the LM plotter, while in GOBO we divided them by three quantiles (low, median, high expression). In both databases, we used the Affymetrix probe 200632_s_ for the analysis.

2. Immunohistochemistry of TNBC tumor biopsies

In collaboration with the Department of Pathological Anatomy at Hospital de Jaén, we investigated the expression of *NDRG1* protein via IHC in 75 TNBC tumor biopsies, incorporated into tissue microarrays provided by the Andalusian Health Service Biobank.

They sectioned formalin-fixed, paraffin-embedded tumor biopsies at 4 µm thickness and performed the deparaffinization, rehydration, and antigen retrieval using standard procedures with EnVision FLEX reagents (Agilent Dako). They conducted antigen retrieval at pH 6.0 for 20 min at 97°C as per the manufacturer's recommendations. Subsequently, they washed the slides in diluted EnVision FLEX Wash Buffer and incubated them in EnVision FLEX Peroxidase-Blocking Reagent solution for 5 min. Following another wash, they incubated the slides with primary antibody against *NDRG1* (1:75 dilution, Cell Signaling) at room temperature (RT) for 20 min using an automated system (Autostainer link 48, Dako). Then, the slides were washed and incubated with Dako EnVision FLEX/HRP for 20 min, and further washed and incubated with EnVision FLEX Substrate Working solution for 8 min. Counterstaining was performed with hematoxylin (EnVision FLEX Hematoxylin). Finally, they dehydrated the slides and mounted the samples in a Leica CV5030 automatic mounter with mounting media (Panreac).

A pathologist analyzed *NDRG1* staining intensity using digital images obtained with a 3DHISTECH preparation scanner. The intensity was scored as 0 (negative), 1+ (weak), 2+ (moderate) and 3+ (intense). Additionally, another score was implemented by multiplying the intensity value (0-3) by the percentage of stained cells (0-100%), resulting in a possible range of 0 to 300.

In collaboration with the biostatistics unit of the "Fundación para la Investigación Biosanitaria de Andalucía Oriental" (FIBAO) at the University Hospital of Jaén, we conducted a comprehensive analysis of the patient samples. In this analysis, continuous variables were characterized using means and standard deviations, while categorical variables were presented as absolute frequencies and relative percentages. We assessed the normality of numeric variables using the Shapiro-Wilk test. To identify correlations between different markers, we performed Spearman correlation analyses.

We calculated patient survival using Kaplan-Meier analysis and used the Log-rank test for comparisons. To determine risk or protective factors associated with mortality, we conducted individual Cox regression analyses, calculating raw hazard ratios (HR) and confidence intervals (CI). For numeric variables, we established thresholds according to Youden's index using receiver operating characteristic (ROC) curves[136].

In constructing the Cox multivariate regression model, we included variables with $p < 0.20$ and calculated adjusted HR and CI. We considered $p < 0.05$ as statistically significant for all analyses. We performed all statistical analyses using IBM SPSS v21.0 software.

3. Cell culture

3.1. Cell lines

We used four established cell lines of TNBC: two obtained from primary breast (SUM159 and BT549), and two from pleural effusion (MDA-MB-231 and MDA-MB-436). All cell lines were acquired from the American Type Culture Collection (ATCC), except SUM159, which was from Asterand Bioscience.

- SUM159 (Asterand ref: SUM159PT) was obtained from an anaplastic carcinoma of a 71 years-old, white, female breast cancer patient.
- BT549 (ATCC ref: HTB-122) was from a mammary gland invasive ductal tumor of a 72-year-old, white, female breast cancer patient.
- MDA-MB-231 (ATCC ref: HTB-26) was isolated from the mammary gland of a 51-year-old white female with adenocarcinoma.
- MDA-MB-436 (ATCC ref: HTB-130) was from an adenocarcinoma in the mammary gland of a 43-year-old, white female.

We also utilized a docetaxel-resistant cell line (SUM159-R) that was generated by our team and previously characterized[137].

To produce R-Spondin enriched media, we used HA-R Spondin 1-Fc 293T cells, which were gently donated by Dr. Silvestre Vicent Cambra and Dr. Mariano Ponz-Sarvisé (Centro de Investigación Médica Aplicada CIMA, Universidad de Navarra, Pamplona, Spain).

To produce Noggin-enriched media, we used HEK293 cells transfected with plasmid pcDNA NEO insert mouse Noggin, which were kindly provided by Dr. Hans Clevers (Hubrect Institute, Netherlands).

3.2. Culture conditions

We performed cell culture under sterile conditions in a Biological Safety Cabinet type 2A with a laminar flow hood (Thermo Fisher Scientific). We maintained all cell lines in media consisting of Dulbecco's Modified Eagle Medium (DMEM; PAN Biotech, Hamburg, Germany; ref: P04-03590) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich; ref: F9665) at 37°C, 5% CO₂, and 90% humidity in a Steri-Cult incubator (Thermo electron corporation).

For maintenance, we changed culture media every two to three days and, when cells reached 80-90% confluency, we passaged them following the next steps: we removed the media, we washed the cells with phosphate-buffered saline (PBS, Merck, ref: 524650) and enzymatically detached them with trypsin-EDTA (Sigma-Aldrich, ref: T4049) for 3 min at 37°C. We inactivated trypsin by the addition of culture media, and we reseeded the cell lines into another 75 or 175 cm² culture flasks. We passed the cell line for a maximum of four months.

3.3. Cryopreservation

To maintain the cell lines, they were frozen in liquid nitrogen. When flasks reached a 90% confluence, we collected the cells as described above and centrifuged them at 300×g for 5 min at RT. We resuspended the cells in 1 ml culture media supplemented with 10% dimethyl sulfoxide (DMSO; AppliChem GmbH, Darmstand; ref: A3672) and transferred them into cryotubes. We stored the cryotubes in isopropanol cryo-freezing containers at -80°C (Thermo Fisher Scientific). After 24 or 48 h, we transferred them into a liquid nitrogen tank (Taylor Wharton 24K K Series Cryostorage System) at -196°C.

3.4. Cell thawing

To recover frozen cells from -80°C or liquid nitrogen, we rapidly thawed cryotubes at 37°C in a water bath, resuspended them in culture media, and centrifuged them at 300×g for 5 min at RT to remove DMSO. We resuspended the pellets in culture media and seeded them in a 75 cm² culture flask.

4. TGF- β stimulation

We stimulated the TGF- β pathway by adding TGF- β 1 (10 ng/ml; Peprotech; ref: 100-21) for 8 h to the cell lines. In the case of the study of ALDH1⁺, CD44⁺/CD24⁻ and “side population” CSC populations of SUM159 and BT549 were stimulated for 14 days (replenishing every 72 h).

5. Gene knockdown

We inhibited *NDRG1* for 48 h by transient transfection with siRNA from Santa Cruz (50 ng/ml; ref: sc-36021) and lipofectamine RNAiMAX (Invitrogen). We used scrambled siRNA (SCR) as the negative control.

6. CLUE query

To conduct drug repurposing, we utilized the CMap database and CMap Query tool (<https://clue.io/>) developed at the Broad Institute.

Specifically, we leveraged the differential expression profile resulting from *NDRG1* inhibition in TGF- β 1-stimulated MDA-MB-231 cells, which we had previously obtained through RNA sequencing. Our objective was to identify compounds capable of inducing similar transcriptional signatures.

We performed two separate queries using genes with logarithmic fold change ($\log_2$ fold change) thresholds of ± 1.5 and ± 1.6 , respectively. We considered compounds with *tau* scores higher than 80, 90, and 96 for General, Summary, and MCF-7 cell line analyses, respectively. We used DrugBank (<https://www.drugbank.ca/>), Drug Information Portal (<https://druginfo.nlm.nih.gov/drugportal/>) PubMed (<https://www.ncbi.nlm.nih.gov/pubmed/>) and ClinicalTrials.gov (<https://clinicaltrials.gov/>) databases for further information and pre-select only FDA-approved drugs that were novel for TNBC treatment.

7. Treatments

We treated cells with efavirenz, ouabain, vinburnine, or docetaxel (MedChemExpress; Monmouth Junction, NJ, USA) at the indicated concentrations, dissolved in DMSO (Sigma-Aldrich). Control cells were treated with 1% DMSO as the vehicle.

8. Viability study

8.1. Repurposed drugs IC₅₀ calculation

We conducted a cell viability study to evaluate the efficacy of repurposed drug candidates and to determine their half-maximal inhibitory concentration (IC₅₀) values. For this purpose, we performed the WST-1 assay, which measures cellular metabolic activity as an indicator of viability, proliferation, and cytotoxicity. This colorimetric method is based on the reduction of the tetrazolium salt WST-1 to formazan dye by the succinate-tetrazolium reductase system in the mitochondrial respiratory chain. Consequently, the absorbance measurement directly correlates with the number of metabolically active cells[138].

In 96-well plates, we seeded 2,000, 1,000, 1,500, and 800 cells of MDA-MB-231, SUM159, BT549, and SUM159-R, respectively, per well and 6 wells per condition. After 24 h, we treated them at increasing concentrations of each compound for 72 h, or vehicle (DMSO). The different range of concentrations is summarized in Table 3.

Table 3. Treating concentrations for cell viability assay and calculated IC₅₀.

	MDA-MB-231	SUM159	BT549	SUM159-R
Efavirenz (μM)	2, 10, 20, 30, 40, 50, 60, 70, 80	2, 10, 20, 30, 40, 50, 60, 70, 80	0.1, 0.25, 0.5, 1, 5, 10, 20, 40	0.5, 1, 2, 5, 10, 20, 40, 60, 80
Ouabain (nM)	25, 50, 75, 100, 150, 250, 500, 1000	10, 25, 50, 75, 100, 150, 250, 500	5, 10, 25, 50, 75, 100, 250, 500	0.5, 1, 2.5, 5, 10, 25, 50, 100, 500
Vinburnine (μM)	0.05, 0.1, 0.25, 0.5, 1, 5, 10, 20, 40	0.05, 0.1, 0.25, 0.5, 1, 5, 10, 20, 40	0.1, 0.25, 0.5, 1, 5, 10, 20, 40	0.05, 0.1, 0.25, 0.5, 1, 5, 10, 20, 40

After three days, we added 10% WST-1 reagent to each well and incubated the plates for 1-2 h at 37°C in darkness, following the manufacturer's instructions.

We then measured the absorbance at 450 nm using an M200 Nanoquant plate reader (Tecan). Cell viability was expressed as normalized absorbance relative to control cells. Finally, we calculated IC₅₀ values through the graphical representation of dose-normalized response curves using GraphPad Prism 9.0.0 software (Figure 12).

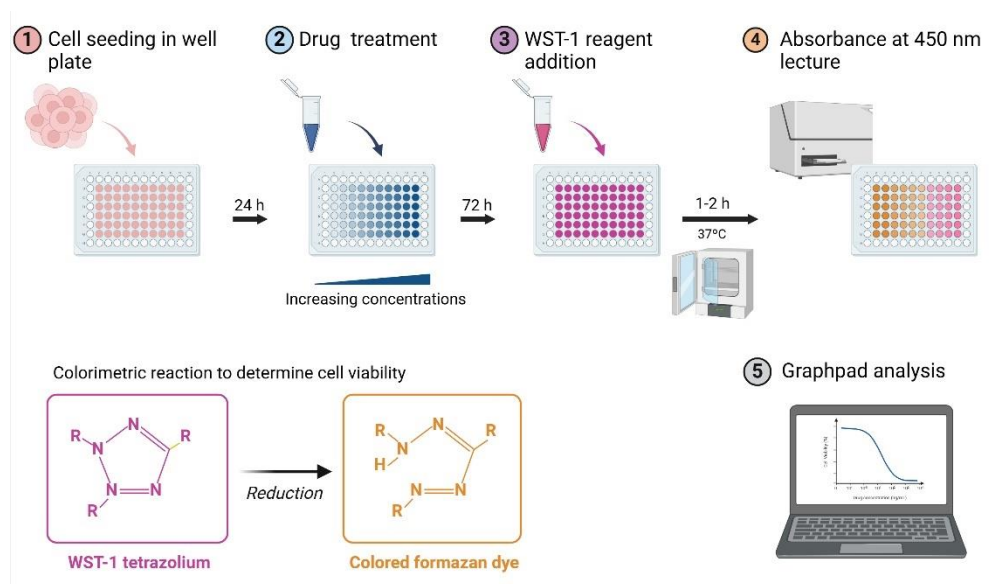


Figure 12. Schematic procedure to assess cell viability using WST-1 reagent.

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8.2. Docetaxel IC₅₀ calculation

In the case of SUM159-R, we studied if repurposed drugs could restore chemosensitivity to docetaxel. For this aim, we calculated the IC₅₀ value for docetaxel in combination with vehicle or repurposed drug treatment for 72 h.

Briefly, in 96-well plates, we seeded 800 SUM159-R cells, per well and 6 wells per condition. After 24 h, we treated them with the vehicle or IC₅₀ value of our repurposed drugs and increasing concentrations (nM) of docetaxel for 72 h: 25, 50, 75, 100, 200, 300, 500, and 750. After 72 h, as described in the previous section, we used WST-1 reagent and GraphPad Prism 9.0.0 software (Figure 12).

9. Gene expression

9.1. RNA extraction

In 6-well plates, we cultured MDA-MB-231 and SUM159 cell lines and treated them with IC50 of repurposed drug candidates for 12 h. We isolated total RNA using GeneJET RNA Purification Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Briefly, we removed the culture media and washed the cells with PBS twice. Then, we added 600 μ l of lysis buffer supplemented with 2% β -mercaptoethanol (Sigma-Aldrich), mixed, and collected in an Eppendorf tube to further vortex for 10 s. We next added 360 μ l of absolute ethanol and mixed each sample by pipetting. To isolate RNA from the rest of the sample, we transferred the mix through GeneJET RNA Purification columns and centrifuged at 12,000 $\times$ g for 1 min. After discarding the flow-through solution, we washed the column with 700 μ l of Wash Buffer 1 (ethanol-supplemented), centrifuged, and discarded the flow-through solution again. Next, we washed the column twice with Wash Buffer 2 (ethanol-supplemented), using 600 μ l and 250 μ l respectively, with centrifugation steps in between. For the final elution, we added 60 μ l of ultra-pure water to each column and centrifuged at 12,000 $\times$ g for 2 min. The extracted RNA was collected in clean Eppendorf tubes and stored at -80°C for further analysis.

9.2. RNA quantification

RNA concentration was quantified with a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific). We measured absorbance at 260 and 280 nm and calculated the OD260/OD280 ratio to assess RNA purity with values between 1.8 and 2.0 indicating high-quality nucleic acids. The instrument calculates RNA concentration based on the principle that one OD260 unit corresponds to 40 mg/ml of nucleic acids.

9.3. cDNA synthesis

mRNA was retrotranscribed to cDNA by using the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. On ice, we combined 2 μ l of iScript Reaction Mix, 0.5 μ l of iScript Reverse Transcriptase, 1 μ g of extracted RNA, and ultra-pure water to a total volume of 10 μ l per sample. After thorough mixing, we conducted the reverse transcription reaction in a thermocycler. The protocol consisted of priming at 25°C for 5 min, reverse

transcription at 46°C for 20 min, and enzyme inactivation at 95°C for 1 min. We then immediately placed the tubes on ice and stored the resulting cDNA at -20°C for future use.

9.4. Real-time quantitative PCR (RT-qPCR)

To perform real-time quantitative PCR (RT-qPCR) of mRNA, we utilized iTaq Universal SYBR Green Supermix (Bio-Rad). On ice, we prepared the reaction mix combining Supermix (2X concentration) with forward and reverse primers (final concentration 500 nM/each primer). In separate tubes, we diluted the cDNA samples to 15 ng/μl using ultra-pure water. Using an automatic multichannel micropipette, we added 6 μl of the reaction mix and 4 μl of diluted cDNA to each well of the RT-qPCR plate. The plate was sealed with a transparent film, centrifuged at 300×g for 1 min at RT, and placed in a QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific). We followed the manufacturer's recommended thermal cycling conditions: initial denaturation and polymerase activation at 95°C for 15 min, followed by 40 amplification cycles of 95°C for 15 s and 60°C for 60 s. mRNA abundance was normalized to *GAPDH* content. Data analysis was performed using the Thermo Fisher cloud tool, applying the $2^{-\Delta\Delta C_t}$ method. Here, ΔC_t represents the difference in comparative threshold cycle (C_t) between the target gene and the normalizer (*GAPDH*) for each sample. The primer sequences used are detailed in Table 4.

Table 4. Oligonucleotide sequences of primers used in single gene expression analysis by RT-qPCR.

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
CXCL10	AAAGCAGTTAGCAAGGAAAG	TCATTGGTCACATTTAGTG
CXCL11	TGCTACAGTTGTTCAAGGCTTCC	GGTACATTATGGAGGCTTCTCAATATC
PODXL	CTACTAGACAGTGTTTCAC	GAGGTCTGTTGAGTCTTTG
OAS1	ATTGTAAGAAGAAGCTTGGG	CAGAGTTGCTGGTAGTTTATG
GAPDH	ATCACCATCTCCAGGAGC	CATGGTTCACACCCATGAC

9.5. RNA sequencing

In 6-well plates, we seeded MDA-MB-231 for TGF-β1 stimulation (8 h) followed by *NDRG1* inhibition using siNDRG1 or SCR control (48 h). Samples were submitted to the Genomic Unit at GENyO (Centre for Genomics and Oncological Research) for RNA sequencing. RNA extraction was performed using the

Illumina Ribo-Zero Plus rRNA Depletion kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions.

RNA sequencing was conducted using a poly-A selection protocol. The final library was validated using the Agilent 2100 Bioanalyzer DNA 1000 kit, yielding a product of approximately 265-300 bp. Sequencing was performed on the NextSeq 500 platform (Illumina).

Next, bioinformatic analysis was carried out by GENyO's bioinformatics unit. Raw data quality control was assessed using FastQC (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>), revealing excellent quality without modifications. RNA sequencing by Expectation Maximization (RSEM)[139] was used for mapping and quantifying gene expression from raw Fast files. Data normalization was performed using the TMM method (NOISeq Package)[140] to ensure comparability between samples. Differential expression analysis was conducted using DESeq2[141] in R studio. Genes with $|\log_2 \text{ fold change}| \geq 1.5$ and FDR-adjusted p -value ≤ 0.05 were considered significant.

10. Protein extraction and immunoblot

To quantify protein expression, we utilized the immunoblot technique. In 6-well plates, we seeded SUM159, BT549, MDA-MB-231, or MDA-MB-436 cell lines and either stimulated with/without TGF- β 1 for 8 h followed by 48 h transfection with siNRG1 or SCR, or treated them with IC50 of repurposed drug candidates for 24 and 72 h.

After the selected time, we removed the media and washed the cells with PBS twice. Using a scalpel, they were lysed with 70 μ l of 1X lysis buffer (Cell Signaling) supplemented with 1X protease/phosphatase inhibitor cocktail (Thermo Fisher Scientific) and vortexed thrice for 30 s every 10 min. Finally, samples were centrifuged at 10,000 $\times$ g for 10 min and collected the supernatant to obtain the protein extract. We quantified the protein concentration using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific) with a bovine serum albumin (BSA) standard curve.

We denatured 30 μ g of protein by mixing with sample buffer (Thermo Fisher Scientific) containing 10% β -mercaptoethanol and heating at 95 °C for 5 min. We separated the samples by SDS-PAGE (sodium dodecyl polyacrylamide gel) electrophoresis in polyacrylamide gels (Bio-Rad) at 200 V for 30 min in a Tris-

Glycine-SDS (TGS) buffer (Bio-Rad). After their transfection onto nitrocellulose membranes (Bio-Rad) at 50 V for 50 min in Tris-Glycine (TG) buffer (Bio-Rad), we blocked the membranes for 1 h at RT with 5% skim milk dissolved in 1X Tris-Buffer saline (TBS) supplemented with 0.1% Tween 20 (VWR International) (TBST).

We incubated the membranes with the corresponding primary antibody (1:1000 dilution) at 4°C overnight in a shaker. The following day, the membranes were washed with TBST for 10 min thrice, incubated with the corresponding secondary antibody (1:2000 dilution) for 1 h at RT, and the washes were repeated. Then, we incubated the membranes with a chemiluminescence reagent: Immobilon Classic, Crescendo (Merk Millipore), or SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific), according to the intensity of the signal. We detected the protein bands with the ImageQuant LAS4000 digital imager (GE Healthcare). We performed the densitometric analysis with ImageJ using GAPDH or β -Actin as loading controls.

Detailed antibody information is summarized in Table 5.

Table 5. List of detailed information for all antibodies.

Bios, Biosciences; ER, estrogen receptor; FC, flow cytometry; HER2: human epidermal growth factor receptor 2; IF, immunofluorescence; IHC, immunohistochemistry; PR, progesterone receptor; Pt, Proteintech; Sig, Signaling; IM, immunoblot.

Protein	Antibody	Commercial brand	Reference	Use
Akt	Mouse monoclonal anti-Akt	BD Bios	610860	IM
Anti-mouse Alexa Fluor 594	Rabbit monoclonal anti-Iba1/AIF-1 (E4O4W) (Alexa Fluor® 594 Conjugate)	Cell Sig	48934	IF
Anti-rabbit Alexa Fluor 488	Anti-rabbit IgG (H+L), F(ab') ₂ Fragment (Alexa Fluor® 488 Conjugate)	Cell Sig	4412	IF
ER	Rabbit monoclonal antibody, clone Sp1	Vitro		IHC
GAPDH (1E6D9)	Mouse monoclonal anti-GAPDH	Pt	HRP-60004	IM
HER2	Rabbit monoclonal antibody	Vitro		IHC
Ki67 (8D5)	Mouse monoclonal anti-Ki67	Cell Sig	9449	IF
MHC Class I (H-2Kd)	MHC Class I (H-2Kd) Monoclonal Antibody (SF1-1.1.1), APC	eBios	17-5957-82	FC
NDRG1 (D8G9)	Rabbit monoclonal anti-NDRG1	Cell Sig	9485	IF, IM
p-Akt (Ser473)	Rabbit monoclonal anti-phospho-Akt	Cell Sig	4060	IF, IM
p-NDRG1 (Thr346, D98G11)	Rabbit monoclonal anti-phospho-NDRG1	Cell Sig	5482	IM
PR	Rabbit monoclonal antibody, clone 16	Vitro		IHC
Slug (C19G7)	Rabbit monoclonal anti-Slug	Cell Sig	9585	IM
Snail (C15D3)	Rabbit monoclonal anti-Snail	Cell Sig	3879	IM
Twist (Twist2C1a)	Mouse monoclonal anti-Twist	Santa Cruz	sc-81417	IM
Vimentin (D21H3)	Rabbit monoclonal anti-Vimentin	Cell Sig	5741	IM

11. Migration assay

We assessed cellular migratory capacity using the wound healing assay.

This was performed following either 8 h-TGF- β stimulation and 48 h *NDRG1* knockdown, or 24 h repurposed drug treatment. In 6-well plates with 80% confluent cell monolayers, we created a wound with a 100 μ l-pipette tip. After washing the monolayer with PBS, we added fresh media. We captured photos

with an Olympus CKX53 microscope at 0 and 14 h post-wounding (or 24 h for SUM159 with repurposed treatments). Finally, we analyzed cell migration by measuring the distance between the two sides of the wound using ImageJ software. We expressed the index relative to control cells (Figure 13).

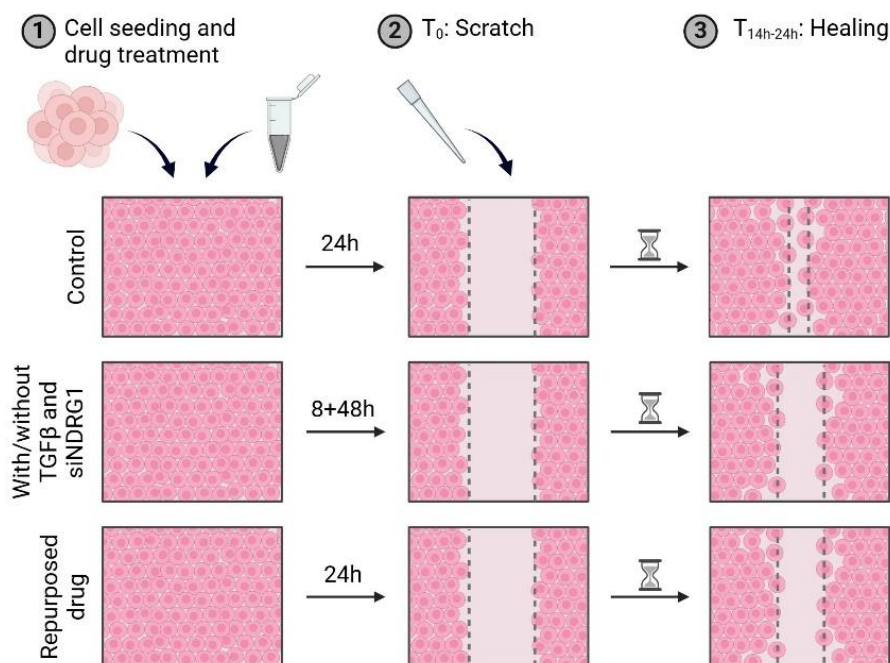


Figure 13. Schematic representation of wound healing assay.

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12. Invasion assay

To evaluate the invasive potential of transfected cells under TGF-β1 stimulation, we used the Cultrex BME Cell Invasion Assay kit (Trevigen), following the manufacturer's protocol.

Before the assay, cells were pre-treated with TGF-β1 for 8 h and transfected for *NDRG1* inhibition for 48 h. Subsequently, we seeded MDA-MB-231 and SUM159 cells onto a 1X-BME-coated transwell chamber at a density of 500,000 cells/ml in 50 μL of serum-free medium. The bottom chamber contained growth medium supplemented with 10% FBS to serve as a chemoattractant. Serum-free medium was used as a negative control.

Following a 16 h incubation period, the quantity of invading cells was determined using Calcein AM, a fluorescent dye that is cleaved by intracellular esterases in

viable cells. Fluorescence was measured at excitation and emission wavelengths of 485 nm and 520 nm, respectively. The number of invading cells was quantified by comparison to a standard curve, providing a precise and reproducible measure of cellular invasion.

13. Mammosphere-efficiency forming assay

To assess the self-renewal capacity of CSCs after treatments, we performed a mammosphere-forming efficiency (MSFE) assay.

We cultured mammospheres in 24-well ultra-low attachment plates at 37°C, 5% CO₂, and 90% humidity using a patented culture media (WO2016020572A1). This media consisted of DMEM/F12 supplemented with 1X B-27 (Invitrogen), 4 ng/ml heparin (Sigma-Aldrich), 10 µg/ml Insulin-Transferrin-Selenium (Invitrogen), 1 mg/ml hydrocortisone (Sigma-Aldrich), 10 ng/ml epidermal growth factor (EGF, Sigma-Aldrich), 10 ng/ml fibroblast growth factor (FGF, Sigma-Aldrich), 10 ng/ml IL-6 (Miltenyi), and 10 ng/ml hepatocellular growth factor (Miltenyi).

We seeded 2,000 cells of MDA-MB-231 and SUM159, 1,000 cells of BT549, and 200 cells of SUM159-R per well, and treated them with repurposed drugs at corresponding doses. Culture times varied by cell line: 3 days for SUM159 and SUM159-R, 5 days for MDA-MB-231 and BT549. We counted mammospheres larger than 50 µm in diameter.

For secondary mammosphere formation, we harvested, dissociated with trypsin-EDTA, and mechanically disrupted by pipetting up and down with a P100 micropipette. We re-seeded cells without additional treatments (500 cells/well for MDA-MB-231 and SUM159-R, 250 cells/well for SUM159 and BT549). Secondary mammospheres were counted after 3 (SUM159), 5 (BT549, SUM159-R), or 7 days (MDA-MB-231). We captured images of both generations using an Olympus CKX53 microscope. MSFE (%) was calculated by dividing the number of counted mammospheres by the number of plated cells.

14. Clonogenic assay

To determine the clonogenic capacity of the cells, we conducted soft-agar colony assays.

In 6-well plates, we seeded MDA-MB-231, SUM159, and BT549 cell lines and treated them with corresponding doses of repurposed drug candidates for 72 h. Subsequently, cells were harvested, counted, and seeded at 10,000 cells per well in 0.5 ml of 0.4% agar layer atop 0.5 ml of 0.8% agar layer in 24-well plates. After solidification, we added 0.5 ml of DMEM supplemented with 10% FBS which we changed twice weekly.

We prepared the layers from a sterile 1.6% agar stock dissolved in ultra-pure water, which we diluted with DMEM supplemented with 10% FBS with or without cells to obtain the 0.4% and 0.8% layers, respectively. We incubated the plates for 15 min at 4°C to allow the solidification of each layer.

Colonies were grown by incubating the plates at 37°C and 5% CO₂ for 21 days (SUM159) and 28 days (BT549 and MDA-MB-231). After incubation, we carefully washed the wells with PBS and stained the colonies with 1 mg/ml iodinitrotetrazolium chloride (Sigma-Aldrich) overnight at 37°C in darkness. The following day, we removed the reagent, washed the plates with PBS twice, and captured images of the dishes with a Leica Si9 magnifying glass. For colony counting, we analyzed these images with ImageJ by converting them to 8-bit format, adjusting thresholds, and using the “analyze particles” function to count colonies with diameters greater than 50 µm.

15. Flow cytometry

We used flow cytometry to evaluate different CSC subpopulations as well as to study the effect on apoptosis following gene knockdown or drug treatments.

15.1. Aldehyde dehydrogenase 1 (ALDH1)

After TGF-β stimulation and *NDRG1* transfection, or 72-h repurposed drug treatment, we measured ALDH1 enzyme activity using Aldefluor assay kit (StemCell Technologies, Vancouver, BC, Canada; ref:01700).

This assay identifies human cells expressing high levels of ALDH by converting the aldefluor reagent (BODIPY-aminoacetaldehyde) to fluorescent BODIPY-aminoacate (BAA) in the presence of ALDH. BAA is retained inside the cells and allows the measure of fluorescence in the cells. The fluorescence intensity correlates with cellular ALDH activity.

We collected the cells, washed them with cold PBS, and centrifuged them at 300×g for 5 min at 4°C. On ice, we resuspended the pellet in 1 ml Aldefluor buffer supplemented with verapamil (24 µg/ml) and added 5 µl of Aldefluor reagent. We transferred 300 µl to another tube containing 5 µl of diethylaminobenzaldehyde (DEAB) reagent, a specific ALDH inhibitor to control background fluorescence. Next, we incubated all samples at 37°C for 40 min in a thermoblock. After incubation, we stopped the reaction with ice and centrifuged the tubes at 300×g for 5 min at 4°C. We finally resuspended each tube with 500 µl of supplemented buffer and added 1 µl of SYTOX blue as a viability indicator for flow cytometric analysis. We acquired data using a FACS Verse flow cytometer (BD Bioscience) and made the analysis using FlowJo software.

15.2. CD44⁺/CD24⁻

Furthermore, we studied the CD44⁺/CD24⁻ cell populations by staining with CD44-PE and anti-CD24-FITC antibodies (BD Biosciences; San José, CA, USA).

After TGF-β stimulation and *NDRG1* silencing, or treatment with the repurposed drug for 72 h, cells were harvested, washed with cold PBS, and centrifuged at 300×g for 5 min at 4°C. We further resuspended the pellet in 100 µl of PBS supplemented with 2% FBS and 10 mM HEPES. Subsequently, we incubated them with anti-CD44 (0.6 µg/test) and anti-CD24 (4 µg/test) antibodies for 15 min at 4°C. Following incubation, we centrifuged the tubes at 300×g for 5 min at 4°C, removed the buffer and resuspended the cells in PBS supplemented with 25 mM HEPES. Before data acquisition using a FACS Verse flow cytometer (BD Bioscience), we used SYTOX blue to discriminate non-viable cells. Flow cytometric data analysis was performed using FlowJo software. We analyzed the data using FlowJo software.

15.3. Side population

We also evaluated the drug-resistant side population using the Hoechst 33342 dye exclusion assay.

Following TGF-β stimulation and *NDRG1* knockdown, or treatment with the repurposed drug for 72 h, cells were collected, washed with PBS, and centrifuged at 300×g for 5 min at 4°C. We resuspended the pellet in 500 µl of DMEM supplemented with 2% FBS and 10 mM HEPES and incubated them with 5 µg/ml Hoechst 33342 (Sigma-Aldrich) for 90 min at 37°C in darkness, with

interval mixing. For control of inhibition, we used verapamil (50 μ M). Post-incubation, we centrifuged and washed the tubes with cold PBS twice. We resuspended the pellet in PBS supplemented with 2% FBS and 10 mM HEPES and used propidium iodide (PI) to exclude non-viable cells during flow cytometric analysis. We excited Hoechst 33342 using a UV laser at 355 nm and detected the emissions at 450/50 nm (Hoechst blue) and 670/30 nm (Hoechst red) using a FACS Aria III cell sorter (BD Biosciences). We performed data analysis using FlowJo software.

15.4. Cell apoptosis

We assessed the effect on cell apoptosis following treatments with docetaxel, repurposed drug candidates or combinations at 24 h, and additionally at 48 h for the BT549 cell line, utilizing the Annexin V Apoptosis Detection Kit with PI (Immunostep; Salamanca, Spain; ref: ANXVKDY-100T).

Apoptosis, a regulated cell death process characterized by morphological changes including loss of membrane asymmetry, was evaluated using Annexin V, a human anticoagulant protein that binds to phosphatidylserine. This phospholipid, typically located in the inner monolayer of the cellular membrane, becomes exposed to the external monolayer during the early stages of apoptosis due to the loss of membrane structure, and Annexin V can bind it. Concurrent staining with Annexin V and the non-vital dye PI enables discrimination between viable cells (double-negative), early apoptotic cells (Annexin V-positive, PI-negative), late apoptotic cells (double-positive), and necrotic cells (Annexin V-negative, PI-positive)[142]. In the selected kit, Annexin V is conjugated to DY634 (detected in the APC channel).

Following the manufacturer's protocol, cells were harvested post-treatment and washed twice with PBS. Cell pellets were resuspended in 100 μ L of Annexin V Binding Buffer, followed by the addition of 5 μ L Annexin V-Dye634 and 5 μ L PI. After a 15-minute incubation at RT in darkness, 400 μ L of Annexin V Binding Buffer was added. Data acquisition was performed within 1 h using a FACSVerse flow cytometer (BD Bioscience). Subsequent analysis was conducted using FlowJo software.

We followed the manufacturer's protocol and, after the corresponding treatment, we collected and washed the cells with PBS twice. Then, we resuspended the pellet in 100 μ L of Annexin V Binding Buffer and added 5 μ L of Annexin V-Dye634 and 5 μ L of PI. After 15 min-incubation at RT in darkness, we

added 400 µl of Annexin V Binding Buffer. Data were acquired within 1 h using a FACS Verse flow cytometer (BD Bioscience). Subsequent analysis was conducted using FlowJo software.

16. Organoid techniques

16.1. Patient samples

The study recruited 15 patients diagnosed with TNBC from the Oncology CMU of the Hospital Clínico San Cecilio in Granada, Spain. After signing the patient's informed consent, a core needle tumor biopsy was collected according to the protocol approved by the Reference Ethics Committee with code PI19/01533/1626-N-19.

16.2. Generation of PDOs

16.2.1. PDOs culture media preparation

The base media is composed of Advanced DMEM/F12 (AdDMEM/F12, Gibco, ref: 11540446) supplemented with 1% penicillin/streptomycin, 10 mM HEPES, and 1X Glutamax (AdDMEM/F12+++). The rest of the growth factors of the PDOs culture media must be freshly prepared to avoid their degradation. The final composition is detailed in Table 6.

Table 6. Composition of PDOs culture media.

EGF, epidermal growth factor; FGF, fibroblast growth factor. Adapted from[128].

Compound	Final concentration
Advanced DMEM/F12	1X
Hepes	10 mM
Primocin	50 µg/ml
GlutaMax 100x	1X
Penicillin/Streptomycin	1X
Nicotinamide	5 mM
N-Acetylcysteine	1.25 mM
B-27 supplement	1X
R-Spondin (homemade)	10%
Heregulin beta 1	5 nM
FGF 7	5 ng/ml
FGF 10	20 ng/ml
EGF	5 ng/ml
Noggin (homemade)	10%
A83-01	0.5 µM
Y-27632*	5 µM
SB202190	0.5 µM

* Y-27632 is added when there are individual cells for 3-4 days

16.2.1.1. R-Spondin-enriched media

We cultured HA-R-Spondin1-Fc 293T cells with 300 µg/ml zeocin for selection. For media production, when the flask was confluent, we replaced the culture media with 25 ml of AdDMEM/F12+++ and incubated them for 1 week at 37°C, 5% CO₂, and 90% humidity. Following incubation, we collected the enriched media in a falcon tube, centrifuged at 300×g for 5 min to remove cellular debris, filtered through a 0.2 µm filter, and stored it at -20°C for long-term maintenance.

16.2.1.2. Noggin-enriched media

We cultured HEK293 cells transfected with plasmid pcDNA NEO insert mouse Noggin with 500 µg/ml geneticin for selection. For media production, when the flask was confluent, we removed the culture media and added 50 ml of AdDMEM/F12++. Following a 1-week incubation, we collected the enriched media in a falcon tube, centrifuged it at 300×g for 5 min to remove cellular debris,

and filtered it through a 0.2 μm sterile filter. For long-term maintenance, we stored the media at -20°C .

16.2.2. Digestion of TNBC biopsy

TNBC biopsies were collected in 50 ml falcon tubes containing 10-15 ml of AdDMEM/F12+++ on ice for transport to the cell culture room. Tissue digestion was performed following the protocol described by Sachs et al.[128].

Under sterile conditions in a Class II Biological Safety, we cut the biopsies into 1-3 mm^3 pieces using two scalpels on an untreated Petri dish. Then, we transferred them to a 50 ml falcon tube containing 5 ml of AdDMEM/F12+++ supplemented with 1.6 U/ml collagenase A (Roche, ref:10103586001) and incubated the tube in an orbital shaker (VWR International) at 37°C and 200 rpm for 1 h to facilitate enzymatic digestion of the tissue, with vortexing every 20 min.

Upon completion of digestion, we added 10 ml of AdDMEM/F12+++ to dilute the collagenase and filtered through a 100 μm sterile filter to remove larger fragments. We centrifuged the tube $300\times g$ for 5 min at 4°C . Subsequent steps were performed on ice to maintain cell viability. We resuspended the pellet in 3 ml of ACK lysis buffer (Gibco, ref: A1049201) and incubated the tube on ice for 3 min to lyse red blood cells. We washed the pellet with AdDMEM/F12+++ twice and transferred the cells into an Eppendorf tube for better visualization.

Using a P200 micropipette, we carefully removed the supernatant, embedded the isolated cells in growth factor-reduced solubilized basement membrane (Matrigel GFR, Corning; Glendale, AZ, USA; ref:354230) or Basement Membrane Extract type 2 (BME, R&D Systems; Minneapolis, MN, USA; ref: 3533-010-02), and plated the cells into 40 μl drops in 24-well plates. After allowing the gelation at 37°C for 30-60 min, we added 500 μl /well of freshly prepared PDOs media to each well[128].

16.2.3. PDOs culture

To maintain the PDOs culture, we changed the media with freshly prepared PDOs media every 3-4 days. We passaged the culture every 7-14 days when PDOs were mature or Matrigel GFR/BME started to lose structural integrity.

For passaging, we carefully aspirated the media and added 500 μl of Tryplex to each well. We mechanically disrupted the Matrigel GFR/BME drop by vigorous

pipetting (6-10 times). Then, to dissociate BME, we performed an additional 10 pipetting cycles with the plate rotated at 45°. For PDOs disaggregation, we incubated the plate at 37° for 5-10 min, followed by further mixing using a micropipette. We checked the dissociation process under a bright-field microscope with incubation times extended up to a maximum of 20 min if single-cell suspensions were not achieved. Post-incubation, we transferred the cells to a 15 ml falcon tube, rinsed them with AdDMEM/F12+++, and centrifuged them at 300×g for 5 min at 4°C. After supernatant removal, we washed the cells with AdDMEM/F12+++ and transferred them to an Eppendorf tube for subsequent seeding as previously described.

16.3. Generation of PDXs

The primary challenge in organoids generation from patient biopsies is the limited sample size, which often yields insufficient viable cells. To overcome this limitation, we established PDX models to increase the amount of tissue for subsequent PDxOs generation.

We develop PDX models from core needle biopsies (Figure 14) obtained from 9 TNBC patients enrolled at the University Hospital Clínico San Cecilio (ibs.GRANADA). We orthotopically implanted the tumor biopsy (1 mm³) into the cleared mammary fat pad of 4-to-8-week-old female NOD SCID gamma mice (NSG, *NOD. Cg-Prkdcscid Il2rgtm1Wjl/SzJ*). After a variable incubation period of 4-8 weeks, 33.3% of the samples successfully grew and generated PDX tumor tissue (generation zero, G0). Upon reaching an appropriate size, we euthanized the mouse, removed the tumors, and dissected them into small (1 mm³) fragments for reimplantation into four new mice to obtain the G1 generation. We repeated this process to generate G2, and when these tumors reached 120-170 mm³, we sacrificed mice and collected the PDX tissue for further procedures.

All *in vivo* experiments were conducted using female NSG mice. We carried out all experimental procedures according to institutional guidelines (Research Ethics Committee of the University of Granada) and international standards (Council of the European Communities). The Institutional Committee for Animal Care and Use of the University of Granada (15/03/2024/045) approved all procedures. All animals were housed and maintained under controlled conditions: 20-24°C, 50% relative humidity, and a 10:14 h light-dark cycle with *ad libitum* access to food and water.



Figure 14. Representative photo of TNBC patient biopsy tissue.

16.4. Immunohistochemistry, FISH, and immunofluorescence of PDXs

Upon G2 tumors reaching 120-170 mm³, we euthanized the mice and collected the tumors. We selected a portion for hormone receptor and HER2 expression analysis to confirm the maintenance of triple-negative status in the PDX model. In collaboration with the anatomopathological department of the University Hospital Clínico San Cecilio, we performed an IHC for a histopathological characterization by the pathologist. Additionally, to assess the presence of NDRG1 protein, we conducted an immunofluorescence (IF).

For these processes, we first prepared the PDX samples. We fixed the PDX tumor in 4% paraformaldehyde (PFA) at 4°C for 24 h. The following day, we washed it twice with PBS, embedded it in paraffin using an automatic tissue processor (TP1020; Leica, Germany), and sectioned it at 4 µm thickness. Next, we deparaffinized the sections with xylene and hydrated by decreasing alcohol concentrations.

For IHC, we retrieved the antigen using Antigen Retrieval fluid 10X EDTA pH 8.0 (Vitro) in a PTLINK module (Vitro). In an Autostainer 480 (Vitro), we used the Master Polymer Plus Detection System (Peroxidase) (Vitro) to perform the staining. Briefly, we washed the sections with PBS and blocked them with 3% hydrogen peroxide for 5 min. We incubated them with primary antibody for 5 to 10 min at RT and then treated the sections with immunodetection solution (biotinylated secondary antibody) for 30 min and developed it using 3,3'-diaminobenzidine (1:50 dilution) (Vitro). We counterstained the sections in Meyer's hematoxylin. Non-immune serum was used as the negative control. The pathologist considered the absence of nuclear staining in neoplastic cells as negative. Images were acquired using a Leica DM 550B microscope.

