

Internship Report - Brisken Lab
Laidlaw Scholars Leadership and Research Program
June-August 2026

The response of Estrogen receptor-positive breast cancer micrometastatic lesions to endocrine therapy

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1. Abstract

This project aims to understand how ER+ breast cancer micrometastatic lesions persist under endocrine therapy, using in vitro, ex vivo, and MIND-model approaches.

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2. Introduction

2.1 Purpose

According to the WHO, in 2024, there were an estimated 2.4 million women diagnosed with breast cancer and 694 000 deaths globally[1]. The most frequent breast cancer cases are hormone receptor (HR)+, in particular Estrogen receptor-positive (ER+), accounting for 75% of all breast cancers[2]. It represents a critical public health issue. Although HR+ has a better prognosis than other types of breast cancer (such as triple-negative, which does not express Estrogen receptor, progesterone receptor and HER2 receptor), the survival rates of ER+ breast cancer patients decrease 5 to 20 years after diagnosis, as many patients develop late recurrence (>10years after diagnosis) due to minimal residual disease and metastatic relapse[3]. By understanding how micrometastatic lesions respond to endocrine therapy, we can understand how they persist under treatment and identify some potential therapy targets to eliminate these persistent cells and prevent metastatic recurrence.

2.2 Background

Estrogen receptor-positive (ER+) breast cancer (BC) is the most common subtype characterized by cells expressing the estrogen receptor. Estrogen activates proliferation of breast cancer cells[4]. These cells spread as dormant disseminated tumour cells by breaking away from the primary tumour site, reaching other areas of the body (bone, brain, lungs, liver, ovaries, etc) and seed as single cells or micrometastatic lesions for decades before giving rise to macrometastasis[5].

ER+ BC patients receive long-term (5-10 years) endocrine therapy to prevent disease progression and recurrence. However, a subset of patients still develop recurrent disease (>5 years post diagnosis). It is unknown how micrometastatic lesions respond to endocrine therapy in patients as they are difficult to detect[6].

To understand these responses, Mouse INtraDuctal MIND models are used to study ER+ BC progression (Figure 1)[7]. Different mouse models exist, the method of injection of the studied tumour or metastasis cells affects the cancer's dissemination (for example injecting in a fat-pad or at the tail vein). To physiologically resemble spontaneous metastasis in patients, employing the MIND models (mouse induced intraductal model) with ER+ tumour cells grafted into the milk ducts of NSG (highly immunocompromised) mice produces a more accurate, slower progression of smaller metastasis.

