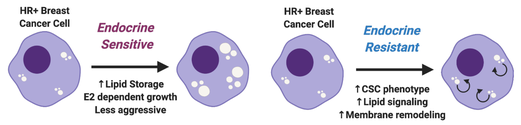
Altered Lipid Biology in HR+ Breast Cancer Endocrine Therapy Resistance

Ashley V. Ward**[1], Duncan Riley[1], Jessica Finlay-Schultz[1], Peter Kabos[2], and Carol A. Sartorius[1]
[1]Department of Pathology, [2]School of Medicine
University of Colorado, Anschutz Medical Campus, Aurora, CO

Background

About 30% of patients with hormone receptor positive (HR+) breast cancer will experience recurrence with therapy resistant disease. HR+ breast cancer typically expresses both estrogen receptor (ER) and progesterone receptor (PR) and is treated with endocrine therapies (ET) to block ER driven growth. Recurrent tumors consistently lose HR positivity and become resistant to these therapies leaving patients with limited therapeutic options. In order to target these populations, we developed a cohort of HR+ breast cancer cell lines with resistance to estrogen withdrawal (EWD, mimicking aromatase inhibition), Tamoxifen (TamR), and Fulvestrant (ICIR). Genetic analyses and lipid droplet staining on parental, EWD, and TamR T47D cells revealed a decrease lipid storage. While HR+ breast cancers exhibit active de novo fatty acid synthesis and visible lipid storage vesicles, this phenotype was lost or reduced in T47D-EWD and T47D-TamR cells.

We hypothesize that ET resistant breast cancers recycle rather than store lipids for metabolic use and membrane production/remodeling.



The following data presents additional models ET resistance to test this hypothesis. By elucidating the role of these changes in lipid biology with ET resistance, we aim to reveal therapeutic targets to improve standard therapies and prevent recurrence for HR+ breast cancer patients.

Objective & Methods

How does endocrine therapy resistance impact breast cancer lipid biology?

Table of Cell Lines

Cell line	ER	PR	Model of ET Resistance	ER Phenotype
T47D	+	+		*Wild type (WT)*
T47D-EWD	-	-	Aromatase Inhibitor	*WT*
T47D-TamR	+	-	Tamoxifen	*WT*
T47D-ICIR	-	-	Fulvestrant	*WT*
UCD4	+	+		*D538G*, ER-activating
UCD4-EWD	+	+	Aromatase Inhibitor	*D538G*
UCD4-TamR	+	-	Tamoxifen	*D538G*
UCD4-ICIR	+	-	Fulvestrant	*D538G*

1. Evaluate effect of endocrine resistance on lipid storage formation.
- Lipid droplet staining with Oil Red O and hematoxylin, light microscopy at 40X objective.

2. Use RNA-seq to identify lipid metabolic transcriptional changes in ET resistant cells compared to parental.
- RNA-sequencing through UCD Genomics Core.

3. Define lipid profile of cells under different forms of ET resistance.
- MTBE extraction, C18 UHPLC-MS, Metaboanalyst software.

4 . Analysis of metabolic phenotypic changes from ET resistance.
- Seahorse XF Mito Stress Test, Agilent Palmitate-BSA FAO substrate, SeahorseXFe96 analyzer.

Fig. 1: ET resistant cell lines alter lipid storage from sensitive parental cells.

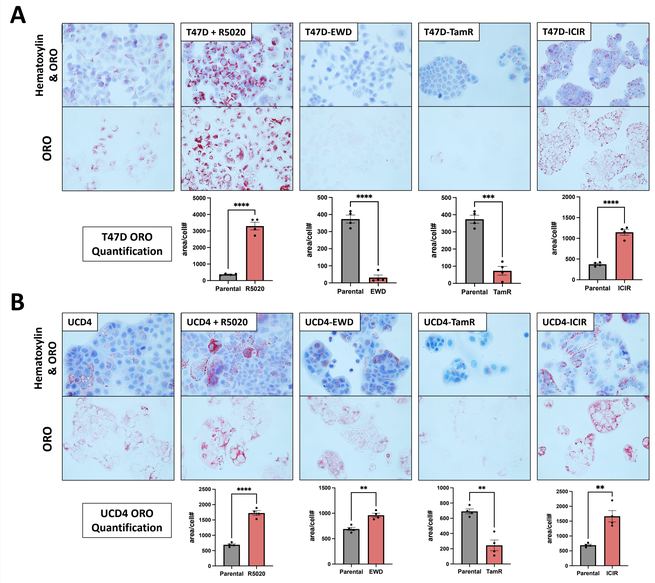


Figure 1. ORO staining of parental and endocrine resistant breast cancer cell lines. A) T47D and **B)** UCD4 parental and resistant cells were plated on coverslips, fixed and stained with hematoxylin and or Oil Red O (ORO) for lipid droplet visualization. Mounted coverslips were imaged 40X on Olympus microscope. ORO staining was quantified (4 fields per condition) on ImageJ and normalized to cell area.

