# Identification of Endogenous Ligands of Estrogen Related Receptors and their role in Breast Cancer

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# TABLE OF CONTENTS

## LIST OF FIGURES, SCHEMES, AND TABLES

### CHAPTER 1-INTRODUCTION



### CHAPTER 2-MANUSCRIPT 1



### CHAPTER 3-MANUSCRIPT 2

**ABSTRACT**

The estrogen-related receptors (ERRs) are orphan nuclear receptors, which consist of three isoforms, ERRα, ERRβ, and ERRγ. It has been reported that ERRs play an essential role in human health and disease, notably in breast cancer cells, where ERRα overexpression is correlated with adverse clinical outcomes in breast cancer patients. Although many efforts have been made to discover the endogenous ligands of ERRs, no endogenous ligand, other than cholesterol, was identified for ERRs. Discovering the endogenous ligand for ERRs is crucial to manipulate their pathway and possibly open a new venue for novel therapeutic strategies for breast cancer treatment or other diseases. Our group previously identified a novel endogenous steroid with an estradienolone-like structure (ED) from human pregnancy urine and blood and demonstrated that ED shows a strong affinity for sex hormone-binding globulin (SHBG) protein. In addition, our team's primary data demonstrated that ED does not show an affinity for the estrogen receptor (ER), but acts as an endogenous ligand of ERRs, and inhibits breast cancer cell proliferation.

In this thesis, I confirmed that ED's structure is distinct from estradiol (E2), a known ligand of ER and that ED does not show an affinity for ER or the glucocorticoid receptor (GR) using a bioluminescence resonance energy transfer (BRET) assay. Moreover, I demonstrated that ED directly binds to ERRα and ERRγ, and decreases their transcriptional activity. Importantly, my findings, consistent with our team's previous data, displayed that ED inhibits cell proliferation in a nanomolar range in ER-positive breast cancer (MCF-7) and triple-negative breast cancer (MDA-MB-231) cells. However, it does not show a significant inhibitory effect on non-tumorigenic epithelial breast cells (MCF-10A). Moreover, I have demonstrated that ED's inhibitory effect on breast cancer cell proliferation is ERRα-dependent. These findings suggest that the ED-ERR interaction represents a druggable pathway, which may have important implications for breast cancer therapy.

In addition, my finding revealed that cholesterol isolated from human pregnancy serum was enriched in beads-GST-ERRα-LBD column and directly binds to ERRα. This data is in line with another group that recently demonstrated that cholesterol isolated from the brain and kidney of mice acts as an agonist of ERRα. It has been shown that obesity and high cholesterol intake are associated with a higher risk of breast cancer recurrence and mortality by reprogramming the cancer cells' metabolic pathways, and statins, known cholesterol-lowering drugs, have been linked to improved breast cancer patient's survival. However, the underlying mechanism by which cholesterol exerts its pathological effect on altering breast cancer cell metabolism is not well-understood. There is also accumulating evidence that ERRα is overexpressed in breast cancer cells and is involved in the regulation of mitochondrial metabolism.

Interestingly, my findings demonstrated that exogenous cholesterol binds directly to ERRα and enhances its interaction with its coactivator, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α). In addition, exogenous cholesterol increases ERRα transcriptional activity in a PGC-1α-dependent manner. This process leads to induced ERRα mRNA and protein levels due to a specific auto-induction and increases ERR's metabolic target genes.

Importantly, my findings demonstrated that exogenous cholesterol increases oxidative phosphorylation (OXPHOS) rates, the TCA cycle intermediates, and glycolysis metabolite levels in breast cancer cells, and these cholesterol-induced effects are mediated via the ERRα pathway. However, exogenous cholesterol does not significantly alter glutaminolysis, pentose phosphate pathway (PPP), or one-carbon metabolism (OCM) metabolite levels. My data further exhibited that exogenous cholesterol enhances the levels of the nicotinamide adenine dinucleotide phosphate-oxidase (NADPH), and increases breast cancer cell proliferation and migration in both ER-positive (ER+) and triple-negative breast cancer (TNBC) cells, and this cholesterol-stimulatory effect is ERRα-dependent in these cells.

Furthermore, considering the strong association between high cholesterol levels and obesity, I analyzed the data available at the Gene Expression Omnibus (GEO) database and observed that basal-like breast tumors of obese breast cancer patients who overexpress ERRα, exhibit significantly increased expression levels of genes related to OXPHOS, the TCA cycle, and detoxifying enzymes compared to the basal-like breast tumors of non-obese breast cancer patients. Together, these findings suggest a possible mechanistic explanation for the cholesterol-induced metabolic alterations and breast cancer risk and might highlight the clinical benefit of cholesterol-lowering drugs in breast cancer patients.

In summary, the identification and functional characterization of endogenous ED as an inverse agonist and cholesterol as an agonist of ERRα, provide molecular tools to better understand the mechanism of action of ERRα, and in particular, ERRα's role in breast cancer cells' metabolic reprogramming. These findings may have potential therapeutic implications to treat breast cancer patients, particularly in TNBC, where ERRα is overexpressed and associated with poor prognosis.