To determine the HER2 status of the PDX, we performed the fluorescence *in situ* hybridization (FISH) using the RBB2/CCP17 FISH Probe Kit (CT-PAC001, CytoTest Inc) following manufacturer's instructions in the deparaffinized

sections of PDX. The pathologist interpreted the results according to the latest ASCO/CAP 2023 recommendations[143].

For IF, we retrieved the antigen at 121°C for 15 min in a sodium citrate buffer solution (pH 6.0). We blocked the sections for 2 h at RT with 5% BSA and incubated them with the anti-NDRG1 primary antibody (1:100 dilution in 0,1% BSA) overnight at 4°C. After PBS washes, we incubated them with the anti-rabbit Alexa Fluor 488 secondary antibody (1:500 dilution in 0.1% BSA) for 2 h at RT in darkness. After PBS washes, we counterstained the nuclei with Hoechst (Sigma-Aldrich) (1:100 dilution in H₂O) for 15 min at RT in darkness. After PBS washes to remove the excess, we mounted slides with mounting medium (Cell Signaling). Images were captured using a Zeiss LSM 710 confocal microscope, and staining intensity analyses were performed using ImageJ software.

Detailed antibody information is summarized in Table 5.

16.5. Establishment of PDxOs

16.5.1. Digestion of PDXs

For the establishment of PDxOs (Figure 15), we harvested G2 tumors upon reaching 120-170 mm³. We euthanized mice by cervical dislocation in a laminar flow hood. We excised the tumors, washed them with PBS in a Petri dish, and transferred them to a 50 ml Falcon tube containing AdDMEM/F12 supplemented with 1% antibiotic/antimycotic solution (10,000 units penicillin, 10 mg streptomycin, and 25 µg amphotericin B per ml; Sigma-Aldrich, ref: A5955) for transport to the cell culture facility.

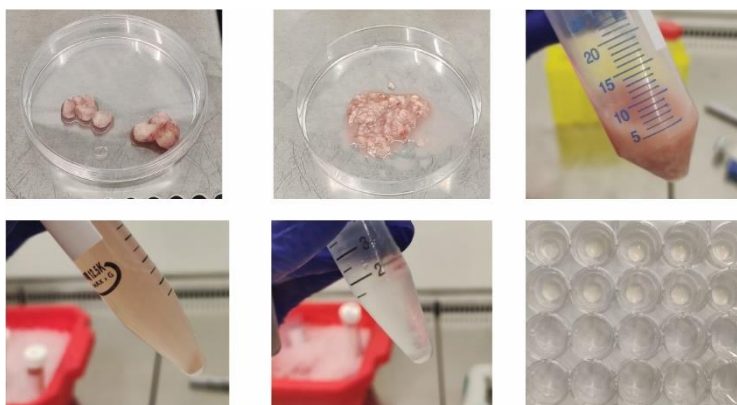


Figure 15. Photos of PDX tumor digestion to obtain PDxOs culture.

Top left: resected tumors, top center: minced tumors, top right: falcon tube containing minced tissue being digested with collagenase A, bottom left: dirty pellet after digestion, bottom center: clean pellet after erythrocyte lysis and PBS washes, bottom right: 24-well plate with Matrigel drops containing cells before the addition of PDxOs culture media.

We adapted the digestion protocol from Sachs et al. and Campaner et al. [128,144]. To minimize cell adhesion, we pre-coated all tubes, pipettes, and tips with FBS.

We minced the tumors into 1-3 mm³ pieces using two scalpels on an untreated Petri dish. We collected the fragments in a 50 ml Falcon tube containing 5 ml of AdDMEM/F12 with 1.6 U/ml collagenase A (Roche, ref:10103586001) for enzymatic digestion. We performed mechanical disruption by pipetting (5-10 cycles) using a 5- or 10-ml pipette, with the tip pressed against the tube bottom to facilitate tumor disaggregation. We continued enzymatic digestion in an orbital shaker at 37°C and 200 rpm for 15 min. After solid fragment precipitation, we collected the supernatant in a separate Falcon tube containing 15 ml of AdDMEM/F12 and preserved it on ice. We performed two to three digestion cycles to enrich the cell pellet.

We filtered the final digestion product and preserved supernatants through a 100 µm mesh to remove large fragments. We washed the filter with 3 ml of AdDMEM/F12 and centrifuged the filtrate at 300×g for 5 min at 4°C. We aspirated the cloudy supernatant containing fat and washed the pellet with 8 ml of cold PBS in a 15 ml Falcon tube. We performed subsequent steps on ice to maintain cell viability. When we observed erythrocyte contamination, we treated the pellet with 2 ml of ACK lysis buffer for 7 min on ice. We diluted the reaction with

cold PBS and centrifuged at 300×g for 5 min at 4°C. We washed the pellet with cold PBS to remove the residual buffer and carefully removed excess supernatant using a P200 pipette.

We embedded isolated cells in Matrigel GFR or BME and plated them as 50 µl drops in 24-well plates. After 30-60 min of gelation at 37°C, we added 500 µl/well of freshly prepared PDxOs culture media (Table 7).

Table 7. Composition of PDxOs culture media.

hEGF, human epidermal growth factor. Adapted from[123,130].

Compound	Final concentration
Advanced DMEM/F12	1X
FBS	5%
HEPES	10 mM
GlutaMax 100x	1X
Penicillin/Streptomycin	1X
hEGF	10 ng/ml
Y-27632	10 µM

16.5.2. PDxOs culture passage

When PDxOs reached maturity and confluence, typically once a week, we passaged them. We began by removing the media and washing each well twice with cold PBS. We then added 500 µL of cold PBS to each well and mechanically disrupted the Matrigel GFR dome containing PDxOs using a P1000 pipette. We collected the suspension in a 15 mL Falcon tube and centrifuged it at 300×g for 5 min at 4°C to remove the PBS. To disaggregate the Matrigel GFR and release the PDxOs, we added 100 µL/well of Cell Recovery solution (Corning, ref:354253) and incubated the suspension for 1 h at 4°C. After incubation, we diluted the solution with cold PBS and centrifuged at 300×g for 5 min at 4°C to obtain a clean pellet of PDxOs.

We then resuspended the PDxOs pellet in trypsin-EDTA, pipetting up and down with P1000 and P200 pipettes, and incubated the suspension at 37°C for 2-5 min. We optimized this incubation time for each tumor sample, preferring shorter durations initially. If compact organoids remained after seeding, we increased the incubation time in subsequent passages. Following incubation, we pipetted the suspension up and down up to 10 times to completely dissociate the organoids into single cells. To halt trypsinization, we added cold PBS to dilute the trypsin, centrifuged the suspension at 300×g for 5 min, and

washed it once with PBS. Finally, we re-embedded the cell pellet, plated it, and added fresh media as previously described.

16.5.3. PDxOs cryopreservation

To address the low viability of thawed organoids, we freeze the contents of 8-12 wells from 24-well plates per cryotube to maintain as many organoids as possible.

We collected the PDxOs as described in the previous section, maintaining them as small organoid clusters rather than dissociating them into individual cells to enhance their post-thaw viability. Instead of embedding the pellet in Matrigel GFR, we resuspended it in 1 mL of FBS supplemented with 10% DMSO and transferred the suspension into cryotubes.

Following a protocol similar to that used for adherent cell lines, we placed the cryotubes in isopropanol cryo-freezing containers and stored them at -80°C. After 24 h, we transferred the cryotubes to a liquid nitrogen tank for long-term storage.

16.5.4. PDxOs thawing

To thaw the cryopreserved PDxOs, we used a protocol similar to that used for adherent cell lines. We rapidly thawed the cryotube into a 37°C water bath and immediately transferred its contents into a Falcon tube containing 10 mL of pre-warmed AdDMEM/F12 to dilute the DMSO. We then centrifuged the tube at 300×g for 5 min at 4°C, carefully aspirated the supernatant, and washed the pellet once with cold PBS to eliminate any residual DMSO. Following this, we seeded the organoids as previously described in section 16.5.1.

16.6. Immunohistochemistry of PDxOs

To characterize the PDxOs, we conducted IHC in collaboration with the anatomopathological department of the University Hospital Clínico San Cecilio, following a similar procedure to that used for PDX samples.

After approximately ten days of growth, when PDxOs reached a diameter of 100 µm, we collected them and released them from Matrigel GFR using Cell Recovery solution as previously described. We fixed PDxOs with 4% PFA at RT for 10 min, followed by two PBS washes. We then embedded the fixed PDxOs in

50 μ L drops of 1% agarose and allowed them to solidify at 4°C overnight. The following day, we processed the agarose drops containing the organoids using an automatic tissue processor (TP1020; Leica, Germany) for paraffin embedding and sectioned them at 4 μ m thickness.

We proceeded with the IHC protocol as described in section 16.4. For PDxOs, we determined HER2 status using IHC instead of FISH.

Detailed antibody information is summarized in Table 5.

16.7. Flow cytometry

To investigate the evolution of the murine cell population in PDxOs cultures, we assessed the expression of murine major histocompatibility complex (MHC) Class I through flow cytometry.

We began by dissociating one confluent well of PDxOs into single cells using the previously described protocol. We then resuspended the cells in DMEM supplemented with 10% FBS and incubated them with anti-Mo MHC Class I (H-2Kd) antibody (1.2 μ g/mL, SF1-1.1.1, Invitrogen) for 15 min at RT in darkness. Following incubation, we washed the cells once with PBS to remove any residual antibody.

We performed data acquisition using a flow cytometer (FACS Verse, BD Bioscience) and conducted subsequent analysis using FlowJo software.

16.7.1. Fluorescence-activated cell sorting (FACS)

To eliminate murine cellular contamination from our PDxOs cultures, we utilized fluorescence-activated cell sorting (FACS). We began by dissociating the PDxOs into single cells using trypsin, as previously described. We then incubated the cell suspension with anti-Mo MHC Class I (H-2Kd) antibody (1.2 μ g/mL, SF1-1.1.1, Invitrogen) for 15 min at RT in darkness. Following incubation, we washed the cells once with PBS to remove any residual antibody.

For the FACS-based purification, we implemented a gating strategy to collect the negative staining population, effectively isolating the human cells from the murine contaminants. After sorting, we cultured the purified human cell population overnight in 24-well ultra-low attachment plates to allow for recovery and promote organoid formation.

The following day, we embedded the cells in Matrigel GFR or BME domes as described in section 16.5.1.

16.8. Cell viability of PDxOs

To evaluate the efficacy of treatments and determine appropriate dosing concentrations, we conducted a cell viability assay using alamarBlue HS (Invitrogen) and calculated the IC₅₀ values (Figure 16).

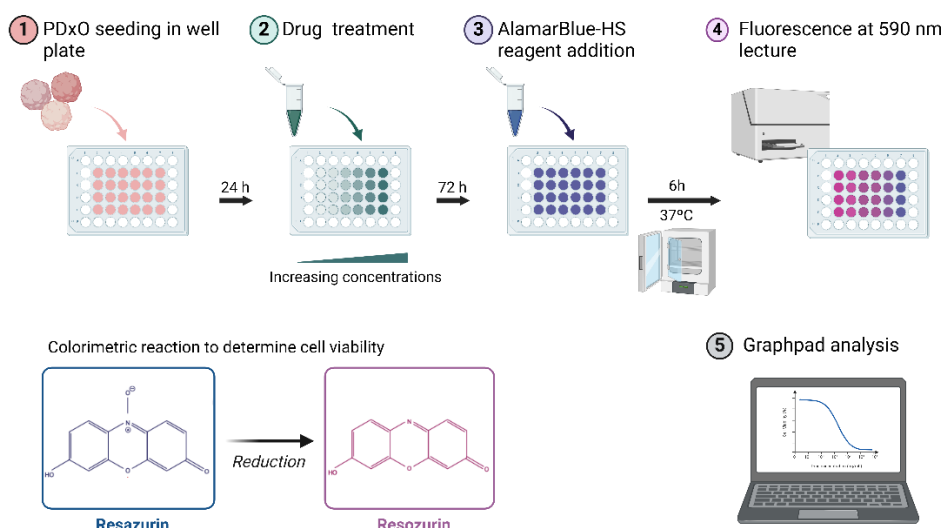


Figure 16. Schematic procedure to assess cell viability of PDxOs using alamarBlue HS reagent.

Created with Biorender.

This assay utilizes a resazurin-based solution that leverages the reducing power of living cells for quantitative viability measurement. In viable cells, the oxidative blue compound resazurin is reduced to resorufin, a red and highly fluorescent compound, allowing for the quantification of cell viability through absorbance or fluorescence readings[145]. After three days of growth, when PDxOs reached a diameter of approximately 30-50 μm , we harvested and re-embedded them into 10 μL drops of Matrigel GFR or BME. We plated these drops in 48-well plates, with five wells per condition. The following day, we replaced the media with 200 μL of fresh PDxOs media containing either vehicle (DMSO) or increasing concentrations of the following drugs:

- Docetaxel (nM): 1, 10, 25, 50.

- Efavirenz (μM): 1, 25, 50, 100.
- Ouabain (nM): 20, 60, 100, 250.
- Vinburnine (μM): 1, 10, 50, 100.

On the third day of treatment, we assessed cell viability by adding 10% alamarBlue HS to each well and incubating the plates for 6 h at 37°C in darkness. We measured fluorescence (emission wavelength: 590 nm) using a M200 Nanoquant plate reader (Tecan). We expressed cell viability as normalized fluorescence relative to control cells and calculated IC₅₀ values through dose-normalized response curve analysis using GraphPad Prism 9.0.0 software.

To investigate the efficacy of combination treatments with docetaxel, we conducted cell viability assays following a similar protocol. We treated PDxOs with one of the following conditions: vehicle (DMSO); IC₅₀ of docetaxel (9 nM), efavirenz (21 μM), ouabain (33 nM), or vinburnine (9 μM); or pairwise combinations of docetaxel's IC₅₀ with each repurposed drug (docetaxel + efavirenz (D+E), docetaxel + ouabain (D+O), or docetaxel + vinburnine (D+V)). We calculated cell viability relative to the vehicle (control) group.

16.9. Immunofluorescence of PDxOs

Due to the limited quantity of PDxOs culture and the challenge of obtaining sufficient protein for immunoblot analysis, we opted to investigate the presence of NDRG1 and the effects of treatments on p-AKT and the proliferation marker Ki67 using IF.

For NDRG1 detection, we allowed PDxOs to grow for 10 days, reaching approximately 100 μm in diameter. We then plated 15 μL drops of PDxOs in 8-well chambers for analysis. For p-AKT and Ki67 assessment, we plated PDxOs after 6 days of growth and treated them for 72 h with either vehicle (DMSO) or the IC₅₀ of each repurposed drug: efavirenz (9 μM), ouabain (33 nM), and vinburnine (9 μM).

To prepare the samples, we aspirated the media, washed the Matrigel GFR drops containing PDxOs, and fixed them with 4% PFA solution for 20 min at RT. We then washed the organoid domes twice with PBS and permeabilized them with 0.2% Triton X-100 for 20 min at RT. Next, we washed the samples twice with PBS and blocked them in 3% BSA for 1 h at RT. Following blockade, we incubated the PDxOs overnight at 4°C with primary antibodies diluted in 0.1% BSA against NDRG1 (1:100 dilution), or p-AKT (1:100 dilution) and Ki67 (1:800 dilution). The

next day, we washed the samples thrice with PBS and incubated them with secondary antibodies anti-rabbit Alexa Fluor 488 (1:500 dilution) and anti-mouse Alexa Fluor 549 (1:500 dilution) for 2 h at RT in darkness.

After incubation, we washed the organoids thrice with PBS, counterstained the nuclei with Hoechst (Sigma-Aldrich) (1:100 dilution in H₂O) for 15 min at RT in darkness, and washed them thrice with PBS to remove excess stain. Finally, we mounted the slides with mounting medium, carefully pressing the Matrigel GFR drop to avoid air bubble formation.

We captured images using a Zeiss LSM 710 confocal microscope and performed staining intensity analyses using ImageJ software.

Detailed antibody information is summarized in Table 5.

17. Statistical analysis of *in vitro* assays

For our statistical analyses and data visualization, we utilized GraphPad Prism 9.0.0 (GraphPad Software Inc.). To assess statistical differences between the two experimental groups, we applied a two-tailed unpaired Student's t-test, comparing individual mean values. We present our results as mean $\pm$ standard deviation (SD). In our analyses, we considered differences statistically significant when the p-value was less than 0.05.

RESULTS

1. Objective I: Study of the role of *NDRG1* after TGF- β stimulation and drug repurposing study by transcriptomic analysis

1.1. *NDRG1* expression and survival of TNBC patients

To confirm the findings of Nagai et al.[69] that correlate overexpression of *NDRG1* in TNBC patients with poorer survival, we first analyzed public databases. Our investigation utilizing the Kaplan-Meier plotter database demonstrated that high *NDRG1* expression was associated with reduced RFS across all breast cancer cases (HR: 1.42; $p = 1.6 \times 10^{-11}$), as well as in specific subtypes: luminal B (HR: 1.27; $p = 0.008$), basal-like (HR: 1.58; $p = 6.6 \times 10^{-5}$), and TNBC (HR: 1.64; $p = 0.008$) as depicted in Figure 17. Interestingly, no significant correlation was observed between high *NDRG1* expression and RFS in Luminal A and HER2+ molecular subtypes.

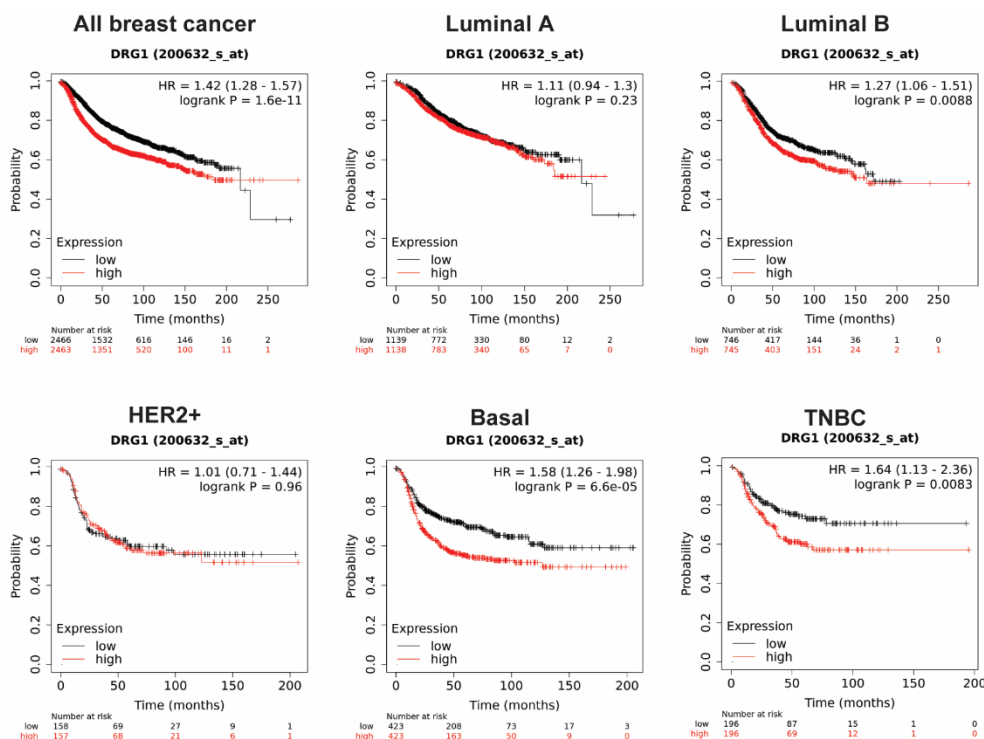


Figure 17. *NDRG1* expression and patient survival in different breast cancer subtypes from KM plotter database.

Kaplan-Meier curves from the KM plotter database comparing high expression of *NDRG1* and relapse-free survival (RFS) in all cases of breast cancer, luminal A, luminal B, HER2-positive (HER2+), basal-like, and TNBC.

Objective I: Study of the role of *NDRG1* after TGF- β stimulation and drug repurposing study by transcriptomic analysis

Further gene set analysis conducted using the GOBO online tool revealed similar outcomes as increased *NDRG1* gene expression was significantly associated with poorer RFS in all breast cancer cases ($p = 0.003$) and in the basal subtype ($p = 0.002$) (Figure 18). Likewise, this correlation was not confirmed in luminal A, luminal B, or HER2+ subtypes.

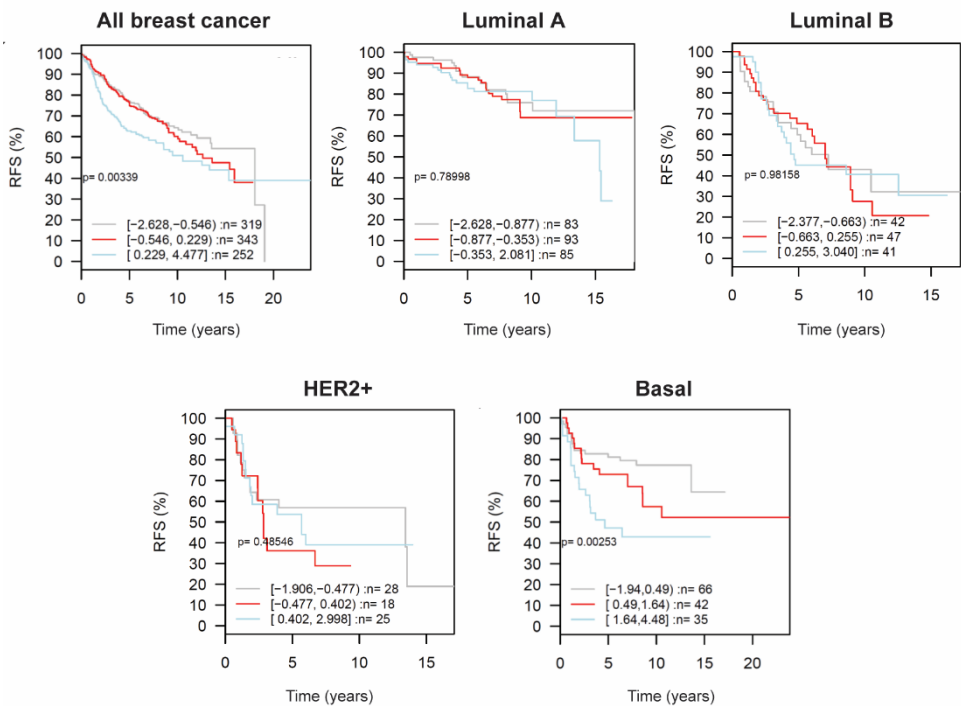


Figure 18. *NDRG1* expression and patient survival in different breast cancer subtypes from GOBO database.

Kaplan-Meier curves analyses by GOBO online tool comparing high expression of *NDRG1* and relapse-free survival (RFS) in all cases of breast cancer, luminal A, luminal B, and HER2-positive (HER2+), basal-like subtypes.

To complete this research, we studied *NDRG1* protein expression from a total cohort of 83 TNBC patients after surgery by IHC ($n=75$), and it was detected in 56 patients ($\sim 75\%$) (Figure 19A). To elucidate the potential prognostic value of *NDRG1* expression, we performed survival analysis on our patient cohort and our results demonstrated that high *NDRG1* expression (score ≥ 199) was significantly associated with reduced cumulative patient survival (mean survival: 6.9 years) compared to lower expression (score < 199) (mean survival:

15.1 years) ($p = 0.021$) (HR = 2.313; 95% CI: 1.08-4.93; $p = 0.030$) as shown in Figure 19B.

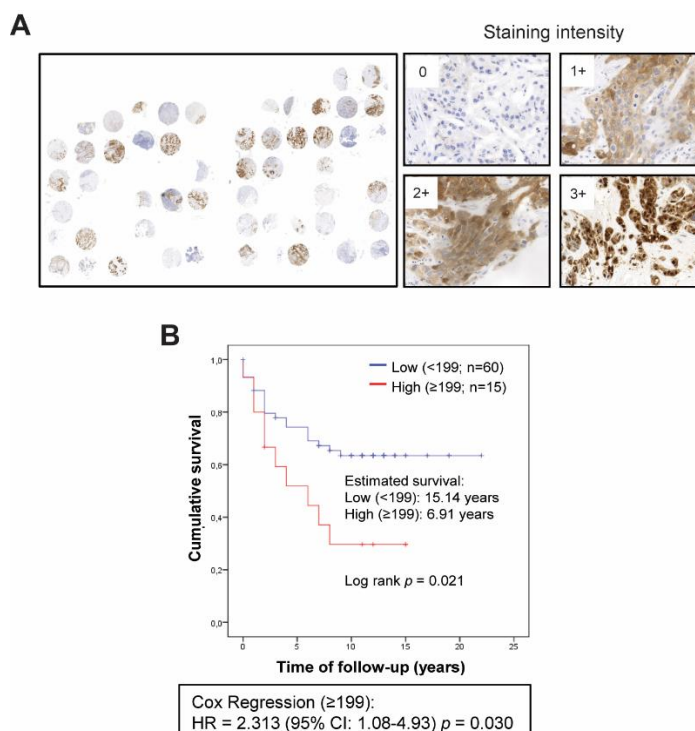


Figure 19. NDRG1 expression and patient survival of our cohort of TNBC patients (n=83).

A Kaplan-Meier curve comparing high expression (score ≥ 199) of NDRG1 protein and cumulative patient survival in tumor tissue of TNBC patients (n= 75). **B** Representative images of negative, 1+, 2+, and 3+ NDRG1 staining intensity (original optical objective: 40 $\times$). HR, hazard ratio; CI, confidence interval.

1.2. NDRG1 pleiotropic activity on EMT, metastasis, and tumor-initiating abilities

The pleiotropic nature of NDRG1 in breast cancer has been a subject of considerable research, with studies reporting its dual role as both a metastasis/tumor progressor and suppressor[55,60]. To address the inconsistencies observed in the results and elucidate the NDRG1 controversy, we focused on our previous results and studied if NDRG1 and/or p-NDRG1 (Thr346) could be involved in tumor aggressiveness mediated by TGF- β 1. Therefore, we hypothesized that NDRG1 might exhibit differential roles depending on the origin of TNBC cell lines (primary tumor versus pleural effusion/metastatic lesion) under TGF- β 1 stimulation.

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Our previous data showed enhanced expression of p-NDRG1 (Thr346) in TNBC cell lines stimulated with TGF- β 1[70], which herein we validated as we found an increased expression of p-NDRG1 (Thr346) at 8 h that was maintained during 48 h and started to decrease at 72 h in two TNBC cell lines (SUM159 and MDA-MB-231) treated with a single stimulation of TGF- β 1 (Figure 20).

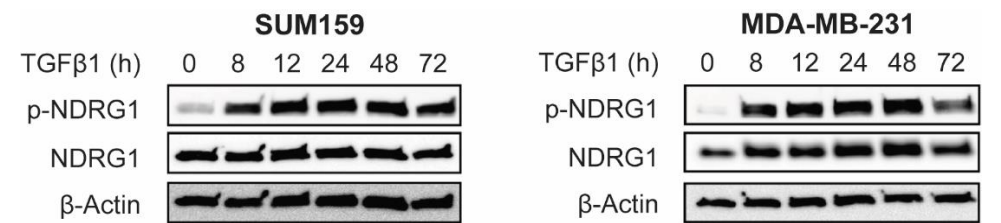


Figure 20. Immunoblot of the effect on *NDRG1* phosphorylation (Thr346) at indicated time points after stimulation with TGF- β 1 in SUM159 and MDA-MB-231 cell lines.

To study our hypothesis, we utilized a diverse panel of TNBC cell lines including SUM159 and BT549 (both established from primary tumors), as well as MDA-MB-231 and MDA-MB-436 (both derived from pleural effusions). These cell lines were treated with TGF- β 1 for 8 h, and then the *NDRG1* gene was inhibited for 48 h (8+48 protocol). We rigorously validated transfection efficiency in each cell line and confirmed the increased expression of both total *NDRG1* and p-NDRG1 (Figure 21).

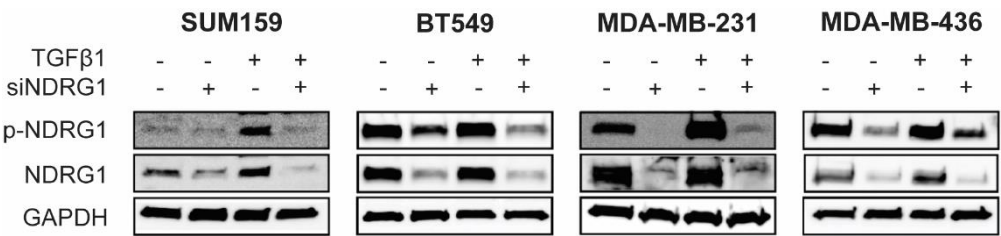
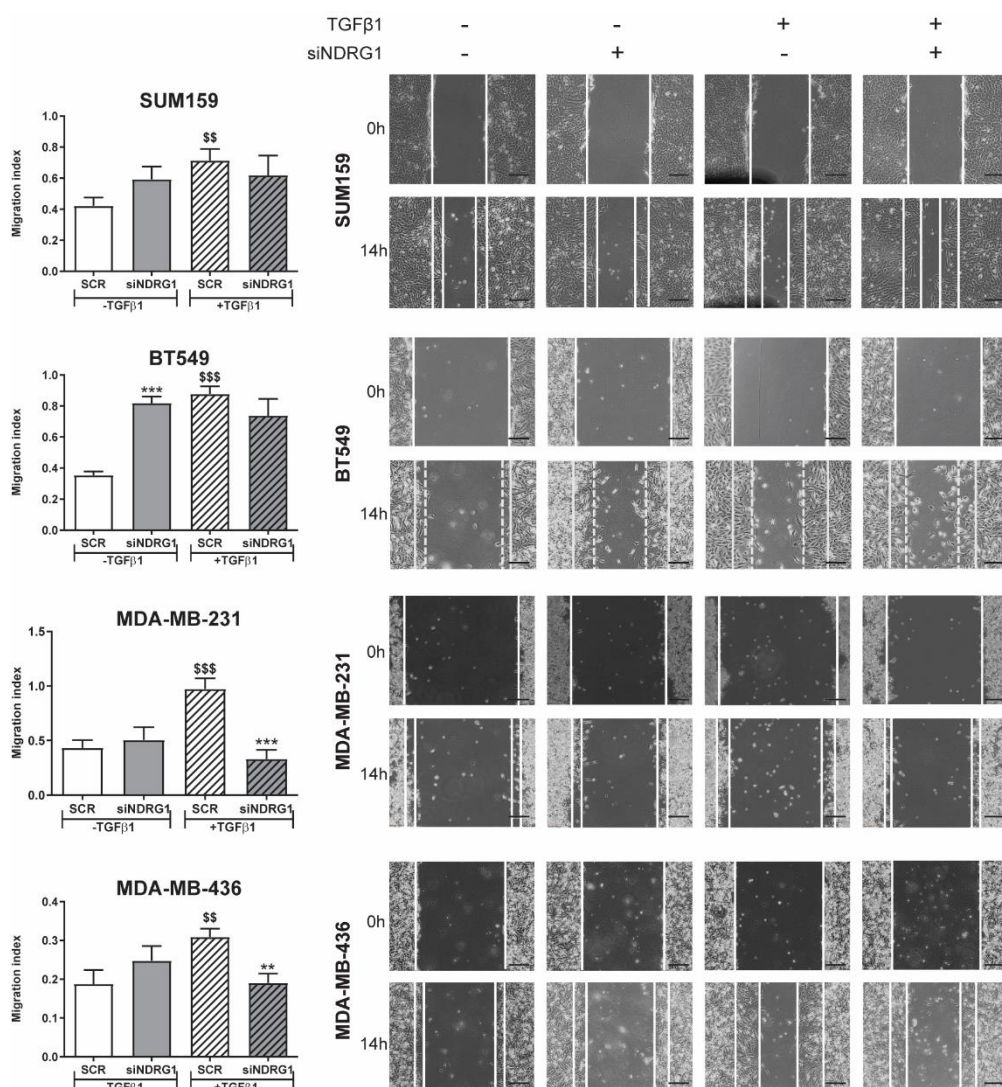


Figure 21. Immunoblot of the effect on p-NDRG1 (Thr346) and total *NDRG1* in SUM159, BT549, MDA-MB-231, and MDA-MB-436 cell lines after stimulation with TGF- β 1 for 8 h followed by *NDRG1* knockdown for 48 h (8+48 protocol).

Next, we analyzed the metastatic characteristics of tumor cells to unravel the role of *NDRG1*. *NDRG1* inhibition did not uniformly decrease migration across the four cell lines tested. Interestingly, migration was even enhanced in BT549 cells. Upon *NDRG1* silencing following TGF- β 1 stimulation, we observed a differential response based on the cell line origin. As depicted in Figure 22, while

no reduction in migration was detected in cells established from primary tumors, a significant decrease was observed in cells derived from pleural effusions when compared to the TGF- β 1-treated control group.



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In parallel, we assessed tumor cell invasion using Matrigel-coated Boyden chambers. *NDRG1* knockdown under TGF- β 1 treatment resulted in reduced invasion specifically in MDA-MB-231 cells, while no significant changes were observed in SUM159 cells (Figure 23).

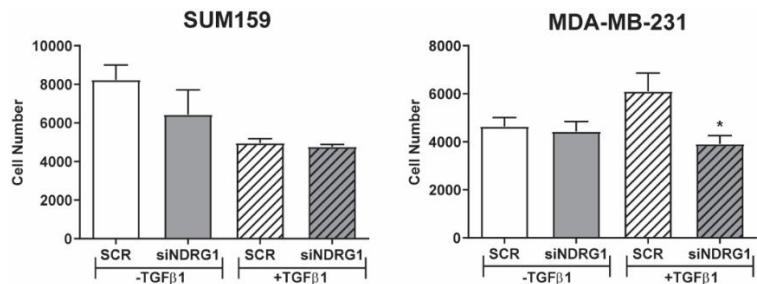


Figure 23. Tumor cell invasion of SUM159 and MDA-MB-231 cell lines after stimulation with TGF- β 1 for 8 h followed by *NDRG1* knockdown for 48 h (8+48 protocol).
Statistically significant differences between siNDRG1 and SCR (scrambled siRNA): * p<0.05.

Complementary, to elucidate the potential differential role of *NDRG1* on EMT upon TGF- β 1 stimulation, we conducted immunoblot analyses on these cell lines (Figure 24). In the absence of TGF- β 1 stimulation, *NDRG1* knockdown exhibited minimal, no effect, or even enhancing effects on EMT marker expression, consistent with its putative role as a metastasis suppressor. However, under TGF- β 1 stimulation, *NDRG1* inhibition correlated with reduced Twist protein levels across all cell lines, while Snail and Slug were downregulated specifically in SUM159 cells.

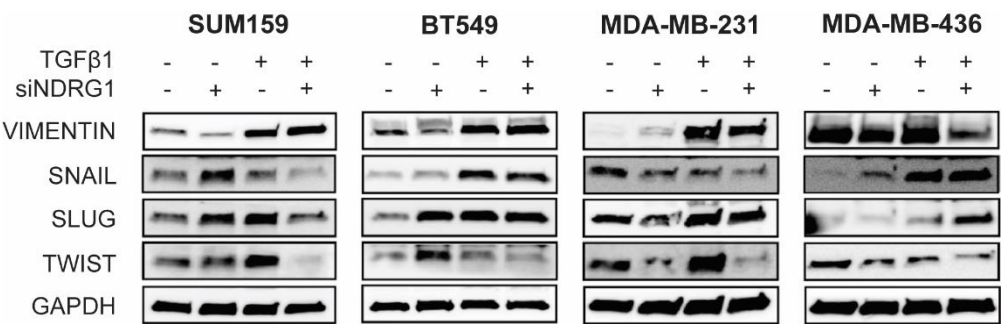


Figure 24. Immunoblot of the effect on EMT markers in SUM159, BT549, MDA-MB-231, and MDA-MB-436 cell lines after stimulation with TGF- β 1 for 8 h followed by *NDRG1* knockdown for 48 h (8+48 protocol).

These findings, coupled with the absence of effects on migration and invasion in primary tumor-derived cells, suggest that Snail, Slug, or Twist are not modulated by *NDRG1* as part of a cellular program to induce metastasis. Interestingly, Vimentin expression was inhibited following *NDRG1* silencing in MDA-MB-231 and MDA-MB-436 cells treated with TGF- β 1, an effect not observed in SUM159 and BT549 cells (Figure 24). These results support the hypothesis of *NDRG1* pleiotropy on identical molecular targets across different cancer types.

Given that Twist is also related to the formation of CSCs, and chemoresistance[146] and this protein was the only consistently reduced in the condition of our interest, we continue assessing the effect on different CSC subpopulations. Specifically, we investigated the impact on ALDH1⁺ cells; CD44⁺/CD24⁻ population; and side population.

Initially, we observed that the 8+48 h protocol did not significantly affect the ALDH1⁺ population in cells derived from primary tumors (SUM159 and BT549) as shown in Figure 25. Whereas in pleural effusion-derived cells (MDA-MB-231 and MDA-MB-436), *NDRG1* inhibition upon TGF- β 1 stimulation reduced this subpopulation of CSCs.

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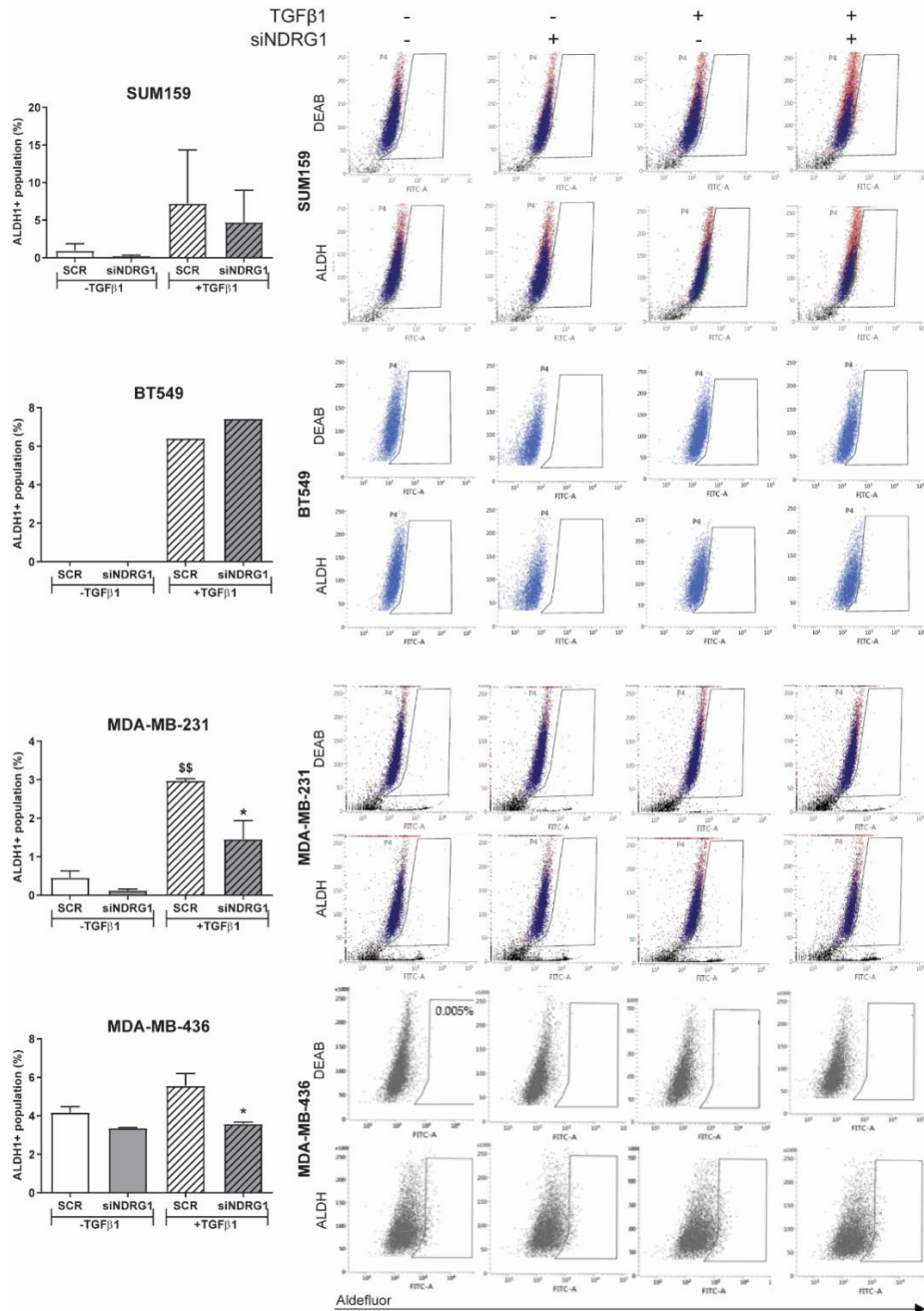


Figure 25. Effect on ALDH1+ CSCs in SUM159, BT549, MDA-MB-231, and MDA-MB-436 cell lines after stimulation with *TGF-β* for 8 h followed by *NDRG1* knockdown for 48 h (8+48 protocol).

Flow cytometric analysis of ALDH1+ CSC population and representative dot plots of flow cytometric analysis of ALDH1+ population and DEAB (diethylaminobenzaldehyde) control. Statistically significant differences between siNDRG1 and SCR (scrambled siRNA): * $p < 0.05$. Statistically significant differences between SCR with and without *TGF-β*1: \$\$ $p < 0.01$.

Given the differential effects of TGF- β 1 exposure duration on CSC induction as prolonged TGF- β 1 treatment induces CSCs and drug resistance, while shorter stimulation facilitates lung colonization by breast cancer cells[147], we postulated that *NDRG1* might exhibit distinct roles in CSC formation depending on the origin of TNBC tumor cells. Specifically, we hypothesized that TNBC cells derived from primary tumors might require prolonged TGF- β 1 exposure for *NDRG1*-mediated CSC formation. However, cells originating from pleural effusions might respond to shorter stimulation periods. To test this hypothesis, we subjected SUM159 and BT549 cells to TGF- β 1 treatment for 14 days, with *NDRG1* knockdown performed on day 12 (14-day protocol). Interestingly, consistent and significant reductions in the ALDH1+ population in both primary tumor cell lines were observed following *NDRG1* knockdown after this longer TGF- β 1 stimulation, when compared to their corresponding negative controls (Figure 26). Consequently, for further CSC studies, we continued with different protocols of stimulation depending on the origin of the cell lines.