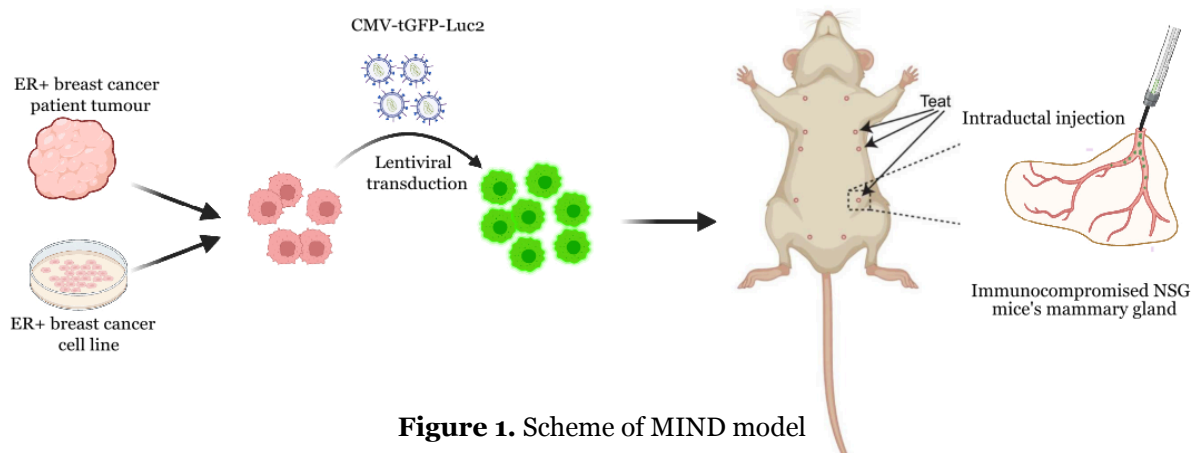


Figure 1. Scheme of MIND model

3. Methods and materials, research design description

3.1 Culture and qPCR analysis of metastatic cells under therapy

Cells from an ER+ breast cancer cell line (MCF7) were cultured and grown under hormone-deprived conditions to mimic endocrine therapy. In patients, aromatase inhibitors (AI) are used as hormonal therapy drugs in post-menopausal women that block the enzyme aromatase from converting androgens into estrogen[8]. The aim is to validate previous in vivo findings (in MIND models) in which micrometastatic lesions responded to estrogen depletion but were persistent. It was found that BRD2 and inflammatory-associated gene expression were increased upon estrogen depletion.

An in vitro model with two conditions was used. The first condition is hormone-deprived and mimics endocrine therapy, similarly to how estrogen is not produced anymore in patients. The medium contains Dimethylsulfoxide (DMSO) and Charcoal striped serum (CSS). The second condition mimics the absence of treatment and is supplemented with estrogen (10 nM of estradiol E2) resuspended in DMSO.

The experiment was reproduced three times with variable conditions:

- 100 000 cells seeded and with 4 days of exposure to E2,
- 50 000 cells seeded exposed to E2 during 6 days,
- 50 000 cells and 4 hours of E2.

For each experiment, both conditions had three replicates (six wells in total).

Analysis was conducted with RNA extraction, reverse transcription to cDNA and qPCR:

Cells were treated with Trizol reagent (Qiagen), the RNA was isolated using 200 µl chloroform and 500 µl isopropanol. RNA concentration was verified to accordingly generate cDNA via reverse transcription with random primers, 10mM dNTP mix, 5X first strand buffer, 0.1 M DTT, RNaseOUT (40 units/µl) and 1 µl M-MLV reverse transcriptase (Invitrogen). Hamilton qPCR (quantitativePCR) was conducted in triplicate, performed with PerfeCta SYBR Green FastMix for iQ (2X) reaction mix, to identify the differences between the assays in the expressions of various genes: ER target, proliferation, inflammation, BRD2 and housekeeping genes for monitoring. The data generated was analysed to produce the figures in R studio.

3.2 Culture and dPCR analysis of metastatic cells under therapy

A variation of the previous experiment was conducted with MCF7 metastatic cells harvested from the lungs of 3 mice and exposed to different conditions (mock and mimicked endocrine therapy via ovariectomy) for 72h to further test responses. RNA was extracted and reverse transcribed to cDNA using the RT_Quantitect kit. A digital PCR was executed on the cDNA

3.3 In vivo Imaging System (IVIS) analysis of metastatic growth under endocrine therapy

To better understand how ER+ micrometastatic lesions react to endocrine therapy, luciferase-expressing cells (expressing *GFP-luc*) from Patient Derived Xenograft (PDX) are studied in MIND models. The xenograft (METS-27) is derived from pleural effusion of a 57 year old patient with Invasive Lobular Carcinoma (ILC) BC (100% ER+, 100% PR+, HER2-, 40% Ki67+). ILC differs from a Non Specific Type (NST) with a low expression of CDH and less E-cadherin causing cells to undergo discohesion: separating from one another, changing shape, and gaining the ability to migrate through surrounding tissues as isolated cells or single-file threads, rather than building a solid, palpable tumour mass.