**RÉSUMÉ**

Les récepteurs associés aux œstrogènes (ERR) sont des récepteurs nucléaires orphelins. Ils sont composés de trois isoformes, l'ERRα, l'ERRβ et l'ERRγ. Il a été rapporté que les ERR jouent un rôle essentiel dans la santé humaine et les maladies et ce, de manière significative au niveau des cellules cancéreuses du sein. On observe dans ces cellules une surexpression de ERRα corrélée à des résultats cliniques défavorables chez les patientes atteintes d'un cancer du sein. Bien que de nombreux efforts ont été faits pour découvrir les ligands endogènes des ERR, aucun ligand endogène autre que le cholestérol n'a été identifié pour les ERR. La découverte du ligand endogène des ERR est cruciale pour manipuler leur voie et, éventuellement, proposer une approche de stratégies thérapeutiques novatrices afin de traiter le cancer du sein ou d'autres maladies. Notre groupe a précédemment identifié un nouveau stéroïde endogène de structure semblable à l'estradiénolone (ED) à partir de l'urine et du sang de femmes enceintes, et a démontré que l'ED montre une forte affinité pour la globuline liant les hormones sexuelles (SHBG). De plus, les données préliminaires de notre équipe ont démontré que l'ED ne montre pas d'affinité pour le récepteur des œstrogènes (ER), mais qu'il agit comme un ligand endogène des ERR et qu'il inhibe la prolifération des cellules cancéreuses du sein.

Dans cette thèse, j'ai confirmé que la structure de l'ED est distincte de l'estradiol (E2), un ligand connu de l'ER et que l'ED ne montre pas d'affinité pour l'ER ou le récepteur des glucocorticoïdes (GR) en utilisant le test de transfert d'énergie de résonance par bioluminescence (BRET). De plus, j'ai démontré que l'ED se lie directement à l'ERRα et l'ERRγ et diminue leur activité transcriptionnelle. Il est important de noter que mes résultats corroborent nos données précédentes. En effet, mes résultats ont montré que l'ED inhibe la prolifération cellulaire dans l'ordre de grandeur du nanomolaire dans les cellules du cancer du sein ER-positif (MCF-7) et du

cancer du sein triple négatif (MDA-MB-231). Cependant, il ne montre pas d'effet inhibiteur significatif sur les cellules épithéliales non-tumorigènes du sein (MCF-10A). De plus, j'ai démontré que l'effet inhibiteur de l'ED sur la prolifération des cellules cancéreuses du sein dépend de l'ERRα. Ces résultats suggèrent que l'interaction ED-ERR représente un caractère thérapeutique, qui peut avoir des implications importantes pour le traitement du cancer du sein.

Mes résultats ont aussi révélé que le cholestérol isolé de sérums de femmes enceintes qui avait été enrichi sur colonnes avec des billes de GST-ERRα-LBD se liait directement à l'ERRα. Ces données sont en accord avec un autre groupe qui a récemment démontré que le cholestérol isolé du cerveau et des reins de souris agit comme un agoniste de ERRα. Il a été démontré que l'obésité et un apport élevé en cholestérol sont associés à un risque accru de récurrence et de mortalité du cancer du sein à travers la reprogrammation des voies métaboliques des cellules cancéreuses. De plus, les statines, des médicaments connus abaissant le taux de cholestérol, ont été associées à l'amélioration de la survie des patientes atteintes d'un cancer du sein. Cependant, le mécanisme sous-jacent par lequel le cholestérol exerce son impact pathologique sur la modification du métabolisme des cellules cancéreuses du sein n'est pas bien compris. De plus en plus de preuves indiquent que l'ERRα est surexprimé dans les cellules cancéreuses du sein et qu'il est impliqué dans la régulation du métabolisme mitochondrial.

Il est intéressant de mentionner que mes résultats ont démontré que le cholestérol exogène se lie directement à l'ERRα et améliore son interaction avec son coactivateur, le récepteur activé par les proliférateurs du peroxysome gamma coactivateur 1-alpha (PGC-1α). À la suite de cette interaction améliorée, le cholestérol exogène augmente l'activité transcriptionnelle du ERRα d'une manière dépendante de PGC-1α. Ce processus conduit à des niveaux d'ARNm et de protéines

d'ERRα induits en raison d'une auto-induction spécifique et augmente les gènes cibles métaboliques d'ERR.

Il est important de noter que mes résultats ont démontré que le cholestérol exogène augmente la phosphorylation oxydative (OXPHOS), les intermédiaires du cycle de TCA et les niveaux de métabolites de glycolyse dans les cellules cancéreuses du sein. Cet effet induit par le cholestérol est médié par la voie ERRα.