Fig. 2: UCD4 cells display enriched lipid-related gene expression with ET resistance.

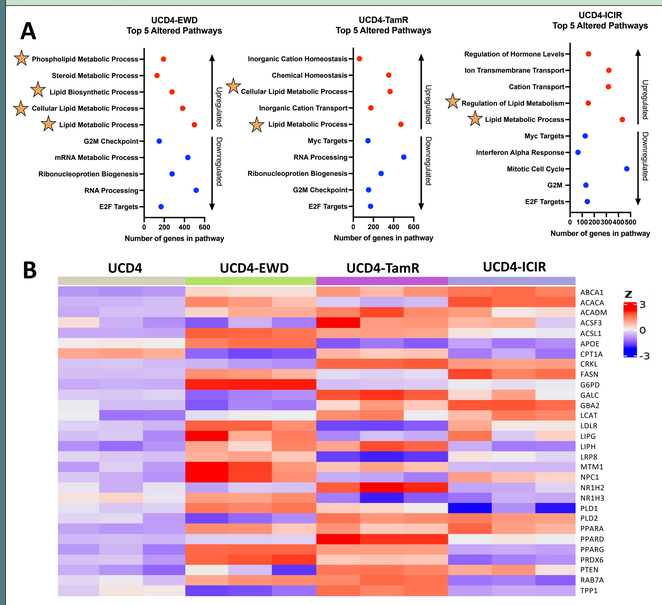
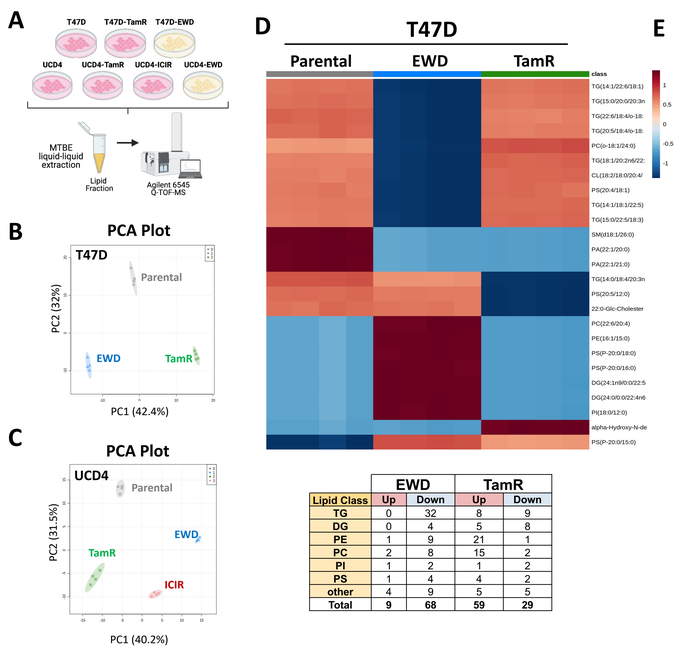


Figure 2. RNA-seq of UCD4 parental and ET resistant cell lines. Sequencing conducted through CU Anschutz Genomics Core, depth 30 million reads/sample**. A)** Top 5 upregulated and downregulated pathways by over-representation (ORA) pathway analysis of UCD4-EWD, -TamR, -and –ICIR cells versus parental cells. **B)** Heatmap of significantly altered genes from GO Lipid Metabolic Process gene set.

Fig. 3: ET resistance shifts global cellular lipidome of HR+ breast cancer cell lines.



Lipid Class	EWD Up	EWD Down	TamR Up	TamR Down
TG	0	32	8	9
DG	0	4	5	8
PE	1	9	21	1
PC	2	8	15	2
PI	1	2	1	2
PS	1	4	4	2
other	4	9	5	5
Total	**9**	**68**	**59**	**29**

Lipid Class	EWD Up	EWD Down	TamR Up	TamR Down	ICIR Up	ICIR Down
PC	25	22	27	25	24	29
PE	11	20	22	9	17	16
PI	4	4	1	6	1	6
PS	7	5	2	10	4	7
Cer	4	9	17	9	9	12
SM	2	0	2	0	2	0
PA	0	3	2	1	2	1
PG	1	1	0	2	0	2
TG	9	6	16	27	24	27
DG	2	6	6	6	7	9
other	11	7	15	16	15	18
Total	**76**	**83**	**110**	**111**	**105**	**127**