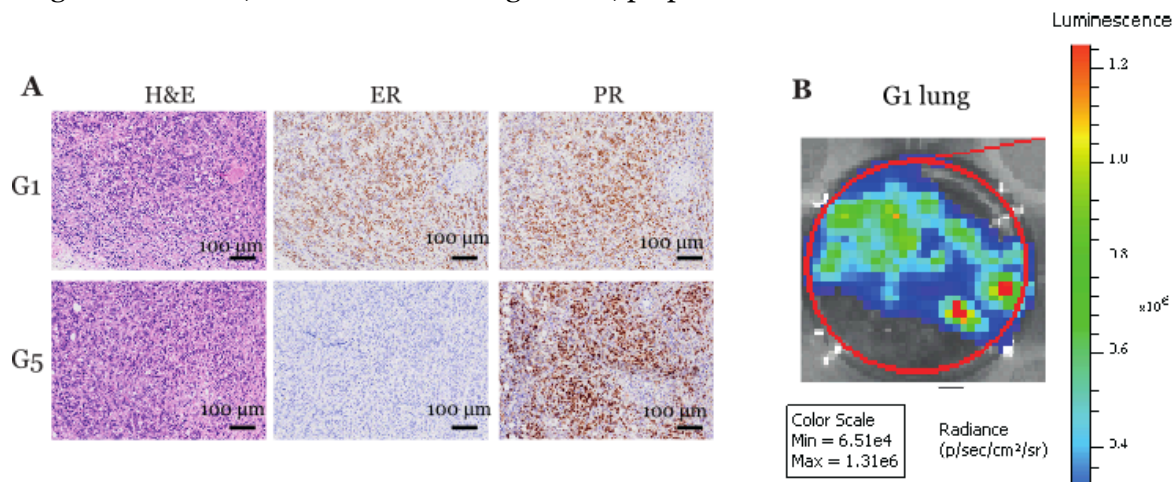


Figure 2. A. PDX tumour histology, immunohistochemistry stainings of H&E, ER and PR
B. IVIS ex-vivo luminescence of metastasis in lungs

Two conditions are compared for the primary tumour and the lung metastases: mock and endocrine therapy mimicked via ovariectomy (OVX), the removal of the ovaries in mice models, which stops the production of estrogen.

To track metastatic progression, the PDX cells in MIND models are transduced with a lentivirus to express a GFP-luciferase reporter gene. Metastatic growth is quantified using an IVIS imaging system, which captures the bioluminescent signal emitted by the organs (brain, lungs, liver, bone, kidneys, uterus/ovaries, peritoneum, and GI tract) of the mice. By drawing regions of interest (ROIs) around individual organs, the system detects and quantifies the bioluminescence levels, directly reflecting the burden of human metastatic cells present in the tissue. This data is then processed through R studio and compared to the results of another PDX (METS-59) which was analysed through the same process.

It is important to note that cancer cells tend to lose the expression of luciferase GFP, therefore some of the metastatic human cells are not detected via IVIS imaging with this analysis. tumour histology (cf. 3.4) is another type of evaluation used to compare the metastasis between the two conditions.

3.4 Analysis and quantification of tumour histology

Tissue samples from primary tumours in mammary glands and metastasis from the same previous PDX (cf. 3.3) with the mock and mimicked endocrine therapy OVX conditions were further examined to assess the effect of endocrine therapy on proliferation. They were stained through immunohistochemistry (IHC) by using antibodies to detect specific proteins (ER, AR, PR, Ki-67, Lamin AC). The software QuPath was used to identify the tissue regions and quantify the total percentage of positive cells, using objects and cell classifiers trained on a representative fraction of the images, with parameters such as size and thresholds adapted to each protein. The files containing the classifiers with their parameters are available on Zenodo “QuPath classifiers for tissues and cells”, <https://doi.org/10.5281/zenodo.23071088> [9]. The resulting figures were created in R studio using the annotation measurements.