Cependant, le cholestérol exogène ne modifie pas de manière significative la glutaminolyse, la voie du pentose phosphate (PPP) et les intermédiaires métaboliques du métabolisme à un carbone (OCM). Mes résultats ont en outre montré que le cholestérol exogène augmente les niveaux de nicotinamide adénine dinucléotide phosphate réduite (NADPH) dans les cellules du cancer du sein ER-positif (ER +) et triple négatif (TNBC), et cet effet dépend de ERRα dans ces cellules

En outre, j'ai analysé les données disponibles dans la base de données Gene Expression Omnibus (GEO) et observé que les patientes obèses atteintes de cancer du sein de type basal, qui surexpriment l'ERRα, démontrent une augmentation significative des niveaux d'expression des gènes liés à l'OXPHOS, au cycle de TCA et aux enzymes détoxifiantes par rapport aux tumeurs mammaires basales non obèses. Comme l'obésité est souvent associée à un taux de cholestérol élevé, ces résultats suggèrent une explication sur le mécanisme possible derrière l'altération métabolique via le risque de cancer du sein induit par le cholestérol et pourraient mettre en évidence l'avantage clinique des médicaments abaissant le taux de cholestérol chez les patientes atteintes du cancer du sein.

Collectivement, l'identification et la caractérisation fonctionnelle de l'ED endogène comme agoniste inverse, et du cholestérol comme agoniste de l'ERRα, fournit des outils moléculaires pour

mieux comprendre le mécanisme d'action de l'ERRα et en particulier, le rôle d'ERRα dans la reprogrammation métabolique des cellules cancéreuses du sein. Ces découvertes peuvent avoir des implications thérapeutiques potentielles pour traiter les patientes atteintes d'un cancer du sein, en particulier TNBC, où l'ERRα est surexprimé et associé à un pronostic défavorable.

# Acknowledgments

First and foremost, I would like to thank my supervisor, Dr. Anie Philip, for her scientific support and for the opportunities that she provided me with during my Ph.D. I am very grateful for the balance between guidance and freedom that I was exposed to in her laboratory. This experience helped me to discover my abilities and to develop the required skills for my future career as an independent researcher. I would like also to thank her for kindly teaching me how to write grant proposals and scientific manuscripts.

I would like also to thank the members of my advisory committee: Dr. Jacques Lapointe (My advisory chair), Dr. Sylvie Mader, and Dr. David P. Labbé who provided me with valuable suggestions and support throughout the course of this work. This thesis would not have been completed without their help.

I am especially grateful to Dr. Mader for her invaluable scientific input and support. She kindly provided me with the breast cancer cell lines (MDA-MB-231, MCF-7, and MCF-10A), and also the opportunity to perform BRET assay (chapter 2, Figure 2B) in her laboratory.

I am also sincerely grateful to Dr. Bertrand Jean-Claude and his great team in the drug discovery platform located in Research Institute-McGill University Health Center (RI-MUHC) for the stimulating scientific discussions and motivation during the course of this work. As a first user of the platform, I had the privilege to be trained and work with high-tech instruments, like high-resolution LC-MS/MS to analyze extracted steroids and lipids isolated from human pregnancy urine and serum samples. Some of these MS data are presented in chapter 2 and 3 of this thesis.

I am also thankful to Dr. Donald Poirier (steroid chemist, Laval University) who provided us with the interpretation of mass spectrometry data related to chapter 2 and chapter 3 of this thesis. In addition, a very special thanks to Dr. Fong-Fu Hsu and Dr. Fujiwara Hideji from Washington

University school of Medicine in St. Louis to kindly host me for the duration of two weeks in Washington University, and provided me with the stimulating discussions, action plans, and intense training on high-tech analytical chemistry instruments. They provided us with the interpretation of mass spectrometry data.

I would like also to thank the Metabolomic core facility located at Goodman cancer research center (McGill University) for providing us with the steady-state metabolomic data.

Many thanks also to our collaborators and colleagues, including Dr. Ruin Moaddel (National Institute on Aging, USA) for the generous gift of the purified human ligand-binding domain of estrogen-related receptor gamma protein; Dr. Donald P. McDonnell (Duke University, USA) for the kind donation of compound 29 (small molecule inhibitor of ERRα); Dr. Morag Park (McGill University) for providing us with triple-negative breast cancer-patient derived xenograft (TNBC-PDX) cells; Dr. Alice Benjamin (Obstetrician and Gynecologist) for providing us with the human pregnancy serum and urine samples.

I am also very grateful to my friends, Dr. Shawn McGuirk (Goodman Cancer Research Center, McGill University) for the scientific discussion, and reviewing chapter 4 of my thesis, and providing me with constructive feedback; Dr. Annie Boisvert (McGill University) for proof reading the French abstract of this thesis.

I would like also to thank my husband, who enormously supported me during the difficult times of my tenure as a Ph.D. student, and without his unconditional love and support, this thesis would have never been accomplished. Thanks for being such an awesome husband and a best friend! I would like also to appreciate the unconditional love from my parents, my sister, and my parents-in-law, who provided me with joyful moments and thoughtful support.