Figure 3. Mass spectrometry analysis shows differential lipid landscapes from varying forms of endocrine resistance. Hydrophobic lipid fractions were isolated from T47D or UCD4 parental, Tamoxifen resistant (TamR), long-term estrogen withdrawal (EWD), and Fulvestrant resistant (ICIR) cell lysates by MTBE liquid-liquid extraction method with a protein crash. Fractions were run in positive ionization mode on an Agilent 6560 IMMS-QTOF or Agilent 6545 Q-TOF-MS: Analytical column (Agilent Zorbax Rapid Resolution HD (RRHD) SB-C18, 1.8µm, 2.1x100mm). Data normalized to protein per sample (T47D) or total useful signal (UCD4), log2 transformed, and autoscaled on Metaboanalyst. **A)** Schematic of metabolomics sample prep and workflow. **B, C)** Principal component analysis of all samples. T47D samples (B) and UCD4 samples (C). Shaded regions represent 95% confidence regions. **D, E)** Heatmap of top 50 significant metabolites for each condition by ANOVA. Euclidean distance measure and Ward clustering method applied. Charts below show number of significantly altered analytes compared to parental. Significance cut-off = adjusted p-value > 0.05. Figures were generated using Metaboanalyst.

Fig. 4: ET resistance differentially shifts mitochondrial metabolism in HR+ breast cancer cell lines.

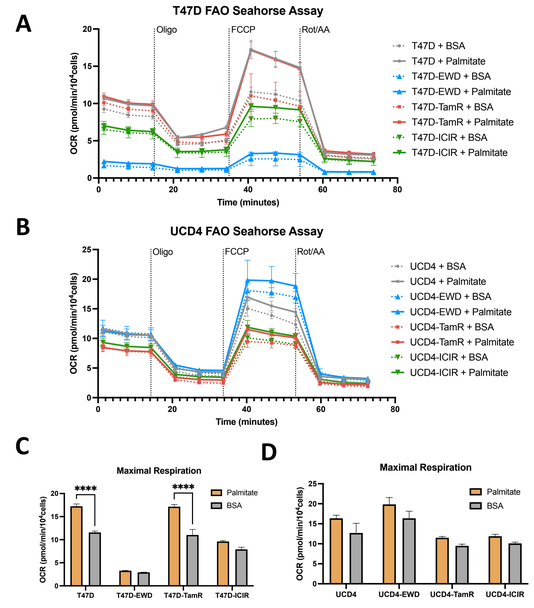


Figure 4. Analysis of significantly altered lipid composition on endocrine resistant samples. Seahorse XF Mito Stress Test on T47D and UCD4 ET resistant cells. Cells were nutrient deprived for 24h and supplemented with 160uM Palmitate-BSA or BSA control 2h before assay. All data normalized to cell count. **A-B)** Normalized oxygen consumption rate (OCR) kinetic graphs of T47D ET resistant cells (A) and UCD4 ET resistant cells (B). **C)** Maximal respiration bar graphs of T47D cell line assay shown in (A). **D)** Maximal respiration bar graphs of UCD4 cell line assay shown in (B). ANOVA, multiple comparisons statistical analysis used, **** p<0.0001.

Conclusions & Future Directions

Conclusions

- In both cell lines, TamR cells lose lipid storage phenotype while ICIR cells gain lipid storage phenotype.
- All ET resistance cells lines display dysregulated lipid metabolic gene transcript expression by pathway analysis and significantly alter global lipidome from parental cells.
- ET resistance does not impact FAO consistently across T47D and UCD4 cell lines.

Phenotype	EWD		TamR		ICIR	
	T47D	UCD4	T47D	UCD4	T47D	UCD4
Lipid storage	X	✓	X	X	✓	✓
Upregulated Lipid Metabolic genes	✓	✓	✓	✓	✓	✓
Altered lipidome from parental cells	✓	✓	✓	✓	✓	✓
FAO by Seahorse	X	✓	✓	X	X	X

Each form of endocrine resistance differential impacts metabolic phenotype and cellular lipidome.

Future Directions

- Assess fatty acid uptake vs. biogenesis in our resistance models.
- Examine phenotype of resistance models in 3D growth conditions.
- Determine role of ESR1 mutation on lipid biology in ET resistance.
- Fatty acid tracing to define membrane remodeling in each model of ET resistance.

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**Correspondence: ashley.2.ward@cuanschutz.edu