4. Results and findings

4.1 Culture and qPCR analysis of metastatic cells under therapy

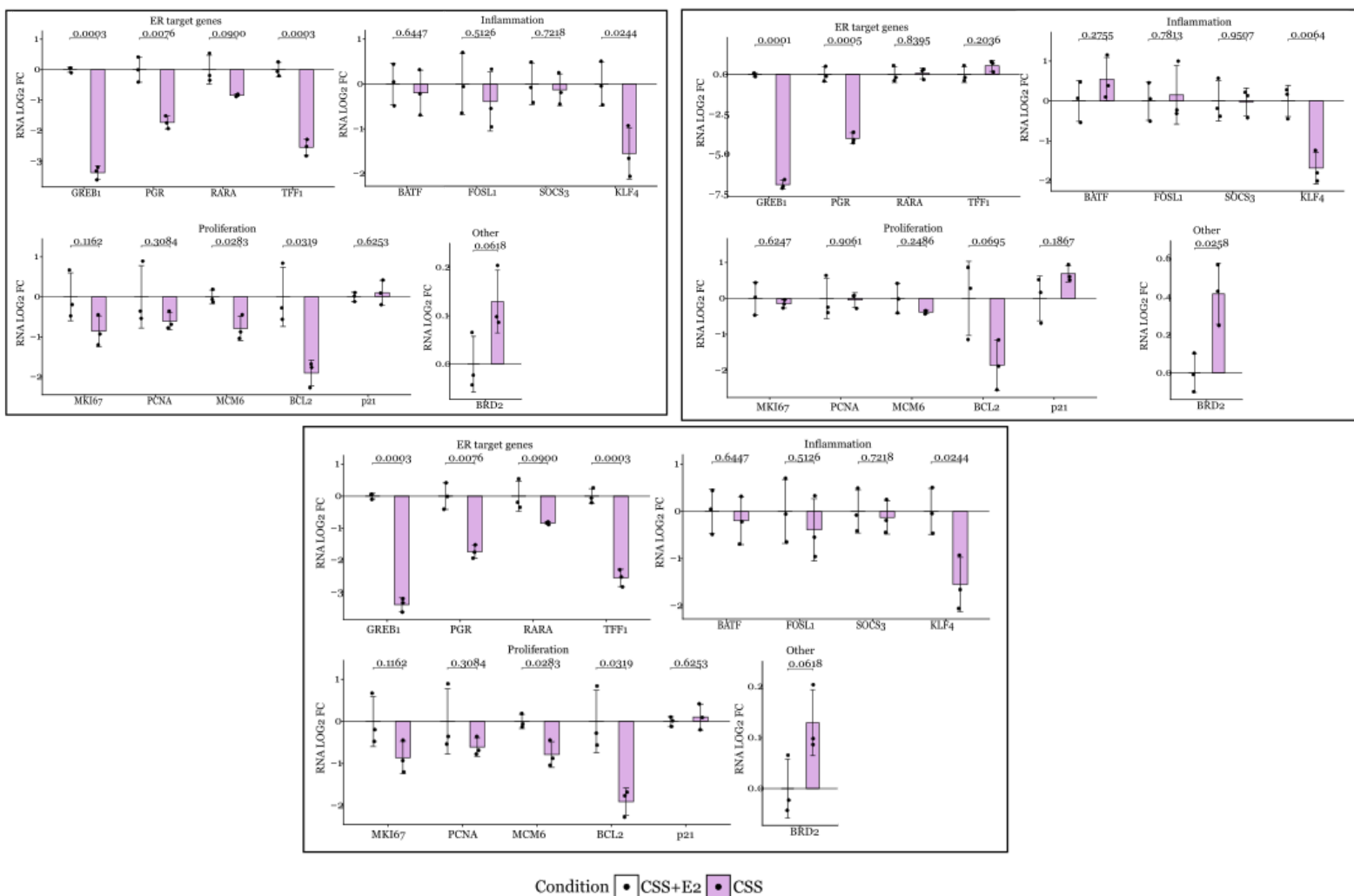


Figure 3. Gene expression analysis **A.** CSS/E2 10 nM 4 days 100 000 cells **B.** CSS/E2 10 nM 6 days 50 000 cells **C.** CSS/E2 10 nM 4 hours 50 000 cells