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# Contributions to original knowledge

This thesis has been prepared following the guidelines specified by the Faculty of Graduate and Postdoctoral Studies of McGill University. The thesis, entitled “Identification of Estrogen Related Receptors’ Endogenous Ligands and their Impact on Breast Cancer” has been prepared in the manuscript-based format. It consists of an Abstract and its French translation (Résumé), and five chapters entitled Chapter 1: Introduction; Chapter 2: Isolation and functional characterization of a novel endogenous inverse agonist of estrogen-related receptors (ERRs) from human pregnancy urine.; Chapter 3: Cholesterol as an endogenous ligand of ERRα promotes ERRα-mediated cellular proliferation and metabolic target gene expression in breast cancer cells.; Chapter 4: Cholesterol-induced metabolic reprogramming in breast cancer cells is mediated via ERRα pathway.; Chapter 5: General Discussion. A bibliography containing the literature cited in each chapter is provided at the end of each chapter.

Unless otherwise stated, I, Faegheh GHANBARI, under the supervision of Dr. Anie Philip, have designed and conducted the experiments. I collected, analyzed, and interpreted the data for the work presented here. My contributions to the body of knowledge described in this thesis are listed as follows:

1. Using a new LC-MS/MS method that I developed, I have confirmed that the endogenous ED’s structure differs from the one of estradiol. However, their mass (272 m/z) is similar.

2. I have confirmed that the endogenous ED does not show affinity to estrogen receptor (ER) and also I have demonstrated that the endogenous ED has no affinity for glucocorticoid receptor (GR).

3. I have revealed that the endogenous ED binds directly to the ligand-binding domain of ERRα and ERRγ, and demonstrated that the endogenous ED shows high affinity to ERRγ-LBD with a small dissociation constant ($k_d$) compared to ERRγ's synthetic ligand, 4-OHT.

4. I have demonstrated, consistent with our team's previous findings that the ED purified from human pregnancy urine is an endogenous ligand that acts as an inverse agonist of ERRα and ERRγ, and that it does so in the nanomolar range compared to the synthetic ligands of ERRs that inhibit ERRs' activity in micromolar range using different cell line and different batch of purified ED.

5. I have confirmed that the endogenous ED inhibits the cell proliferation of MCF-7 and MDA-MB-231 cells in a dose-dependent manner, with even lower $IC_{50}$ than the ones our group previously demonstrated using a different batch of purified ED and also different cell proliferation assay.

6. I have demonstrated that the endogenous ED inhibits breast cancer cell proliferation via ERRα pathway.

7. I have confirmed that the purified ED does not show any effect on the cell proliferation of non-tumorigenic breast epithelial cells (MCF-10A).

8. I have demonstrated that a compound with the mass 273 m/z (consistent with our team's previous MS data of purified ED), and another distinct compound with the mass 333 m/z were enriched in beads-GST-ERRγ affinity column, and my data indicated that a possible structure of the compound with the mass 273 m/z (predicted and chemically synthesized previously by our team, and also predicted based on my MS data) shows high affinity to SHBG and ERRγ-LBD.

9. I have established that cholesterol, isolated from human pregnancy serum, acts as an endogenous agonist of ERRα, and it increases ERRα's transcriptional activity in a PGC-1α dependent manner, consistent with another study, in which cholesterol was isolated from brain and kidney of mice.

10. I have demonstrated that the exogenous cholesterol enhances the interaction of ERRα with its coactivator PGC-1α, which may result in induction in ERRα and its metabolic target genes expression levels, as well as detoxifying enzymes in MCF-7 and MDA-MB-231 cells, and that this cholesterol-induced effect is ERRα dependent in these cells.

11. I have further demonstrated that the exogenous cholesterol increases a cascade of metabolic pathways, such as aerobic glycolysis, OXPHOS, the TCA cycle, and the expression of 6PGD involved in the pentose phosphate pathway in MDA-MB-231 and TNBC-PDX cells via ERRα axis. However, it increases all the above metabolic pathways, except aerobic glycolysis, in MCF-7 cells in an ERRα dependent manner. Furthermore, cholesterol does not alter glutaminolysis, PPP, and OCM intermediate levels in breast cancer cells.

12. I have shown that exogenous cholesterol enhances NADPH levels. However, inhibition of ERRα decreases NADPH levels, and adding cholesterol does not rescue its effect.

13. I have also demonstrated that the exogenous cholesterol increases breast cancer cell proliferation and migration in TNBC and ER+ breast cancer cells in an ERRα dependent manner.

14. I have demonstrated that the gene expression levels of OXPHOS and the TCA cycle, detoxifying enzymes, and the key enzymes involved in the PPP and OCM pathway (G6PD and

GART, respectively) increase in obese basal-like breast cancer tumors compared to the non-obese patients using the Gene Expression Omnibus (GEO) database.