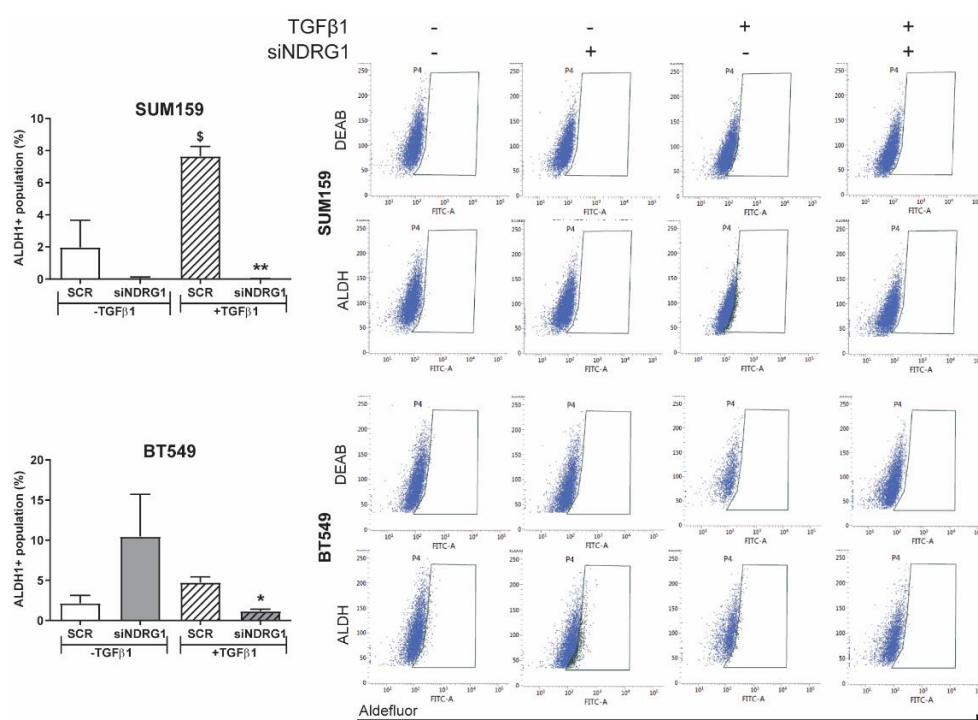


Figure 26. Effect on ALDH1+ CSCs in SUM159 and BT549 cell lines after stimulation with TGF- β 1 for 12 days followed by *NDRG1* knockdown for 48 h (14 days protocol).

Flow cytometric analysis of ALDH1+ CSC population and representative dot plots of flow cytometric analysis of ALDH1+ population and DEAB (diethylaminobenzaldehyde) control. Statistically significant differences between siNDRG1 and SCR (scrambled siRNA): * $p < 0.05$, ** $p < 0.01$. Statistically significant differences between SCR with and without TGF- β 1: \$ $p < 0.05$.

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Then, regarding the CD44⁺/CD24^{low/-} population, we noticed a marked decrease specifically in SUM159 cells after *NDRG1* knockdown, while minimal or no changes were detected in MDA-MB-231 and MDA-MB-436 cells, respectively, as described in Figure 27.

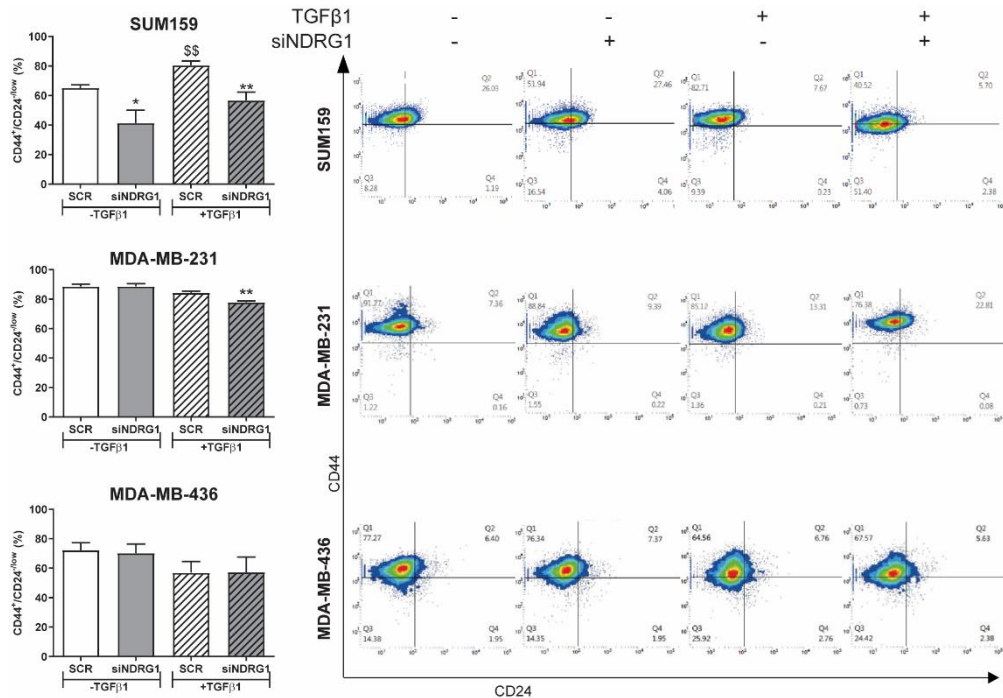


Figure 27. Effect on CD44⁺/CD24^{low/-} population after stimulation with TGF- β 1 and *NDRG1* inhibition in SUM159 (14 days protocol), MDA-MB-231 and MDA-MB-436 cell lines (8+48 protocol).

Flow cytometric analysis of and their representative dot plots. Statistically significant differences between siNDRG1 and SCR (scrambled siRNA): * p<0.05, ** p<0.01. Statistically significant differences between SCR with and without TGF- β 1: \$\$ p<0.01.

In the same way, the side population was modulated exclusively in SUM159 cells (Figure 28). Hence, collectively, these data provide valuable insights into the extent of *NDRG1*'s pleiotropism and suggest its involvement in maintaining diverse CSC subpopulations in TNBC. The specific effects appear to be contingent upon the tissue of origin of the cell lines (primary tumor or pleural effusion) and their degree of differentiation into a more aggressive phenotype induced by TGF- β 1 stimulation.

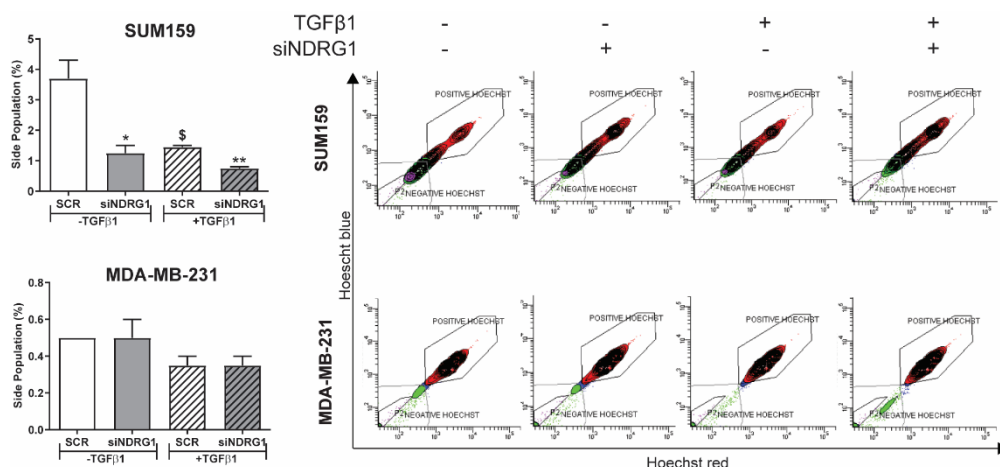


Figure 28. Effect on side population after stimulation with TGF-β1 and *NDRG1* inhibition in SUM159 (14 days protocol), and MDA-MB-231 cell lines (8+48 protocol).

Flow cytometric analysis of and their representative dot plots. Statistically significant differences between siNDRG1 and SCR (scrambled siRNA): * $p < 0.05$, ** $p < 0.01$. Statistically significant differences between SCR with and without TGF-β1: \$ $p < 0.05$.

Taken together, these results support the hypothesis of NDRG1 pleiotropy in different cancer types and cellular contexts. The differential effects observed between primary tumor-derived and pleural effusion-derived cell lines suggest that NDRG1's role in metastasis, EMT, and maintaining diverse CSC subpopulations may be contingent upon the stage of cancer progression and their degree of differentiation into a more aggressive phenotype induced by TGF-β1 stimulation. Our findings contribute to our understanding of the complex and context-dependent functions of NDRG1 in TNBC progression and may have implications for targeted therapeutic strategies.

1.3. Selection of drug candidates based on *NDRG1* knockdown in TGF-β1-stimulated TNBC cells

Considering NDRG1's pleiotropic nature and the potential for unexpected and undesired effects from direct inhibition, we hypothesized that repurposed treatments could be identified based on RNA sequencing of the cellular state associated with *NDRG1* inhibition under TGF-β stimulation. This approach aims to leverage differential gene expression profiles to identify repurposed drugs using the CMap tool, potentially replicating the beneficial effects observed with *NDRG1* inhibition after TGF-β stimulation. By doing so, we seek to standardize

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the unique NDRG1-associated condition observed in specific patients, effectively broadening the accessibility of this therapeutic approach.

Initially, we conducted an RNA sequencing analysis to elucidate the differential gene expression signature in TGF- β 1-stimulated MDA-MB-231 cells with and without *NDRG1* knockdown. To ensure a stringent differential gene expression profile for subsequent CMap querying, we curated the data based on statistical significance and a limited expression level by selecting a log₂ fold change threshold of ± 1.5 (Table 8).

Table 8. RNA sequencing and selection in the dataset of Clue.io.

Invalid gene: Not valid HUGO symbol or Entrez ID, not used in the query. Valid gene: Valid HUGO symbol or Entrez ID and part of BING space, used in the query. Valid but not used in query: Valid HUGO symbol or Entrez ID not part of BING space, not used in query

Gene Stable ID	Gene name	log ₂ fold change	p-value	p-adjusted	Included
ENSG00000077984	<i>CST7</i>	2,89120048	4,07776E-08	9,86935E-07	Valid
ENSG00000127884	<i>ECHS1</i>	2,45256643	3,47696E-66	2,90326E-63	Valid
ENSG00000172667	<i>ZMAT3</i>	2,29875241	7,1603E-41	2,61575E-38	Valid
ENSG00000134602	<i>STK26</i>	2,23385713	7,97858E-09	2,19975E-07	Valid
ENSG00000187068	<i>C3orf70</i>	2,20673159	3,17693E-05	0,000400628	Invalid
ENSG00000272916	<i>AC022400.6</i>	2,17164743	5,90976E-06	9,00718E-05	Invalid
ENSG00000269028	<i>MTRNR2L12</i>	2,16038535	0,004222804	0,024341509	Invalid
ENSG00000171241	<i>SHCBP1</i>	2,03930148	4,08754E-54	2,2754E-51	Valid
ENSG00000167081	<i>PBX3</i>	2,02932733	8,83305E-31	1,87742E-28	Valid
ENSG00000006576	<i>PHTF2</i>	1,94997015	8,54906E-38	2,70104E-35	Valid
ENSG00000114125	<i>RNF7</i>	1,9390751	3,41329E-34	9,0685E-32	Valid
ENSG00000177432	<i>NAP1L5</i>	1,91394891	0,000115589	0,001219626	Valid but not used
ENSG00000184992	<i>BRI3BP</i>	1,86130108	1,19247E-23	1,78718E-21	Valid but not used
ENSG00000163734	<i>CXCL3</i>	1,8046993	1,50571E-05	0,000208305	Valid
ENSG00000186517	<i>ARHGAP30</i>	1,79168104	0,000368629	0,00327951	Valid but not used
ENSG00000197296	<i>FITM2</i>	1,79166835	2,10743E-13	1,19592E-11	Valid but not used
ENSG00000180758	<i>GPR157</i>	1,72757544	2,21528E-13	1,2421E-11	Valid
ENSG00000064666	<i>CNN2</i>	1,71878634	1,38528E-43	5,78356E-41	Valid
ENSG00000163378	<i>EOGT</i>	1,68472568	4,2987E-26	7,17883E-24	Valid
ENSG00000108468	<i>CBX1</i>	1,67039729	1,86546E-44	9,08635E-42	Valid
ENSG00000095752	<i>IL11</i>	1,66833307	2,58955E-85	4,32454E-82	Valid
ENSG00000197111	<i>PCBP2</i>	1,66264088	1,14098E-43	4,94004E-41	Valid
ENSG00000114450	<i>GNB4</i>	1,65441225	1,30324E-31	2,98722E-29	Valid but not used
ENSG00000185787	<i>MORF4L1</i>	1,63448712	2,84584E-70	2,77232E-67	Valid
ENSG00000188167	<i>TMPE</i>	1,63346165	2,17887E-10	7,83722E-09	Valid but not used
ENSG00000274290	<i>HIST1H2BE</i>	1,625234	5,38313E-08	1,27686E-06	Valid but not used
ENSG00000092820	<i>EZR</i>	1,61388354	3,42907E-63	2,67239E-60	Valid

ENSG00000179598	PLD6	1,61151246	0,000248738	0,002348745	Valid but not used
ENSG00000185129	PURA	1,5674716	3,9245E-32	9,36275E-30	Valid
ENSG00000123685	BATF3	1,56086421	0,00307458	0,019006787	Valid
ENSG00000188706	ZDHHC9	1,55807413	1,09073E-10	4,0999E-09	Valid but not used
ENSG00000184743	ATL3	1,55387829	1,18988E-70	1,26452E-67	Valid but not used
ENSG00000170540	ARL6IP1	1,54432197	1,4697E-92	4,29521E-89	Valid
ENSG00000152749	GPR180	1,53378367	8,9653E-16	6,84996E-14	Valid but not used
ENSG00000114999	TTL	1,52815181	8,44732E-39	2,9924E-36	Valid but not used
ENSG00000166471	TMEM41B	1,52033822	2,23942E-43	9,02717E-41	Valid
ENSG0000009335	UBE3C	1,51727035	1,06835E-87	2,0815E-84	Valid
ENSG00000147676	MAL2	-1,5191934	1,6147E-11	6,8144E-10	Valid but not used
ENSG00000283378	BX088645.1	-1,53083195	0,003324681	0,020250479	Invalid
ENSG00000109113	RAB34	-1,53207516	3,66075E-56	2,37745E-53	Valid but not used
ENSG00000164100	NDST3	-1,53704935	0,00205093	0,013778949	Valid
ENSG00000131781	FMO5	-1,57141185	0,004109014	0,023791173	Valid
ENSG00000112697	TMEM30A	-1,57967709	1,90913E-71	2,23177E-68	Valid
ENSG00000179546	HTR1D	-1,58429356	7,7899E-05	0,00087057	Valid
ENSG00000167578	RAB4B	-1,63264198	3,83641E-05	0,000472081	Valid
ENSG00000111731	C2CD5	-1,64657179	2,74304E-22	3,60293E-20	Valid
ENSG00000168702	LRP1B	-1,64744293	0,000351929	0,0031477	Valid
ENSG00000089127	OAS1	-1,65931207	0,001287315	0,009399569	Valid
ENSG00000133135	RNF128	-1,68888367	3,99036E-08	9,69798E-07	Valid
ENSG00000142634	EFHD2	-1,71662877	1,13227E-23	1,71899E-21	Valid
ENSG00000165806	CASP7	-1,78198167	1,21515E-16	1,04449E-14	Valid
ENSG00000170004	CHD3	-1,8897193	1,17821E-50	6,26058E-48	Valid
ENSG00000132842	AP3B1	-1,95005961	1,17996E-89	2,75874E-86	Valid
ENSG00000128567	PODXL	-2,04031867	2,12474E-38	7,09662E-36	Valid
ENSG00000164023	SGMS2	-2,07587609	2,50071E-44	1,16933E-41	Valid but not used
ENSG00000170801	HTRA3	-2,22884304	0,000117975	0,001243579	Valid but not used
ENSG00000169248	CXCL11	-2,36664521	3,11037E-09	9,20511E-08	Valid
ENSG00000104419	NDRG1	-2,79571715	1,4719E-143	1,7206E-139	Valid
ENSG00000138135	CH25H	-2,92520295	2,00569E-06	3,41288E-05	Valid
ENSG00000169245	CXCL10	-3,09717104	6,756E-18	6,75023E-16	Valid
ENSG00000257411	AC034102.2	-6,75313076	0,007516582	0,03882848	Invalid
ENSG00000256514	AP003419.1	-6,98521401	4,86647E-05	0,000577521	Invalid

As anticipated, *NDRG1* was downregulated ($\log_2$ fold change = -2,79571715). Notably, we observed the suppression of other interesting targets such as *CXCL10* and *CXCL11*, *PODXL*, and *OAS1*.

In our approach to identifying perturbational compounds, we conducted **two distinct searches** utilizing the CMap tool. The first search incorporated genes

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with a $\log_2$ fold change over ± 1.5 , while the second search applied a more stringent threshold of ± 1.6 , effectively eliminating genes with less pronounced differential expressions. It is important to note that when inputting genes into the CMap database, only gene names and their directional change in expression (increase or decrease) are considered. The magnitude of change, as represented by the $\log_2$ fold change value, cannot be directly specified. Consequently, the CMap tool assigns equal weight to all input genes when identifying perturbational compounds. To address this limitation and obtain a more comprehensive perspective, we conducted these two separate searches.

The first search, utilizing the ± 1.5 threshold, provided a broader selection of genes, while the second search, with the ± 1.6 threshold, offered a more focused gene set. By using this dual strategy, we sought to balance between inclusivity and specificity in our gene selection, potentially uncovering a wider range of perturbational compounds while also identifying those most closely aligned with the more pronounced gene expression changes. Furthermore, another limitation is that some genes were classified as 'valid but not used' or 'invalid' by the CMap tool, potentially affecting the accuracy of the subsequent drug repurposing analysis (Table 8).

Given our objective to replicate the antitumor transcriptional characteristics observed upon *NDRG1* inhibition following TGF- β 1 stimulation, we focused on PCLs and compounds exhibiting positive connectivity scores. Our analysis emphasized three categories of results: *General*, *Summary*, and MCF-7, as the only breast cancer cell line available in the CMap tool.

In our analysis of **PCLs** (Figure 29), that recapitulate compounds with the same mechanism of action, *NDRG1* knockdown was associated with events such as MAPK signaling (Figure 29A, 29B, 29D, 29E), Wnt family modulation (Figure 29B, 29D), histone-modifying EMSY complex loss of function (Figure 29A-E), and expression of TGF- β receptor inhibitors (Figure 29F). Comparison of the ± 1.5 and ± 1.6 $\log_2$ fold change query results revealed higher connectivity scores and greater similarity across different categories in the ± 1.6 $\log_2$ fold change results.

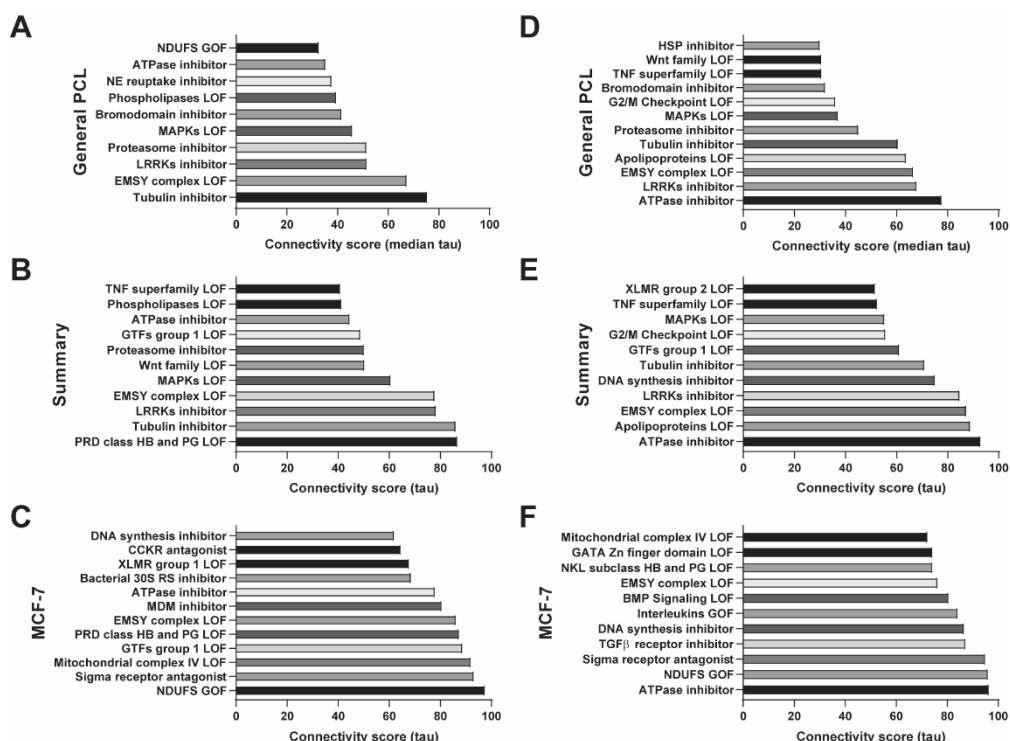


Figure 29. Perturbagen classes (PCLs) topmost connectivity scores of ± 1.5 (A) and ± 1.6 (B) $\log_2$ fold change query in the categories of General, Summary, and MCF-7 of CMap.

Bone Morphogenetic Protein (BMP); Cholecystokinin Receptor (CCKR); Gain Of Function (GOF); General Transcription Factors (GTFs); Homeoboxes (HB); Heat Shock Protein (HSP); Loss Of Function (LOF); Leucine Rich Repeat Kinases (LRRKs); Mitogen-Activated Protein Kinases (MAPKs); Murine Double Minute (MDM); NADH ubiquinone oxidoreductase core subunits (NDUFS); Norepinephrine (NE); NK-Like (NKL); Pseudogenes (PG); ribosomal subunit (RS); Transforming Growth Factor (TGF); Tumor Necrosis Factor (TNF); X-Linked Mental Retardation (XLMR).

Notably, ATPase inhibitors emerged as the strongest result across all three categories in the ± 1.6 - $\log_2$ fold change query (Figure 29D-F), suggesting that a more restrictive gene expression threshold yields more precise results. It is noteworthy that ATPase inhibitors also appeared in the ± 1.5 threshold results (Figure 29A-C), albeit occupying the ninth position, a considerably less prominent placement compared to their top ranking with the ± 1.6 threshold. This suggests that the ± 1.5 threshold may lead CMap to offer PCLs that modulate the expression profile of genes with lower fold changes.

This concept is further supported by the behavior of tubulin inhibitors, which exhibit an inverse pattern. In Figure 29A, in General PCLs, tubulin inhibitors

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appear as the top-ranked, while they drop to the fifth position in the ± 1.6 -log₂ fold change PCLs results (Figure 29D). A similar trend is observed in the *Summary* category, where tubulin inhibitors shift from the second position to the sixth (Figure 29B, 29E). In other words, tubulin inhibitors emerge as a relevant class with the ± 1.5 threshold but lose prominence with the ± 1.6 threshold, indicating that they primarily affect differentially expressed genes with lower fold changes, which are less interesting for our study.

These findings collectively reinforce the notion that applying a more stringent log₂ fold change threshold (± 1.6) in our gene selection process yields more relevant and focused results in the context of identifying potential therapeutic compounds for TNBC. This approach allows us to prioritize genes with more pronounced expression changes, potentially leading to the identification of more impactful drug candidates.

In addition, we later focused on **compounds** with connectivity scores higher than 80, 90, and 96 for *General*, *Summary*, and MCF-7 cell line analyses, respectively (Figure 30). The comparison of the ± 1.6 and ± 1.5 queries revealed that the ± 1.6 query yielded a greater number of drugs with strong connectivity scores. Remarkably, some drugs with high connectivity scores in the ± 1.5 query (Figure 30A) were absent in the ± 1.6 query (Figure 30B), such as tropisetron, ABT-751, and vincristine in the *General* category, and MK-7108, epothilone, and tropisetron in the *Summary* category. This absence suggests that the connectivity of these drugs was primarily based on genes with a ± 1.5 change, likely contributing less to the desired outcome. Consequently, we proceeded with the ± 1.6 results for our subsequent investigations.

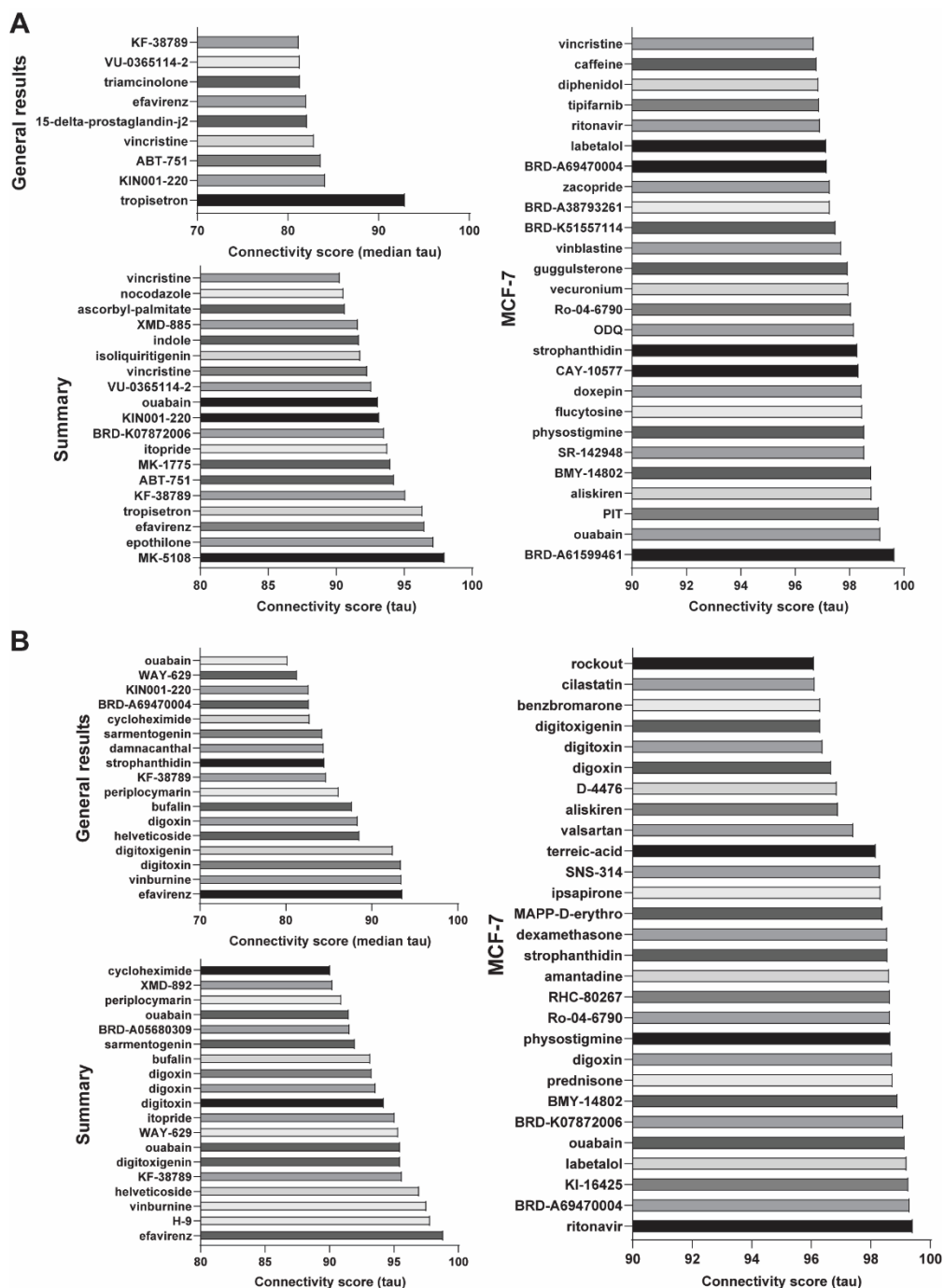


Figure 30. Compounds with connectivity scores higher than 80, 90, and 96 of ± 1.5 (A) and ± 1.6 (B) $\log_2$ fold change query in the categories of General, Summary, and MCF-7 of CMap.

Objective I: Study of the role of NDRG1 after TGF-β stimulation and drug repurposing study by transcriptomic analysis

As depicted in Figure 30B, in the ±1.6 query, the top two compounds in both *General* and *Summary* results were efavirenz and vinburnine. Moreover, several ATPase inhibitors, including digitoxigenin, digitoxin, digoxin, and ouabain, appeared consistently across categories, aligning with their top PCL ranking (Figure 29D, 29E). Although MCF-7 cell line results (Figure 30B) presented a more diverse set of drugs, there still were common compounds with the *General* and *Summary* categories, particularly in cardiac glycosides such as ouabain, digoxin, digitoxin, and digitoxigenin.

From the array of drugs presented in Figure 30B, we considered only those that were FDA-approved and novel for breast cancer treatment, excluding drugs currently under investigation in breast cancer clinical trials. We systematically identified several promising candidates, carefully avoiding those with redundant mechanisms of action. Our selection process culminated in the proposal of three primary candidates, as summarized in Table 9. Based on the *General* and *Summary* results, we prioritized efavirenz and vinburnine, while ouabain was selected as the ATPase inhibitor demonstrating the highest connectivity score in the MCF-7 cell line analysis.

Table 9. Summary of repurposed drug selection.

HIV: human immunodeficiency virus.

	Description	Related disease	Connectivity score
Efavirenz	HIV reverse transcriptase inhibitor	HIV	Summary / General 98.87 / 93.63
Ouabain	ATPase inhibitor	Hypertension, arrhythmia	MCF-7 99.17
Vinburnine	Allosteric modulator of muscarinic acetylcholine receptors	Cerebral stroke	Summary / General 97.57 / 93.53

2. Objective II: Validation of efficacy in TNBC cell lines

2.1. Cell viability and IC50 calculation

To evaluate the efficacy of our repurposed drug candidates in TNBC, we initially assessed their impact on the cell viability of three distinct cell lines over a 72-h treatment period. Although the gene expression data used for drug repurposing was derived from TGF-β1-treated cells, the effects of this cytokine were

inherently incorporated within the expression profile and, consequently, the connectivity mapping. Therefore, drug treatments were administered without TGF- β 1 supplementation.

All cell lines exhibited sensitivity to the three repurposed drugs with their IC₅₀ values calculated. In the MDA-MB-231 cell line (Figure 31), efavirenz exhibited an IC₅₀ of 15.4 μ M, while ouabain demonstrated the most potent effect with an IC₅₀ of 54.0 nM. Vinburnine showed intermediate potency with an IC₅₀ of 1.0 μ M.

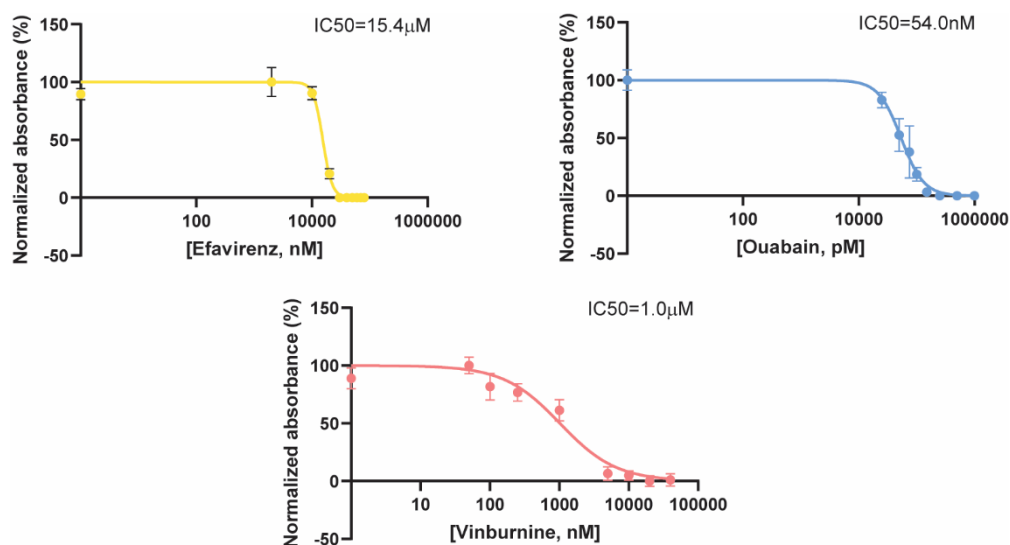


Figure 31. IC₅₀ of efavirenz, ouabain, and vinburnine in MDA-MB-231 cell line after 72-h treatments.

Results were normalized to the vehicle control.

The SUM159 cell line displayed similar sensitivity patterns, with efavirenz, ouabain, and vinburnine yielding IC₅₀ values of 13.9 μ M, 38.0 nM, and 1.0 μ M, respectively, as depicted in Figure 32.

Objective II: Validation of efficacy in TNBC cell lines

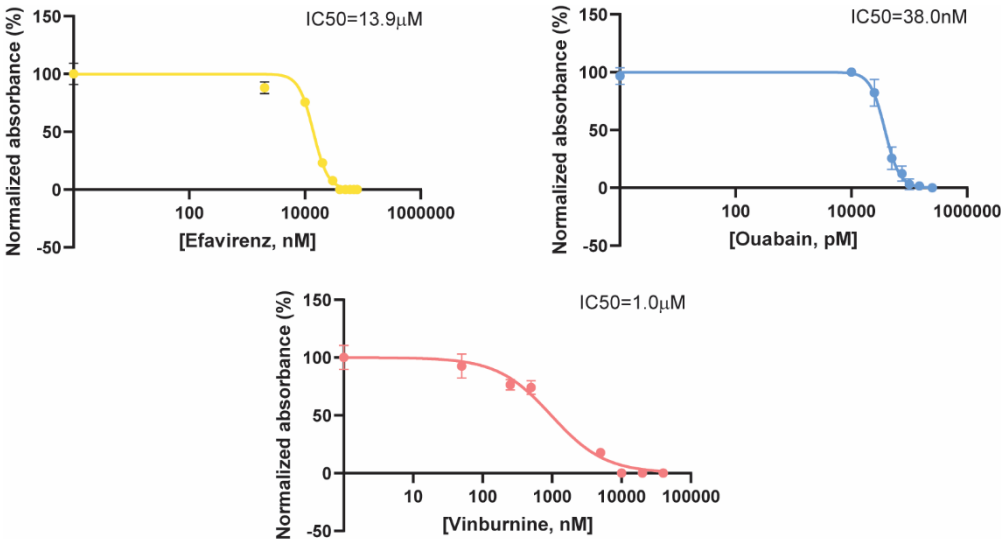


Figure 32. IC₅₀ of efavirenz, ouabain, and vinburnine in SUM159 cell line after 72-h treatments.

Results were normalized to the vehicle control.

Interestingly, the BT549 cell line exhibited differential sensitivity to the compounds as illustrated in Figure 33. It was notably more sensitive to efavirenz, with an IC₅₀ of 1.6 μM, and showed the highest sensitivity to ouabain among all cell lines tested, with an IC₅₀ of 22.7 nM. Conversely, BT549 demonstrated increased resistance to vinburnine, with an IC₅₀ of 5.6 μM.

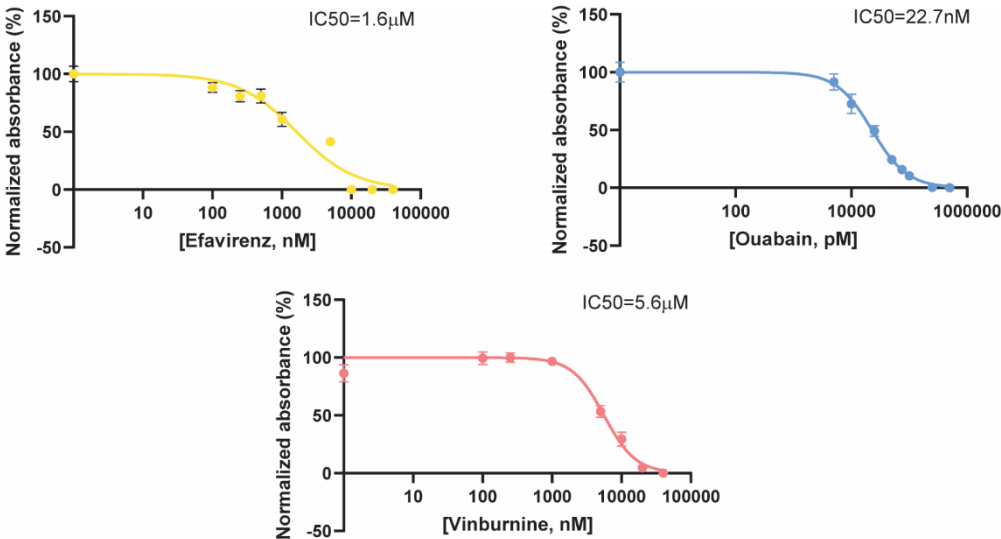


Figure 33. IC₅₀ of efavirenz, ouabain, and vinburnine in BT549 cell line after 72-h treatments.

Results were normalized to the vehicle control.

Across all cell lines, ouabain consistently displayed the most potent cytotoxic effect, achieving the lowest IC₅₀ values among all drugs tested. Given the established utility of IC₅₀ data in evaluating drug efficacy and potency, we selected these concentrations as the rational basis for establishing specific treatment doses in subsequent experiments, as summarized in Table 10.

Table 10. IC₅₀ calculated values and selected treatment doses for repurposed drug candidates.

	IC ₅₀ value			Treatment dose		
	MDA-MB-231	SUM159	BT549	MDA-MB-231	SUM159	BT549
Efavirenz (μM)	15.4	13.9	1.6	15	15	2
Ouabain (nM)	54.0	38.0	22.7	54	38	23
Vinburnine (μM)	1.0	1.0	5.6	1	1	6

2.2. Cell apoptosis

Subsequently, we investigated the apoptosis-inducing potential of our repurposed drugs after 24 h of treatment. As shown in Figure 34, efavirenz emerged as the most effective candidate for increasing the total apoptotic population in all three cell lines. We further explored the effects of drug combinations: efavirenz + ouabain (EO), efavirenz + vinburnine (EV), and ouabain + vinburnine (OV). In MDA-MB-231 cells, EO and EV combinations induced higher levels of apoptosis compared to individual agents. Conversely, in SUM159 cells, where efavirenz induced approximately 10% apoptosis, no significant enhancement was observed with the combinations.

Objective II: Validation of efficacy in TNBC cell lines

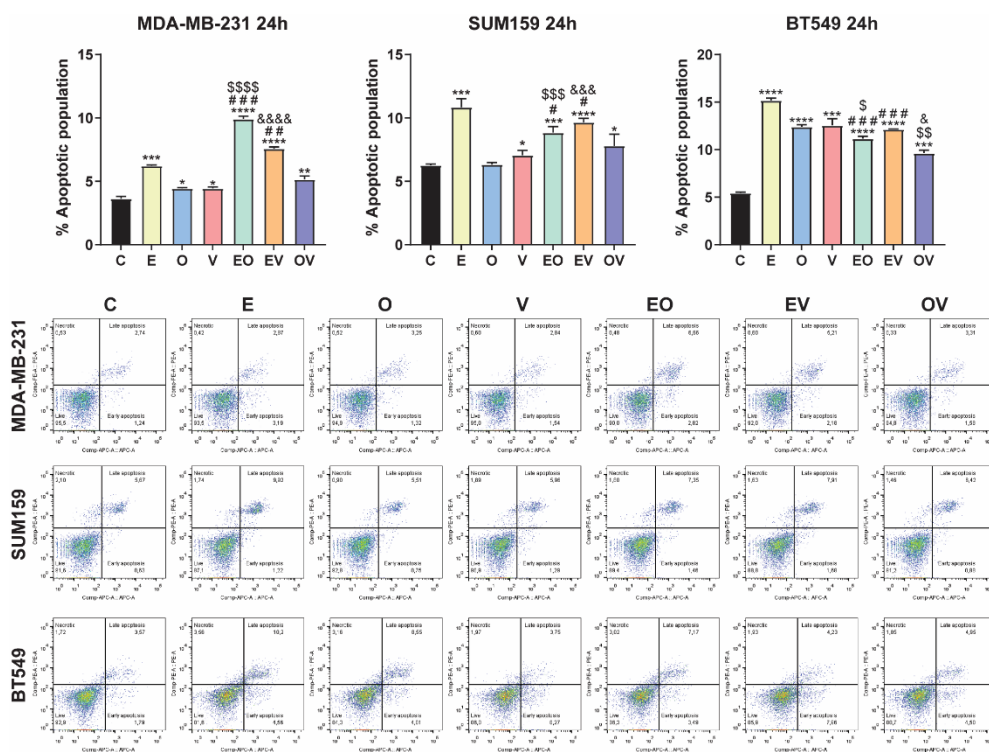


Figure 34. Effect of repurposed drugs on apoptosis after 24-h treatments in MDA-MB-231, SUM159, and BT549 cell lines.

Analysis of total apoptotic population after 24-h treatments in MDA-MB-231, SUM159, and BT549 cell lines and representative dot plots of Annexin V/PI FACS analysis. Viable cells (Annexin V and PI negative cells); early apoptotic cells (Annexin V positive and PI negative cells); late apoptotic cells (Annexin V and PI positive); and necrotic cells (Annexin V negative and PI positive). Statistically significant differences with the vehicle control (C): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Statistically significant differences with E (efavirenz): # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$. Statistically significant differences with O (ouabain): \$ $p < 0.05$, \$\$ $p < 0.01$, \$\$\$ $p < 0.001$, \$\$\$\$ $p < 0.0001$. Statistically significant differences with V (vinburnine): & $p < 0.05$, && $p < 0.001$, &&& $p < 0.0001$. Data are presented as mean $\pm$ SD.

Focusing on BT549 cells, all treatments substantially induced apoptosis at 24 h, potentially masking the effects of the combinations. Hence, to determine whether the observed effects on apoptosis were sustained, we conducted further analyses at 48 h post-treatment. We observed that vinburnine increased apoptosis to 17%, while the EO combination approximately doubled the apoptotic population compared to the individual monotherapies (Figure 35).

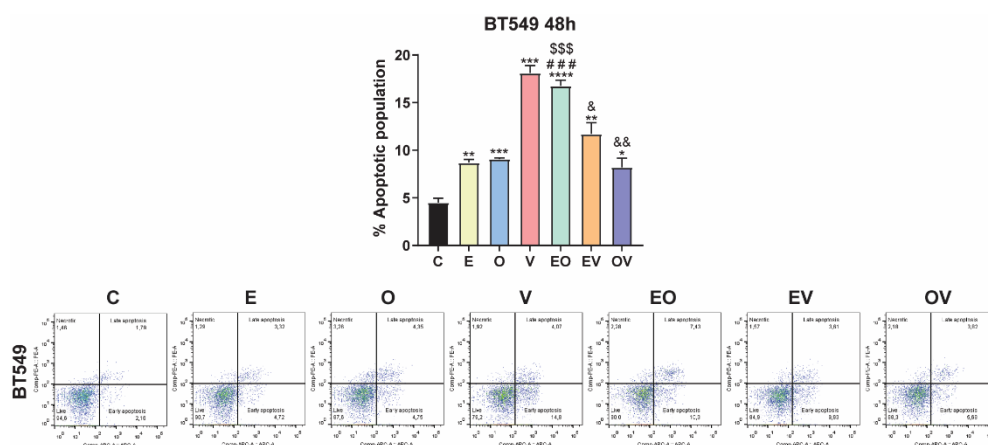


Figure 35. Effect of repurposed drugs on apoptosis after 48-h treatments in BT549 cell line.