Across all three conditions, estrogen deprivation consistently suppressed ER target genes (*GREB1*, *PGR*, *RARA*, *TFF1*), proliferation genes (*MKI67*, *PCNA*, *MCM6*, *BCL2*) and inflammation (*BATF*, *FOSL1*, *SOCS3*, *KLF4*). The expression of *BRD2* was elevated under CSS conditions compared to controls, indicating an adaptive upregulation of *BRD2* during therapy-induced growth arrest.

4.2 Culture and dPCR analysis of metastatic cells under therapy

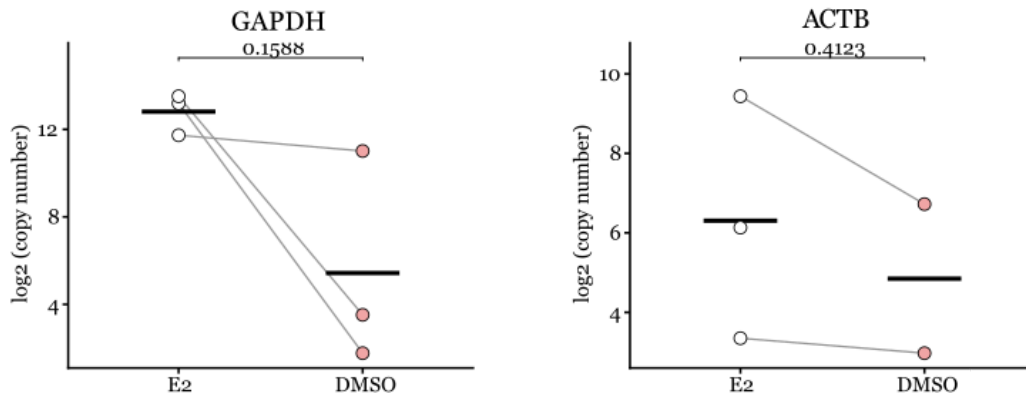


Figure 4. dPCR paired analysis of gene expression between mock (E2) and mimicked endocrine therapy (DMSO) in mets from lungs. Paired analysis reveals a consistent reduction in absolute transcript abundance for *GAPDH* and *ACTB*. This global decrease in reference gene copy numbers reflects a general metabolic and transcriptional quiescence in lung metastatic cells subjected to hormone depletion.