Some of the data presented in this thesis are reported in the articles below as followed:

1) **Ghanbari F**, Hebert-Losier A, Barry J, Poirier D, Giguere V, Mader S, Philip A. Isolation and functional characterization of a novel endogenous inverse agonist of estrogen-related receptors (ERRs) from human pregnancy urine. *J Steroid Biochem Mol Biol* 2019; 191: 105352.
2) **Ghanbari F**, Mader S, Philip A. Cholesterol as an Endogenous Ligand of ERRα Promotes ERRα-Mediated Cellular Proliferation and Metabolic Target Gene Expression in Breast Cancer Cells. *Cells* 2020; 9: 1765.
3) **Ghanbari F**, Morag P, Philip A. Cholesterol-induced metabolic reprogramming in breast cancer cells is mediated via ERRα pathway. Manuscript to be submitted shortly.

Other manuscripts, not reported in this thesis;

1) **Ghanbari F**, Hsu F, Fujiwara H, Poirier D, Philip A. Identification of the potential endogenous ligands of ERRγ using human pregnancy urine. In preparation.

# Contribution of Authors

1) CHAPTER 2-MANUSCRIPT 1**: Ghanbari F, Hebert-Losier A, Barry J, Poirier D, Giguere V, Mader S, Philip A. Isolation and functional characterization of a novel endogenous inverse agonist of estrogen-related receptors (ERRs) from human pregnancy urine.** ***J Steroid Biochem Mol Biol*** **2019; 191: 105352.**

**The candidate F.G.**: designed, performed the experiment, analyzed, and plotted the data related to Figure 1, Figure 2B, Figure 3, Figure 4C, Figure 6, and schematic Figure 7. under Dr. Philip's supervision. The candidate (**F.G.**) contributed to data interpretation, writing, and reviewing the manuscript.

**A.H.**: Performed experiments for Figure 4A and B, Figure 5, and also provided initial data for ED purification (Figure 1A-C) and for the luciferase reporter assay (Figure 4C) and breast cancer cell proliferation (Figure 6 A-D) using a different batch of purified ED and a different proliferation assay than the one reported in this manuscript.

**J.B.:** Contributed to the execution of experiments in 4A and 4B, and the initial results on luciferase reporter assay (Figure 4C)

**D.P.:** Interpreted mass spectrometry (MS) data presented in Figure 1D in this manuscript, and confirmed the structural features. And also D.P provided initial MS data for the endogenous ED (Figure 1D), using different LC/MS method and different batch of purified ED than the one reported in this manuscript.

**V.G.**: Supervised J.B. and contributed to the interpretation of data (Figure 4).

**S.M.:** Supervised A.H. to design and perform breast cancer cell proliferation assay (Figure 6A-D) using a different batch of purified ED and using different assay than the one reported in this manuscript, and contributed to data interpreting, and reviewed the manuscript.

**A.P.:** Supervised the candidate F.G. and A.H., performed the experiment and plotted Figure 2A, contributed to the data interpretation, wrote the original draft of the manuscript, reviewed the manuscript.

2) CHAPTER 3-MANUSCRIPT 2**: Ghanbari F, Mader S, Philip A. Cholesterol as an Endogenous Ligand of ERRα Promotes ERRα-Mediated Cellular Proliferation and Metabolic Target Gene Expression in Breast Cancer Cells. *Cells* 2020; 9: 1765.**

**The candidate F.G.**: Designed, performed, analyzed, interpreted, and plotted the data under Dr. Philip's supervision, and wrote the original draft of the manuscript, and participated in reviewing the manuscript. The interpretation of mass spectrometry data was done by Dr. Donald Poirier (Laval University) **A.P.:** Supervised F.G., wrote and reviewed the manuscript. **S.M.**: Contributed to data interpretation and manuscript reviewing.

3) CHAPTER 4-MANUSCRIPT 3: **Ghanbari F, Park M, Philip A. Cholesterol-induced metabolic reprogramming in breast cancer cells is mediated via ERRα pathway. Manuscript to be submitted shortly.**

**The candidate F.G.**: Designed and conceptualized the project under Dr. Philip's supervision. The candidate performed all the experiments except steady-state metabolomic analysis, which was done in a metabolomic core facility (Goodman Cancer Research Center, McGill University). The candidate analyzed, interpreted, plotted, wrote, and reviewed the manuscript. **A.P.**: Supervised the candidate,

revised the manuscript. **P.M.**: Developed the protocol and generated the TNBC-PDX cells and established the culture conditions for those cells.