Flow cytometric analysis of total apoptotic population after 48-h treatments in BT549 cell line and representative dot plots of Annexin V/PI FACS analysis. Viable cells (Annexin V and PI negative cells); early apoptotic cells (Annexin V positive and PI negative cells); late apoptotic cells (Annexin V and PI positive); and necrotic cells (Annexin V negative and PI positive). Statistically significant differences with the vehicle control (C): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Statistically significant differences with E (efavirenz): ### $p < 0.001$. Statistically significant differences with O (ouabain): \$\$\$ $p < 0.001$. Statistically significant differences with V (vinburnine): & $p < 0.05$, && $p < 0.01$. Data are presented as mean $\pm$ SD.

These findings demonstrate the potential efficacy of our repurposed drug candidates, both as monotherapies and in combinations, inducing cytotoxicity and apoptosis in TNBC cell lines, warranting further investigation into their mechanisms of action and potential clinical applications.

2.3. CSC subpopulations

Due to the importance that CSCs present in TNBC and their contribution to the aggressiveness profile of this subtype compared to other breast cancers[35], we assessed the efficacy of our repurposed drugs and their combinations targeting various CSC populations.

Initially, as shown in Figure 36, we studied the ALDH1+ population after 72 h of treatment via flow cytometry. In MDA-MB-231 cells, all treatments resulted in a significant reduction of the ALDH1+ population, whereas SUM159 cells exhibited similar results, except for efavirenz monotherapy, which did not yield a significant difference compared to the control. Interestingly, in BT549 cells, vinburnine enhanced the ALDH1+ population, while the EV combination showed

Objective II: Validation of efficacy in TNBC cell lines

no significant effect. Fortunately, treatments with efavirenz, ouabain, EO, and OV nearly eliminated the ALDH1+ population in BT549 cells, mirroring the effects observed in the other two cell lines. Notably, the EO and OV combinations demonstrated superior efficacy across all cell lines.

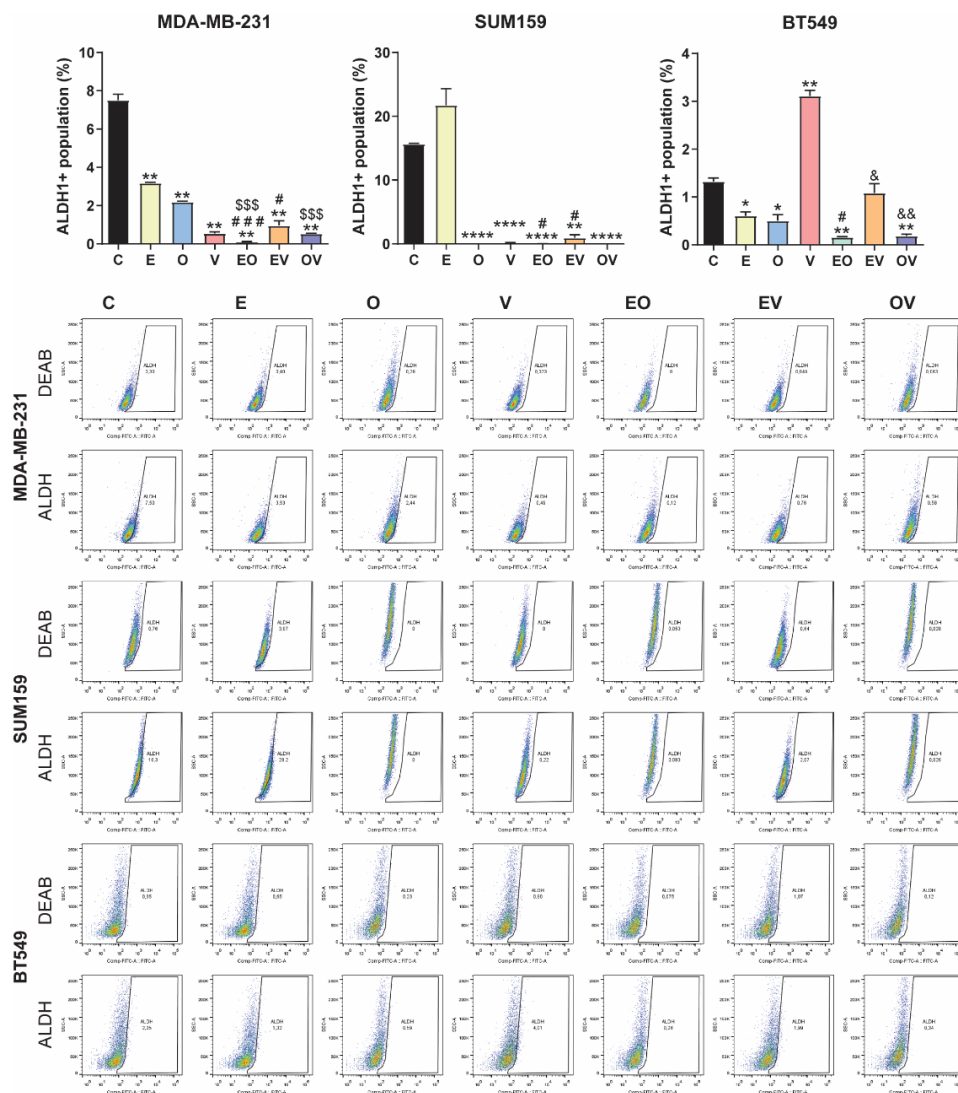


Figure 36. Effect of repurposed drugs on ALDH1+ CSC population after 72-h treatments in MDA-MB-231, SUM159, and BT549 cell lines.

Flow cytometric analysis of ALDH1+ CSC population and representative dot plots of flow cytometric analysis of ALDH1+ population and DEAB control after 72-h treatments in MDA-MB-231, SUM159, and BT549 cell lines. Statistically significant differences with the vehicle control (C): * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. Statistically significant differences with E (efavirenz): # $p < 0.05$, ### $p < 0.001$. Statistically significant differences with O (ouabain): \$\$\$ $p < 0.001$. Statistically significant differences with V (vinburnine): & $p < 0.05$, && $p < 0.01$. Data are presented as mean $\pm$ SD.

Next, we investigated the invasive CD44^{high}/CD24⁻ population (Figure 37). In contrast to the ALDH1+ population, we observed heterogeneous responses across treatments and cell lines. MDA-MB-231 cells exhibited the most consistent results, with all treatment groups demonstrating statistically significant decreases in the CD44^{high}/CD24⁻ population. In SUM159 cells, vinburnine and EV reduced this population, while EO and OV paradoxically increased it. BT549 cells displayed an inverse trend compared to SUM159, with vinburnine producing no significant changes, while efavirenz, ouabain, and their combination decreased the CD44^{high}/CD24⁻ population.

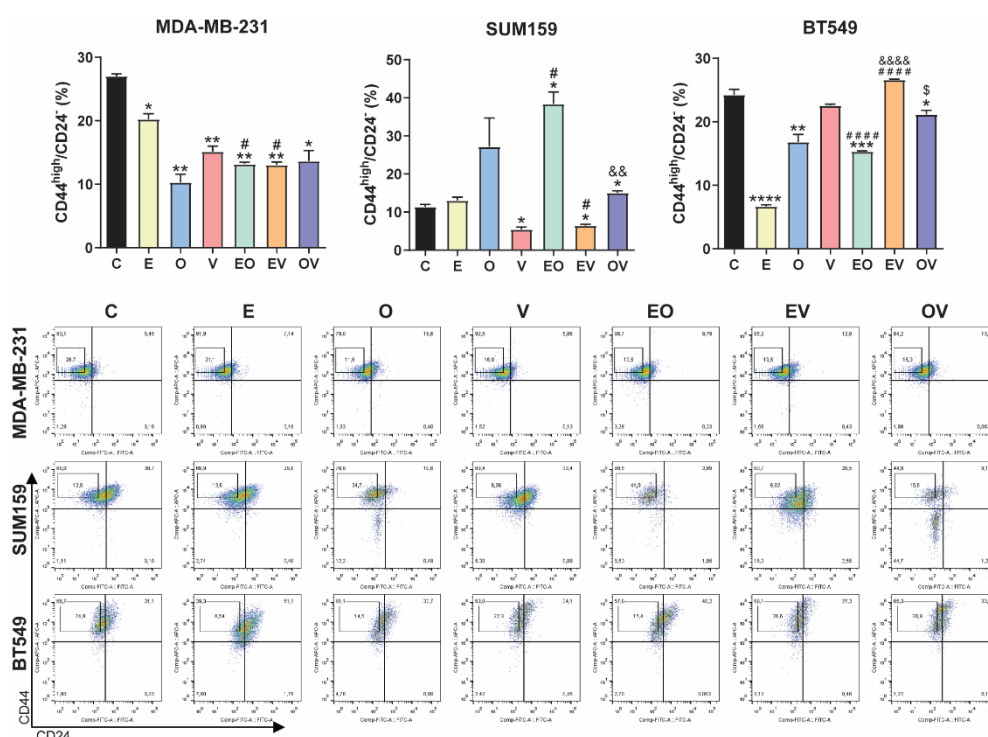


Figure 37. Effect of repurposed drugs on CD44^{high}/CD24⁻ CSC population after 72-h treatments in MDA-MB-231, SUM159, and BT549 cell lines.

Flow cytometric analysis of CD44^{high}/CD24⁻ population and representative dot plots of flow cytometric analysis of CD44^{high}/CD24⁻ population after 72-h treatments in MDA-MB-231, SUM159, and BT549 cell lines. Statistically significant differences with the vehicle control (C): * p<0.05, ** p<0.01, **** p<0.0001. Statistically significant differences with E (efavirenz): # p<0.05, #### p<0.0001. Statistically significant differences with O (ouabain): \$ p<0.05. Statistically significant differences with V (vinburnine): && p<0.01, &&&& p<0.0001. Data are presented as mean ± SD.

Objective II: Validation of efficacy in TNBC cell lines

Furthermore, we examined the effects of our treatments on the side population in MDA-MB-231 and SUM159 cell lines (Figure 38). We observed a decrease in this population with all treatments, except for OV and the outstanding increase of EV in MDA-MB-231 cells.

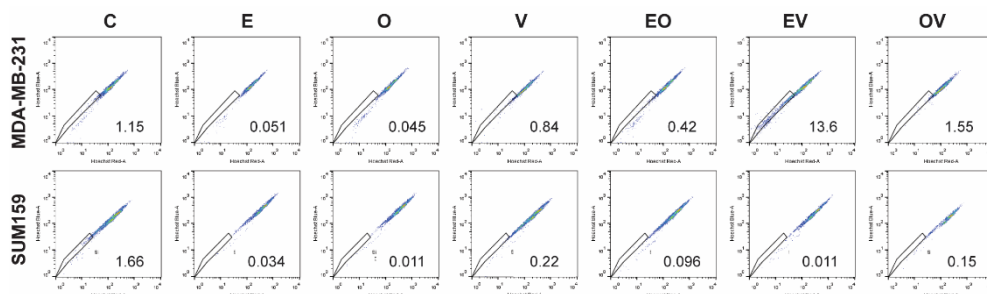


Figure 38. Effect of repurposed drugs on side population after 72-h treatments in MDA-MB-231 and SUM159 cell lines.

Flow cytometric analysis of side population and dot plots of flow cytometric analysis of side population after 72-h treatments in MDA-MB-231 and SUM159 cell lines. C, control; E, efavirenz; O, ouabain; V, vinburnine.

Collectively, these data suggest that our repurposed drugs effectively reduce various CSC populations, particularly in MDA-MB-231 cells. The differential responses observed across cell lines and CSC populations underscore the complex nature of TNBC and highlight the potential of these repurposed drugs to target multiple aspects of tumor heterogeneity.

2.4. CSC self-renewal

In order to evaluate the self-renewal capacity of TNBC cells under treatment, we conducted MSFE assays. As illustrated in Figure 39, a trend towards decreased primary MSFE was observed across all cell lines, with SUM159 exhibiting the most pronounced effect.

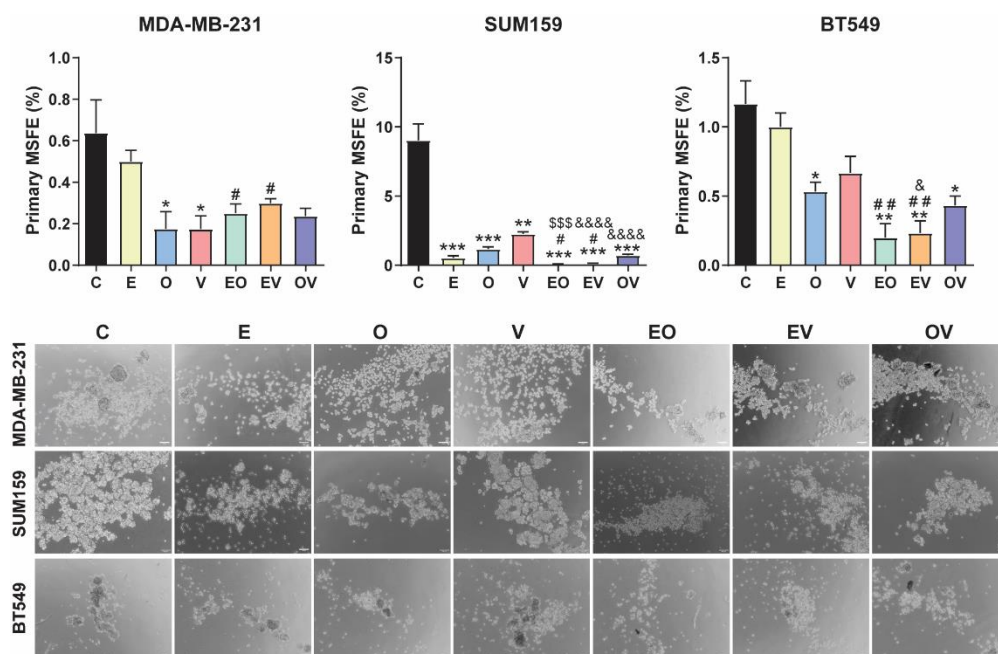


Figure 39. Effect of repurposed drugs on mammosphere-forming efficiency (MSFE) in primary mammospheres after treatments in MDA-MB-231, SUM159, and BT549 cell lines.

Graphic representation of primary MSFE analyses and representative images of primary generation of mammospheres of MDA-MB-231, SUM159, and BT549 cell lines. Original objective 4x. Scale bar 100 μ m. Statistically significant differences with the vehicle control (C): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Statistically significant differences with E (efavirenz): # $p < 0.05$, ## $p < 0.01$. Statistically significant differences with O (ouabain): \$\$\$ $p < 0.001$. Statistically significant differences with V (vinburnine): & $p < 0.05$, &&& $p < 0.0001$. Data are presented as mean $\pm$ SD.

In fact, more compelling results were obtained in the second generation of mammospheres (Figure 40), where cells were not directly treated, thus reflecting the long-term impact of the drugs on self-renewal capacity. In MDA-MB-231 cells, all treatments significantly reduced second-generation MSFE, while in SUM259, only efavirenz and its combinations demonstrated efficacy. BT549 cells showed statistically significant decreases in MSFE with vinburnine, EO, and OV treatments. Indeed, the EO combination emerged as the most effective across all cell lines, despite the variable efficacy of efavirenz and ouabain as single agents.

Objective II: Validation of efficacy in TNBC cell lines

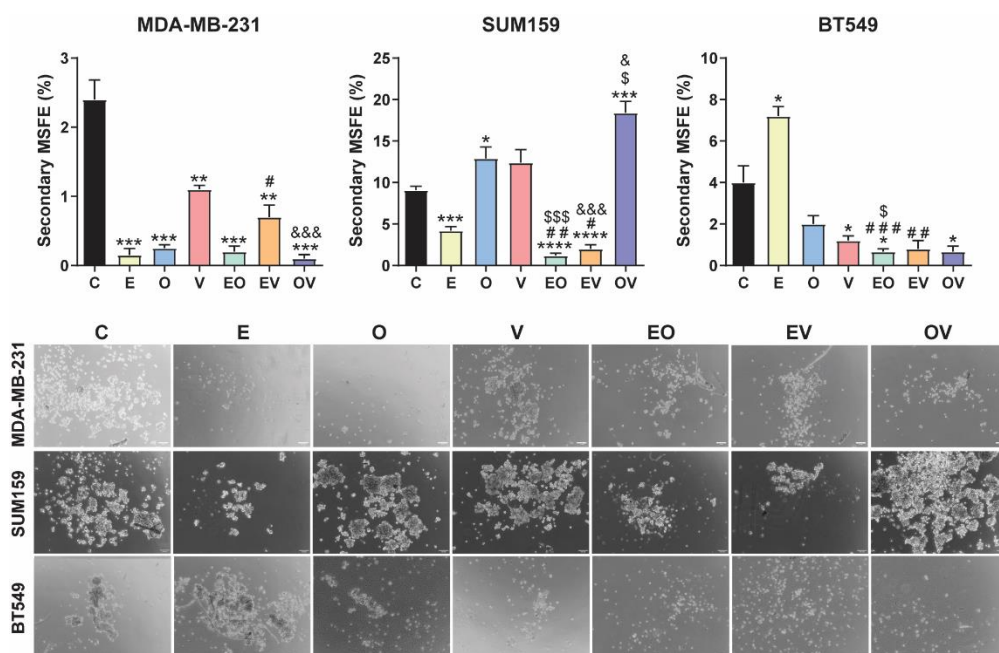


Figure 40. Effect of repurposed drugs on mammosphere-forming efficiency (MSFE) in secondary mammospheres in MDA-MB-231, SUM159, and BT549 cell lines.

Graphic representation of secondary MSFE analyses and representative images of secondary generation of mammospheres of MDA-MB-231, SUM159, and BT549 cell lines. Original objective 4x. Scale bar 100µm. Statistically significant differences with the vehicle control (C): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Statistically significant differences with E (efavirenz): # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$. Statistically significant differences with O (ouabain): \$ $p < 0.05$, \$\$\$ $p < 0.001$. Statistically significant differences with V (vinburnine): & $p < 0.05$, && $p < 0.001$. Data are presented as mean $\pm$ SD.

2.5. Clonogenicity

To assess the clonogenicity capacity of TNBC under our repurposed treatments, we performed the soft-agar colony assay. BT549 cells failed to form proper colonies, consistent with previous reports[137]. As depicted in Figure 41, in MDA-MB-231 cells, all treatments significantly reduced colony formation, with EO and OV combinations showing the greatest efficacy. However, SUM159 cells exhibited no changes with ouabain and vinburnine monotherapies, slight decreases with efavirenz, EV, and OV, and the most pronounced reduction with EO treatment ($p < 0.001$).

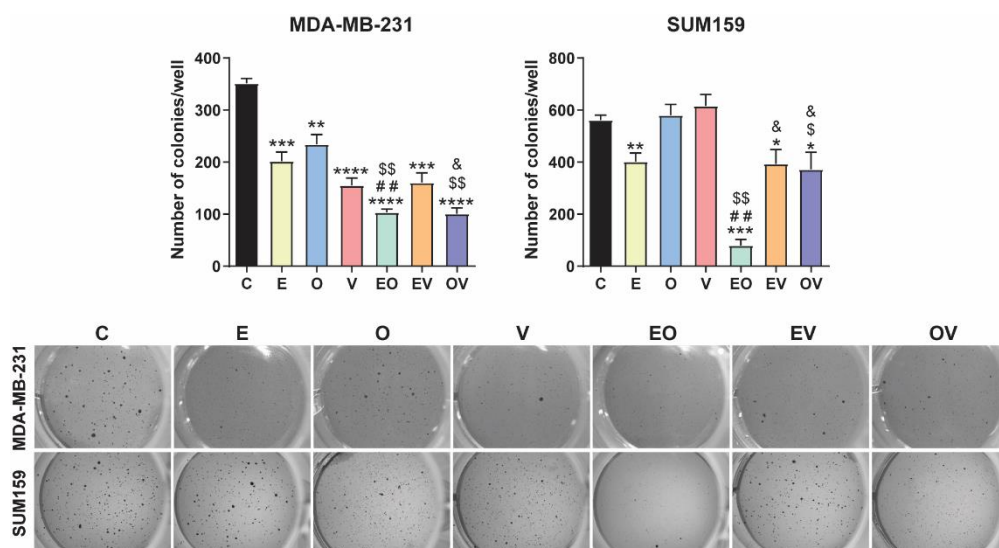


Figure 41. Effect of repurposed drugs on soft-agar colony formation 72-h treatments in MDA-MB-231 and SUM159 cell lines.

Graphic representation of the number of soft-agar colonies/well and their representative images of MDA-MB-231 and SUM159 cell lines. Statistically significant differences with the vehicle control (C): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Statistically significant differences with E (efavirenz): ## $p < 0.01$. Statistically significant differences with O (ouabain): \$ $p < 0.05$, \$\$ $p < 0.01$. Statistically significant differences with V (vinburnine): & $p < 0.05$. Data are presented as mean $\pm$ SD.

2.6. Cell migration

Lastly, cell migration, evaluated using a wound healing assay, revealed unexpected results in MDA-MB-231 cells, where only ouabain, vinburnine, and EO treatments significantly decreased the migration index. Conversely, SUM159 cells showed notable reductions in migration with efavirenz, ouabain, EO, and EV treatments, while all therapies significantly reduced migration in BT549 cells (Figure 42). These findings stand in contrast to the migration results observed with *NDRG1* inhibition and TGF- β stimulation, where primary tumor-derived cell lines (SUM159 and BT549) did not exhibit significant reductions in migration (Figure 22).

Objective II: Validation of efficacy in TNBC cell lines

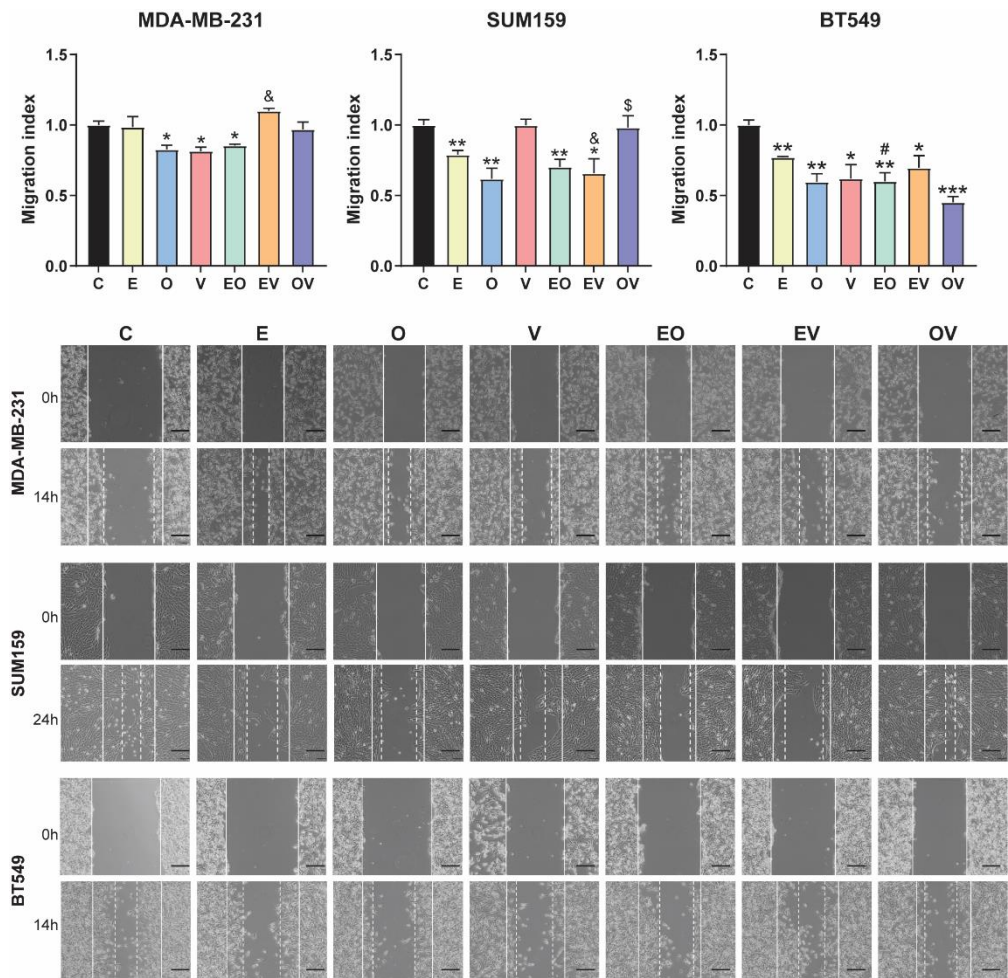


Figure 42. Effect of repurposed drugs on cell migration after 24-h treatments in MDA-MB-231, SUM159, and BT549 cell lines.

Graph analyses show tumor cell migration normalized to vehicle control and representative images of tumor cell migration after 24-h treatments in MDA-MB-231, SUM159, and BT549 cell lines. Results were normalized to the vehicle control. Scale bar 200 μm. Statistically significant differences with the vehicle control (C): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Statistically significant differences with E (efavirenz): # $p < 0.05$. Statistically significant differences with O (ouabain): \$ $p < 0.05$. Statistically significant differences with V (vinburnine): & $p < 0.05$. Data are presented as mean $\pm$ SD.

In summary, our comprehensive analysis of various CSC markers and functional assays consistently identified EO as the most potent combination therapy, yielding superior results across all cell lines and experimental paradigms. This study addresses a significant gap in the literature regarding the combination of repurposed drugs in cancer research and provides compelling evidence for

potential synergistic effects. The EO combination demonstrated enhanced anticancer efficacy compared to monotherapies, significantly reducing nearly all CSC phenotypic properties evaluated.

2.7. Inhibition of p-AKT and EMT markers

Our CMap drug repurposing approach was based on a transcriptional signature. Consequently, our selected drugs were not required to directly inhibit NDRG1. However, we evaluated the expression of both total NDRG1 and its phosphorylated form at Thr346 (p-NDRG1) via immunoblot analysis after 24 and 72 h of treatment in the absence of TGF- β (Figure 43). As anticipated, no consistent trend of NDRG1 inhibition was observed across the cell lines following treatment.

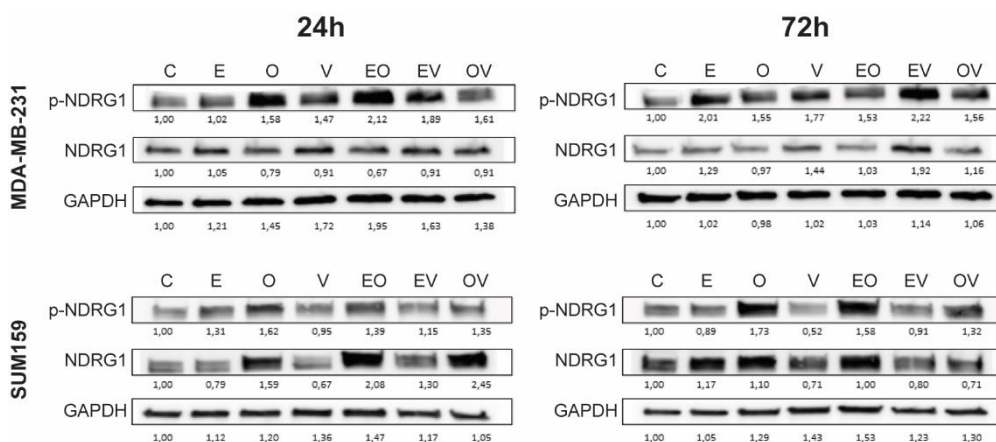


Figure 43. Effect of repurposed drugs on NDRG1 expression after 24-h and 72-h treatments in MDA-MB-231 and SUM159 cell lines.

Immunoblot of p-NDRG1 (Thr346) and total NDRG1 after 24-h and 72-h treatments in MDA-MB-231 and SUM159 cell lines. C, control; E, efavirenz; O, ouabain; V, vinburnine.

We aimed to elucidate the antitumor mechanisms of our candidate molecules. We considered that this drug repurposing method predicts molecules that can mimic the queried transcriptional signature. Subsequently, we investigated four genes whose expression was decreased in the original query using RT-qPCR (Table 8): *CXCL10*, *CXCL11*, *PODXL*, and *OAS1* (Figure 44). After 12-h treatments, in MDA-MB-231 cells, *CXCL10* expression significantly decreased with efavirenz, ouabain, and vinburnine (* $p < 0.05$) and the combination EO (** $p < 0.01$). In contrast, in SUM159, this reduction was only statistically significant

Objective II: Validation of efficacy in TNBC cell lines

with ouabain (** $p < 0.01$). Secondly, *CXCL11* expression decreased following treatment with EO and EV in MDA-MB-231 cells (* $p < 0.05$) and ouabain in SUM159 cells (** $p < 0.01$). *PODXL* expression showed a significant reduction in MDA-MB-231 cells treated with EV (* $p < 0.05$). Interestingly, *OAS1* expression was significantly enhanced in SUM159 cells treated with EV (* $p < 0.05$).

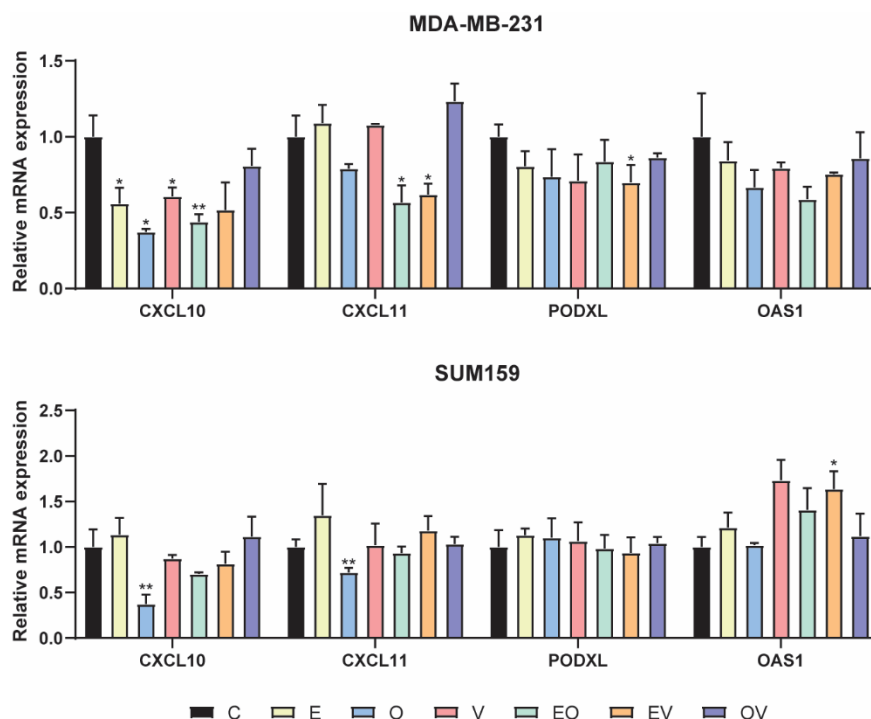


Figure 44. Effect of repurposed drugs on relative mRNA expression of *CXCL10*, *CXCL11*, *PODXL*, and *OAS1* after 12-h treatments in MDA-MB-231 and SUM159 cell lines.

Results were normalized to the vehicle control. Statistically significant differences with the control vehicle: * $p < 0.05$, ** $p < 0.01$. C, control; E, efavirenz; O, ouabain; V, vinburnine.

Given that the chemokines *CXCL10* and *CXCL11* are known to activate the AKT pathway via CXCR3[148–150], we hypothesized that our repurposed candidates might exert their anticancer activities through modulation of the **AKT pathway**. Consequently, via immunoblot, we studied the expression of both the total and the phosphorylated form at Ser473 (p-AKT) after 24 h and 72 h of treatment (Figure 45).

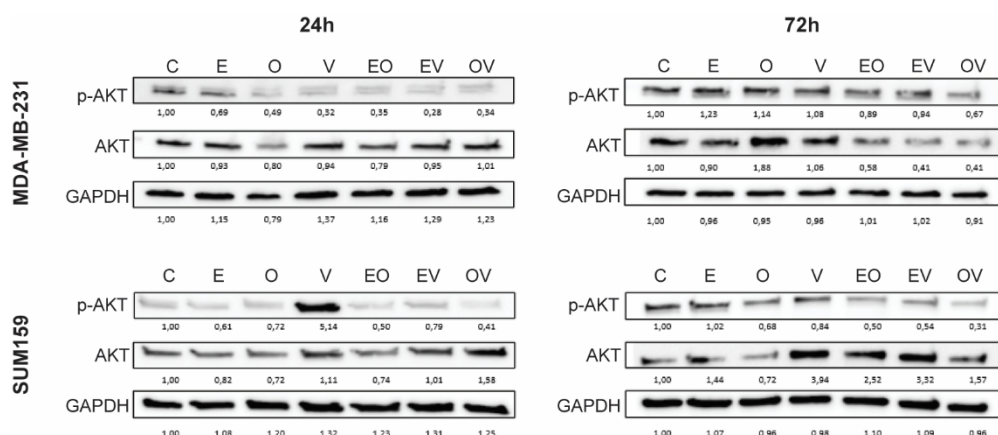


Figure 45. Effect of repurposed drugs on AKT expression after 24-h and 72-h treatments in MDA-MB-231 and SUM159 cell lines.

Immunoblot of p-AKT (Ser473) and total AKT after 24-h and 72-h treatments in MDA-MB-231 and SUM159 cell lines. C, control; E, efavirenz; O, ouabain; V, vinburnine.

At 24 h post-treatment, a general decrease in p-AKT was observed in MDA-MB-231 and SUM159 cells across all treatments, except for an increase following vinburnine treatment in SUM159 cells. Nonetheless, this pattern of p-AKT reduction was not uniformly maintained at 72 h. For instance, in MDA-MB-231 cells, p-AKT expression returned to baseline levels at 72 h, while the three combination treatments (EO, EV, and OV) induced a reduction in total AKT. Whereas in SUM159 cells, p-AKT expression was restored with efavirenz treatment, ouabain, EO, EV, and OV sustained the reduction observed at 24 h. Notably, the previous increase in p-AKT induced by vinburnine at 24 h was no longer apparent at 72 h. The observed temporal and cell line-specific variations in AKT activation suggest that the repurposed drug candidates may exert their effects through multiple mechanisms.

Subsequently, we evaluated the **expression of EMT markers** (Snail, Slug, and Vimentin), which are known downstream targets of the AKT pathway[148,149].

As illustrated in Figure 46, after 24 and 72 h of treatment, all interventions reduced Slug expression in all cell lines, except for efavirenz monotherapy at 24 h in MDA-MB-231 and at 72 h in BT549.

Objective II: Validation of efficacy in TNBC cell lines

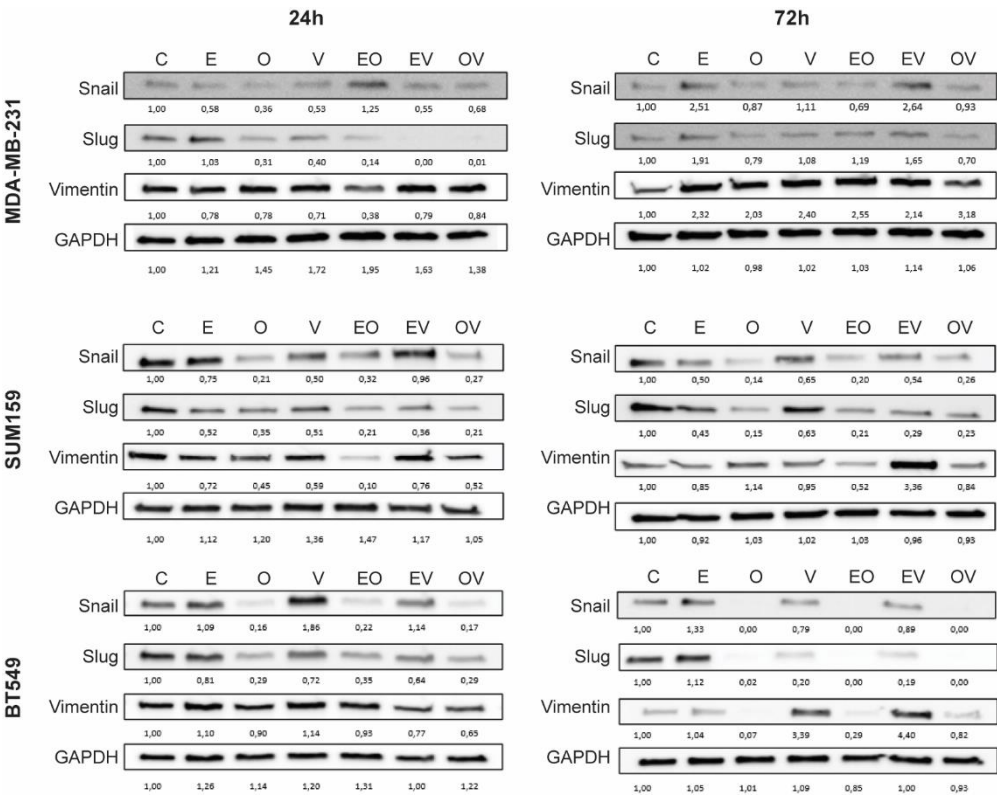


Figure 46. Effect of repurposed drugs on EMT markers after 24-h and 72-h treatments in MDA-MB-231, SUM159 and BT549 cell lines.

Immunoblot of Snail, Slug, and Vimentin after 24-h and 72-h treatments in MDA-MB-231, SUM159, and BT549 cell lines. C, control; E, efavirenz; O, ouabain; V, vinburnine.

Regarding Snail expression, after 24 h of treatment, ouabain, EO and OV consistently reduced the protein expression across all three cell lines, except for EO in MDA-MB-231. At 72 h post-treatment, while the SUM159 cell line maintained similar patterns of Snail modulation as observed at 24 h, in MDA-MB-231, EO did produce a reduction in its expression. Furthermore, the BT549 cell line exhibited distinct responses at 72 h. Ouabain, EO, and OV treatments completely ablated the expression of Snail and Slug, as evidenced by the absence of detectable protein bands.

Lastly, Vimentin expression exhibited cell line-specific variations after 24 h of treatment. In SUM159 cells, ouabain, EO, and OV treatments reduced its expression, with EO demonstrating a particularly pronounced decrease. This robust reduction in Vimentin expression following EO treatment was also observed in MDA-MB-231 cells but was notably absent in BT549 cells. At 72 h

post-treatment, diverse responses were observed across cell lines. MDA-MB-231 cells exhibited a general enhancement in Vimentin expression. In contrast, SUM159 cells showed a more varied response: EO and OV treatments resulted in decreased Vimentin expression, while EV treatment induced an increase in Vimentin levels. Notably, the BT549 cell line again exhibited a remarkable reduction in this EMT marker expression after monotherapy and combinations with ouabain (EO and OV), similar to the profile of Snail and Slug proteins.

These differences found across the three TNBC cell lines of the study highlight the complex dynamic nature of EMT signaling modulation by our repurposed drug candidates and the importance of precision medicine. Collectively, these results, in conjunction with our previous findings, consistently demonstrate that the EO combination was the most effective treatment in reducing both the expression of AKT pathway components and EMT markers across all three TNBC cell lines. The comprehensive suppression of key signaling molecules and EMT markers by EO underscores its potential as a promising therapeutic strategy for TNBC, which could target multiple aspects of tumor progression and metastasis.

2.8. Docetaxel-resistant cell line

As previously described in the "Materials and Methods" section 3.1, our research team established a docetaxel-resistant cell line (SUM159-R)[137]. We subsequently evaluated the cell viability of this resistant line following 72 h of treatment with our repurposed drug candidates.

Figure 47 illustrates that SUM159-R exhibited distinct sensitivity profiles compared to the parental SUM159 cells. Notably, SUM159-R displayed enhanced sensitivity to efavirenz and ouabain, with IC₅₀ values of 8.5 μ M and 18.9 nM, respectively. Conversely, these cells demonstrated increased resistance to vinburnine, with an IC₅₀ of 4.0 μ M.

Objective II: Validation of efficacy in TNBC cell lines

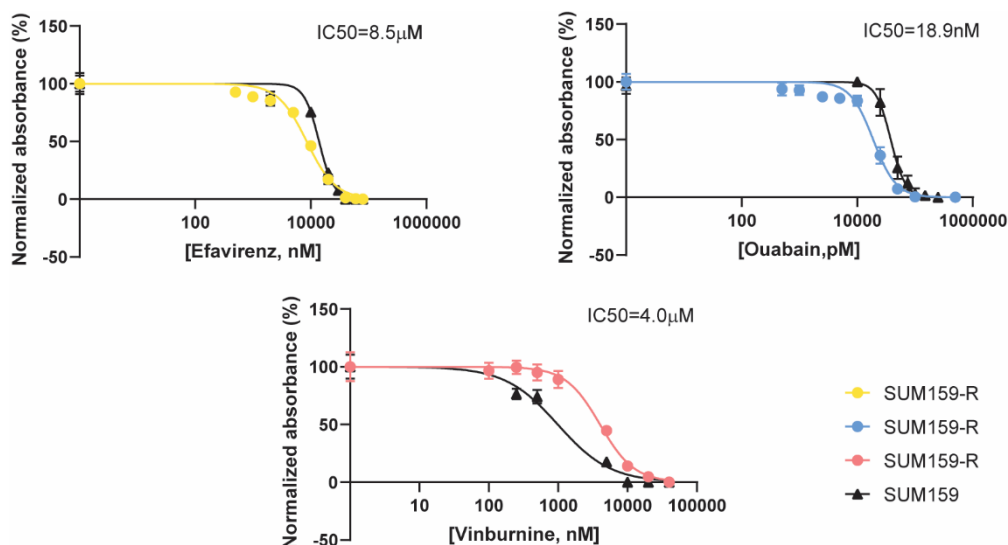


Figure 47. IC₅₀ of efavirenz, ouabain, and vinburnine after 72-h treatments in SUM159 and SUM159-R cell lines.

Black line represents SUM159 and color lines represent SUM159-R cell line. Calculated IC₅₀ value of SUM159-R cell line. Results were normalized to the vehicle control.

Then, we assessed the impact of our repurposed treatments on the self-renewal capacity of SUM159-R cells. As illustrated in Figure 48, all treatments effectively reduced both primary and secondary MSFE. Notably, efavirenz monotherapy emerged as the most efficacious in suppressing self-renewal, followed by ouabain. This consistent suppression of self-renewal capacity across all treatments underscores the promise of these repurposed drugs in targeting cancer stem cell-like properties in chemoresistant TNBC.

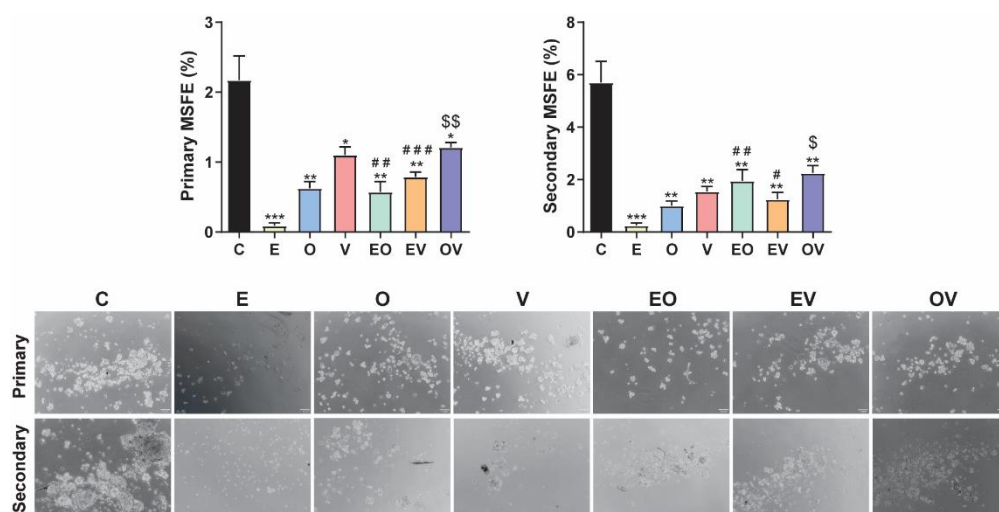


Figure 48. Effect of repurposed drugs on mammosphere-forming efficiency (MSFE) in primary after 72-h treatments and secondary mammospheres in SUM159-R cell line.