4.3 In vivo Imaging System (IVIS) analysis of metastatic growth under endocrine therapy

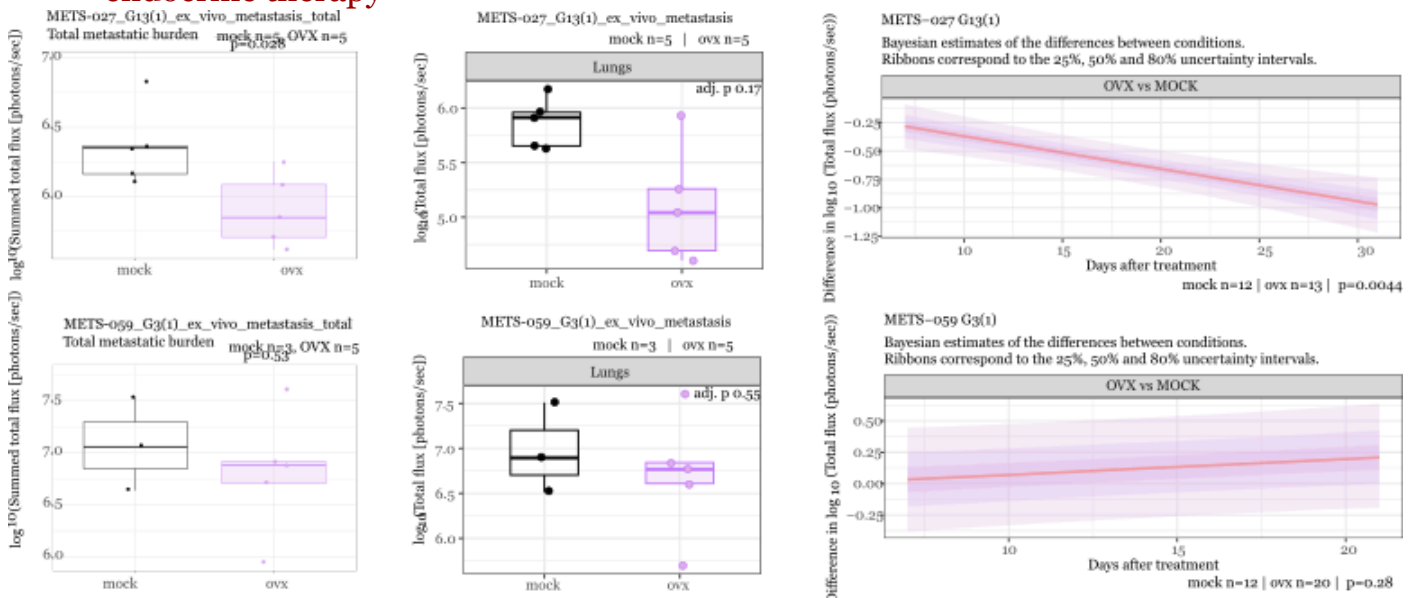


Figure 5. In Vivo Imaging System (IVIS) analysis of metastatic growth under endocrine therapy **A.** Mets 27 total metastatic burden **B.** Mets 27 lung metastasis **C.** Mets 27 bayesian estimates of growth difference **D.** Mets 59 total metastatic burden **E.** Mets 59 lung metastasis **F.** Mets 59 bayesian estimates of growth difference

In METS-27 models, OVX treatment induced a statistically significant reduction in both total metastatic burden and lung bioluminescence flux. In contrast, METS-59 exhibited no significant change in total metastatic signal between mock and OVX groups. This highlights inter-patient response differences, which could reflect pre-existing endocrine resistance or prior therapy exposure in METS-59. In both analyses, some cells also might not have been detected as they tend to lose the expression of GFP.