# Abbreviations

| | |
|---|---|
| ABCA1 | ATP-binding cassette subfamily A1 |
| ABCG1 | ATP-binding cassette subfamily G member 1 |
| ACO2 | Aconitase 2, mitochondrial |
| AF-1 | Activation function 1 |
| AF-2 | Activation function 1 |
| Akt | Protein kinase B |
| AP-1 | Activator protein 1 |
| ApoE | Apolipoprotein E |
| APT5L | ATP synthetase subunit g, mitochondial |
| AR | Androgen receptors |
| ATP | Adenosine triphosphates |
| ATP5D | ATP synthetase subunit delta, mitochondrial |
| BAT | Brown adipose tissue |
| BC | Breast cancer |
| BMI | Body Mass Index |
| BPA | Bisphenol A |
| BRAF | Photo-oncogene B-Raf |
| BRET | Bioluminescence Resonance Energy Transfer |
| BSA | Bovine serum albumin |
| CCNE1 | Cyclin E1 |
| CDK 4/6 | Cyclin-dependent kinase 4/6 |

| | |
|---|---|
| cDNA | Complementary DNA |
| ChIP | Chromatin Immunoprecipitation |
| ChIP-seq | ChIP-Sequencing |
| Chol | Cholesterol |
| co-IP | Co-immunoprecipitation |
| Compound A | N-[(2Z)-3-(4,5-dihydro-1,3-thiazol-2-yl)-1,3-thiazolidin-2-yl idene]-5H dibenzo[a,d][7]annulen-5-amine |
| COX5B | Cytochrome c oxidase subunit 5B, mitochondrial |
| Cpd29 | Compound 29 (4-[4-(2,4-Dioxothiazolidin-5- ylidenemethyl)2-methoxyphenoxy]-3-trifluoromethylbenzonitrile) |
| CS | Citrate synthesase |
| CYP19 | Aromatase |
| DBD | DNA-binding domain |
| DES | Diethylstilbestrol |
| DHEAS | Dehydroepiandrosterone sulfate |
| DHT | Dihydrotestosterone |
| DNA | Deoxyribonucleic acid |
| E2 | 17β-estradiol |
| $EC_{50}$ | Half-maximal effective concentrations |
| ECAR | Extracellular acidification rate |
| ED | Estradienolone-like structure |
| EGF | Epidermal growth factor |
| ER | Estrogen receptor |
| ER+ | ER-positive breast cancer |

| | |
|---|---|
| ERBB2 | Erythroblastic oncogene B (gene coding for HER2) |
| ERE | ER response elements |
| ERRE | ERR response element |
| ERRs | Estrogen-related receptors |
| ESI | Electrospray ionization source |
| ESRRA | Gene coding for ERR alpha |
| ET | Endocrine therapy |
| ETC | Electron transport chain |
| F1-.6 bis P | Fructose-1,6-bisphosphate |
| FAO | Fatty acid oxidation |
| FBS | Fetal Bovin serum |
| FDG-PET | 18F-deoxyglucose-positron emission tomography () |
| FH | Fumarase |
| FOXO1 | Forkhead box O1 |
| FPPS | Farnesyl pyrophosphate synthase |
| G6PD | Glucose-6-phosphate dehydrogenase |
| GA3P | Glyceraldehyde 3-Phosphate |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| GART | Phosphoribosylglycinamide transformylase |
| GC/MS | Gas chromatography-mass spectrometry |
| GEO | Gene Expression Omnibus |
| GGPPS | Geranylgeranyl pyrophosphate synthase |
| GLS2 | Liver glutaminase |

| | |
|---|---|
| GLUD1 | Glutamate dehydrogenase 1 |
| GR | Glucocorticoid receptor |
| GRIP1 | Glucocorticoid receptor-interacting protein 1 |
| GSH | Reduced Glutathione |
| GSST | Oxidized Glutathione |
| GST | Glutathione-S-transferases |
| GSTM1 | Glutathione S-transferase Mu 1 |
| 27HC | 27-hydroxycholesterol |
| HCD | High cholesterol diet |
| HCT | Hydrocortisone |
| HDAC8 | Histone deacetylase 8 |
| HDL | High-density lipoprotein |
| HER2 | Human epidermal growth factor receptor2 |
| HFHC | High-fat, high-cholesterol |
| HIF-1 | Hypoxia-inducible factor 1 |
| HK2 | Hexokinase-2 |
| HMGCR | 3-hydroxy-3-methyl-glutaryl-CoA reductase |
| HSD17B1 | 17β-hydroxysteroid dehydrogenase type 1 |
| IB | Immunoblot |
| $IC_{50}$ | Half-maximal inhibitory concentration |
| ICC | Immunocytochemistry |
| IDH2 | Isocitrate dehydrogenase 2 |
| IDH3A | Isocitrate dehydrogenase 3A |

| | |
|---|---|
| IDOL | Inducible degrader of LDLR |
| IGF-1R | Insulin-like growth factor 1 receptor |
| IgG | Immunoglobulin G |
| IPTG | Isopropyl-β-D- thiogalactopyranoside |
| IRB | Institutional review board |
| $K_d$ | Dissociation constant |
| Kras | Kristen Rat Sarcoma |
| LBD | Ligand binding domain |
| LC-MS/MS | Liquid chromatography-tandem mass spectrometry |
| LDHB | Lactate dehydrogenase B |
| LDL | Low-density lipoprotein-cholesterol |
| LDL-R | Low-density lipoprotein receptor |
| Lova | Lovastatin |
| LXRs | Liver X receptors |
| MAO-B | Monoamine oxidase B |
| MCAD | Medium-chain acyl-coenzyme A dehydrogenase |
| MEF | Myocyte enhancer factor |
| MET | Mesenchymal-to-epithelial transition |
| MR | Mineralocorticoid |
| MRM | Multiple reaction monitoring |
| mRNA | Messenger RNA |
| MS | Mass spectrometry |
| MSC | Mesenchymal stem cells |