Graphic representation of primary and secondary MSFE analyses, and representative images of generation of mammospheres of SUM159-R cell line. Original objective 4x. Scale bar 100µm. Statistically significant differences with the vehicle control (C): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Statistically significant differences with E (efavirenz): # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$. Statistically significant differences with O (ouabain): \$ $p < 0.05$, \$\$ $p < 0.01$. Data are presented as mean $\pm$ SD. V, vinburnine.

Lastly, we investigated the potential of our repurposed drugs to modulate docetaxel sensitivity and potentially reverse chemoresistance. Interestingly, while efavirenz and ouabain did not significantly alter docetaxel sensitivity, co-treatment with vinburnine resulted in a marked reduction in the IC₅₀ of docetaxel compared to the vehicle control (Figure 49). This finding highlights the potential of vinburnine as a chemosensitizing agent in docetaxel-resistant TNBC cells.

Objective II: Validation of efficacy in TNBC cell lines

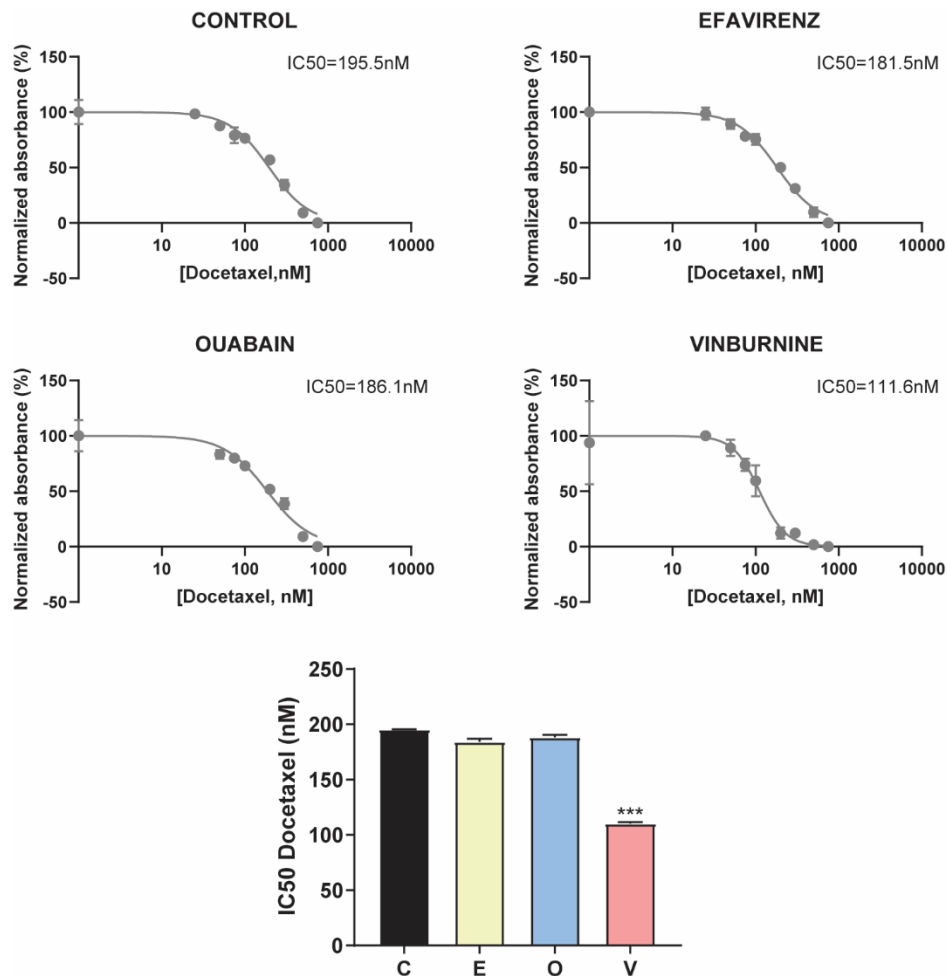


Figure 49. IC₅₀ of docetaxel in MDA-MB-231 cell line after 72-h treatments. Results were normalized to the vehicle control. Statistically significant differences with the vehicle: *** p<0.001. Data are presented as mean ± SD. C, control; E, efavirenz; O, ouabain; V, vinburnine.

3. Objective III: Generation of organoid cultures derived from patients (PDOs)

3.1. PDOs

To validate the effectiveness of our repurposed drugs and advance towards a translational approach we endeavored to assess their effects on PDOs from TNBC patients. As detailed in the "Materials and Methods" section 16.2, we attempted to establish PDOs from six TNBC biopsies samples through enzymatic digestion, according to Sachs et al.[128].

As shown in Figure 50, none of these samples yielded long-term established PDOs. In some instances, such as Patient A (day 0), viable cells were initially collected, while in others, like Patient E (day 0), cell aggregates were obtained post-digestion. Conversely, samples from Patients C, D, and F resulted in heterogeneous digestion products with significant debris. Unfortunately, the sample from Patient B yielded a minimal cell pellet after digestion, resulting in an almost empty culture (Patient B, day 0).

Although subsequent passages were not usually performed to preserve the limited viable material, photographic documentation revealed a consistent reduction in cell numbers over time across all samples (Patient A, day 12; Patient B, days 15 and 23; Patient C, day 11; Patient D, day 9; Patient E, days 7 and 11).

In conclusion, even though we could observe some organoids, the long-term establishment of PDOs from these TNBC biopsy samples was not accomplished. We posit that the primary factor contributing to this result was the insufficient quantity of tissue obtained from TNBC biopsies. This limitation highlights the challenges inherent in developing patient-derived models for TNBC.

Objective III: Generation of organoid cultures derived from patients (PDOs)

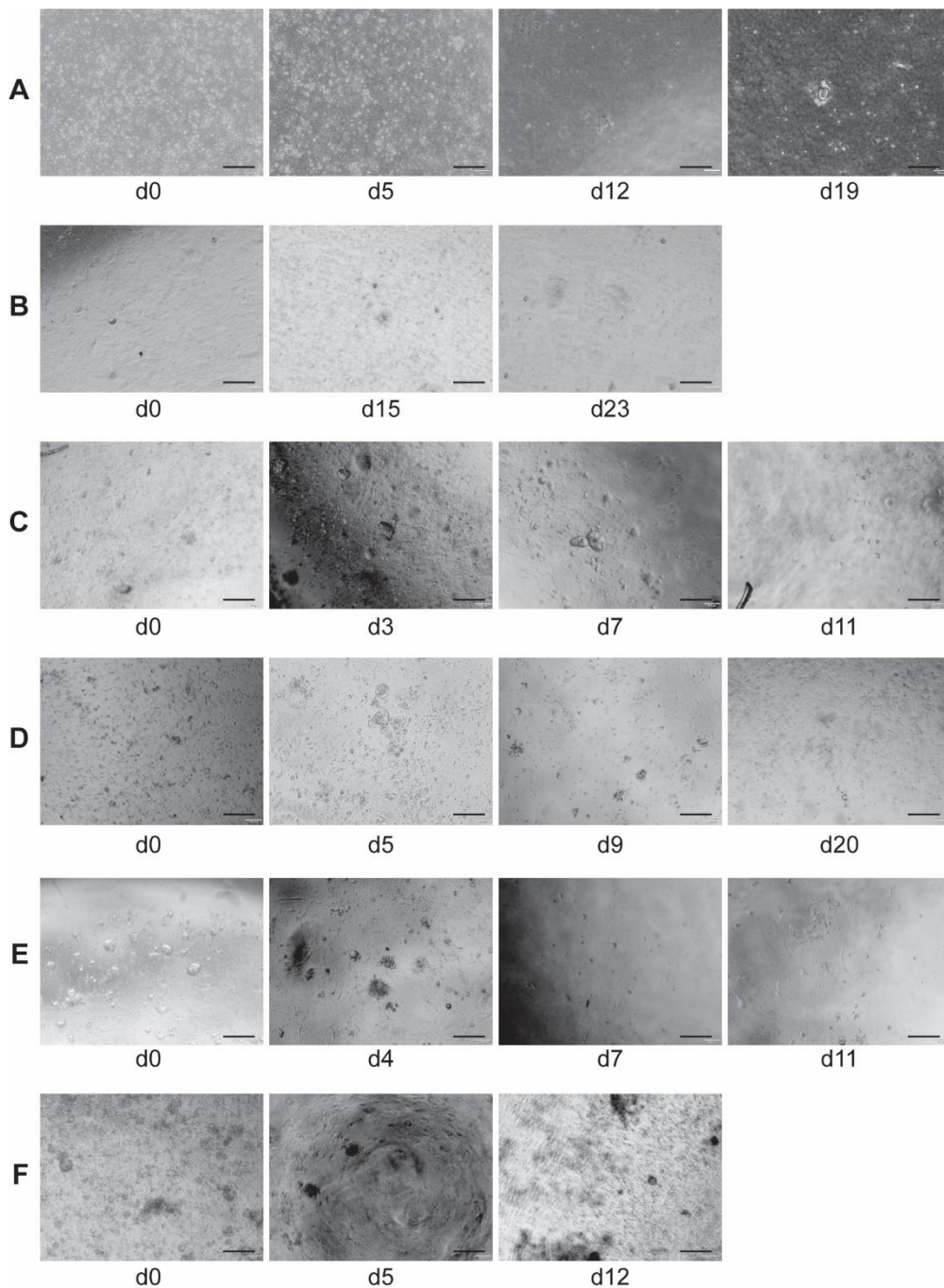


Figure 50. Progressive states of unsuccessful establishments of TNBC PDOs.

Representative bright-field microscope photos of PDOs cell cultures from TNBC patients (A-F) through the days (d). Original objective 4x. Scale bar 200 μ m

3.2. PDxOs

To overcome the obstacle of the small amount of tissue obtained from TNBC biopsies, we focused our attention on the production of PDxOs. Of the nine samples that were collected, we successfully generated three models. Hence, our ratio of PDxOs engraftment is 33.3%. UGR03 recapitulated cystic characteristics while UGR01 and UGR05 represented solid and encapsulated PDxOs.

3.2.1. UGR03

3.2.1.1. *Establishment of PDxOs cultures*

In our endeavor to establish PDxOs from tumor samples, we initiated the process by enzymatically digesting the corresponding PDxOs. Subsequently, we monitored the culture morphology and the nature of the cells that composed the culture.

As illustrated in Figure 51A, after 8 days in culture, no organoid formation was observed in UGR03. However, by day 29, we noted a reduction in the density of individual cells and an increased proportion of organoids with diameters exceeding 50 μm . To assess the evolution of the murine cell population, we conducted flow cytometric analysis using the H-2Kd MHC marker. The results revealed a predominant murine population (approximately 75%) that persisted after two passages (Figure 51B). Consequently, we were unable to successfully establish human organoids from the UGR03 model.

Objective III: Generation of organoid cultures derived from patients (PDOs)

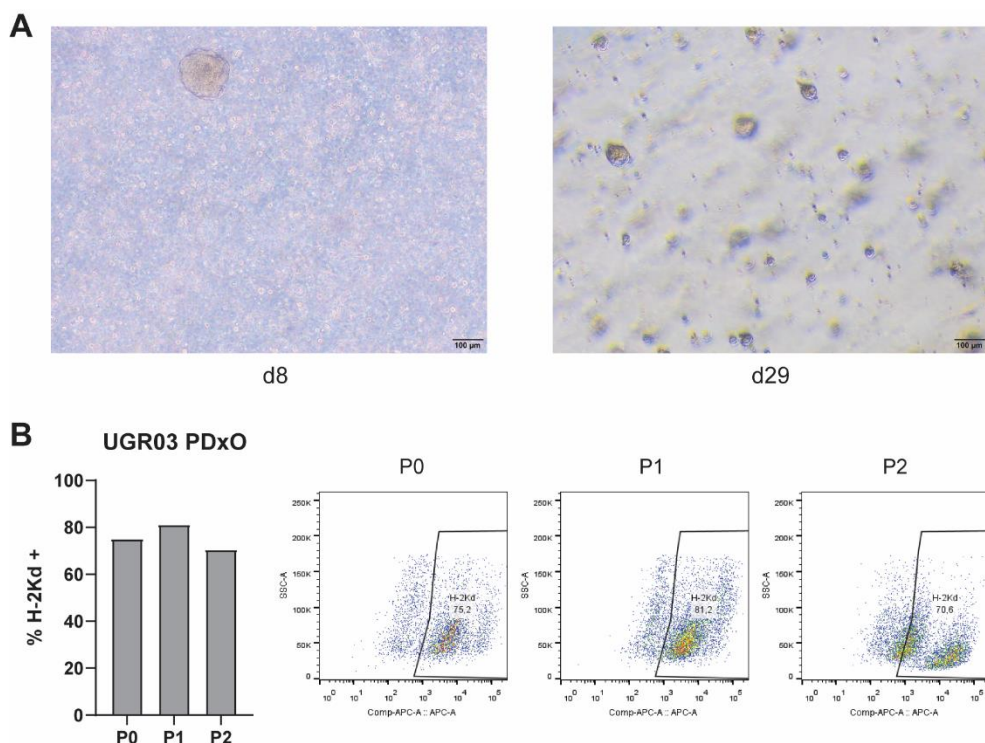


Figure 51. Representative photos of UGR03 PDxOs culture through the days (d) (A) and study of murine population evolution along the passages (B).

Flow cytometric analysis and plots of the H-2Kd positive staining population of UGR03 PDxOs after tumor digestion and passages (P) 0, 1, and 2. Original objective 4x. Scale bar 100µm.

3.2.2. UGR05

3.2.2.1. Establishment of PDxOs cultures

Regarding the UGR05 model, the PDxOs culture exhibited optimal growth in terms of morphology and density, as evidenced in Figure 52A at 26 days post-initiation, following multiple passages. Nonetheless, we observed a progressive increase in the proportion of murine cells within the culture by flow cytometric analysis (Figure 52B). Specifically, the murine population expanded from 26% at passage 2 to 47% at passage 4, ultimately exceeding 90% by passage 6.

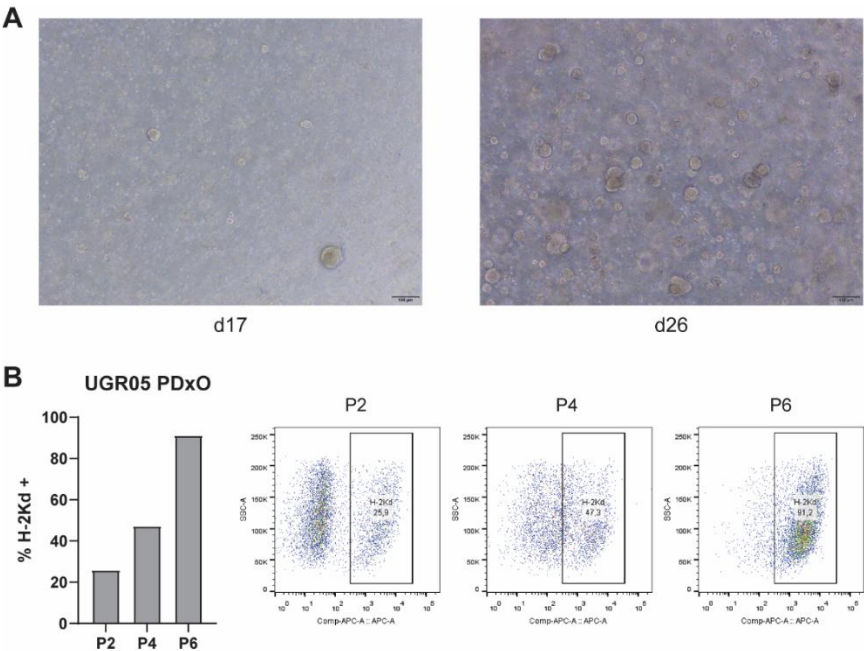


Figure 52. Representative photos of UGR05 PDxOs culture through the days (d) (A) and study of murine population evolution along the passages (B).

Flow cytometric analysis and plots of the H-2Kd positive staining population of UGR01 PDxOs after tumor digestion and passages (P) 2, 4, and 6. Original objective 4x. Scale bar 100µm.

The gradual overgrowth of murine cells highlights the need for refined methodologies to selectively promote the expansion of human tumor cells while minimizing the proliferation of murine stromal components in such experimental systems. For this reason, to enrich the human tumor cell population of UGR05, we performed a negative selection based on the murine-specific marker by FACS (Figure 53).

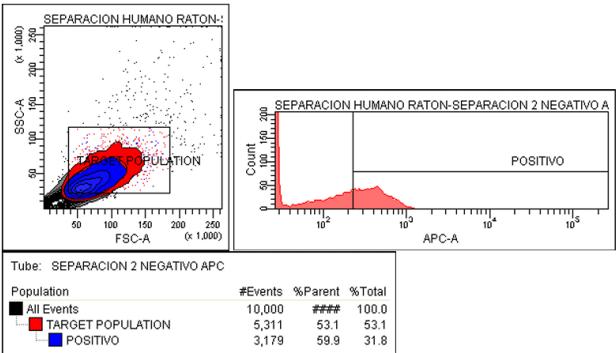


Figure 53. Flow cytometry plots of the FACS selection of UGR05 PDX digestion.

Objective III: Generation of organoid cultures derived from patients (PDOs)

Subsequently, we monitored the growth of the cellular pellet obtained from the sorting process to the PDxOs (Figure 54A). The culture exhibited favorable but slow growth, with organoids reaching diameters exceeding 100 μm of diameter (d17). In passage four, to verify the cellular composition of the developing PDxOs culture, we conducted a flow cytometry analysis. The results revealed that 72.6% of the culture consisted of murine cells (Figure 54B). This fact suggested that the FACS selection might have been contaminated with highly proliferative mouse cells, which had outgrown the human population. Consequently, the human cells could not develop a stable PDxO culture.

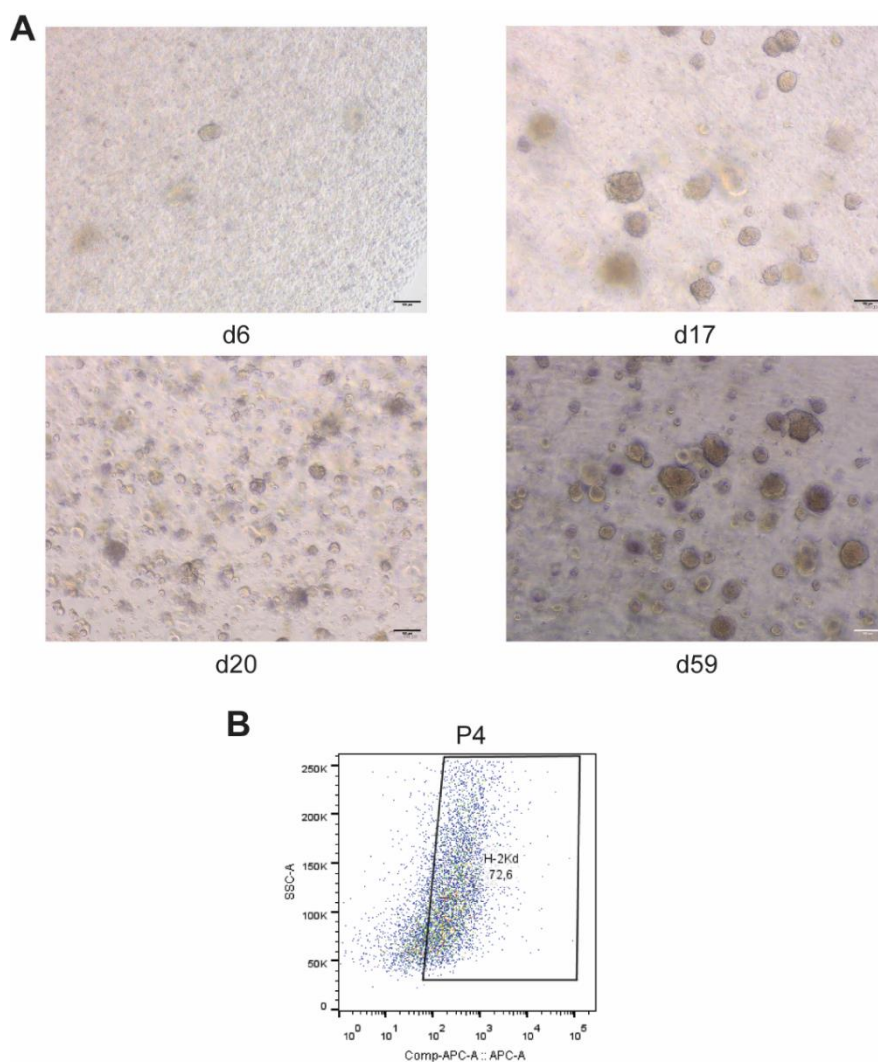


Figure 54. Representative photos of the second attempt of UGR05 PDxOs culture through the days (d) (A) and study of murine population in passage (P) four after FACS selection (B).

Original objective 4x. Scale bar 100 μm .

Despite UGR05 being a successful PDX model, we were compelled to exclude it from further studies due to our inability to maintain a long-term human PDxOs culture. This outcome underscores the challenges in establishing pure human-derived organoid cultures from PDX models.

3.2.3. UGR01

3.2.3.1. Establishment of PDxOs cultures

In the case of UGR01 model, good growth and proliferation were noticed, as illustrated in Figure 55A. PDxOs grew in a high density and an increase in the amount of culture material was produced through the passages. Moreover, in flow cytometry analysis we obtained a reduction in the murine population over the passages, reaching negligible levels by passages 3-5 (Figure 55B). Consequently, UGR01 has successfully been established as a long-term human PDxOs culture.

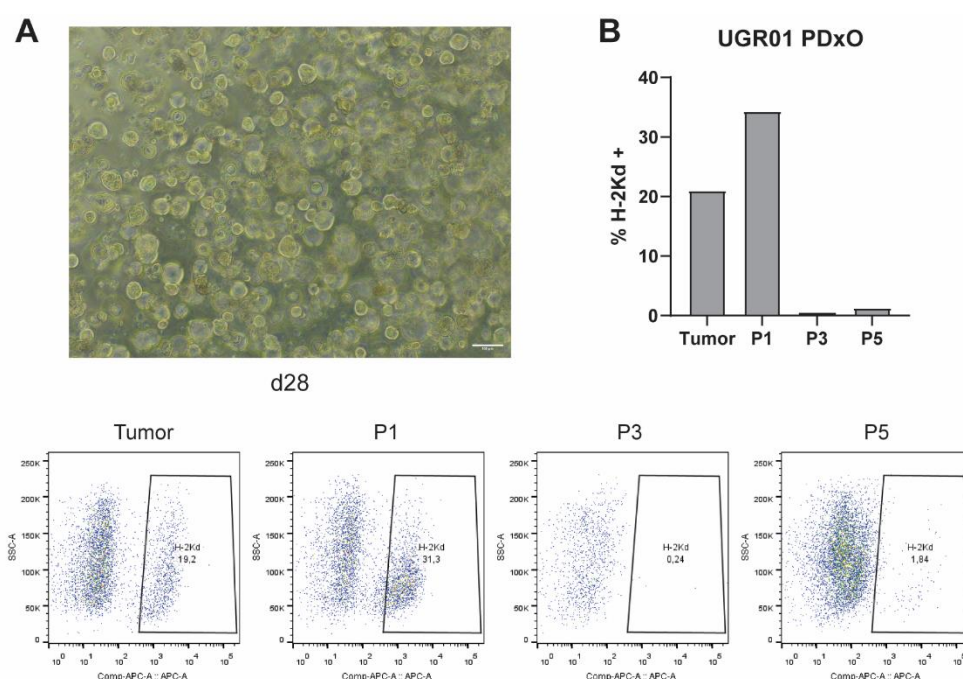


Figure 55. Representative photos of UGR01 PDxOs culture at day (d) 28 and study of murine population evolution along the passages.

Flow cytometric analysis and plots of the H-2Kd positive staining population of UGR01 PDxOs after tumor digestion and passages (P) 1, 3, and 5. Original objective 4x. Scale bar 100µm.

Objective III: Generation of organoid cultures derived from patients (PDOs)

In summary, the establishment of PDxOs presents significant challenges, with various obstacles potentially impeding the successful cultivation of human PDX-derived organoid cultures. In our study, we attempted to generate PDxOs from three distinct TNBC PDX models. Despite the inherent difficulties, we successfully established one viable human PDxOs model suitable for further investigation into our research.

3.2.3.2. Assessment of histopathological features and *NDRG1* expression

To characterize the culture, we assessed if PDX and PDxOs faithfully recapitulated the histological features of the original patient tumor. A pathologist confirmed the absence of hormone receptors (PR and ER) in both PDX and PDxOs by IHC, whereas the absence of HER2 amplification was confirmed by FISH in PDX and IHC in the organoids' culture (Figure 56).

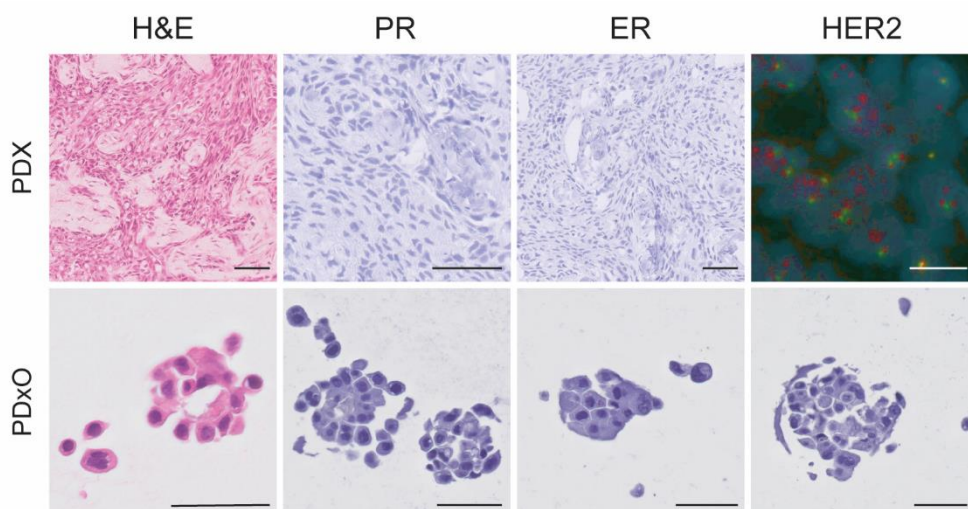


Figure 56. Assessment of histopathological features of UGR01 PDX and PDxOs.

Hematoxylin and eosin (H&E) staining, immunohistochemistry of progesterone receptor (PR) and estrogen receptor (ER) of UGR01 PDX and PDxOs, assessment of negative HER2 amplification by immunohistochemistry of UGR01 PDxOs and by FISH showing two copies of the gene (red) and centromere 17 (green) per nucleus (blue) of UGR01 PDX. Scale bar of immunohistochemistry images 50 μ m. Scale bar of FISH image 10 μ m.

Then, given our previous findings demonstrating a correlation between elevated *NDRG1* protein expression and diminished survival rates in TNBC patients, we

evaluated NDRG1 expression in the UGR01 sample. Confocal microscopy analysis confirmed the basal expression of NDRG1 in the PDX model and its preservation in the PDxOs culture (Figure 57).

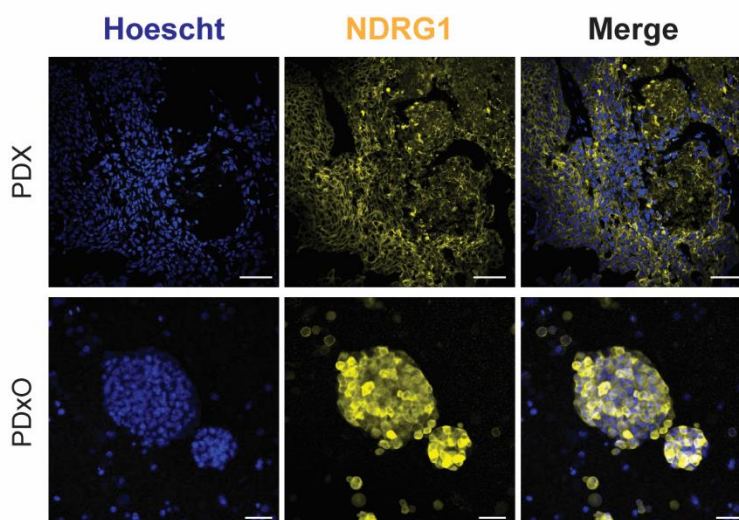


Figure 57. Basal presence of NDRG1 protein in UGR01 PDX and PDxOs.

Representative confocal images of NDRG1 (yellow) in the PDX model and PDxOs (UGR01). Scale bar 50 μ m.

In summary, our results indicate that the UGR01 model represents a suitable platform for our drug repurposing study, providing a robust foundation for validating the translational potential of our identified drug candidates in the context of TNBC.

4. Objective IV: Assessment of the efficacy of repurposed drugs on organoids

4.1. UGR01

4.1.1. Cell viability and IC₅₀ calculation

To test our repurposed drug candidates, we assessed cell viability after 72 h of treatment. As illustrated in Figure 58, the IC₅₀ value obtained for ouabain in UGR01 PDxOs was comparable to those observed in TNBC adherent cell lines,

Objective IV: Assessment of the efficacy of repurposed drugs on organoids

with a value of 33.5 nM. Conversely, slightly elevated IC₅₀ values were observed for efavirenz and vinburnine in PDxOs, at 21.5 μ M and 9.1 μ M, respectively.

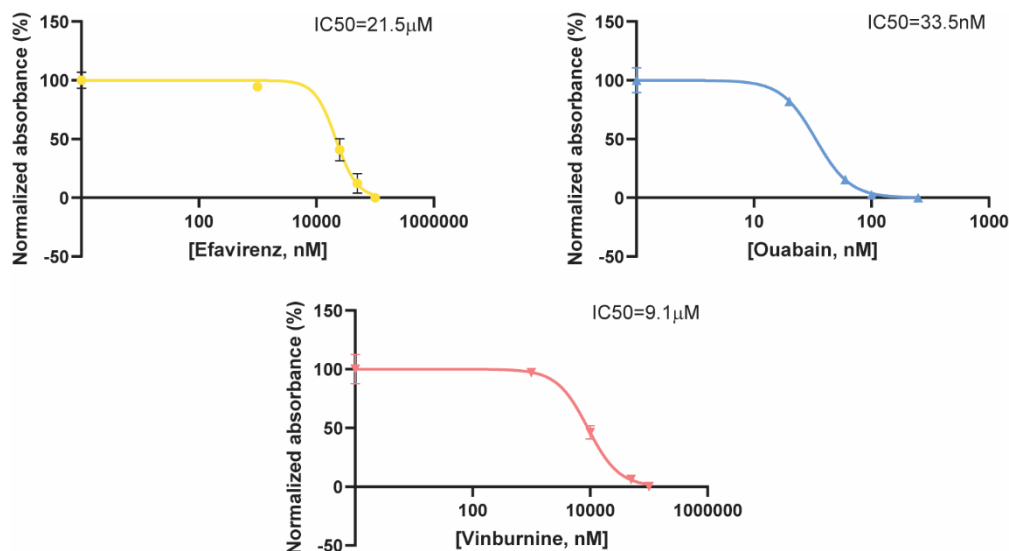


Figure 58. IC₅₀ of efavirenz, ouabain, and vinburnine in the UGR01 PDxOs model after 72-h treatments.

Results were normalized to the vehicle control.

4.1.2. Inhibition of p-AKT and Ki67

The antineoplastic potential of efavirenz, ouabain, and vinburnine was further corroborated by a substantial reduction in Ki67 expression, as demonstrated by IF staining (Figure 59). Moreover, confocal microscopy data revealed a marked decrease in p-AKT levels following 72 h of treatment with all three monotherapies compared to the control (Figure 59). This reduction in p-AKT immunostaining aligns with the previously observed mechanism of action for these compounds in attached TNBC cell lines (Figure 45).

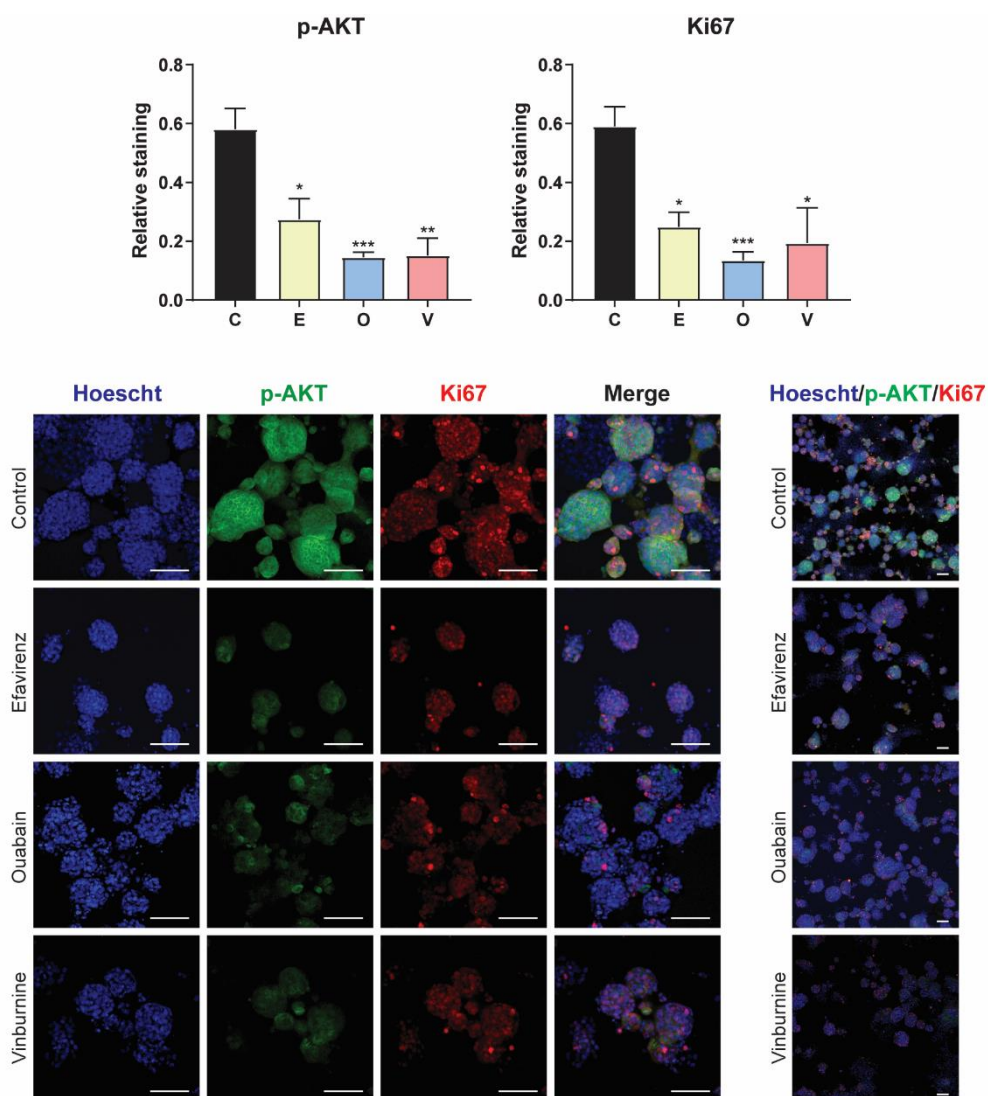


Figure 59. Effect on p-AKT and Ki67 of UGR01 PDxOs after 72-h treatments.

Representative confocal images (original optical objective: 10X) and quantification of p-AKT (green) and Ki67 (red) in UGR01 PDxOs after 72-h treatments. Scale bar 100 µm. Statistically significant differences with the vehicle control (C): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. E, efavirenz; O, ouabain; V, vinburnine.

4.1.3. Combination with docetaxel

Aimed to facilitate clinical translation, we investigated the combination of efavirenz, ouabain, and vinburnine with the taxane docetaxel. For that purpose,

Objective IV: Assessment of the efficacy of repurposed drugs on organoids

the IC₅₀ of this chemotherapy was determined in UGR01 PDxOs cultures. Our analysis revealed that the IC₅₀ value was 8.8 nM, as depicted in Figure 60.

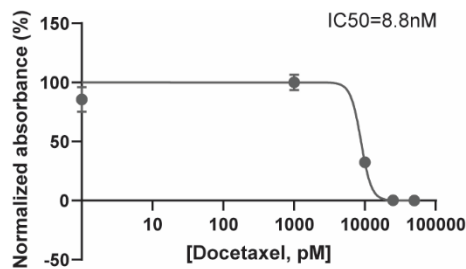


Figure 60. IC₅₀ of docetaxel in the UGR01 PDxOs model after 72-h treatments.
Results were normalized to the vehicle control.

To assess whether the combination of docetaxel with the repurposed drugs could enhance chemotherapeutic efficacy, we evaluated the impact of these combinations on cell viability in the UGR01 PDxOs model. At the PDxOs density analyzed, monotherapy with vinburnine significantly reduced cell viability ($p < 0.0001$) compared to the vehicle control, whereas docetaxel, efavirenz, and ouabain did not exhibit significant effects as single agents (Figure 61). However, when the repurposed drugs were combined with docetaxel, organoid viability was significantly compromised: D+E ($p < 0.01$), D+O, and D+V ($p < 0.0001$).

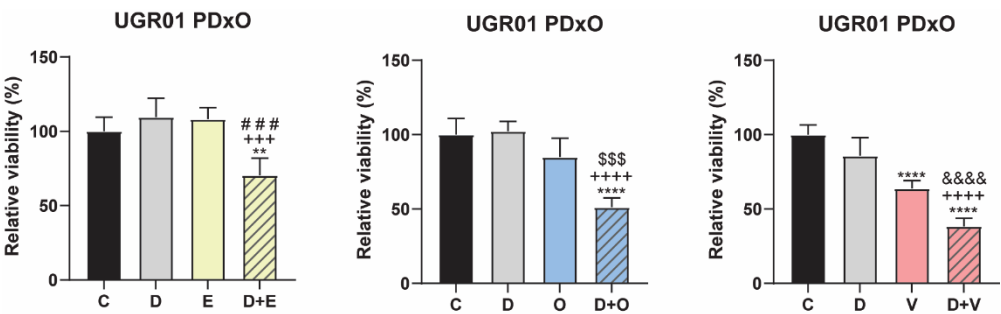


Figure 61. Effect of combinations of docetaxel combined with repurposed drugs on cell viability of UGR01 PDxOs after 72-h treatments.

Relative viability of UGR01 PDxOs after 72-h IC₅₀ treatments of vehicle control (C), docetaxel (D), efavirenz (E), ouabain (O), vinburnine (V), or combinations (D+E, D+O, D+V). Results were normalized to the vehicle control. Statistically significant differences with the vehicle: ** $p < 0.01$, **** $p < 0.0001$. Statistically significant differences with docetaxel: +++ $p < 0.001$, ++++ $p < 0.0001$. Statistically significant differences with E: ### $p < 0.001$. Statistically significant differences with O: \$\$\$ $p < 0.001$. Statistically significant differences with V: &&&& $p < 0.0001$.

In conclusion, our findings suggest a potential synergistic effect between the repurposed drug candidates and docetaxel, validating their potential as adjuvant therapies for TNBC treatment.

DISCUSSION

1. TGF- β governs the pleiotropic activity of NDRG1 in TNBC

TNBC represents the most aggressive subtype of breast cancer, characterized by its drug resistance and propensity for metastasis primarily attributed to the subpopulation of CSCs[35,151]. While chemotherapy remains the primary therapeutic approach, recent approvals of novel targeted therapies for mTNBC have emerged[23]. These include PARP inhibitors for patients harboring *BRCA* mutations[7,22] and immune checkpoint inhibitors such as pembrolizumab and atezolizumab for PD-L1-positive tumors[23]. However, these advancements remain insufficient, as only a subset of patients can benefit from them. For instance, merely 20% of TNBC patients exhibit elevated expression of PD-L1 markers[7]. Consequently, there is a critical need to identify new prognostic biomarkers and therapeutic targets, develop targeted therapies against CSCs to mitigate the origin of metastases and recurrences, and ultimately improve patient survival.

Our investigation initially focused on **NDRG1 as a potential marker of interest**, examining its association with breast cancer patient survival. Our analysis of public datasets (Kaplan-Meier plotter and GOBO) consistently revealed **elevated *NDRG1* gene expression in aggressive breast cancer subtypes**, including basal-like, HER2+, and TNBC, significantly correlating with diminished overall survival. Furthermore, to increase the value of the study, **our cohort of 75 TNBC tumor biopsies also confirmed this prognostic correlation**. These findings align with recent studies that have also observed a malignant relationship between higher NDRG1 expression and aggressive forms of breast cancer[152–154]. Consequently, we present NDRG1 as a novel marker of aggressiveness in TNBC patients, which could be readily assessed using IHC by hospital pathologists.

It is noteworthy that previous investigations have applied varying cutoff values to discriminate between high and low NDRG1 expression. For instance, Villodre et al. utilized an H-score median of 120 in a NDRG1 study on 64 inflammatory breast cancer specimens (16 TNBC) post-neoadjuvant treatment[154]. Conversely, in their posterior analysis of tissue microarrays from 216 breast cancer patients (including 97 TNBC cases) post-neoadjuvant therapy, they used a threshold value of 160[152]. In contrast, our study established the limit at 199. This variability in cutoff values, even within the same research group, underscores the potential for significant discrepancies across different hospitals and countries, emphasizing that it **is imperative to standardize assessment** methodologies. Considering these findings, we propose a future

investigation of NDRG1 in TNBC with a larger sample size to enhance its prognostic value and establish more robust criteria for its clinical application.

NDRG1 exhibits pleiotropic effects across breast cancer subtypes, with notable inconsistencies observed **even within the TNBC subtype**. This variability underscores the complexity of establishing a unified understanding of the role of NDRG1 across diverse cell types, populations, and patient cohorts. More specifically, investigations by Sevinsky et al.[155] and Mishra et al.[153] demonstrated that *NDRG1* inhibition decreased proliferation in MDA-MB-231 and MDA-MB-468 cells, but not in SUM159[153,155], or in the triple-negative inflammatory breast cancer cell lines SUM149 and BCX010[152]. Furthermore, *NDRG1* knockdown showed no significant effect on the migration of MDA-MB-231 and SUM159 cells[153], a finding we independently confirmed. Conversely, metastatic properties and tumor-initiating cell populations were inhibited in SUM149 and BCX010 cells upon *NDRG1* knockdown[152].