4.4 Analysis and quantification of tumour histology

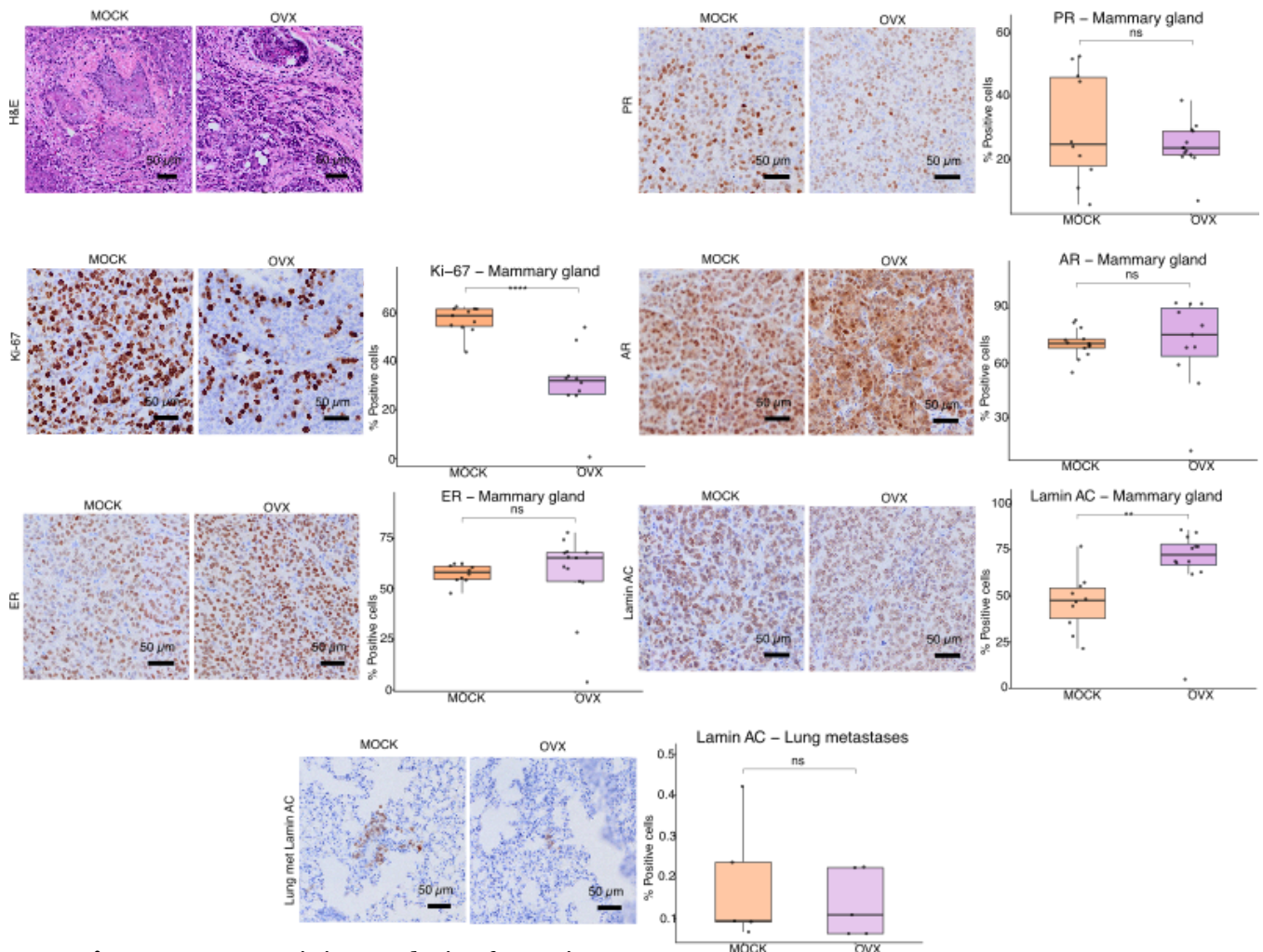


Figure 6. IHC staining analysis of proteins

In primary mammary gland tumours, OVX treatment produced a significant drop in Ki-67-positive proliferating cells, confirming primary tumour growth suppression and indicating less growth and cell division. In lung tissues, human-specific Lamin AC staining showed no significant difference in percentage of positive tumour cells between mock and OVX conditions. This indicates that while endocrine therapy effectively halts primary tumour division, persistent micrometastatic human cells remain present in distant lung tissues. This result could also be partially due to the low number of cells in the micrometastasis, the light staining on lamin AC and variability. To better study the effect of the mimicked endocrine therapy, reproducing the experiment with more biological replicates could produce more significant results.

5. Discussion, conclusion

While mimicked endocrine therapy (estrogen deprivation and ovariectomy) effectively suppresses primary tumour proliferation and reduces metastatic load in sensitive PDX models like METS-27, micrometastatic lesions persist in distant organs such as lungs. The adaptive upregulation of BRD2 observed during estrogen depletion suggests that persistent micrometastatic cells utilize epigenetic survival mechanisms. Furthermore, the divergent responses between METS-27 and METS-59 highlight the impact of patient heterogeneity and pre-existing Estrogen Receptor (ER) mutations associated with acquired resistance. Variance in lung Lamin AC quantification and transgene silencing (GFP/luciferase) can highlight the necessity of combining IVIS bioluminescence imaging with IHC quantification for analysis. Ultimately, validating these findings across additional patient-derived xenografts within the MIND model will be crucial to further understand metastatic survival pathways and develop effective combined therapies to eradicate persistent disease.

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