| | |
|---|---|
| mTOR | Mammalian target of rapamycin |
| MTR | 5-Methyltetrahydrofolate-Hemocysteine Methyltransferase |
| MYC | c-MYC |
| NADPH | Nicotinamide adenine dinucleotide phosphate-oxidase |
| N-BPs | Nitrogen-containing bisphosphonates |
| NCOA | Nuclear receptor coactivator |
| NDUFB7 | Ubiquinone oxidoreductase subunit B7 |
| NMR | Nuclear magnetic resonance |
| NR3B1 | Nuclear Receptor subfamily 3 group B member 1 (ERRα) |
| NR3B2 | Nuclear Receptor subfamily 3 group B member 2 (ERRβ) |
| NR3B3 | Nuclear Receptor subfamily 3 group B member 3 (ERRγ) |
| NRFs | Nuclear respiratory factors |
| NRs | Nuclear receptors |
| 4-OHT | 4-hydroxytamoxifen |
| OCM | One-carbon metabolism |
| OCR | Oxygen consumption rate |
| OD | Optical density |
| OXPHOS | Oxidative phosphorylation |
| 2PG/3PG | 2-phosphoglycerate/3-phosphoglycerate |
| 6PGD | 6-Phosphogluconate dehydrogenase |
| Park2 | ubiquitin-protein ligase Parkin |
| PCAF | p300 coactivator associated factor |
| PDK4 | Pyruvate dehydrogenase kinase isoenzyme 4 |

| | |
|---|---|
| PEPCK | Phosphoenolpyruvate carboxykinase |
| PGC-1α/β | Peroxisome Proliferator-Activated Receptor gamma coactivator-1 alpha and beta |
| PI3K | Phosphoinositide 3-kinase |
| PKCδ | Protein kinase Cδ |
| PPARγ | Peroxisome proliferator-activated receptor γ |
| PPP | Pentose phosphate pathway |
| PR | Progesterone receptor |
| PROX1 | Prospero homeobox protein-1 |
| p-Serine | phospho-Serine |
| RAR | Retinoic acid receptor |
| Ras | Rat sarcoma |
| RIP140 | Receptor-interacting protein-140 |
| RLucII | Renilla Luciferase II |
| RNA | Ribonucleic acid |
| RNA-PolII | RNApolymerase II |
| RNA-seq | RNA-sequencing |
| ROS | Reactive oxygen species |
| RTK | Receptor tyrosine kinase |
| SD | Standard deviation |
| siRNA | Small interference ribonucleic acid |
| SDS | Sodium dodecyl sulfate |
| SDS-PAGE | SDS-polyacrylamide gel electrophoresis |
| SERDs | Selective estrogen down-regulators |

| | |
|---|---|
| SERM | Endogenous ER modulator |
| SERMs | Estrogen receptor modulators |
| SHBG | Sex hormone-binding globulin protein |
| Sirt1 | Sirtuin 1 homolog |
| SOD2 | Superoxide dismutase 2 |
| SPP1 | Secreted phosphoprotein 1 |
| SRC | Steroid receptor coactivator family |
| SREBP1 and 2 | Sterol regulatory element-binding protein 1 and 2 |
| SREs | Sterol response elements |
| ST | Syncytial trophoblast |
| SULT2A1 | Steroid sulfotransferase |
| TAM | Tamoxifen |
| TCA cycle | Tricarboxylic acid cycle |
| TCF | T-cell factor |
| TCGA | The Cancer Genome Atlas analyses |
| TCL | Total cell lysates |
| TFF1 | Trefoil Factor-1 |
| TFs | Transcription factors |
| TMS | Trimethylsilyl |
| TBNC | Triple negative breast cancer |
| TNBC-PDX | TNBC-patient-derived xenograft |
| UHPLC | Ultra-High Performance Liquid Chromatography |
| VEGF | Vascular endothelial growth factor |

| | |
|---|---|
| VLDL | Very low-density lipoprotein-cholesterol |
| WAT | White adipose tissue |
| WNT11 | Wingless-type MMTV integration site family, member 11 |
| WT | Wild type |
| XCT790 | ((2E)-3-(4-{[2,4-bis(trifluoromethyl)benzyl]oxy}-3-methoxyphenyl)-2-cyano-N-[5-(trifluoromethyl)-1,3,4-thiadiazol-2-yl]acrylamide) |
| YY-1 | Yin Yang-1 |