Without a clear mechanistic explanation, these discrepancies among cell lines **highlight the necessity for elucidating the underlying molecular determinants**. Villodre et al. extended their research using SUM149 and BCX010 cells, justifying their focus based on the intrinsic aggressive nature of these lines, particularly their migratory capacity and enrichment in tumor-initiating cells[152]. Nonetheless, this rationale does not fully account for the negative results obtained in other aggressive, metastatic, and tumor-initiating cell-enriched lines such as MDA-MB-231 or SUM159, as reported by Mishra et al.[153].

Motivated by our previous findings, which revealed an increased expression of the phosphorylated active form of NDRG1 (Thr346) following TGF- β 1 induction, we directed our focus towards elucidating the role of NDRG1 in TNBC aggressiveness within the context of this activated pathway[70]. This approach aims to provide insights into the molecular mechanisms underlying the observed variability in NDRG1 function across TNBC cell lines and potentially reconcile the inconsistencies reported in previous studies.

Under TGF- β stimulation, our findings among the four selected cell lines reveal that **NDRG1** plays a significant role in the metastatic capabilities of TNBC cells. Specifically, it is **more relevant in cell lines derived from distant metastatic sites**, as we observed more significant results in MDA-MB-231 and MDA-MB-436 than in SUM159 and BT549 cells. This note aligns with the concept that breast tumor cells isolated from pleural effusions exhibit distinct gene expression

profiles compared to their primary tumor counterparts. This includes notable upregulation of metastasis-related pathways like TGF- β signaling[156].

We also studied the process of EMT as TGF- β signaling is a well-established driver[157]. We suggest that the differential impact of *NDRG1* on metastatic potential may be attributed, in part, to the reduced expression of Vimentin observed in metastasis-derived cell lines following *NDRG1* silencing in the presence of TGF- β 1 stimulation. Vimentin is a well-established marker associated with tumor cell invasion and metastasis[158], and its downregulation in this context suggests a potential mechanism by which *NDRG1* modulates metastatic behavior in these cells. Interestingly, our data demonstrated that *NDRG1* silencing leads to the **inhibition of Twist expression** across all examined cell lines and suppression of Snail and Slug in SUM159 cells. These findings suggest that *NDRG1* may not primarily induce metastasis through the modulation of TGF- β -induced EMT[157]. Instead, the role of *NDRG1* in promoting metastasis appears to be more complex and potentially independent of classical EMT pathways in the context of active TGF- β signaling.

To further elucidate the effects of *NDRG1* inhibition in the context of an active TGF- β pathway, we focused on CSCs. This assessment is given the well-established role of TGF- β , Twist, and Vimentin sustaining CSC generation, as well as drug resistance, and tumor progression[146,147]. Similar to migration and EMT markers, our findings regarding the maintenance of different **CSC subtypes were dependent on the origin of the cell lines** and their degree of differentiation into a more aggressive phenotype induced by TGF- β 1 stimulation. Notably, primary-tumor-derived cell lines required intense stimulation to observe *NDRG1*'s role in CSC maintenance. Particularly, upon TGF- β stimulation, *NDRG1* maintained ALDH1+ CSCs across all cell lines, while CD44⁺/CD24⁻ and side population CSCs were maintained in primary-tumor-derived cells.

The observed differences among CSC subtypes can be attributed to CSC heterogeneity and their relationship with the EMT process. As introduced above, CSCs can exist in alternative mesenchymal-like (CD44⁺/CD24⁻), epithelial-like (ALDH1+) states, as well as intermediate epithelial/mesenchymal/CSC phenotypes (e.g., CD44⁺/CD24⁻/ALDH1+)[39]. Breast tumor cells exhibiting complete epithelial-like or mesenchymal-like states demonstrate reduced self-renewal capacity or cell plasticity, respectively[159]. In contrast, cells with a hybrid phenotype are crucial for tumorigenicity in basal breast cancer,

displaying enhanced plasticity, stemness, self-renewal, and generating progenies of drug-resistant ALDH1+ cells[36,159,160]. In this context of heterogeneity, it is important to note that some molecular pathways such as TGF- β can mediate and trigger CSC generation and EMT process. Consequently, the dissemination of proliferative cells that can mediate metastases is increased[161]. In corroboration with our findings and involving the NDRG1 pathway, two independent research studies reported a notable absence of brain metastases of aggressive breast cancer cell lines following *NDRG1* inhibition[152,162].

Collectively, our results suggest that **NDRG1 may play a significant role in the EMT and CSC processes**. Prolonged exposure of tumor cells to TGF- β 1 at the primary site appears to initiate EMT activation and subsequently maintain diverse CSC phenotypes. This cascade of events promotes distant invasiveness and chemoresistance through the generation and preservation of heterogeneous populations of tumor-initiating cells, including those with slow-cycling features (ALDH1+, CD44⁺/CD24⁻, and side population). It is noteworthy to address the importance of the study of these mechanisms that provide aggressiveness to TNBC. Taking knowledge of the involvement of TGF- β and NDRG1, it is crucial to provide targeted therapies to potentially reduce this progression.

2. Signature-based drug repurposing to treat TNBC

Overall, this nuanced understanding of NDRG1's function in TNBC highlights **the importance of considering the cellular context** when **evaluating potential therapeutic targets and developing strategies** to combat metastatic disease in TNBC. In particular, the origin of the tumor cells and the activation status of key signaling pathways.

Subsequently, aimed at eradicating these differences and **standardizing the cellular context**, instead of searching for direct NDRG1 inhibitors, we contemplate a drug repurposing study based on a favorable and anti-tumor gene expression. Specifically, we used the previously studied differential signature in TGF- β 1-stimulated TNBC cells with and without *NDRG1* knockdown. Besides, identifying already FDA-approved drugs to treat TNBC, in other words, drug repositioning, allows a faster clinical translation and a more efficient way than *the novo* discovery of compounds[72].

Given that in cell lines derived from pleural effusion, a brief TGF- β 1 stimulation was sufficient to elicit the role of *NDRG1* in this pathway, we opted to study MDA-MB-231 gene expression under these conditions (protocol 8+48 h) via RNA sequencing. Among down-regulated genes of this differential signature, we emphasized the inhibition of pro-inflammatory cytokines *CXCL10* and *CXCL11*, which are associated with CSC and EMT[148–150]; and *PODXL*, a protein involved in tumor cell migration and tumorsphere formation[163]. Besides, *OAS1* is an enzyme implicated in innate immune response whose overexpression predicts poor survival in breast cancer patients[164], and whose downregulation decreased cell motility in MDA-MB-231 cells[165], correlating with the observed reduction in migration.

We then proceeded with a **CMap search** by inputting this differential signature. We did not perform the strategy called “gene reversion” which consists on the introduction of a malignant signature and search for candidates that can reverse this expression[166,167]. In contrast, our interest lies in results with positive scores as we aim to **mimic the antitumor transcriptional characteristics** observed upon *NDRG1* inhibition following TGF- β stimulation. We conducted two queries based on log₂ fold change limits of ± 1.5 and ± 1.6 , obtaining more homogeneous results in the second query, suggesting that the more stringent query yields more precise results.

In the ± 1.6 **PCLs analysis**, we observed mechanisms of action that correlate with an antitumor perspective. Particularly, **MAPKs, Wnt, and histone-modifying EMSY complex** are pathways whose aberrant regulation contributes to malignant processes[48,168,169]. As the results exhibited loss of function, we considered that this supports our hypothesis and repurposed drugs based on their similarity to *NDRG1* knockdown profile may exert an anti-tumorigenic effect. An examination of the top PCLs results from the ± 1.6 query revealed **ATPase inhibitors**, also referred to as cardiac glycosides, as particularly noteworthy. This family has emerged as a promising candidate in cancer research, demonstrating efficacy across a spectrum of malignancies[170,171], including colorectal[172], leukemia[173], prostate cancer[174], or breast cancer[175]. The antitumor characteristics of these molecules are multidisciplinary, with studies demonstrating their ability to induce various modes of cell death, encompassing apoptosis, autophagy, and immunogenic cell death[170,171]. While the precise mechanisms underlying their anticancer effects remain to be fully elucidated, current evidence suggests the Na⁺/K⁺ ATPase pump plays a crucial role in regulating several key molecular pathways

implicated in cancer progression, such as EGFR signaling, MAPK cascades, and the PI3K/Akt/mTOR axis[176]. The prominence of ATPase inhibitors as top disruptive agents across the *General*, *Summary*, and MCF-7 categories in our analysis underscores their significant potential as therapeutic agents in cancer treatment, warranting further investigation into their molecular targets and clinical applications.

Regarding **MCF-7 results** (the sole breast cancer cell line in CMap), **TGF- β receptor inhibitors** appear in the $\pm 1.6\text{-log}_2$ fold change results. This fact suggests that *NDRG1* inhibition in the presence of TGF- β 1 in breast cancer induces an expression change similar to that triggered by TGF- β inhibitors. As above introduced, TGF- β regulates the cell cycle and acts as a pro-tumoral mediator in advanced stages, promoting EMT[177]. In breast cancer, its overexpression is associated with tumor progression and poor prognosis, and the blockade of its signaling decreases the aggressiveness of this cancer type[178]. Park et al. demonstrated the efficacy of an innovative TGF- β type I receptor inhibitor that suppressed EMT *in vitro* and decreased lung metastasis in TNBC mouse models[179]. Consequently, the presence of TGF- β inhibitors in the PCLs potentially reinforces our initial hypothesis regarding the importance of *NDRG1* in this signaling pathway.

The objective of the present research is to **identify and repurpose potential therapeutic** candidates for TNBC. Therefore, we proceeded to evaluate compounds based on our CMap analysis. Consistent with our PCLs findings, the $\pm 1.5\text{-log}_2$ fold change query yielded fewer drugs and weaker connectivity scores; thus, we focused on the $\pm 1.6\text{-log}_2$ fold change results for subsequent analyses. As emphasized in Results section 1.3, the $\pm 1.6\text{-log}_2$ fold change query revealed several ATPase inhibitors (e.g., digitoxigenin, digitoxin, digoxin, and ouabain) among the top candidates. Notably, the two highest-ranking compounds in both *General* and *Summary* results were efavirenz, a non-nucleoside reverse transcriptase inhibitor used in human immunodeficiency virus (HIV) treatment[180], and vinburnine (also known as (-)-eburnamonine), a vasodilating alkaloid and allosteric modulator of muscarinic acetylcholine receptors[181,182]. The MCF-7 cell line analysis demonstrated a similar prevalence of ATPase inhibitors (ouabain, digoxin, digitoxin, and digitoxigenin). Based on these findings, we selected ouabain, the highest-ranking cardiac glycoside in the MCF-7 results, for further investigation.

In alignment with drug repurposing criteria[71,72,75], we selected three FDA-approved compounds that are novel in the context of TNBC treatment: **efavirenz**, which has been previously investigated in TNBC[183,184]; **ouabain**, which has shown potential as a treatment for various cancer types, including lung[185] and breast cancer[176,185,186]; and **vinburnine**, which has demonstrated antiproliferative effects in leukemia[187]. Intriguingly, ouabain and vinburnine have emerged as promising candidates in other cancer-related CMap investigations. While the cardiac glycoside was identified through leukemic stem cell signatures[188], vinburnine was determined in studies against pancreatic adenocarcinoma, where researchers analyzed an autophagy-related gene signature[189]. In the same way, in head and neck squamous cell carcinoma research, vinburnine was highlighted when investigators input an antitumor gene signature associated with the inhibition of the *Homeobox B7* gene, which is frequently overexpressed in cancer and known to promote tumorigenesis[190]. This selection strategy aims to leverage existing FDA-approved drugs with established safety profiles, potentially expediting the drug development process for TNBC treatment.

3. Repurposed drugs target CSCs and EMT via the AKT pathway

In our investigation of repurposed drug candidates for TNBC treatment, all three candidates successfully demonstrated significant **compromise of cell viability** across the three tested adherent cell lines. The calculated IC₅₀ values for efavirenz, ouabain, and vinburnine align with previously reported ranges in the literature[176,183,191], validating the robustness of our findings. For instance, our results for **efavirenz** in MDA-MB-231 cells yielded an IC₅₀ of 15.4 μ M after 72 h of treatment, comparable to the 27.89 μ M value after 4 days of treatment[183]. Notably, this research also confirmed the absence of anti-proliferative effects in immortalized normal breast tissue cell lines (MCF-10A), supporting the potential safety profile of this drug in healthy tissue[183]. Similarly, our findings for **ouabain** in MDA-MB-231 cells showed an IC₅₀ of 54.0 nM after 3 days of treatment, which is in line with the 21.2 nM reported by Khajah et al. after 4 days[176]. Their study also utilized MCF-10A cells to demonstrate that higher concentrations were required to decrease proliferation in non-cancerous breast epithelial cells[176].

We further substantiated the cytotoxic effects of these compounds through **apoptosis assays**, revealing a significant increase in the apoptotic cell

population across all three TNBC cell lines examined. The apoptosis-inducing effect of efavirenz aligns with previous findings reported in breast cancer[183] and melanoma cell lines[192], underscoring its potential as a broad-spectrum anticancer agent. Furthermore, nanomolar concentrations of ouabain have been reported to enhance cell apoptosis in various cancer types, including renal and lung cancer[193], leukemia[194], and TNBC[186]. Regarding our results, the ATP inhibitor produced a slight increase in the apoptotic population in the MDA-MB-231 cell line at 24 h and a more pronounced enhancement in BT549 at both time points tested (24 h and 48 h), while no effect was observed in SUM159 after 24-h treatment. This variety among cell lines could be a consequence of the molecular heterogeneity characteristic of TNBC.

Given the paucity of studies exploring **the combination of repurposed drugs** in cancer research, we explored **potential synergistic effects** by analyzing combinations of efavirenz, ouabain, and vinburnine. Strikingly, the EO combination demonstrated enhanced apoptosis induction compared to monotherapies. This enhanced effect was particularly pronounced in MDA-MB-231 cells at 24 h and BT549 cells at 48 h post-treatment.

As sustained in the prior investigation of the NDRG1 pathway, we continue the assessments studying the behavior of **CSCs**[35,151] to extend the effects beyond the initial cytotoxic effects on TNBC cells. To this end, we investigated the three distinct CSC subpopulations previously studied with *NDRG1* inhibition and assessed the self-renewal, clonogenicity, and migration capacities of TNBC cell lines following treatment with our repurposed drug candidates and their pair-combinations.

Notably, all treatments led to a marked reduction in the **ALDH1+ population** across the tested cell lines. In MDA-MB-231 cells, the decrease observed after efavirenz treatment aligned with findings reported by Chiou et al.[184]. Furthermore, the reduction effect of ouabain has also been assessed against ALDH1+ osteosarcoma cells[195]. The **CD44^{high}/CD24⁻ population** showed variable responses across cell lines, with the most pronounced effects observed in MDA-MB-231 cells, where significant decreases were noted across all treatment groups. Interestingly, while Chiou et al. did not observe differences in the CD44⁺/CD24⁻ subpopulation with efavirenz treatment, our study demonstrated a reduction in the CD44^{high}/CD24⁻ subpopulation in this cell line[184]. However, the effects against the SUM159 cell line do not correspond to the reduction observed when *NDRG1* was inhibited after TGF- β stimulation.

We hypothesize that, as for drug repurposing, we used the gene signature from MDA-MB-231, we might obtain differences in the other cell lines. Preliminary investigations into the side population revealed decreases with all treatments except for EV and OV combinations in MDA-MB-231 cells. Furthermore, this study represents the first investigation into the effects of vinburnine treatment on CSCs. The sole existing data in the literature pertains to the reduction observed in CD34⁺CD38⁻ and CD34⁺CD10⁻ subpopulations in acute and chronic lymphocytic leukemia, as well as acute myelogenous leukemia, following administration of a vinburnine derivative ((-)-15-methylene-eburnamnine)[187]. Our findings collectively demonstrate that the repurposed drugs exhibit significant efficacy in reducing diverse CSC populations, with particularly pronounced effects observed in the MDA-MB-231 cell line.

Moreover, the MDA-MB-231 cell line also obtained better results with all treatments in **MSFE and colony formation** compared to SUM159 and BT549 cells. In contrast, the effect on **cell migration** was more pronounced in the BT549 cell line, although good results were also exhibited in the other two cell lines. Remarkably, ouabain highlights the monotherapy that reduced migration in all three TNBC cell lines. This fact is in concordance with the observations in osteosarcoma[195], gastric[196], and lung cancer[197]. Furthermore, it is important to mention that these primary tumor-derived cell lines (SUM159 and BT549) did not exhibit significant reductions in migration when *NDRG1* was inhibited after TGF- β stimulation. However, it seems that the application of a drug repurposing methodology mimicking the gene expression profile of the metastatic MDA-MB-231 cell line has enhanced migration effects in SUM159 and BT549 cell lines. Whereas only metastatic MDA-MB-231 and MDA-MB-436 demonstrated superior results under the stimulation + inhibition protocol (8+48 h), repurposed drug candidates indeed produced significant reductions in cell migration across all cell lines. Their ability to reduce migration in SUM159 and BT549 cells, despite their initial resistance to *NDRG1* inhibition and TGF- β stimulation, underscores the potential of this repurposing strategy to overcome cell line-specific limitations and target a broader spectrum of TNBC subtypes.

Our study provides compelling evidence for the anticancer potential of the three repurposed drugs in TNBC treatment. Furthermore, our results indicate that the **most potent therapy was EO**, as this combination was able to reduce almost all CSC properties in the tested TNBC cell lines. The comprehensive impact on

cell viability, apoptosis induction, and CSC populations, coupled with the synergistic effects observed in drug combinations, opens new avenues for therapeutic intervention in this challenging cancer subtype.

In our exploration of the **mechanistic foundations** underlying the efficacy of these repurposed drug candidates, we have uncovered compelling results that merit in-depth discussion. Our findings revealed that these compounds do not achieve their therapeutic effects through direct inhibition of NDRG1, a conclusion that corroborates observations reported by other researchers utilizing the CMap methodology[115,198]. This discovery assumes particular significance when considered within the context of our initial research, which elucidated the intricate and heterogeneous role of NDRG1 in TNBC pathogenesis.

Our investigations have demonstrated that NDRG1-mediated aggressiveness in TNBC is modulated through TGF- β stimulation. Concurrently, other researchers have posited that NDRG1 can function as a tumor suppressor in certain contexts[50]. This duality in the role of NDRG1 underscores the potential advantages of therapeutic approaches that **do not directly target this protein but reproduce its signature**. The absence of direct NDRG1 inhibition by our repurposed drug candidates confirms the value of transcriptional-based drug repurposing methods. This type of methodology confers significant clinical benefits, as drug candidates potentially mitigate undesirable effects that could arise from indiscriminate NDRG1 suppression. This nuanced understanding of the role of NDRG1 in TNBC biology highlights the complexity inherent in drug repurposing strategies for oncology. It emphasizes the critical importance of conducting thorough mechanistic studies to elucidate the precise mode of action of the repurposed compounds. The fact that these drugs exert their anti-tumor effects without directly inhibiting NDRG1 suggests the involvement of alternative molecular pathways or targets, thus we focused on other related genes to unravel the mechanism of action.

Further, we explored alternative targets among the inhibited genes of the differential signature, as the CMap tool provides candidates that might recreate the inserted query. Although little differences were obtained in the mRNA expression analyses of *CXCL10*, *CXCL11*, *PODXL*, and *OAS1*, we hypothesized that our compounds could exert their anticancer activities through AKT because *CXCL10* and *CXCL11* are known activators of the AKT pathway through

CXCR3[148–150]. Our findings revealed a **significant reduction in AKT phosphorylation** upon treatment, corroborating previous studies on efavirenz in pancreatic cancer[199] and ouabain in the lung[197], gastric cancers[196], and glioma[200].

Moreover, the observed diminution in EMT markers aligns with the established role of PODXL in EMT through the PI3K/AKT pathway[201] and with the modulation of the EMT-related protein OAS1 by AKT[202,203]. Interestingly, drugs can reduce Snail and Slug expression in MDA-MB-231, while *NDRG1* inhibition upon TGF- β stimulation did not exert any changes on this cell line. This highlights the value of the potential effects that drugs can produce as they exert a complex interplay with the signaling cascades in cancer progression.

Our findings provide **additional support for the anticancer properties** of these repurposed drugs. Efavirenz has been demonstrated to reduce the expression of long interspersed nuclear element-1 (LINE-1) retrotransposon, which is notably overexpressed in TNBC cell lines[183]. Ouabain has been shown to attenuate STAT3 signaling in lung and cervical cancer[204], while vinburnine appears to exert its cytotoxic effects primarily through oxidative stress pathways[187]. Collectively, these observations substantiate the multifaceted anticancer activity of our repurposed drug candidates, particularly in reducing CSC phenotypic properties and migratory potential.

The potent effect of the EO combination in reducing stemness markers correlates strongly with the observed decrease in AKT and EMT-related proteins. This finding is consistent with the well-established relationship between the AKT pathway and CSC maintenance and propagation[205–207]. The **synergistic effect observed with the EO combination underscores the potential of combining repurposed drugs** to enhance therapeutic efficacy in targeting aggressive breast cancer subtypes.

One of the primary challenges in TNBC is the development of **chemoresistance** and tumor recurrence. Despite chemotherapy being the first-line treatment for TNBC, with initial response rates often surpassing those of other breast cancer subtypes, the emergence of resistance remains a critical obstacle to long-term patient survival[7,208]. To address this issue, we investigated the effects of our repurposed drug candidates on a docetaxel-resistant cell line we had already established, SUM159-R[137].

Our results revealed intriguing patterns of drug sensitivity in the docetaxel-resistant TNBC cells. Notably, the calculated IC50 values for efavirenz and

ouabain were lower in the resistant cell line compared to the parental line. This suggests an enhanced sensitivity to these compounds in docetaxel-resistant cells. This observation aligns with the concept of **collateral sensitivity**, where resistance to one drug can lead to increased vulnerability to others, potentially due to alterations in cellular pathways or membrane transport systems[208].

Furthermore, **vinburnine** demonstrated a remarkable ability to **enhance docetaxel sensitivity in resistant cells**, offering a promising strategy for overcoming chemoresistance in TNBC. This synergistic effect could be attributed to the potential of vinburnine to modulate drug efflux pumps[208], as we observed in the reduction produced by these molecules in the side population of SUM159. Furthermore, in the parental line, vinburnine highly decreases ALDH1⁺ and CD44^{high}/CD24⁻ populations, which are also related to chemoresistance[33,34,37]. The capacity to re-sensitize resistant cells to standard chemotherapy agents represents a significant advancement in addressing one of the major challenges in TNBC treatment.

Importantly, all three repurposed drug candidates significantly reduced MSFE in the chemoresistant cell line, suggesting that these compounds may target CSC populations in this acquired phenotype, often implicated in chemoresistance and tumor recurrence[208].

Our current findings are based on a limited set of experiments and **require further investigation** such as the assessment of CSC subpopulations. However, they provide a foundation for a new hypothesis: in cases where resistance to taxane-based chemotherapy has developed, we could propose two alternative second-line treatment strategies. The first option involves utilizing efavirenz or ouabain as monotherapy, leveraging their enhanced efficacy in docetaxel-resistant cells. The second approach combines docetaxel with vinburnine, capitalizing on vinburnine's ability to restore sensitivity to docetaxel in resistant cells.

These preliminary results underscore the potential of drug repurposing to address chemoresistance in TNBC. However, it is crucial to emphasize the need for comprehensive follow-up studies like mechanistic investigations, to fully elucidate the molecular basis of these observations such as their impact on known resistance mechanisms.

4. Organoids represent a highly valuable but challenging model

As introduced above, PDOs have emerged in cancer research as a novel model system that recapitulates the genetic complexity and heterogeneity of the original tumor tissue. This approach offers a patient-specific platform without the need for animal models. However, in the present study, **we were unable to successfully establish long-term PDOs cultures** from any of the six TNBC biopsy samples received.

Sachs et al. were among the first researchers to optimize large-scale breast cancer PDOs establishment, reporting a success rate of 80% (95/155). They attributed unsuccessful attempts to initial culture medium compositions or improper sample collection[128]. Building upon this methodology, Rosenbluth et al. achieved a remarkable 95% efficiency in establishing breast PDOs (n=78) from both healthy and tumor tissue[209]. However, Campaner et al. reported a more modest establishment rate of 53% for breast cancer PDOs, with a 60% success rate specifically for TNBC tumors (3/5)[144]. Similarly, Chen et al. established 99 PDOs from 132 breast cancer samples, achieving a 75% success rate. They attributed some failures to improper cryopreservation of tumor samples[127].

A key distinction between these studies and our work lies in the **quantity of sample** material available for processing. While Sachs et al. simply referred to "breast cancer tissue"[128], Rosenbluth et al. clarified that they processed tissue fragments derived from mastoplasty reduction or prophylactic mastectomy procedures, suggesting access to substantial tumor samples[209]. Campaner et al. specified working with 1-2 cm³ resections[144]. Notably, Chen et al. not only processed tumor resections but also generated organoids from tumor biopsies with a 65% success rate (11/17), although they observed reduced growth after the second passage in some cases. Their methodology differed slightly from Sachs et al., using collagenase II with 2-6 h digestion times and a modified culture medium incorporating Wnt3a-conditioned medium, hydrocortisone, estradiol, and insulin[127]. Furthermore, Campaner et al. observed a correlation between PDOs maintenance and the Ki67 index of the original tumor[144].

In our study with TNBC biopsies, **we successfully generated PDOs and maintained them in culture for a short period**, i.e. we obtained a primary culture[210]. However, we did not establish long-term cultures, as we could not extend the culture for several passages[128,210]. Moreover, the quantity of

PDOs was insufficient to proceed with subsequent experimentation. We propose that the **limited size of the samples** was one of the challenges we faced while optimizing this novel procedure in our laboratory. This highlights the importance of both sample quantity and quality in the successful establishment of PDOs, particularly when working with small biopsy specimens.

In collaboration with medical professionals, we encountered limitations in acquiring tumor specimens from surgical resections due to the clinical management of patients. First, it is crucial to acknowledge that TNBC accounts for approximately 15-20% of breast cancer cases[5], resulting in a restricted pool of potential participants for research studies. Furthermore, the standard of care for large tumors involves neoadjuvant chemotherapy to reduce tumor size and facilitate subsequent surgical resection[30]. TNBC tumors typically exhibit high chemosensitivity[7], leading to significant tumor reduction following neoadjuvant treatment. Consequently, the residual tissue obtained during surgical resection is often minimal and prioritized for pathological evaluation to inform patient diagnosis and prognosis. This clinical approach, while beneficial for patient outcomes, presents challenges for obtaining adequate tumor samples for research purposes.

In order to achieve a higher amount of tissue to digest, we consider **PDXs as an alternative source**. While acknowledging the inherent limitations of PDX models, including potential sample loss due to engraftment rate, ethical concerns associated with animal experimentation, time and cost implications, and the introduction of murine variability in cellular and genetic complexity[121,124], we proceeded with this approach. From 9 samples, we established three PDX models, yielding an engraftment rate of 33.3%. This rate was slightly higher than those reported by other researchers, who achieved success rates of 23.0%[211] or 26.9%[212] in establishing TNBC PDX models.

UGR03 model did not grow adequately *in vitro* to form organoids. UGR03, a predominantly cystic PDX model[213], produced excessive debris following digestion, and despite washing and passaging, the cells lacked sufficient viability to establish a PDxOs line. In fact, UGR03 PDX was not considered a proper model for further research as the nature of its characteristics does not allow a comprehensive study of therapies.

We adhered to the protocols documented by the research group led by Dr. Alana L. Welm[123,130], as they are the pioneers in establishing a large-paired collection of breast cancer PDX and PDxOs, along with their subsequent

genomic study. Consequently, we assessed the **murine population** in our PDxOs. Isolated cells from UGR03 were predominantly of murine origin. In the case of **UGR05**, despite initially presenting only 26% of murine cells, this proportion increased to 90% with subsequent passages. This phenomenon has been reported to occur in some models where the human fraction is less proliferative[130]. Although Scherer et al. reported that most of their PDxOs models showed a decrease in murine population over passages, they also observed **models with persistent murine cells** and even entirely murine PDxOs[130], which aligns with our findings for UGR05. In an effort to eliminate this cell population, we performed a modified version of FACS selection method. Scherer et al. proposed a double-antibody strategy consisting on staining stroma-like mouse cells (anti-mouse CD90.2, anti-mouse CD29) and human cells or human epithelial cells (anti-human CD326/EpCAM, anti-human CD298)[130]. However, we simplified this method as we stained mouse cells with anti-Mo MHC Class I (H-2Kd) antibody. Although we collected the non-murine population, the selected cells exhibited limited proliferative capacity. Subsequent flow cytometry analysis showed that the PDxOs culture was predominantly composed of murine cells. This observation suggests that murine cellular contamination, probably due to the negative selection, displayed superior proliferative capabilities compared to the human cell fraction. Consistent with our initial attempts to establish the UGR05 PDxOs model, the murine fraction demonstrated robust growth. Despite our initial success in isolating human cells, we encountered significant challenges in the long-term maintenance and expansion of human PDxOs. Consequently, the UGR05 PDxO model was considered unsuitable for further experimentation, notwithstanding the favorable characteristics exhibited by the PDX model.

UGR01 proved to be an **optimal working model**, demonstrating robust proliferation and allowing us to establish enough cultures for necessary experimentation. Moreover, the murine population naturally diminished over time, as reported by Scherer et al.[130].

In our project, we aimed to obtain purely human organoids from PDXs, following publications in this field[123,130]. Altman et al. also proceeded with this approach as they used a mouse cell depletion after PDX digestion. Nonetheless, they did not confirm the human nature of the cell culture in subsequent passages[214] as Scherer et al. recommended [130]. Furthermore, not all researchers provide detailed information about the composition of the cell population of their breast cancer PDxOs[215–217]. This absence of analysis

suggests that a segment of the scientific community may not consider such stringent criteria necessary. Consequently, PDxOs, like widely accepted hybrid PDX models, could represent a valid **hybrid model** with high utility for certain assays, as they **maintain fidelity to the original tumor** characteristics.

Following the establishment of long-term human PDxOs culture, we proceeded to characterize the **UGR01 model**. Previous work utilizing UGR01 PDX had already confirmed the preservation of TNBC characteristics observed in the patient[218]. As anticipated[123], IHC analysis of the PDxOs culture, evaluated by a pathologist, also **confirmed the TNBC diagnosis**. Furthermore, the high expression of NDRG1 in both PDX and PDxOs models correlates with reduced survival among TNBC patients, as demonstrated in the initial phase of this project.

The three repurposed drugs under investigation demonstrated a significant **reduction in cellular proliferation**, as evidenced by decreased Ki67 expression in IHC analyses[219]. Additionally, we observed a corroborative **reduction in p-AKT** levels, consistent with our findings on attached TNBC cell lines.

To facilitate clinical translation and address the fact that the repurposed drug combinations are not FDA-approved, we continued the assessment of the efavirenz, ouabain, and vinburnine in **combination with the taxane** docetaxel, as previously studied in the chemoresistant cell line. In this case, the UGR01 PDxOs model exerts sensitivity to the taxane as the calculated IC₅₀ value was 8.8 nM. We next studied if the combinations could decrease cell viability. At the PDxOs density analyzed, we observed **compromised organoid viability** when the repurposed drugs were combined with docetaxel, compared to monotherapies. This finding suggests a potential synergistic effect between the repurposed drug candidates and docetaxel, validating their potential as co-adjuvants in TNBC treatment. Furthermore, an *in vivo* study in mice yielded interesting results when combining docetaxel and efavirenz. Efavirenz, known as CYP3A4 inducer, did not significantly change the exposure to docetaxel. This is noteworthy because docetaxel is primarily metabolized by the CYP3A4 enzyme family. Hence, these findings suggest a favorable safety profile of this combination as the chemotherapy dose remains unaffected by metabolic signaling[220].

These results collectively **underscore the value of our UGR01 PDxOs model** in recapitulating TNBC characteristics and its utility in evaluating novel therapeutic strategies. The observed synergistic effects between repurposed drugs and

standard chemotherapy highlight a promising avenue for enhancing TNBC treatment efficacy, potentially offering new options for this challenging breast cancer subtype.

In summary, this study presents a comprehensive investigation, spanning from the **elucidation of the protumor mechanism** of a protein with pleiotropic behavior in the cancer field to the **repurposing of drugs with validation** that includes a 3D organoid model. While drug repurposing is initially perceived as a straightforward approach, it presents a complex landscape of challenges and opportunities in the realm of cancer research. Investigation of compounds that have already undergone extensive *in vitro* testing and clinical toxicity profiles offer significant advances in streamlining certain aspects of the research process. Nevertheless, it is crucial to emphasize that comprehensive studies on the efficacy and mechanisms of these repurposed drugs in the context of the targeted disease remain imperative. Careful consideration of appropriate dosing regimens is equally crucial[71]. The current regulatory environment surrounding new-use patents for repurposed drugs introduces additional layers of complexity. Ambiguities in legislation, especially regarding pre-existing published scientific information, often deter pharmaceutical industries from pursuing drug repurposing initiatives[72]. This hesitancy underscores the need for clearer guidelines and incentives to foster innovation in this field.

In our investigation, we explored the potential of three repurposed drugs presenting different original indications and mechanisms of action. If we consider the synergistic effects of their paired combinations, EO is highlighted as the best choice for reducing aggressiveness markers on attached TNBC cell lines. In the validation on TNBC PDxOs, the three combinations with docetaxel were similarly effective, suggesting its prospective translational power.

In the field of ATPase inhibitors, we particularly focused on **ouabain**, obtaining outstanding results for TNBC treatment. Despite extensive research on these compounds across various cancer types, the transition to clinical trials has been notably absent. This gap between preclinical promise and clinical application may be attributed to a combination of factors, including insufficient motivation, inadequate funding, or a lack of consistently positive results to justify further development. Moreover, the high toxicity associated with chronic administration of cardiac glycosides[221,222] presents a barrier to its clinical translation, potentially dampening enthusiasm for its therapeutic potential.

Contrastingly, **vinburnine** represents a less explored avenue in the literature, underscoring the diverse landscape of drug repurposing candidates and the need for comprehensive investigations even for compounds with limited prior research in oncology. However, we herein present promising results of its effects reducing stemness and migration. Vinburnine also provides a re-sensibilization to chemotherapy in our docetaxel-resistant model and significantly reduces cell viability in combination with the taxane in PDxOs.

Efavirenz emerges also as a promising candidate in our study. Our positive results and the substantial body of evidence in the literature demonstrate its efficacy against cancer, particularly at concentrations below the toxicity thresholds[223,224]. This observation suggests a favorable safety profile for potential anticancer applications. Encouragingly, a recent search of clinical trials revealed an ongoing study in China (NCT05076682) investigating efavirenz for reversing immune resistance in TNBC. This development not only validates our findings but also instills optimism regarding the prospects of drug repurposing in oncology.

CONCLUSIONS

1. The pleiotropic activity of NDRG1 is mediated by TGF- β signaling, promoting metastasis and/or CSC maintenance at various stages of tumor progression.
2. The selected drug candidates—efavirenz, ouabain, and vinburnine—and their combinations markedly impacted the CSC phenotype in TNBC cell lines, highlighting their high potential as a therapy. Furthermore, repurposed drug treatments and NDRG1 inhibition under TGF- β stimulation showed varying effects, suggesting additional therapeutic properties of drugs derived from the MDA-MB-231 gene expression signature.
3. The absence of direct NDRG1 inhibition following treatment with our drug candidates highlights the value of signature-based drug repurposing, achieving similar functional outcomes while avoiding direct inhibition of potentially problematic proteins.
4. The repurposed drugs inhibited p-AKT expression, likely through the reduction of *CXCL10* and *CXCL11*. This inhibition may be responsible for the observed suppression of CSCs and EMT, suggesting a novel mechanism for their anti-cancer effects.
5. Although the long-term establishment of TNBC PDOs was not achieved due to limitations such as insufficient starting tissue and its intrinsic proliferation capacity, we successfully developed PDxOs that accurately recapitulate original tumor characteristics.
6. Efavirenz, ouabain, and vinburnine demonstrated efficacy in reducing cell proliferation through inhibition of AKT signaling in the TNBC PDxOs model UGR01, validating the results obtained in established TNBC cell lines.

CONCLUSIONES

1. La actividad pleiotrópica de NDRG1 está mediada por la señalización de TGF- β , promoviendo la metástasis y/o el mantenimiento de las CSC en diferentes etapas de la progresión tumoral.
2. Los fármacos candidatos seleccionados —efavirenz, ouabaína y vinburnina— y sus combinaciones impactaron notablemente sobre el fenotipo de las CSC en líneas celulares de TNBC, destacando su alto potencial como terapia. Además, los tratamientos con fármacos reposicionados y la inhibición de NDRG1 bajo estimulación con TGF- β mostraron efectos variables, sugiriendo propiedades terapéuticas adicionales de los fármacos derivados de la firma de expresión génica de MDA-MB-231.
3. La ausencia de inhibición directa de NDRG1 tras el tratamiento con nuestros candidatos resalta el valor del reposicionamiento de fármacos basado en firmas, logrando resultados funcionales similares mientras se evita la inhibición directa de proteínas potencialmente problemáticas.
4. Los fármacos reposicionados inhibieron la expresión de p-AKT, probablemente a través de la reducción de CXCL10 y CXCL11. Esta inhibición puede ser responsable de la supresión observada de CSC y EMT, sugiriendo un nuevo mecanismo para sus efectos anticancerígenos.
5. Aunque no se logró el establecimiento de PDOs de TNBC a largo plazo debido a limitaciones como insuficiente tejido inicial y su capacidad intrínseca de proliferación, desarrollamos con éxito PDxOs que recapitulan con precisión las características del tumor original.
6. Efavirenz, ouabaína y vinburnina demostraron eficacia en la reducción de la proliferación celular a través de la inhibición de la señalización de AKT en el modelo PDxOs de TNBC UGR01, validando los resultados obtenidos en líneas celulares establecidas de TNBC.

BIBLIOGRAPHY

- [1] W.H. Organization, Breast Cancer, (n.d.). <https://www.who.int/news-room/fact-sheets/detail/breast-cancer> (accessed September 5, 2024).
- [2] J. Ferlay, M. Colombet, I. Soerjomataram, D.M. Parkin, M. Piñeros, A. Znaor, F. Bray, Cancer statistics for the year 2020: An overview, *Int. J. Cancer* 149 (2021) 778–789. <https://doi.org/10.1002/ijc.33588>.
- [3] W.H. Organization, GCO Cancer Today, (n.d.). <https://gco.iarc.who.int/today/en/dataviz/> (accessed September 6, 2024).
- [4] A. Goldhirsch, E.P. Winer, A.S. Coates, R.D. Gelber, M. Piccart-Gebhart, B. Thürlimann, H.-J. Senn, K.S. Albain, F. André, J. Bergh, H. Bonnefoi, D. Bretel-Morales, H. Burstein, F. Cardoso, M. Castiglione-Gertsch, A.S. Coates, M. Colleoni, A. Costa, G. Curigliano, N.E. Davidson, A. Di Leo, B. Ejlertsen, J.F. Forbes, R.D. Gelber, M. Gnant, A. Goldhirsch, P. Goodwin, P.E. Goss, J.R. Harris, D.F. Hayes, C.A. Hudis, J.N. Ingle, J. Jassem, Z. Jiang, P. Karlsson, S. Loibl, M. Morrow, M. Namer, C. Kent Osborne, A.H. Partridge, F. Penault-Llorca, C.M. Perou, M.J. Piccart-Gebhart, K.I. Pritchard, E.J.T. Rutgers, F. Sedlmayer, V. Semiglazov, Z.-M. Shao, I. Smith, B. Thürlimann, M. Toi, A. Tutt, M. Untch, G. Viale, T. Watanabe, N. Wilcken, E.P. Winer, W.C. Wood, Personalizing the treatment of women with early breast cancer: highlights of the St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2013, *Ann. Oncol.* 24 (2013) 2206–2223. <https://doi.org/10.1093/annonc/mdt303>.
- [5] L. Yin, J.-J. Duan, X.-W. Bian, S. Yu, Triple-negative breast cancer molecular subtyping and treatment progress, *Breast Cancer Res.* 22 (2020) 61. <https://doi.org/10.1186/s13058-020-01296-5>.
- [6] B.D. Lehmann, J.A. Bauer, X. Chen, M.E. Sanders, A.B. Chakravarthy, Y. Shyr, J.A. Pietenpol, Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies, *J. Clin. Invest.* 121 (2011) 2750–2767. <https://doi.org/10.1172/JCI45014>.
- [7] M. Capuzzo, V. Celotto, M. Santorsola, A. Fabozzi, L. Landi, F. Ferrara, A. Borzacchiello, V. Granata, F. Sabbatino, G. Savarese, M. Cascella, F. Perri, A. Ottaiano, Emerging treatment approaches for triple-negative breast cancer, *Med. Oncol.* 41 (2024) 1–21. <https://doi.org/10.1007/s12032-023-02257-6>.
- [8] B.D. Lehmann, B. Jovanović, X. Chen, M. V. Estrada, K.N. Johnson, Y. Shyr, H.L. Moses, M.E. Sanders, J.A. Pietenpol, Refinement of Triple-Negative Breast Cancer Molecular Subtypes: Implications for Neoadjuvant Chemotherapy Selection, *PLoS One* 11 (2016) e0157368. <https://doi.org/10.1371/journal.pone.0157368>.
- [9] M.D. Burstein, A. Tsimelzon, G.M. Poage, K.R. Covington, A. Contreras,

- S.A.W. Fuqua, M.I. Savage, C. Kent Osborne, S.G. Hilsenbeck, J.C. Chang, G.B. Mills, C.C. Lau, P.H. Brown, C. Lau, J. Chang, C.K. Osborne, S. Hilsenbeck, G. Mills, S. Fuqua, G. Poage, K. Covington, P. Brown, Comprehensive Genomic Analysis Identifies Novel Subtypes and Targets of Triple-negative Breast Cancer, *Clin Cancer Res* 21 (2015) 1688–1698. <https://doi.org/10.1158/1078-0432.CCR-14-0432>.
- [10] Y.-R. Liu, Y.-Z. Jiang, X.-E. Xu, K.-D. Yu, X. Jin, X. Hu, W.-J. Zuo, S. Hao, J. Wu, G.-Y. Liu, G.-H. Di, D.-Q. Li, X.-H. He, W.-G. Hu, Z.-M. Shao, Comprehensive transcriptome analysis identifies novel molecular subtypes and subtype-specific RNAs of triple-negative breast cancer, *Breast Cancer Res.* 18 (2016) 33. <https://doi.org/10.1186/s13058-016-0690-8>.
- [11] L. Mosca, A. Ilari, F. Fazi, Y.G. Assaraf, G. Colotti, Taxanes in cancer treatment: Activity, chemoresistance and its overcoming, *Drug Resist. Updat.* 54 (2021) 100742. <https://doi.org/10.1016/j.drup.2020.100742>.
- [12] S. Al-Mahmood, J. Sapiezynski, O.B. Garbuzenko, T. Minko, Metastatic and triple-negative breast cancer: challenges and treatment options, *Drug Deliv. Transl. Res.* 8 (2018) 1483–1507. <https://doi.org/10.1007/s13346-018-0551-3>.
- [13] R. Mattioli, A. Ilari, B. Colotti, L. Mosca, F. Fazi, G. Colotti, Doxorubicin and other anthracyclines in cancers: Activity, chemoresistance and its overcoming, *Mol. Aspects Med.* 93 (2023) 101205. <https://doi.org/10.1016/j.mam.2023.101205>.
- [14] A. Emadi, R.J. Jones, R.A. Brodsky, Cyclophosphamide and cancer: Golden anniversary, *Nat. Rev. Clin. Oncol.* 6 (2009) 638–647. <https://doi.org/10.1038/nrclinonc.2009.146>.
- [15] R. Torrisi, M. Zuradelli, E. Agostinetto, G. Masci, A. Losurdo, R. De Sanctis, A. Santoro, Platinum salts in the treatment of BRCA-associated breast cancer: A true targeted chemotherapy?, *Crit. Rev. Oncol. Hematol.* 135 (2019) 66–75. <https://doi.org/10.1016/j.critrevonc.2019.01.016>.
- [16] C.M. Walko, C. Lindley, Capecitabine: a review., *Clin. Ther.* 27 (2005) 23–44. <https://doi.org/10.1016/j.clinthera.2005.01.005>.
- [17] V. Heinemann, Role of Gemcitabine in the Treatment of Advanced and Metastatic Breast Cancer, *Oncology* 64 (2003) 191–206. <https://doi.org/10.1159/000069315>.
- [18] J. Cortes, P. Schöffski, B.A. Littlefield, Multiple modes of action of eribulin mesylate: Emerging data and clinical implications, *Cancer Treat. Rev.* 70 (2018) 190–198. <https://doi.org/10.1016/j.ctrv.2018.08.008>.
- [19] N.F. Dybdal-Hargreaves, A.L. Risinger, S.L. Mooberry, Eribulin Mesylate:

- Mechanism of Action of a Unique Microtubule-Targeting Agent, *Clin. Cancer Res.* 21 (2015) 2445–2452. <https://doi.org/10.1158/1078-0432.CCR-14-3252>.
- [20] R.K. Gregory, I.E. Smith, Vinorelbine--a clinical review., *Br. J. Cancer* 82 (2000) 1907–13. <https://doi.org/10.1054/bjoc.2000.1203>.
- [21] V. Lorusso, A. Latorre, F. Giotta, Chemotherapy Options beyond the First Line in HER-Negative Metastatic Breast Cancer, *J. Oncol.* 2020 (2020) 1–17. <https://doi.org/10.1155/2020/9645294>.
- [22] J.H. Park, J.H. Ahn, S.B. Kim, How shall we treat early triple-negative breast cancer (TNBC): From the current standard to upcoming immuno-molecular strategies, *ESMO Open* 3 (2018) 1–16. <https://doi.org/10.1136/esmoopen-2018-000357>.
- [23] S.A. Im, A. Gennari, Y.H. Park, J.H. Kim, Z.F. Jiang, S. Gupta, T.H. Fadjari, K. Tamura, M.Y. Mastura, M.L.T. Abesamis-Tiambeng, E.H. Lim, C.H. Lin, A. Sookprasert, N. Parinyanitikul, L.M. Tseng, S.C. Lee, P. Caguioa, M. Singh, Y. Naito, R.A. Hukom, B.K. Smruti, S.S. Wang, S.B. Kim, K.H. Lee, H.K. Ahn, S. Peters, T.W. Kim, T. Yoshino, G. Pentheroudakis, G. Curigliano, N. Harbeck, Pan-Asian adapted ESMO Clinical Practice Guidelines for the diagnosis, staging and treatment of patients with metastatic breast cancer, *ESMO Open* 8 (2023). <https://doi.org/10.1016/j.esmoop.2023.101541>.
- [24] A.R. Schreiber, C.L. O'Bryant, P. Kabos, J.R. Diamond, The emergence of targeted therapy for HER2-low triple-negative breast cancer: a review of fam-trastuzumab deruxtecan, *Expert Rev. Anticancer Ther.* 23 (2023) 1061–1069. <https://doi.org/10.1080/14737140.2023.2257885>.
- [25] C. Yang, C. Brezden-Masley, A.A. Joy, S. Sehdev, S. Modi, C. Simmons, J.-W. Henning, Targeting HER2-low in metastatic breast cancer: an evolving treatment paradigm, *Ther. Adv. Med. Oncol.* 15 (2023) 17588359231175440. <https://doi.org/10.1177/17588359231175440>.
- [26] C.G. Willett, Y. Boucher, E. di Tomaso, D.G. Duda, L.L. Munn, R.T. Tong, D.C. Chung, D. V Sahani, S.P. Kalva, S. V Kozin, M. Mino, K.S. Cohen, D.T. Scadden, A.C. Hartford, A.J. Fischman, J.W. Clark, D.P. Ryan, A.X. Zhu, L.S. Blaszkowsky, H.X. Chen, P.C. Shellito, G.Y. Lauwers, R.K. Jain, Direct evidence that the VEGF-specific antibody bevacizumab has antivasular effects in human rectal cancer, *Nat. Med.* 10 (2004) 145–147. <https://doi.org/10.1038/nm988>.
- [27] C. Thomssen, J.-Y. Pierga, K.I. Pritchard, L. Biganzoli, H. Cortes-Funes, K. Petráková, B. Kaufman, A. Duenne, I. Smith, First-Line Bevacizumab-Containing Therapy for Triple-Negative Breast Cancer: Analysis of 585 Patients Treated in the ATHENA Study, *Oncology* 82 (2012) 218–227.

<https://doi.org/10.1159/000336892>.

- [28] R. Bell, J. Brown, M. Parmar, M. Toi, T. Suter, G.G. Steger, X. Pivot, J. Mackey, C. Jackisch, R. Dent, P. Hall, N. Xu, L. Morales, L. Provencher, R. Hegg, L. Vanlemmens, A. Kirsch, A. Schneeweiss, N. Masuda, F. Overkamp, D. Cameron, Final efficacy and updated safety results of the randomized phase III BEATRICE trial evaluating adjuvant bevacizumab-containing therapy in triple-negative early breast cancer, *Ann. Oncol.* 28 (2017) 754–760. <https://doi.org/10.1093/annonc/mdw665>.
- [29] Y. Boucher, A.S. Kumar, J.M. Posada, E. Gjini, K. Pfaff, M. Lipschitz, A. Lako, D.G. Duda, S.J. Rodig, F.S. Hodi, R.K. Jain, Bevacizumab improves tumor infiltration of mature dendritic cells and effector T-cells in triple-negative breast cancer patients, *Npj Precis. Oncol.* 5 (2021) 62. <https://doi.org/10.1038/s41698-021-00197-w>.
- [30] K.H. Park, S. Loibl, J. Sohn, Y.H. Park, Z. Jiang, H. Tadjoeidin, S. Nag, S. Saji, M. Md. Yusof, E.M.B. Villegas, E.H. Lim, Y.S. Lu, S. Ithimakin, L.M. Tseng, T. Dejthevaporn, T.W.W. Chen, S.C. Lee, C. Galvez, S. Malwinder, T. Kogawa, J. Bajpai, B. Brahma, S. Wang, G. Curigliano, T. Yoshino, S.B. Kim, G. Pentheroudakis, S.A. Im, F. Andre, J.B. Ahn, N. Harbeck, Pan-Asian adapted ESMO Clinical Practice Guidelines for the diagnosis, treatment and follow-up of patients with early breast cancer, *ESMO Open* 9 (2024). <https://doi.org/10.1016/j.esmoop.2024.102974>.
- [31] A. Ahmad, Pathways to Breast Cancer Recurrence, *ISRN Oncol.* 2013 (2013) 1–16. <https://doi.org/10.1155/2013/290568>.
- [32] M. Cojoc, K. Mäbert, M.H. Muders, A. Dubrovskaya, A role for cancer stem cells in therapy resistance: Cellular and molecular mechanisms, *Semin. Cancer Biol.* 31 (2015) 16–27. <https://doi.org/10.1016/j.semcancer.2014.06.004>.
- [33] N. Fultang, M. Chakraborty, B. Peethambaran, Regulation of cancer stem cells in triple negative breast cancer, *Cancer Drug Resist.* 4 (2021) 321–342. <https://doi.org/10.20517/cdr.2020.106>.
- [34] W. Li, H. Ma, J. Zhang, L. Zhu, C. Wang, Y. Yang, Unraveling the roles of CD44/CD24 and ALDH1 as cancer stem cell markers in tumorigenesis and metastasis, *Sci. Rep.* 7 (2017) 1–15. <https://doi.org/10.1038/s41598-017-14364-2>.
- [35] M.D. Brooks, M.L. Burness, M.S. Wicha, Therapeutic Implications of Cellular Heterogeneity and Plasticity in Breast Cancer, *Cell Stem Cell* 17 (2015) 260–271. <https://doi.org/10.1016/j.stem.2015.08.014>.
- [36] C. Ginestier, M.H. Hur, E. Charafe-Jauffret, F. Monville, J. Dutcher, M. Brown, J. Jacquemier, P. Viens, C.G. Kleer, S. Liu, A. Schott, D. Hayes, D.

- Birnbaum, M.S. Wicha, G. Dontu, ALDH1 Is a Marker of Normal and Malignant Human Mammary Stem Cells and a Predictor of Poor Clinical Outcome, *Cell Stem Cell* 1 (2007) 555–567. <https://doi.org/10.1016/j.stem.2007.08.014>.
- [37] H. Tomita, K. Tanaka, T. Tanaka, A. Hara, Aldehyde dehydrogenase 1A1 in stem cells and cancer, *Oncotarget* 7 (2016) 11018–11032. <https://doi.org/10.18632/oncotarget.6920>.
- [38] T. Reya, S.J. Morrison, M.F. Clarke, I.L. Weissman, Stem cells, cancer, and cancer stem cells, *Nature* 414 (2001) 105–111. <https://doi.org/10.1038/35102167>.
- [39] S. Liu, Y. Cong, D. Wang, Y. Sun, L. Deng, Y. Liu, R. Martin-Trevino, L. Shang, S.P. McDermott, M.D. Landis, S. Hong, A. Adams, R. D'Angelo, C. Ginestier, E. Charafe-Jauffret, S.G. Clouthier, D. Birnbaum, S.T. Wong, M. Zhan, J.C. Chang, M.S. Wicha, Breast cancer stem cells transition between epithelial and mesenchymal states reflective of their normal counterparts, *Stem Cell Reports* 2 (2014) 78–91. <https://doi.org/10.1016/j.stemcr.2013.11.009>.
- [40] M.A. Goodell, K. Brose, G. Paradis, A.S. Conner, R.C. Mulligan, Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo., *J. Exp. Med.* 183 (1996) 1797–1806. <https://doi.org/10.1084/jem.183.4.1797>.
- [41] C. Hirschmann-Jax, A.E. Foster, G.G. Wulf, J.G. Nuchtern, T.W. Jax, U. Gobel, M.A. Goodell, M.K. Brenner, A distinct “side population” of cells with high drug efflux capacity in human tumor cells., *Proc. Natl. Acad. Sci. U. S. A.* 101 (2004) 14228–14233. <https://doi.org/10.1073/pnas.0400067101>.
- [42] E.M. Grasset, M. Dunworth, G. Sharma, M. Loth, J. Tandurella, A. Cimino-Mathews, M. Gentz, S. Bracht, M. Haynes, E.J. Fertig, A.J. Ewald, Triple-negative breast cancer metastasis involves complex epithelial-mesenchymal transition dynamics and requires vimentin, *Sci. Transl. Med.* 14 (2022) eabn7571. <https://doi.org/10.1126/scitranslmed.abn7571>.
- [43] F. Font-Clos, S. Zapperi, C.A.M. La Porta, Classification of triple negative breast cancer by epithelial mesenchymal transition and the tumor immune microenvironment, *Sci. Rep.* 12 (2022) 9651. <https://doi.org/10.1038/s41598-022-13428-2>.
- [44] L. Zhang, F. Zhou, A. García de Vinuesa, E.M. de Kruijf, W.E. Mesker, L. Hui, Y. Drabsch, Y. Li, A. Bauer, A. Rousseau, K.-A. Sheppard, C. Mickanin, P.J.K. Kuppen, C.X. Lu, P. ten Dijke, TRAF4 Promotes TGF- β Receptor Signaling and Drives Breast Cancer Metastasis, *Mol. Cell* 51

- (2013) 559–572. <https://doi.org/10.1016/j.molcel.2013.07.014>.
- [45] Y. Hao, D. Baker, P. ten Dijke, TGF- β -Mediated Epithelial-Mesenchymal Transition and Cancer Metastasis, *Int. J. Mol. Sci.* 20 (2019) 2767. <https://doi.org/10.3390/ijms20112767>.
 - [46] J. Xu, S. Lamouille, R. Derynck, TGF- β -induced epithelial to mesenchymal transition, *Cell Res.* 19 (2009) 156–172. <https://doi.org/10.1038/cr.2009.5>.
 - [47] W. Xue, L. Yang, C. Chen, M. Ashrafizadeh, Y. Tian, R. Sun, Wnt/ β -catenin-driven EMT regulation in human cancers, *Cell. Mol. Life Sci.* 81 (2024) 79. <https://doi.org/10.1007/s00018-023-05099-7>.
 - [48] S.-G. Pohl, N. Brook, M. Agostino, F. Arfuso, A.P. Kumar, A. Dharmarajan, Wnt signaling in triple-negative breast cancer, *Oncogenesis* 6 (2017) e310–e310. <https://doi.org/10.1038/oncsis.2017.14>.
 - [49] X. Hu, Q. Zhang, W. Xing, W. Wang, Role of microRNA/lncRNA Intertwined With the Wnt/ β -Catenin Axis in Regulating the Pathogenesis of Triple-Negative Breast Cancer, *Front. Pharmacol.* 13 (2022). <https://doi.org/10.3389/fphar.2022.814971>.
 - [50] E.S. Villodre, A.P.N. Nguyen, B.G. Debeb, NDRGs in Breast Cancer: A Review and In Silico Analysis, *Cancers (Basel)*. 16 (2024) 1342. <https://doi.org/10.3390/cancers16071342>.
 - [51] L.-L. Han, L. Hou, M.-J. Zhou, Z. Ma, D.-L. Lin, L. Wu, Y. Ge, Aberrant NDRG1 methylation associated with its decreased expression and clinicopathological significance in breast cancer, *J. Biomed. Sci.* 20 (2013) 52. <https://doi.org/10.1186/1423-0127-20-52>.
 - [52] K.C. Park, S. V Menezes, D.S. Kalinowski, S. Sahni, P.J. Jansson, Z. Kovacevic, D.R. Richardson, Identification of differential phosphorylation and sub-cellular localization of the metastasis suppressor, NDRG1, *Biochim. Biophys. Acta - Mol. Basis Dis.* 1864 (2018) 2644–2663. <https://doi.org/10.1016/j.bbadis.2018.04.011>.
 - [53] J.M. García-Martínez, D.R. Alessi, mTOR complex 2 (mTORC2) controls hydrophobic motif phosphorylation and activation of serum- and glucocorticoid-induced protein kinase 1 (SGK1), *Biochem. J.* 416 (2008) 375–385. <https://doi.org/10.1042/BJ20081668>.
 - [54] T.P. Ellen, Q. Ke, P. Zhang, M. Costa, NDRG1, a growth and cancer related gene: regulation of gene expression and function in normal and disease states, *Carcinogenesis* 29 (2007) 2–8. <https://doi.org/10.1093/carcin/bgm200>.
 - [55] S. Bandyopadhyay, Y. Wang, R. Zhan, S.K. Pai, M. Watabe, M. Iizumi, E.

- Furuta, S. Mohinta, W. Liu, S. Hirota, S. Hosobe, T. Tsukada, K. Miura, Y. Takano, K. Saito, T. Commes, D. Piquemal, T. Hai, K. Watabe, The Tumor Metastasis Suppressor Gene Drg-1 Down-regulates the Expression of Activating Transcription Factor 3 in Prostate Cancer, *Cancer Res.* 66 (2006) 11983–11990. <https://doi.org/10.1158/0008-5472.CAN-06-0943>.
- [56] Z. Chen, D. Zhang, F. Yue, M. Zheng, Z. Kovacevic, D.R. Richardson, The Iron Chelators Dp44mT and DFO Inhibit TGF- β -induced Epithelial-Mesenchymal Transition via Up-Regulation of N-Myc Downstream-regulated Gene 1 (NDRG1), *J. Biol. Chem.* 287 (2012) 17016–17028. <https://doi.org/10.1074/jbc.M112.350470>.
- [57] C. Kim-Fuchs, S. Winterhalder, A. Winter, T. Malinka, D. Born, S. Schäfer, D. Stroka, B. Gloor, D. Candinas, E. Angst, The Silencing of N-myc Downstream-Regulated Gene-1 in an Orthotopic Pancreatic Cancer Model Leads to More Aggressive Tumor Growth and Metastases, *Dig. Surg.* 31 (2014) 135–142. <https://doi.org/10.1159/000363065>.
- [58] S. V Menezes, L. Fouani, M.L.H. Huang, B. Geleta, S. Maleki, A. Richardson, D.R. Richardson, Z. Kovacevic, The metastasis suppressor, NDRG1, attenuates oncogenic TGF- β and NF- κ B signaling to enhance membrane E-cadherin expression in pancreatic cancer cells, *Carcinogenesis* 40 (2019) 805–818. <https://doi.org/10.1093/carcin/bgy178>.
- [59] X. Wangpu, X. Yang, J. Zhao, J. Lu, S. Guan, J. Lu, Z. Kovacevic, W. Liu, L. Mi, R. Jin, J. Sun, F. Yue, J. Ma, A. Lu, D.R. Richardson, L. Wang, M. Zheng, The metastasis suppressor, NDRG1, inhibits “stemness” of colorectal cancer via down-regulation of nuclear β -catenin and CD44, *Oncotarget* 6 (2015) 33893–33911. <https://doi.org/10.18632/oncotarget.5294>.
- [60] S. Bandyopadhyay, S.K. Pai, S. Hirota, S. Hosobe, Y. Takano, K. Saito, D. Piquemal, T. Commes, M. Watabe, S.C. Gross, Y. Wang, S. Ran, K. Watabe, Role of the putative tumor metastasis suppressor gene Drg-1 in breast cancer progression, *Oncogene* 23 (2004) 5675–5681. <https://doi.org/10.1038/sj.onc.1207734>.
- [61] M. Godbole, T. Togar, K. Patel, B. Dharavath, N. Yadav, S. Janjuha, N. Gardi, K. Tiwary, P. Terwadkar, S. Desai, R. Prasad, H. Dhamne, K. Karve, S. Salunkhe, D. Kawle, P. Chandrani, S. Dutt, S. Gupta, R.A. Badwe, A. Dutt, Up-regulation of the kinase gene SGK1 by progesterone activates the AP-1–NDRG1 axis in both PR-positive and -negative breast cancer cells, *J. Biol. Chem.* 293 (2018) 19263–19276. <https://doi.org/10.1074/jbc.RA118.002894>.
- [62] J.T. Murray, D.G. Campbell, N. Morrice, G.C. Auld, N. Shpiro, R. Marquez, M. Pegg, J. Bain, G.B. Bloomberg, F. Grahmmer, F. Lang, P. Wulff, D.

- Kuhl, P. Cohen, Exploitation of KESTREL to identify NDRG family members as physiological substrates for SGK1 and GSK3, *Biochem. J.* 384 (2004) 477–488. <https://doi.org/10.1042/BJ20041057>.
- [63] W. Liu, F. Xing, M. Iizumi-Gairani, H. Okuda, M. Watabe, S.K. Pai, P.R. Pandey, S. Hirota, A. Kobayashi, Y.-Y. Mo, K. Fukuda, Y. Li, K. Watabe, N-myc downstream regulated gene 1 modulates Wnt- β -catenin signalling and pleiotropically suppresses metastasis, *EMBO Mol. Med.* 4 (2012) 93–108. <https://doi.org/10.1002/emmm.201100190>.
- [64] J. Cheng, H.-Y. Xie, X. Xu, J. Wu, X. Wei, R. Su, W. Zhang, Z. Lv, S. Zheng, L. Zhou, NDRG1 as a biomarker for metastasis, recurrence and of poor prognosis in hepatocellular carcinoma, *Cancer Lett.* 310 (2011) 35–45. <https://doi.org/10.1016/j.canlet.2011.06.001>.
- [65] W.-J. Lu, M.-S. Chua, W. Wei, S.K. So, NDRG1 promotes growth of hepatocellular carcinoma cells by directly interacting with GSK-3 β and Nur77 to prevent β -catenin degradation, *Oncotarget* 6 (2015) 29847–29859. <https://doi.org/10.18632/oncotarget.4913>.
- [66] H. Ureshino, Y. Murakami, K. Watari, H. Izumi, A. Kawahara, M. Kage, T. Arao, K. Nishio, K. Yanagihara, H. Kinoshita, M. Kuwano, M. Ono, N-myc Downstream Regulated Gene 1 (NDRG1) Promotes Metastasis of Human Scirrhous Gastric Cancer Cells through Epithelial Mesenchymal Transition, *PLoS One* 7 (2012) e41312. <https://doi.org/10.1371/journal.pone.0041312>.
- [67] Y. Murakami, K. Watari, T. Shibata, M. Uba, H. Ureshino, A. Kawahara, H. Abe, H. Izumi, N. Mukaida, M. Kuwano, M. Ono, N-myc Downstream-regulated Gene 1 Promotes Tumor Inflammatory Angiogenesis through JNK Activation and Autocrine Loop of Interleukin-1 α by Human Gastric Cancer Cells, *J. Biol. Chem.* 288 (2013) 25025–25037. <https://doi.org/10.1074/jbc.M113.472068>.
- [68] A. Li, X. Zhu, C. Wang, S. Yang, Y. Qiao, R. Qiao, J. Zhang, Upregulation of NDRG1 predicts poor outcome and facilitates disease progression by influencing the EMT process in bladder cancer, *Sci. Rep.* 9 (2019) 5166. <https://doi.org/10.1038/s41598-019-41660-w>.
- [69] M.A. Nagai, R. Gerhard, J.H.T.G. Fregnani, S. Nonogaki, R.B. Rierger, M.M. Netto, F.A. Soares, Prognostic value of NDRG1 and SPARC protein expression in breast cancer patients, *Breast Cancer Res. Treat.* 126 (2011) 1–14. <https://doi.org/10.1007/s10549-010-0867-2>.
- [70] A. González-González, E. Muñoz-Muela, J.A. Marchal, F.E. Cara, M.P. Molina, M. Cruz-Lozano, G. Jiménez, A. Verma, A. Ramírez, W. Qian, W. Chen, A.J. Kozielski, O. Elemento, M.D. Martín-Salvago, R.J. Luque, C. Rosa-Garrido, D. Landeira, M. Quintana-Romero, R.R. Rosato, M.A.

- García, C.L. Ramirez-Tortosa, H. Kim, C. Rodriguez-Aguayo, G. Lopez-Berestein, A.K. Sood, J.A. Lorente, P. Sánchez-Rovira, J.C. Chang, S. Granados-Principal, Activating Transcription Factor 4 Modulates TGF β -Induced Aggressiveness in Triple-Negative Breast Cancer via SMAD2/3/4 and mTORC2 Signaling, *Clin. Cancer Res.* 24 (2018) 5697–5709. <https://doi.org/10.1158/1078-0432.CCR-17-3125>.
- [71] S. Pushpakom, F. Iorio, P.A. Eyers, K.J. Escott, S. Hopper, A. Wells, A. Doig, T. Williams, J. Latimer, C. McNamee, A. Norris, P. Sanseau, D. Cavalla, M. Pirmohamed, Drug repurposing: progress, challenges and recommendations, *Nat. Rev. Drug Discov.* 18 (2019) 41–58. <https://doi.org/10.1038/nrd.2018.168>.
- [72] Y. Xia, M. Sun, H. Huang, W.-L. Jin, Drug repurposing for cancer therapy, *Signal Transduct. Target. Ther.* 9 (2024) 92. <https://doi.org/10.1038/s41392-024-01808-1>.
- [73] Y. Hua, X. Dai, Y. Xu, G. Xing, H. Liu, T. Lu, Y. Chen, Y. Zhang, Drug repositioning: Progress and challenges in drug discovery for various diseases, *Eur. J. Med. Chem.* 234 (2022) 114239. <https://doi.org/10.1016/j.ejmech.2022.114239>.
- [74] D. Sun, W. Gao, H. Hu, S. Zhou, Why 90% of clinical drug development fails and how to improve it?, *Acta Pharm. Sin. B* 12 (2022) 3049–3062. <https://doi.org/10.1016/j.apsb.2022.02.002>.
- [75] B.A. Akhoun, H. Tiwari, A. Nargotra, In Silico Drug Design Methods for Drug Repurposing, in: *Silico Drug Des.*, Elsevier, 2019: pp. 47–84. <https://doi.org/10.1016/b978-0-12-816125-8.00003-1>.
- [76] M. Ávalos-Moreno, A. López-Tejada, J.L. Blaya-Cánovas, F.E. Cara-Lupiañez, A. González-González, J.A. Lorente, P. Sánchez-Rovira, S. Granados-Principal, Drug Repurposing for Triple-Negative Breast Cancer, *J. Pers. Med.* 10 (2020) 200. <https://doi.org/10.3390/jpm10040200>.
- [77] E.W. Su, T.M. Sanger, Systematic drug repositioning through mining adverse event data in ClinicalTrials.gov, *PeerJ* 5 (2017) e3154. <https://doi.org/10.7717/peerj.3154>.
- [78] A. Subramanian, R. Narayan, S.M. Corsello, D.D. Peck, T.E. Natoli, X. Lu, J. Gould, J.F. Davis, A.A. Tubelli, J.K. Asiedu, D.L. Lahr, J.E. Hirschman, Z. Liu, M. Donahue, B. Julian, M. Khan, D. Wadden, I.C. Smith, D. Lam, A. Liberzon, C. Toder, M. Bagul, M. Orzechowski, O.M. Enache, F. Piccioni, S.A. Johnson, N.J. Lyons, A.H. Berger, A.F. Shamji, A.N. Brooks, A. Vrcic, C. Flynn, J. Rosains, D.Y. Takeda, R. Hu, D. Davison, J. Lamb, K. Ardlie, L. Hogstrom, P. Greenside, N.S. Gray, P.A. Clemons, S. Silver, X. Wu, W.-N. Zhao, W. Read-Button, X. Wu, S.J. Haggarty, L. V Ronco, J.S. Boehm, S.L. Schreiber, J.G. Doench, J.A. Bittker, D.E. Root, B. Wong, T.R. Golub, A

- Next Generation Connectivity Map: L1000 Platform and the First 1,000,000 Profiles, Cell 171 (2017) 1437-1452.e17. <https://doi.org/10.1016/j.cell.2017.10.049>.
- [79] Broad Institute, Clue Connectopedia. What is the Connectivity Map?, (2017). https://clue.io/connectopedia/cmap_overview (accessed September 25, 2024).
- [80] Broad Institute, Clue Connectopedia. What is the total gene space accessible by L1000?, (2017). https://clue.io/connectopedia/l1000_gene_space (accessed September 25, 2024).
- [81] Broad Institute, Clue Connectopedia. What are landmark genes?, (2017). https://clue.io/connectopedia/what_are_landmark_genes (accessed September 25, 2024).
- [82] Broad Institute, Clue Connectopedia. How do I find “valid” gene names for use with the Query App?, (2018). https://clue.io/connectopedia/finding_valid_gene_names (accessed September 25, 2024).
- [83] Broad Institute, Clue Connectopedia. How do I interpret connectivity scores, and what is a “good” score?, (2018). https://clue.io/connectopedia/connectivity_scores (accessed September 25, 2024).
- [84] Broad Institute, Clue Connectopedia. What are the perturbagen types and controls in the CMap dataset?, (2017). https://clue.io/connectopedia/perturbagen_types_and_controls (accessed September 25, 2024).
- [85] Broad Institute, Clue Connectopedia. What are Perturbagen Classes (PCLs)?, (2018). <https://clue.io/connectopedia/pcls> (accessed September 25, 2024).
- [86] Broad Institute, Clue Connectopedia. Which cell lines are in the Core CMap Cell Panel (CCCP)?, (2018). https://clue.io/connectopedia/core_cmap_cell_panel (accessed September 25, 2024).
- [87] Broad Institute, Clue Connectopedia. What are the connectivity map algorithms?, (2019). https://clue.io/connectopedia/cmap_algorithms (accessed September 25, 2024).
- [88] M. Rampurwala, K.B. Wisinski, R. O’Regan, Role of the androgen receptor in triple-negative breast cancer, Clin. Adv. Hematol. Oncol. 14 (2016) 186–93. <http://www.ncbi.nlm.nih.gov/pubmed/27058032>.

- [89] M. Kirkby, A.M. Popatia, J.R. Lavoie, L. Wang, The Potential of Hormonal Therapies for Treatment of Triple-Negative Breast Cancer, *Cancers* (Basel). 15 (2023) 4702. <https://doi.org/10.3390/cancers15194702>.
- [90] V.N. Barton, N.C. D'Amato, M.A. Gordon, H.T. Lind, N.S. Spoelstra, B.L. Babbs, R.E. Heinz, A. Elias, P. Jedlicka, B.M. Jacobsen, J.K. Richer, Multiple Molecular Subtypes of Triple-Negative Breast Cancer Critically Rely on Androgen Receptor and Respond to Enzalutamide In Vivo, *Mol. Cancer Ther.* 14 (2015) 769–778. <https://doi.org/10.1158/1535-7163.MCT-14-0926>.
- [91] J.L. Christenson, J.B. Trepel, H.Y. Ali, S. Lee, J.R. Eisner, E.S. Baskin-Bey, A.D. Elias, J.K. Richer, Harnessing a Different Dependency: How to Identify and Target Androgen Receptor-Positive Versus Quadruple-Negative Breast Cancer, *Horm. Cancer* 9 (2018) 82–94. <https://doi.org/10.1007/s12672-017-0314-5>.
- [92] A. Gucalp, T.A. Traina, Targeting the androgen receptor in triple-negative breast cancer, *Curr. Probl. Cancer* 40 (2016) 141–150. <https://doi.org/10.1016/j.currprobcancer.2016.09.004>.
- [93] R. Huang, J. Han, X. Liang, S. Sun, Y. Jiang, B. Xia, M. Niu, D. Li, J. Zhang, S. Wang, W. Wei, Q. Liu, W. Zheng, G. Zhang, Y. Song, D. Panga, Androgen Receptor Expression and Bicalutamide Antagonize Androgen Receptor Inhibit β -Catenin Transcription Complex in Estrogen Receptor-Negative Breast Cancer, *Cell. Physiol. Biochem.* 43 (2017) 2212–2225. <https://doi.org/10.1159/000484300>.
- [94] D.R. Cochrane, S. Bernales, B.M. Jacobsen, D.M. Cittelly, E.N. Howe, N.C. D'Amato, N.S. Spoelstra, S.M. Edgerton, A. Jean, J. Guerrero, F. Gómez, S. Medicherla, I.E. Alfaro, E. McCullagh, P. Jedlicka, K.C. Torkko, A.D. Thor, A.D. Elias, A.A. Protter, J.K. Richer, Role of the androgen receptor in breast cancer and preclinical analysis of enzalutamide, *Breast Cancer Res.* 16 (2014) R7. <https://doi.org/10.1186/bcr3599>.
- [95] E.I. Obeid, S.D. Conzen, The role of adrenergic signaling in breast cancer biology, *Cancer Biomarkers* 13 (2013) 161–169. <https://doi.org/10.3233/CBM-130347>.
- [96] S.W. Cole, A.K. Sood, Molecular Pathways: Beta-Adrenergic Signaling in Cancer, *Clin. Cancer Res.* 18 (2012) 1201–1206. <https://doi.org/10.1158/1078-0432.CCR-11-0641>.
- [97] A. Chang, C.P. Le, A.K. Walker, S.J. Creed, C.K. Pon, S. Albold, D. Carroll, M.L. Halls, J.R. Lane, B. Riedel, D. Ferrari, E.K. Sloan, β 2 -Adrenoceptors on tumor cells play a critical role in stress-enhanced metastasis in a mouse model of breast cancer, *Brain. Behav. Immun.* 57 (2016) 106–115. <https://doi.org/10.1016/j.bbi.2016.06.011>.

- [98] A. Spini, G. Roberto, R. Gini, C. Bartolini, L. Bazzani, S. Donnini, S. Crispino, M. Ziche, Evidence of β -blockers drug repurposing for the treatment of triple negative breast cancer: A systematic review, *Neoplasma* 66 (2019) 963–970. <https://doi.org/10.4149/neo>.
- [99] A. Chang, E. Botteri, R.D. Gillis, L. Löfling, C.P. Le, A.I. Ziegler, N.-C. Chung, M.C. Rowe, S.A. Fabb, B.J. Hartley, C.J. Nowell, S. Kurozumi, S. Gandini, E. Munzone, E. Montagna, N. Eikelis, S.E. Phillips, C. Honda, K. Masuda, A. Katayama, T. Oyama, S.W. Cole, G.W. Lambert, A.K. Walker, E.K. Sloan, Beta-blockade enhances anthracycline control of metastasis in triple-negative breast cancer, *Sci. Transl. Med.* 15 (2023) eadf1147. <https://doi.org/10.1126/scitranslmed.adf1147>.
- [100] A. Montoya, C.N. Amaya, A. Belmont, N. Diab, R. Trevino, G. Villanueva, S. Rains, L.A. Sanchez, N. Badri, S. Otoukesh, A. Khammanivong, D. Liss, S.T. Baca, R.J. Aguilera, E.B. Dickerson, A. Torabi, A.K. Dwivedi, A. Abbas, K. Chambers, B.A. Bryan, Z. Nahleh, Use of non-selective β -blockers is associated with decreased tumor proliferative indices in early stage breast cancer, *Oncotarget* 8 (2017) 6446–6460. <https://doi.org/10.18632/oncotarget.14119>.
- [101] J.-H. Ma, L. Qin, X. Li, Role of STAT3 signaling pathway in breast cancer, *Cell Commun. Signal.* 18 (2020) 33. <https://doi.org/10.1186/s12964-020-0527-z>.
- [102] S. Fu, X. Chen, H.-W. Lo, J. Lin, Combined bazedoxifene and paclitaxel treatments inhibit cell viability, cell migration, colony formation, and tumor growth and induce apoptosis in breast cancer, *Cancer Lett.* 448 (2019) 11–19. <https://doi.org/10.1016/j.canlet.2019.01.026>.
- [103] H. Li, H. Xiao, L. Lin, D. Jou, V. Kumari, J. Lin, C. Li, Drug Design Targeting Protein–Protein Interactions (PPIs) Using Multiple Ligand Simultaneous Docking (MLSD) and Drug Repositioning: Discovery of Raloxifene and Bazedoxifene as Novel Inhibitors of IL-6/GP130 Interface, *J. Med. Chem.* 57 (2014) 632–641. <https://doi.org/10.1021/jm401144z>.
- [104] J. Tian, X. Chen, S. Fu, R. Zhang, L. Pan, Y. Cao, X. Wu, H. Xiao, H.-J. Lin, H.-W. Lo, Y. Zhang, J. Lin, Bazedoxifene is a novel IL-6/GP130 inhibitor for treating triple-negative breast cancer, *Breast Cancer Res. Treat.* 175 (2019) 553–566. <https://doi.org/10.1007/s10549-019-05183-2>.
- [105] E. Oh, Y. Kim, H. An, D. Sung, T.-M. Cho, L. Farrand, S. Jang, J.H. Seo, J.Y. Kim, Flubendazole elicits anti-metastatic effects in triple-negative breast cancer via STAT3 inhibition, *Int. J. Cancer* 143 (2018) 1978–1993. <https://doi.org/10.1002/ijc.31585>.
- [106] Z.-J. Hou, X. Luo, W. Zhang, F. Peng, B. Cui, S.-J. Wu, F.-M. Zheng, J. Xu, L.-Z. Xu, Z.-J. Long, X.-T. Wang, G.-H. Li, X.-Y. Wan, Y.-L. Yang, Q. Liu,

- Flubendazole, FDA-approved anthelmintic, targets breast cancer stem-like cells, *Oncotarget* 6 (2015) 6326–6340. <https://doi.org/10.18632/oncotarget.3436>.
- [107] J. Liu, X. Chen, T. Ward, M. Pegram, K. Shen, Combined niclosamide with cisplatin inhibits epithelial-mesenchymal transition and tumor growth in cisplatin-resistant triple-negative breast cancer, *Tumor Biol.* 37 (2016) 9825–9835. <https://doi.org/10.1007/s13277-015-4650-1>.
- [108] Y.-C. Wang, T.-K. Chao, C.-C. Chang, Y.-T. Yo, M.-H. Yu, H.-C. Lai, Drug Screening Identifies Niclosamide as an Inhibitor of Breast Cancer Stem-Like Cells, *PLoS One* 8 (2013) e74538. <https://doi.org/10.1371/journal.pone.0074538>.
- [109] L. Lu, J. Dong, L. Wang, Q. Xia, D. Zhang, H. Kim, T. Yin, S. Fan, Q. Shen, Activation of STAT3 and Bcl-2 and reduction of reactive oxygen species (ROS) promote radioresistance in breast cancer and overcome of radioresistance with niclosamide, *Oncogene* 37 (2018) 5292–5304. <https://doi.org/10.1038/s41388-018-0340-y>.
- [110] S.S. V., B.K. Palaka, G. Vel, R. Venkatesan, D.R. Ampasala, L. Periyasamy, Identification of novel inhibitors of signal transducer and activator of transcription 3 over signal transducer and activator of transcription 1 for the treatment of breast cancer by in-silico and in-vitro approach, *Process Biochem.* 82 (2019) 153–166. <https://doi.org/10.1016/j.procbio.2019.04.018>.
- [111] A.J. Schech, A.A. Kazi, R.A. Gilani, A.H. Brodie, Zoledronic Acid Reverses the Epithelial–Mesenchymal Transition and Inhibits Self-Renewal of Breast Cancer Cells through Inactivation of NF- κ B, *Mol. Cancer Ther.* 12 (2013) 1356–1366. <https://doi.org/10.1158/1535-7163.MCT-12-0304>.
- [112] P.D. Ottewell, H. Monkkonen, M. Jones, D. V Lefley, R.E. Coleman, I. Holen, Antitumor Effects of Doxorubicin Followed by Zoledronic Acid in a Mouse Model of Breast Cancer, *JNCI J. Natl. Cancer Inst.* 100 (2008) 1167–1178. <https://doi.org/10.1093/jnci/djn240>.
- [113] T. Ishikawa, K. Akazawa, Y. Hasegawa, H. Tanino, J. Horiguchi, D. Miura, M. Hayashi, N. Kohno, Survival outcomes of neoadjuvant chemotherapy with zoledronic acid for HER2-negative breast cancer, *J. Surg. Res.* 220 (2017) 46–51. <https://doi.org/10.1016/j.jss.2017.05.066>.
- [114] M.K. Asiedu, F.D. Beauchamp-Perez, J.N. Ingle, M.D. Behrens, D.C. Radisky, K.L. Knutson, AXL induces epithelial-to-mesenchymal transition and regulates the function of breast cancer stem cells, *Oncogene* 33 (2014) 1316–1324. <https://doi.org/10.1038/onc.2013.57>.
- [115] M.-A. Goyette, R. Cusseddu, I. Elkholi, A. Abu-Thuraia, N. El-Hachem, B.

- Haibe-Kains, J.-P. Gratton, J.-F. Côté, AXL knockdown gene signature reveals a drug repurposing opportunity for a class of antipsychotics to reduce growth and metastasis of triple-negative breast cancer, *Oncotarget* 10 (2019) 2055–2067. <https://doi.org/10.18632/oncotarget.26725>.
- [116] A.J. Burke, F.J. Sullivan, F.J. Giles, S.A. Glynn, The yin and yang of nitric oxide in cancer progression, *Carcinogenesis* 34 (2013) 503–512. <https://doi.org/10.1093/carcin/bgt034>.
- [117] S. Granados-Principal, Y. Liu, M.L. Guevara, E. Blanco, D.S. Choi, W. Qian, T. Patel, A.A. Rodriguez, J. Cusimano, H.L. Weiss, H. Zhao, M.D. Landis, B. Dave, S.S. Gross, J.C. Chang, Inhibition of iNOS as a novel effective targeted therapy against triple-negative breast cancer, *Breast Cancer Res.* 17 (2015) 25. <https://doi.org/10.1186/s13058-015-0527-x>.
- [118] B. Dave, S. Granados-Principal, R. Zhu, S. Benz, S. Rabizadeh, P. Soon-Shiong, K.-D. Yu, Z. Shao, X. Li, M. Gilcrease, Z. Lai, Y. Chen, T.H.-M. Huang, H. Shen, X. Liu, M. Ferrari, M. Zhan, S.T.C. Wong, M. Kumaraswami, V. Mittal, X. Chen, S.S. Gross, J.C. Chang, Targeting RPL39 and MLF2 reduces tumor initiation and metastasis in breast cancer by inhibiting nitric oxide synthase signaling, *Proc. Natl. Acad. Sci.* 111 (2014) 8838–8843. <https://doi.org/10.1073/pnas.1320769111>.
- [119] D. Dávila-González, D.S. Choi, R.R. Rosato, S.M. Granados-Principal, J.G. Kuhn, W.-F. Li, W. Qian, W. Chen, A.J. Kozielski, H. Wong, B. Dave, J.C. Chang, Pharmacological Inhibition of NOS Activates ASK1/JNK Pathway Augmenting Docetaxel-Mediated Apoptosis in Triple-Negative Breast Cancer, *Clin. Cancer Res.* 24 (2018) 1152–1162. <https://doi.org/10.1158/1078-0432.CCR-17-1437>.
- [120] Y.S. DeRose, G. Wang, Y.-C. Lin, P.S. Bernard, S.S. Buys, M.T.W. Ebbert, R. Factor, C. Matsen, B.A. Milash, E. Nelson, L. Neumayer, R.L. Randall, I.J. Stijleman, B.E. Welm, A.L. Welm, Tumor grafts derived from women with breast cancer authentically reflect tumor pathology, growth, metastasis and disease outcomes, *Nat. Med.* 17 (2011) 1514–1520. <https://doi.org/10.1038/nm.2454>.
- [121] G.J. Yoshida, Applications of patient-derived tumor xenograft models and tumor organoids, *J. Hematol. Oncol.* 13 (2020) 4. <https://doi.org/10.1186/s13045-019-0829-z>.
- [122] M. Bleijs, M. van de Wetering, H. Clevers, J. Drost, Xenograft and organoid model systems in cancer research, *EMBO J.* 38 (2019) e101654. <https://doi.org/10.15252/embj.2019101654>.
- [123] K.P. Guillen, M. Fujita, A.J. Butterfield, S.D. Scherer, M.H. Bailey, Z. Chu, Y.S. DeRose, L. Zhao, E. Cortes-Sanchez, C.-H. Yang, J. Toner, G. Wang,

- Y. Qiao, X. Huang, J.A. Greenland, J.M. Vahrenkamp, D.H. Lum, R.E. Factor, E.W. Nelson, C.B. Matsen, J.M. Poretti, R. Rosenthal, A.C. Beck, S.S. Buys, C. Vaklavas, J.H. Ward, R.L. Jensen, K.B. Jones, Z. Li, S. Oesterreich, L.E. Dobrolecki, S.S. Pathi, X.Y. Woo, K.C. Berrett, M.E. Wadsworth, J.H. Chuang, M.T. Lewis, G.T. Marth, J. Gertz, K.E. Varley, B.E. Welm, A.L. Welm, A human breast cancer-derived xenograft and organoid platform for drug discovery and precision oncology, *Nat. Cancer* 3 (2022) 232–250. <https://doi.org/10.1038/s43018-022-00337-6>.
- [124] X.Y. Woo, J. Giordano, A. Srivastava, Z.-M. Zhao, M.W. Lloyd, R. de Bruijn, Y.-S. Suh, R. Patidar, L. Chen, S. Scherer, M.H. Bailey, C.-H. Yang, E. Cortes-Sanchez, Y. Xi, J. Wang, J. Wickramasinghe, A. V Kossenkova, V.W. Rebecca, H. Sun, R.J. Mashl, S.R. Davies, R. Jeon, C. Frech, J. Randjelovic, J. Rosains, F. Galimi, A. Bertotti, A. Lafferty, A.C. O’Farrell, E. Modave, D. Lambrechts, P. Ter Brugge, V. Serra, E. Marangoni, R. El Botty, H. Kim, J.-I. Kim, H.-K. Yang, C. Lee, D.A. 2nd Dean, B. Davis-Dusenbery, Y.A. Evrard, J.H. Doroshov, A.L. Welm, B.E. Welm, M.T. Lewis, B. Fang, J.A. Roth, F. Meric-Bernstam, M. Herlyn, M.A. Davies, L. Ding, S. Li, R. Govindan, C. Isella, J.A. Moscow, L. Trusolino, A.T. Byrne, J. Jonkers, C.J. Bult, E. Medico, J.H. Chuang, Conservation of copy number profiles during engraftment and passaging of patient-derived cancer xenografts., *Nat. Genet.* 53 (2021) 86–99. <https://doi.org/10.1038/s41588-020-00750-6>.
- [125] J.R. Whittle, M.T. Lewis, G.J. Lindeman, J.E. Visvader, Patient-derived xenograft models of breast cancer and their predictive power, *Breast Cancer Res.* 17 (2015) 17. <https://doi.org/10.1186/s13058-015-0523-1>.
- [126] J. Drost, H. Clevers, Organoids in cancer research, *Nat. Rev. Cancer* 18 (2018) 407–418. <https://doi.org/10.1038/s41568-018-0007-6>.
- [127] P. Chen, X. Zhang, R. Ding, L. Yang, X. Lyu, J. Zeng, J.H. Lei, L. Wang, J. Bi, N. Shao, D. Shu, B. Wu, J. Wu, Z. Yang, H. Wang, B. Wang, K. Xiong, Y. Lu, S. Fu, T.K. Choi, N.W. Lon, A. Zhang, D. Tang, Y. Quan, Y. Meng, K. Miao, H. Sun, M. Zhao, J. Bao, L. Zhang, X. Xu, Y. Shi, Y. Lin, C. Deng, Patient-Derived Organoids Can Guide Personalized-Therapies for Patients with Advanced Breast Cancer, *Adv. Sci.* 8 (2021) e2101176. <https://doi.org/10.1002/advs.202101176>.
- [128] N. Sachs, J. de Lig, O. Kopper, E. Gogola, G. Bounova, F. Weeber, A.V. Balgobind, K. Wind, A. Gracanin, H. Begthel, J. Korving, R. van Boxtel, A.A. Duarte, D. Lelieveld, A. van Hoeck, R.F. Ernst, F. Blokzijl, I.J. Nijman, M. Hoogstraat, M. van de Ven, D.A. Egan, V. Zinzalla, J. Moll, S.F. Boj, E.E. Voest, L. Wessels, P.J. van Diest, S. Rottenberg, R.G.J. Vries, E. Cuppen, H. Clevers, A Living Biobank of Breast Cancer Organoids Captures Disease Heterogeneity, *Cell* 172 (2018) 373–386.e10.

<https://doi.org/10.1016/j.cell.2017.11.010>.

- [129] K.K. Dijkstra, C.M. Cattaneo, F. Weeber, M. Chalabi, J. van de Haar, L.F. Fanchi, M. Slagter, D.L. van der Velden, S. Kaing, S. Kelderman, N. van Rooij, M.E. van Leerdam, A. Depla, E.F. Smit, K.J. Hartemink, R. de Groot, M.C. Wolkers, N. Sachs, P. Snaebjornsson, K. Monkhorst, J. Haanen, H. Clevers, T.N. Schumacher, E.E. Voest, Generation of Tumor-Reactive T Cells by Co-culture of Peripheral Blood Lymphocytes and Tumor Organoids, *Cell* 174 (2018) 1586-1598.e12. <https://doi.org/10.1016/j.cell.2018.07.009>.
- [130] S.D. Scherer, L. Zhao, A.J. Butterfield, C.-H. Yang, E. Cortes-Sanchez, K.P. Guillen, B.E. Welm, A.L. Welm, Breast cancer PDxO cultures for drug discovery and functional precision oncology., *STAR Protoc.* 4 (2023) 102402. <https://doi.org/10.1016/j.xpro.2023.102402>.
- [131] S. Karkampouna, F. La Manna, A. Benjak, M. Kiener, M. De Menna, E. Zoni, J. Grosjean, I. Klima, A. Garofoli, M. Bolis, A. Vallergera, J.-P. Theurillat, M.R. De Filippo, V. Genitsch, D. Keller, T.H. Booi, C.U. Stirnimann, K. Eng, A. Sboner, C.K.Y. Ng, S. Piscuoglio, P.C. Gray, M. Spahn, M.A. Rubin, G.N. Thalmann, M. Kruithof-de Julio, Patient-derived xenografts and organoids model therapy response in prostate cancer., *Nat. Commun.* 12 (2021) 1117. <https://doi.org/10.1038/s41467-021-21300-6>.
- [132] S.M. Leto, M. Ferri, F. Sassi, E.R. Zanella, F. Cottino, V. Vurchio, I. Catalano, A. Ferrero, C.C. Zingaretti, C. Marchiò, E. Grassi, L. Trusolino, A. Bertotti, Synthetic Lethal Interaction with BCL-XL Blockade Deepens Response to Cetuximab in Patient-Derived Models of Metastatic Colorectal Cancer, *Clin. Cancer Res.* 29 (2023) 1102–1113. <https://doi.org/10.1158/1078-0432.CCR-22-2550>.
- [133] Y. Aizawa, K. Takada, J. Aoyama, D. Sano, S. Yamanaka, M. Seki, Y. Kuze, J.A. Ramilowski, R. Okuda, Y. Ueno, Y. Nojima, Y. Inayama, H. Hatakeyama, T. Hatano, H. Takahashi, G. Nishimura, S. Fujii, Y. Suzuki, H. Taniguchi, N. Oridate, Establishment of experimental salivary gland cancer models using organoid culture and patient-derived xenografting, *Cell. Oncol.* 46 (2023) 409–421. <https://doi.org/10.1007/s13402-022-00758-6>.
- [134] C. Lago, A. Federico, G. Leva, N.L. Mack, B. Schwalm, C. Ballabio, M. Ganesello, L. Abballe, I. Giovannoni, S. Reddel, S. Rossi, N. Leone, A. Carai, A. Mastronuzzi, A. Bisio, A. Soldano, C. Quintarelli, F. Locatelli, M. Kool, E. Miele, L. Tiberi, Patient- and xenograft-derived organoids recapitulate pediatric brain tumor features and patient treatments., *EMBO Mol. Med.* 15 (2023) e18199. <https://doi.org/10.15252/emmm.202318199>.

- [135] L. Zhou, N. Yao, L. Yang, K. Liu, Y. Qiao, C. Huang, R. Du, Y.T. Yeung, W. Liu, D. Cheng, Z. Dong, X. Li, DUSP4 promotes esophageal squamous cell carcinoma progression by dephosphorylating HSP90 β ., *Cell Rep.* 42 (2023) 112445. <https://doi.org/10.1016/j.celrep.2023.112445>.
- [136] J. V Carter, J. Pan, S.N. Rai, S. Galandiuk, ROC-ing along: Evaluation and interpretation of receiver operating characteristic curves, *Surgery* 159 (2016) 1638–1645. <https://doi.org/10.1016/j.surg.2015.12.029>.
- [137] J. Calahorra, J.L. Blaya-Cánovas, O. Castellini-Pérez, E. Aparicio-Puerta, C. Cives-Losada, J.J.G. Marin, M. Rementeria, F.E. Cara, A. López-Tejada, C. Griñán-Lisón, F. Aulicino, I. Berger, J.A. Marchal, V. Delgado-Almenta, S. Granados-Principal, Unlocking the effective alliance of β -lapachone and hydroxytyrosol against triple-negative breast cancer cells, *Biomed. Pharmacother.* 174 (2024) 116439. <https://doi.org/10.1016/j.biopha.2024.116439>.
- [138] MERCK, Cell Proliferation Reagent WST-1, (n.d.). <https://www.sigmaaldrich.com/ES/en/product/roche/cellproro?srsId=AfmBOoqXbnYjx9AloDrQPf1ZAKRZQ0Mvo4bwnTHdNoMECKsDOYISFgai> (accessed October 29, 2024).
- [139] B. Li, C.N. Dewey, RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome, *BMC Bioinformatics* 12 (2011) 323. <https://doi.org/10.1186/1471-2105-12-323>.
- [140] S. Tarazona, P. Furió-Tarí, D. Turrà, A. Di Pietro, M.J. Nueda, A. Ferrer, A. Conesa, Data quality aware analysis of differential expression in RNA-seq with NOISeq R/Bioc package, *Nucleic Acids Res.* 43 (2015) gkv711. <https://doi.org/10.1093/nar/gkv711>.
- [141] M.I. Love, S. Anders, V. Kim, W. Huber, RNA-Seq workflow: gene-level exploratory analysis and differential expression, *F1000Research* 4 (2015) 1070. <https://doi.org/10.12688/f1000research.7035.1>.
- [142] Immunostep, Immunistep. DY634 Annexin V Apoptosis Detection Kit with PI, (2024). <https://immunostep.com/es/product/dy634-annexin-v-apoptosis-detection-kit-with-pi/> (accessed July 20, 2024).
- [143] M. Ivanova, F.M. Porta, M. D’Ercole, C. Pescia, E. Sajjadi, G. Cursano, E. De Camilli, O. Pala, G. Mazzarol, K. Venetis, E. Guerini-Rocco, G. Curigliano, G. Viale, N. Fusco, Standardized pathology report for HER2 testing in compliance with 2023 ASCO/CAP updates and 2023 ESMO consensus statements on HER2-low breast cancer, *Virchows Arch.* 484 (2024) 3–14. <https://doi.org/10.1007/s00428-023-03656-w>.
- [144] E. Campaner, A. Zannini, M. Santorsola, D. Bonazza, C. Bottin, V. Cancila, C. Tripodo, M. Bortul, F. Zanconati, S. Schoeftner, G. Del Sal, Breast

- Cancer Organoids Model Patient-Specific Response to Drug Treatment, *Cancers* (Basel). 12 (2020) 3869. <https://doi.org/10.3390/cancers12123869>.
- [145] ThermoFisher Scientific, Reactivo de viabilidad celular HS alamarBlueTM, (n.d.). <https://www.thermofisher.com/order/catalog/product/es/es/A50101> (accessed September 30, 2024).
- [146] J. Shi, Y. Wang, L. Zeng, Y. Wu, J. Deng, Q. Zhang, Y. Lin, J. Li, T. Kang, M. Tao, E. Rusinova, G. Zhang, C. Wang, H. Zhu, J. Yao, Y.-X. Zeng, B.M. Evers, M.-M. Zhou, B.P. Zhou, Disrupting the Interaction of BRD4 with Diacetylated Twist Suppresses Tumorigenesis in Basal-like Breast Cancer, *Cancer Cell* 25 (2014) 210–225. <https://doi.org/10.1016/j.ccr.2014.01.028>.
- [147] Y. Katsuno, D.S. Meyer, Z. Zhang, K.M. Shokat, R.J. Akhurst, K. Miyazono, R. Derynck, Chronic TGF- β exposure drives stabilized EMT, tumor stemness, and cancer drug resistance with vulnerability to bitopic mTOR inhibition, *Sci. Signal.* 12 (2019). <https://doi.org/10.1126/scisignal.aau8544>.
- [148] H. Zhou, J. Wu, T. Wang, X. Zhang, D. Liu, CXCL10/CXCR3 axis promotes the invasion of gastric cancer via PI3K/AKT pathway-dependent MMPs production, *Biomed. Pharmacother.* 82 (2016) 479–488. <https://doi.org/10.1016/j.biopha.2016.04.069>.
- [149] Z. Wang, X. Ao, Z. Shen, L. Ao, X. Wu, C. Pu, W. Guo, W. Xing, M. He, H. Yuan, J. Yu, L. Li, X. Xu, Tnf- α augments cxcl10/cxcr3 axis activity to induce epithelial-mesenchymal transition in colon cancer cell, *Int. J. Biol. Sci.* 17 (2021) 2683–2702. <https://doi.org/10.7150/ijbs.61350>.
- [150] E. Tsutsumi, J. Stricklin, E.A. Peterson, J.A. Schroeder, S. Kim, Cxcl10 Chemokine Induces Migration of ING4-Deficient Breast Cancer Cells via a Novel Cross Talk Mechanism between the Cxcr3 and Egfr Receptors, *Mol. Cell. Biol.* 42 (2022). <https://doi.org/10.1128/mcb.00382-21>.
- [151] Y. Li, H. Zhang, Y. Merkher, L. Chen, N. Liu, S. Leonov, Y. Chen, Recent advances in therapeutic strategies for triple-negative breast cancer, *J. Hematol. Oncol.* 15 (2022) 121. <https://doi.org/10.1186/s13045-022-01341-0>.
- [152] E.S. Villodre, X. Hu, B.L. Eckhardt, R. Larson, L. Huo, E.C. Yoon, Y. Gong, J. Song, S. Liu, N.T. Ueno, S. Krishnamurthy, S. Pusch, D. Tripathy, W.A. Woodward, B.G. Debeb, NDRG1 in Aggressive Breast Cancer Progression and Brain Metastasis, *JNCI J. Natl. Cancer Inst.* 114 (2022) 579–591. <https://doi.org/10.1093/jnci/djab222>.

- [153] A. Mishra, M. Bonello, A. Byron, S.P. Langdon, A.H. Sims, Evaluation of Gene Expression Data From Cybrids and Tumours Highlights Elevated NDRG1 -Driven Proliferation in Triple-Negative Breast Cancer, *Breast Cancer Basic Clin. Res.* 14 (2020) 1178223420934447. <https://doi.org/10.1177/1178223420934447>.
- [154] E.S. Villodre, Y. Gong, X. Hu, L. Huo, E.C. Yoon, N.T. Ueno, W.A. Woodward, D. Tripathy, J. Song, B.G. Debeb, NDRG1 Expression Is an Independent Prognostic Factor in Inflammatory Breast Cancer, *Cancers (Basel)*. 12 (2020) 3711. <https://doi.org/10.3390/cancers12123711>.
- [155] C.J. Sevinsky, F. Khan, L. Kokabee, A. Darehshouri, K.R. Maddipati, D.S. Conklin, NDRG1 regulates neutral lipid metabolism in breast cancer cells, *Breast Cancer Res.* 20 (2018) 55. <https://doi.org/10.1186/s13058-018-0980-4>.
- [156] S. Konstantinovskiy, Y. Smith, S. Zilber, H. Tuft Stavnes, A.-M. Becker, J.M. Nesland, R. Reich, B. Davidson, Breast Carcinoma Cells in Primary Tumors and Effusions Have Different Gene Array Profiles, *J. Oncol.* 2010 (2010) 1–14. <https://doi.org/10.1155/2010/969084>.
- [157] V. Das, S. Bhattacharya, C. Chikkaputtaiah, S. Hazra, M. Pal, The basics of epithelial–mesenchymal transition (EMT): A study from a structure, dynamics, and functional perspective, *J. Cell. Physiol.* 234 (2019) 14535–14555. <https://doi.org/10.1002/jcp.28160>.
- [158] M.G. Mendez, S.-I. Kojima, R.D. Goldman, Vimentin induces changes in cell shape, motility, and adhesion during the epithelial to mesenchymal transition, *FASEB J.* 24 (2010) 1838–1851. <https://doi.org/10.1096/fj.09-151639>.
- [159] A. Grosse-Wilde, A. Fouquier d’Hérouël, E. McIntosh, G. Ertaylan, A. Skupin, R.E. Kuestner, A. del Sol, K.-A. Walters, S. Huang, Stemness of the hybrid Epithelial/Mesenchymal State in Breast Cancer and Its Association with Poor Survival, *PLoS One* 10 (2015) e0126522. <https://doi.org/10.1371/journal.pone.0126522>.
- [160] C. Kröger, A. Afeyan, J. Mraz, E.N. Eaton, F. Reinhardt, Y.L. Khodor, P. Thiru, B. Bieri, X. Ye, C.B. Burge, R.A. Weinberg, Acquisition of a hybrid E/M state is essential for tumorigenicity of basal breast cancer cells, *Proc. Natl. Acad. Sci.* 116 (2019) 7353–7362. <https://doi.org/10.1073/pnas.1812876116>.
- [161] P. Gener, J. Seras-Franzoso, P.G. Callejo, F. Andrade, D. Rafael, F. Martínez, S. Montero, D. Arango, J. Sayós, I. Abasolo, S. Schwartz, Dynamism, Sensitivity, and Consequences of Mesenchymal and Stem-Like Phenotype of Cancer Cells, *Stem Cells Int.* 2018 (2018) 1–12. <https://doi.org/10.1155/2018/4516454>.

- [162] A.S. Berghoff, Y. Liao, M.A. Karreman, A. Ilhan-Mutlu, K. Gunkel, M.R. Sprick, C. Eisen, T. Kessler, M. Osswald, S. Wünsche, M. Feinauer, B. Gril, F. Marmé, L.L. Michel, Z. Bago-Horvath, F. Sahm, N. Becker, M.O. Breckwoldt, G. Solecki, M. Gömmel, L. Huang, P. Rübmann, C.M. Thome, M. Ratliff, A. Trumpp, P.S. Steeg, M. Preusser, W. Wick, F. Winkler, Identification and Characterization of Cancer Cells That Initiate Metastases to the Brain and Other Organs, *Mol. Cancer Res.* 19 (2021) 688–701. <https://doi.org/10.1158/1541-7786.MCR-20-0863>.
- [163] K.A. Snyder, M.R. Hughes, B. Hedberg, J. Brandon, D.C. Hernaez, P. Bergqvist, F. Cruz, K. Po, M.L. Graves, M.E. Turvey, J.S. Nielsen, J.A. Wilkins, S.R. McColl, J.S. Babcook, C.D. Roskelley, K.M. McNagny, Podocalyxin enhances breast tumor growth and metastasis and is a target for monoclonal antibody therapy, *Breast Cancer Res.* 17 (2015) 46. <https://doi.org/10.1186/s13058-015-0562-7>.
- [164] Y. Zhang, C. Yu, Prognostic characterization of OAS1/OAS2/OAS3/OASL in breast cancer., *BMC Cancer* 20 (2020) 575. <https://doi.org/10.1186/s12885-020-07034-6>.
- [165] N. Marino, J.W. Collins, C. Shen, N.J. Caplen, A.S. Merchant, Y. Gökmen-Polar, C.P. Goswami, T. Hoshino, Y. Qian, G.W.J. Sledge, P.S. Steeg, Identification and validation of genes with expression patterns inverse to multiple metastasis suppressor genes in breast cancer cell lines., *Clin. Exp. Metastasis* 31 (2014) 771–786. <https://doi.org/10.1007/s10585-014-9667-0>.
- [166] J. Li, S. Zheng, B. Chen, A.J. Butte, S.J. Swamidass, Z. Lu, A survey of current trends in computational drug repositioning., *Brief. Bioinform.* 17 (2016) 2–12. <https://doi.org/10.1093/bib/bbv020>.
- [167] Y. Huang, S. Wang, X. Zhang, C. Yang, S. Wang, H. Cheng, A. Ke, C. Gao, K. Guo, Identification of Fasudil as a collaborator to promote the anti-tumor effect of lenvatinib in hepatocellular carcinoma by inhibiting GLI2-mediated hedgehog signaling pathway, *Pharmacol. Res.* 200 (2024) 107082. <https://doi.org/10.1016/j.phrs.2024.107082>.
- [168] W. Jiang, X. Wang, C. Zhang, L. Xue, L. Yang, Expression and clinical significance of MAPK and EGFR in triple-negative breast cancer., *Oncol. Lett.* 19 (2020) 1842–1848. <https://doi.org/10.3892/ol.2020.11274>.
- [169] L. Hughes-Davies, D. Huntsman, M. Ruas, F. Fuks, J. Bye, S.-F. Chin, J. Milner, L.A. Brown, F. Hsu, B. Gilks, T. Nielsen, M. Schulzer, S. Chia, J. Ragaz, A. Cahn, L. Linger, H. Ozdag, E. Cattaneo, E.S. Jordanova, E. Schuurin, D.S. Yu, A. Venkitaraman, B. Ponder, A. Doherty, S. Aparicio, D. Bentley, C. Theillet, C.P. Ponting, C. Caldas, T. Kouzarides, EMSY links the BRCA2 pathway to sporadic breast and ovarian cancer., *Cell* 115

- (2003) 523–535. [https://doi.org/10.1016/s0092-8674\(03\)00930-9](https://doi.org/10.1016/s0092-8674(03)00930-9).
- [170] K. Regulska, M. Regulski, B. Karolak, M. Murias, B. Stanis, Can cardiovascular drugs support cancer treatment? The rationale for drug repurposing., *Drug Discov. Today* 24 (2019) 1059–1065. <https://doi.org/10.1016/j.drudis.2019.03.010>.
- [171] X. Geng, F. Wang, D. Tian, L. Huang, E. Streater, J. Zhu, H. Kurihara, R. He, X. Yao, Y. Zhang, J. Tang, Cardiac glycosides inhibit cancer through Na/K-ATPase-dependent cell death induction, *Biochem. Pharmacol.* 182 (2020) 114226. <https://doi.org/10.1016/j.bcp.2020.114226>.
- [172] C.-M. Xie, W.Y. Chan, S. Yu, J. Zhao, C.H.K. Cheng, Bufalin induces autophagy-mediated cell death in human colon cancer cells through reactive oxygen species generation and JNK activation., *Free Radic. Biol. Med.* 51 (2011) 1365–1375. <https://doi.org/10.1016/j.freeradbiomed.2011.06.016>.
- [173] M. Tailler, L. Senovilla, E. Lainey, S. Thépot, D. Métivier, M. Sébert, V. Baud, K. Billot, P. Fenaux, L. Galluzzi, S. Boehrer, G. Kroemer, O. Kepp, Antineoplastic activity of ouabain and pyrithione zinc in acute myeloid leukemia., *Oncogene* 31 (2012) 3536–3546. <https://doi.org/10.1038/onc.2011.521>.
- [174] W. Zhao, G. Li, Q. Zhang, M. Chen, L. He, Z. Wu, Y. Zhang, M. Fan, Y. Liang, W. Zhang, F. Zeng, F. Deng, Cardiac glycoside neriifolin exerts anti-cancer activity in prostate cancer cells by attenuating DNA damage repair through endoplasmic reticulum stress., *Biochem. Pharmacol.* 209 (2023) 115453. <https://doi.org/10.1016/j.bcp.2023.115453>.
- [175] X. Li, J. Zheng, S. Chen, F. Meng, J. Ning, S. Sun, Oleandrin, a cardiac glycoside, induces immunogenic cell death via the PERK/eIF2 α /ATF4/CHOP pathway in breast cancer, *Cell Death Dis.* 12 (2021) 314. <https://doi.org/10.1038/s41419-021-03605-y>.
- [176] M.A. Khajah, P.M. Mathew, Y.A. Luqmani, Na⁺/K⁺ ATPase activity promotes invasion of endocrine resistant breast cancer cells., *PLoS One* 13 (2018) e0193779. <https://doi.org/10.1371/journal.pone.0193779>.
- [177] S. Haque, J.C. Morris, Transforming growth factor- β : A therapeutic target for cancer, *Hum. Vaccin. Immunother.* 13 (2017) 1741–1750. <https://doi.org/10.1080/21645515.2017.1327107>.
- [178] D.-D. Li, L. Deng, S.-Y. Hu, F.-L. Zhang, D.-Q. Li, SH3BGR2 exerts a dual function in breast cancer growth and metastasis and is regulated by TGF- β 1, *Am. J. Cancer Res.* 10 (2020) 1238–1254. <http://www.ncbi.nlm.nih.gov/pubmed/32368399>.
- [179] C.-Y. Park, K.N. Min, J.-Y. Son, S.-Y. Park, J.-S. Nam, D.-K. Kim, Y.Y. Sheen,

- An novel inhibitor of TGF- β type I receptor, IN-1130, blocks breast cancer lung metastasis through inhibition of epithelial–mesenchymal transition, *Cancer Lett.* 351 (2014) 72–80. <https://doi.org/10.1016/j.canlet.2014.05.006>.
- [180] F. Maggiolo, Efavirenz., *Expert Opin. Pharmacother.* 8 (2007) 1137–1145. <https://doi.org/10.1517/14656566.8.8.1137>.
- [181] J. Proska, S. Tucek, Positive allosteric action of eburnamonine on cardiac muscarinic acetylcholine receptors., *Eur. J. Pharmacol.* 305 (1996) 201–205. [https://doi.org/10.1016/0014-2999\(96\)00169-0](https://doi.org/10.1016/0014-2999(96)00169-0).
- [182] J. Jakubík, L. Bačáková, E.E. El-Fakahany, S. Tuček, Positive cooperativity of acetylcholine and other agonists with allosteric ligands on muscarinic acetylcholine receptors, *Mol. Pharmacol.* 52 (1997) 172–179. <https://doi.org/10.1124/mol.52.1.172>.
- [183] P.-T. Chiou, S. Ohms, P.G. Board, J.E. Dahlstrom, D. Rangasamy, M.G. Casarotto, Efavirenz as a potential drug for the treatment of triple-negative breast cancers, *Clin. Transl. Oncol.* 23 (2021) 353–363. <https://doi.org/10.1007/s12094-020-02424-5>.
- [184] P.-T. Chiou, S. Ohms, P.G. Board, J.E. Dahlstrom, D. Rangasamy, M.G. Casarotto, The Antiviral Drug Efavirenz in Breast Cancer Stem Cell Therapy, *Cancers (Basel)*. 13 (2021) 6232. <https://doi.org/10.3390/cancers13246232>.
- [185] J. Shen, Y. Zhan, H. Li, Z. Wang, Ouabain impairs cancer metabolism and activates AMPK-Src signaling pathway in human cancer cell lines, *Acta Pharmacol. Sin.* 41 (2020) 110–118. <https://doi.org/10.1038/s41401-019-0290-0>.
- [186] K. Winnicka, K. Bielawski, A. Bielawska, W. Mityk, Apoptosis-mediated cytotoxicity of ouabain, digoxin and proscillaridin A in the estrogen independent MDA-MB-231 breast cancer cells., *Arch. Pharm. Res.* 30 (2007) 1216–1224. <https://doi.org/10.1007/BF02980262>.
- [187] D.C. Gunasekara, M.M. Zheng, T. Mojtahed, J.R. Woods, T.E. Fandy, M. V. Riofski, C.A. Glackin, H.E. Hassan, J. Kirshner, D.A. Colby, 15-Methylene-Eburnamonine Kills Leukemic Stem Cells and Reduces Engraftment in a Humanized Bone Marrow Xenograft Mouse Model of Leukemia, *ChemMedChem* 11 (2016) 2392–2397. <https://doi.org/10.1002/cmdc.201600334>.
- [188] I. Laverdière, M. Boileau, A.L. Neumann, H. Frison, A. Mitchell, S.W.K. Ng, J.C.Y. Wang, M.D. Minden, K. Eppert, Leukemic stem cell signatures identify novel therapeutics targeting acute myeloid leukemia, *Blood Cancer J.* 8 (2018) 52. <https://doi.org/10.1038/s41408-018-0087-2>.

- [189] J. Deng, Q. Zhang, L. Lv, P. Ma, Y. Zhang, N. Zhao, Y. Zhang, Identification of an autophagy-related gene signature for predicting prognosis and immune activity in pancreatic adenocarcinoma, *Sci. Rep.* 12 (2022) 7006. <https://doi.org/10.1038/s41598-022-11050-w>.
- [190] X. Wu, J. Li, T. Yan, X. Ke, X. Li, Y. Zhu, J. Yang, Z. Li, HOXB7 acts as an oncogenic biomarker in head and neck squamous cell carcinoma, *Cancer Cell Int.* 21 (2021) 393. <https://doi.org/10.1186/s12935-021-02093-6>.
- [191] M. V. Riofski, J.P. John, M.M. Zheng, J. Kirshner, D.A. Colby, Exploiting the facile release of trifluoroacetate for the α -methylenation of the sterically hindered carbonyl groups on (+)-sclareolide and (-)-eburnamonine, *J. Org. Chem.* 76 (2011) 3676–3683. <https://doi.org/10.1021/jo102114f>.
- [192] A. Argaw-Denboba, E. Balestrieri, A. Serafino, C. Cipriani, I. Bucci, R. Sorrentino, I. Sciamanna, A. Gambacurta, P. Sinibaldi-Vallebona, C. Matteucci, HERV-K activation is strictly required to sustain CD133+ melanoma cells with stemness features, *J. Exp. Clin. Cancer Res.* 36 (2017) 20. <https://doi.org/10.1186/s13046-016-0485-x>.
- [193] Y. Xiao, C. Meng, J. Lin, C. Huang, X. Zhang, Y. Long, Y. Huang, Y. Lin, Ouabain targets the Na⁺/K⁺-ATPase α 3 isoform to inhibit cancer cell proliferation and induce apoptosis, *Oncol. Lett.* 14 (2017) 6678–6684. <https://doi.org/10.3892/ol.2017.7070>.
- [194] J. Poohadsuan, G.A. O'Doherty, W. Owattanapanich, S. Kungwankiatichai, Y. Rojanasakul, S. Issaragrisil, S. Luanpitpong, Cardiac glycoside ouabain efficiently targets leukemic stem cell apoptotic machinery independent of cell differentiation status, *Cell Commun. Signal.* 21 (2023) 283. <https://doi.org/10.1186/s12964-023-01317-8>.
- [195] W. Guo, B. Wei, T. Cheng, X. Xu, F. Ruan, M. Xiang, The Na⁺/K⁺ ATPase Inhibitor Ouabain Attenuates Stemness and Chemoresistance of Osteosarcoma Cells, *Med. Sci. Monit.* 25 (2019) 9426–9434. <https://doi.org/10.12659/MSM.919266>.
- [196] H.-Y. Chen, M.-D. Yang, Y.-C. Chou, Y.-S. Ma, S.-F. Peng, C.-L. Liao, P.-Y. Chen, T.-C. Hsia, J.-C. Lien, C.-H. Chen, Ouabain Suppresses Cell Migration and Invasion in Human Gastric Cancer AGS Cells Through the Inhibition of MMP Signaling Pathways, *Anticancer Res.* 41 (2021) 4365–4375. <https://doi.org/10.21873/anticancer.15241>.
- [197] V. Pongrakhananon, P. Chunhacha, P. Chanvorachote, Ouabain suppresses the migratory behavior of lung cancer cells, *PLoS One* 8 (2013) e68623. <https://doi.org/10.1371/journal.pone.0068623>.

- [198] O.-S. Kwon, H. Lee, H.-J. Kong, E.-J. Kwon, J.E. Park, W. Lee, S. Kang, M. Kim, W. Kim, H.-J. Cha, Connectivity map-based drug repositioning of bortezomib to reverse the metastatic effect of GALNT14 in lung cancer, *Oncogene* 39 (2020) 4567–4580. <https://doi.org/10.1038/s41388-020-1316-2>.
- [199] M. Hecht, T. Harrer, M. Büttner, M. Schwegler, S. Erber, R. Fietkau, L. V Distel, Cytotoxic effect of efavirenz is selective against cancer cells and associated with the cannabinoid system, *AIDS* 27 (2013) 2031–2040. <https://doi.org/10.1097/QAD.0b013e3283625444>.
- [200] X.-S. Yang, Z. Xu, T.-L. Yi, R. Xu, J. Li, W. Zhang, S. Zhang, H.-T. Sun, Z.-Q. Yu, H.-X. Xu, Y. Tu, S.-X. Cheng, Ouabain suppresses the growth and migration abilities of glioma U-87MG cells through inhibiting the Akt/mTOR signaling pathway and downregulating the expression of HIF-1 α , *Mol. Med. Rep.* 17 (2018) 5595–5600. <https://doi.org/10.3892/mmr.2018.8587>.
- [201] H. Kusumoto, Y. Shintani, R. Kanzaki, T. Kawamura, S. Funaki, M. Minami, I. Nagatomo, E. Morii, M. Okumura, Podocalyxin influences malignant potential by controlling epithelial–mesenchymal transition in lung adenocarcinoma, *Cancer Sci.* 108 (2017) 528–535. <https://doi.org/https://doi.org/10.1111/cas.13142>.
- [202] G.J. Seo, A. Yang, B. Tan, S. Kim, Q. Liang, Y. Choi, W. Yuan, P. Feng, H.-S. Park, J.U. Jung, Akt Kinase-Mediated Checkpoint of cGAS DNA Sensing Pathway, *Cell Rep.* 13 (2015) 440–449. <https://doi.org/https://doi.org/10.1016/j.celrep.2015.09.007>.
- [203] D. Luo, M. Fang, L. Shao, J. Wang, Y. Liang, M. Chen, X. Gui, J. Yan, W. Wang, L. Yu, X. Fan, Q. Wu, The EMT-Related Genes GALNT3 and OAS1 are Associated with Immune Cell Infiltration and Poor Prognosis in Lung Adenocarcinoma, *Front. Biosci.* 28 (2023) 271. <https://doi.org/10.31083/j.fbl2810271>.
- [204] J. Du, L. Jiang, F. Chen, H. Hu, M. Zhou, Cardiac Glycoside Ouabain Exerts Anticancer Activity via Downregulation of STAT3, *Front. Oncol.* 11 (2021) 684316. <https://doi.org/10.3389/fonc.2021.684316>.
- [205] J. Zhou, J. Wulfkühle, H. Zhang, P. Gu, Y. Yang, J. Deng, J.B. Margolick, L.A. Liotta, E. 3rd Petricoin, Y. Zhang, Activation of the PTEN/mTOR/STAT3 pathway in breast cancer stem-like cells is required for viability and maintenance., *Proc. Natl. Acad. Sci. U. S. A.* 104 (2007) 16158–16163. <https://doi.org/10.1073/pnas.0702596104>.
- [206] A. Dubrowska, S. Kim, R.J. Salamone, J.R. Walker, S.-M. Maira, C. García-Echeverría, P.G. Schultz, V.A. Reddy, The role of PTEN/Akt/PI3K signaling in the maintenance and viability of prostate cancer stem-like cell

- populations., *Proc. Natl. Acad. Sci. U. S. A.* 106 (2009) 268–273. <https://doi.org/10.1073/pnas.0810956106>.
- [207] L. Yang, P. Shi, G. Zhao, J. Xu, W. Peng, J. Zhang, G. Zhang, X. Wang, Z. Dong, F. Chen, H. Cui, Targeting cancer stem cell pathways for cancer therapy., *Signal Transduct. Target. Ther.* 5 (2020) 8. <https://doi.org/10.1038/s41392-020-0110-5>.
- [208] M. Nedeljković, A. Damjanović, Mechanisms of Chemotherapy Resistance in Triple-Negative Breast Cancer—How We Can Rise to the Challenge, *Cells* 8 (2019) 957. <https://doi.org/10.3390/cells8090957>.
- [209] J.M. Rosenbluth, R.C.J. Schackmann, G.K. Gray, L.M. Selfors, C.M.-C. Li, M. Boedicker, H.J. Kuiken, A. Richardson, J. Brock, J. Garber, D. Dillon, N. Sachs, H. Clevers, J.S. Brugge, Organoid cultures from normal and cancer-prone human breast tissues preserve complex epithelial lineages, *Nat. Commun.* 11 (2020) 1711. <https://doi.org/10.1038/s41467-020-15548-7>.
- [210] S. Granados-Principal, A. Daddaoua, O. Martinez-Agustin, Capítulo 10: La célula como factoría biotecnológica y herramienta terapéutica: cultivo in vitro de células de mamífero, in: *AVICAM (Ed.), Man. Biotecnol. Para El Grado Farm.*, 2022: pp. 175–196.
- [211] G. V Echeverria, S. Cai, Y. Tu, J. Shao, E. Powell, A.B. Redwood, Y. Jiang, A. McCoy, A.L. Rinkenbaugh, R. Lau, A.J. Trevarton, C. Fu, R. Gould, E.E. Ravenberg, L. Huo, R. Candelaria, L. Santiago, B.E. Adrada, D.L. Lane, G.M. Rauch, W.T. Yang, J.B. White, J.T. Chang, S.L. Moulder, W.F. Symmans, S.G. Hilsenbeck, H. Piwnica-Worms, Predictors of success in establishing orthotopic patient-derived xenograft models of triple negative breast cancer, *Npj Breast Cancer* 9 (2023) 2. <https://doi.org/10.1038/s41523-022-00502-1>.
- [212] H.S. Park, J.D. Lee, J.Y. Kim, S. Park, J.H. Kim, H.J. Han, Y.A. Choi, A.R. Choi, J.H. Sohn, S. Il Kim, Establishment of chemosensitivity tests in triple-negative and BRCA-mutated breast cancer patient-derived xenograft models, *PLoS One* 14 (2019) e0225082. <https://doi.org/10.1371/journal.pone.0225082>.
- [213] J. Massé, C. Truntzer, R. Boidot, E. Khalifa, G. Pérot, V. Velasco, L. Mayeur, C. Billerey-Larmonier, L. Blanchard, H. Charitansky, I. Soubeyran, R. Iggo, L. Arnould, G. MacGrogan, Solid-type adenoid cystic carcinoma of the breast, a distinct molecular entity enriched in NOTCH and CREBBP mutations, *Mod. Pathol.* 33 (2020) 1041–1055. <https://doi.org/10.1038/s41379-019-0425-3>.
- [214] J.E. Altman, A.L. Olex, E.K. Zboril, C.J. Walker, D.C. Boyd, R.K. Myrick, N.S. Hairr, J.E. Koblinski, M. Puchalapalli, B. Hu, M.G. Dozmorov, X.S. Chen, Y.

- Chen, C.M. Perou, B.D. Lehmann, J.E. Visvader, J.C. Harrell, Single-cell transcriptional atlas of human breast cancers and model systems., *Clin. Transl. Med.* 14 (2024) e70044. <https://doi.org/10.1002/ctm2.70044>.
- [215] O. Saatci, A. Kaymak, U. Raza, P.G. Ersan, O. Akbulut, C.E. Banister, V. Sikirzhytski, U.M. Tokat, G. Aykut, S.A. Ansari, H.T. Dogan, M. Dogan, P. Jandaghi, A. Isik, F. Gundogdu, K. Kosemehmetoglu, O. Dizdar, S. Aksoy, A. Akyol, A. Uner, P.J. Buckhaults, Y. Riazalhosseini, O. Sahin, Targeting lysyl oxidase (LOX) overcomes chemotherapy resistance in triple negative breast cancer, *Nat. Commun.* 11 (2020) 2416. <https://doi.org/10.1038/s41467-020-16199-4>.
- [216] M. Cetin, O. Saatci, A.-H. Rezaeian, C.N. Rao, C. Beneker, K. Sreenivas, H. Taylor, B. Pederson, I. Chatzistamou, B. Buckley, S. Lessner, P. Angel, C. McInnes, O. Sahin, A highly potent bi-thiazole inhibitor of LOX rewires collagen architecture and enhances chemoresponse in triple-negative breast cancer, *Cell Chem. Biol.* (2024). <https://doi.org/10.1016/j.chembiol.2024.06.012>.
- [217] I. Segatto, M.C. Mattevi, G.L. Rampioni Vinciguerra, N. Crestan, L. Musco, A. Favero, A. Dall'Acqua, G. Di Giustino, G. Mungo, S. D'Andrea, C. Gava, F. Ruggiero, M. Dugo, L. Gerratana, F. Puglisi, S. Massarut, R. Bomben, M. Callari, T. Perin, G. Baldassarre, B. Belletti, A comprehensive luminal breast cancer patient-derived xenografts (PDX) library to capture tumor heterogeneity and explore the mechanisms of resistance to CDK4/6 inhibitors, *J. Pathol.* 264 (2024) 434–447. <https://doi.org/10.1002/path.6358>.
- [218] J.L. Blaya-Cánovas, C. Griñán-Lisón, I. Blancas, J.A. Marchal, C. Ramírez-Tortosa, A. López-Tejada, K. Benabdellah, M. Cortijo-Gutiérrez, M.V. Cano-Cortés, P. Graván, S.A. Navarro-Marchal, J. Gómez-Morales, V. Delgado-Almenta, J. Calahorra, M. Agudo-Lera, A. Sagarzazu, C.J. Rodríguez-González, T. Gallart-Aragón, C. Eich, R.M. Sánchez-Martín, S. Granados-Principal, Autologous patient-derived exhausted nano T-cells exploit tumor immune evasion to engage an effective cancer therapy, *Mol. Cancer* 23 (2024) 83. <https://doi.org/10.1186/s12943-024-01997-x>.
- [219] R. Yerushalmi, R. Woods, P.M. Ravdin, M.M. Hayes, K.A. Gelmon, Ki67 in breast cancer: prognostic and predictive potential., *Lancet. Oncol.* 11 (2010) 174–183. [https://doi.org/10.1016/S1470-2045\(09\)70262-1](https://doi.org/10.1016/S1470-2045(09)70262-1).
- [220] M.A. Rudek, C.Y. Chang, K. Steadman, M.D. Johnson, N. Desai, J.F. Deeken, Combination antiretroviral therapy (cART) component ritonavir significantly alters docetaxel exposure, *Cancer Chemother. Pharmacol.* 73 (2014) 729–736. <https://doi.org/10.1007/s00280-014-2399-7>.
- [221] A.M. Katz, Effects of digitalis on cell biochemistry: Sodium pump

- inhibition, *J. Am. Coll. Cardiol.* 5 (1985) 16A-21A. [https://doi.org/10.1016/S0735-1097\(85\)80459-9](https://doi.org/10.1016/S0735-1097(85)80459-9).
- [222] K. Karaś, A. Sałkowska, J. Dastyh, R.A. Bachorz, M. Ratajewski, Cardiac glycosides with target at direct and indirect interactions with nuclear receptors, *Biomed. Pharmacother.* 127 (2020) 110106. <https://doi.org/10.1016/j.biopha.2020.110106>.
- [223] C. Marzolini, A. Telenti, L.A. Decosterd, G. Greub, J. Biollaz, T. Buclin, Efavirenz plasma levels can predict treatment failure and central nervous system side effects in HIV-1-infected patients, *AIDS* 15 (2001) 71–75. <https://doi.org/10.1097/00002030-200101050-00011>.
- [224] L.F. López-Cortés, R. Ruiz-Valderas, A. Marín-Niebla, R. Pascual-Carrasco, M. Rodríguez-Díez, M.J. Lucero-Muñoz, Therapeutic drug monitoring of efavirenz: trough levels cannot be estimated on the basis of earlier plasma determinations, *J. Acquir. Immune Defic. Syndr.* 39 (2005) 551–6. <http://www.ncbi.nlm.nih.gov/pubmed/16044006>.