# CHAPTER 1

# INTRODUCTION

### 1.1. Nuclear receptors

Nuclear receptors (NRs) are transcription factors (TFs) that regulate several biological processes, including metabolism, reproduction, and inflammation. Most NRs are controlled endogenously by small lipophilic ligands such as steroids, retinoids, and phospholipids. However, these receptors also contain "orphan" members, for which no ligand has yet been identified [1]. When ligands bind the ligand-binding domain (LBD) of these receptors, they induce conformational changes within the receptor, which in turn bind the DNA response element, using the DNA-binding domain (DBD) of the receptors [2, 3]. Upon recruitment to specific DNA elements in the promoter or distal enhancers of their target genes, nuclear receptors induce a series of coordinated events, including recruitment of co-factors, leading to chromatin modification and ultimately to recruitment of RNApolymerase II (RNA-PolII) transcriptional machinery [4].

Since the NRs, including orphan nuclear receptors, play a critical role in regulating thousands of genes, their activity is highly controlled [5, 6]. Any dysregulation in NRs causes numerous diseases such as cancer, diabetes, and chronic inflammation [7, 8]. Therefore, the identification of endogenous ligands modulating the activity of orphan nuclear receptors represents a significant area of research in the field of nuclear receptors. It has already been shown that the activity of most NRs, including orphan nuclear receptors, can be positively or negatively regulated by small synthetic molecules [9]. This feature makes NRs attractive targets for drug development.

### 1.2. Estrogen-related-receptors (ERRs)

#### 1.2.1. Fundamentals

The Estrogen-related receptors (ERRs) include three members: ERRα (NR3B1, ESRRA gene), ERRβ (NR3B2, ESRRB gene), and ERRγ (NR3B3, ESRRG gene). ERRs belong to a superfamily known as nuclear orphan receptors, which compose of a small number

of cloned receptors for which no ligands have been discovered [10, 11]. However, recently cholesterol was identified as an endogenous ligand of ERRα [31]. ERRα and ERRβ were initially detected by the screening of cDNA libraries using probes associated with the DNA binding domain of the human estrogen receptor α (ERα)[12]. ERRγ was later discovered via a yeast two-hybrid screen using a transcriptional coactivator, glucocorticoid receptor-interacting protein 1 (GRIP1) [13]. It is important to note that ERRs belong to group III of the nuclear receptor superfamily, which also includes ERα, ERβ, glucocorticoid (GR; NR3C1), mineralocorticoid (MR; NR3C2), progesterone (PR; NR3C3), and androgen (AR; NR3C4) receptors. This finding strongly suggests that the ERRs are genuinely steroid receptors [14] (Figure 1 A). Sequence comparison analyses have indicated that while the ERs and the ERRs formed one branch of group III, the four other steroid receptors were classified into another branch of group III [14]. Even though this nuclear receptor was named the Estrogen-Related Receptor, and despite its DNA-binding domain high similarity with that of ERα, it was soon understood that unlike ER, its activity is not regulated by natural estrogens [14] (Figure 1 B).

### 1.2.2. Structural features of ERRs:

ERRs' structural features are kinship to NRs, as shown in Figure 1 C. The structural features include an activation function (AF)-1 domain, a DNA-binding domain (DBD), a ligand-binding domain (LBD), and an AF-2 domain. The N-terminus of ERRs' structure consists of the AF-1 domain, which contains conserved motifs subject to posttranslational phosphorylation and sumoylation, involved in transcriptional activity [15, 16]. The ERRs' DBDs compose two highly conserved zinc figure motifs that carry the receptor to a specific DNA sequence (TCAAGGTCA) termed ERR response element (ERRE). Interestingly, all three isoforms of ERR's subfamily share an approximately similar DBD. Therefore, many genes can be regulated by more than one of the ERR members. Moreover, it has been reported that ERRs are able to bind the ERREs as a monomer, homodimer, or heterodimer [17, 18].

However, the proportion of ERREs within target genes bound by various ERR isoforms is not understood yet, and depending on the context, it likely differs for each cell type. Although DBD of ERRs and ERα share a high similarity, ERRs do not bind specifically to the palindromic sequence of ER response elements (ERE). Importantly, ERRα's affinity to bind ERREs is affected by the acetylation status of 4 Lys residues in the $Zn^{2+}$ finger and C-terminal extension of the DBD [19].

The C-terminal LBD of the ERRs is composed of a well-conserved AF-2 helix motif necessary for cofactor interactions. It is interesting to mention that ERRs can activate transcription without exogenous ligands. This constitutive activity of ERRs is due to a conformational adaptation by the LBD of ERRs in the absence of ligands that supports the recruitment of the cofactors and initiate transcriptional activity of ERRs [20, 21]. The crystal structure-study of the LBD of ERRs reveals that amino acids with bulky side chains occupy these receptors' ligand-binding pockets. It has been demonstrated that they mimic a ligand-bound conformation to facilitate cofactor recruitment by ERR [22].

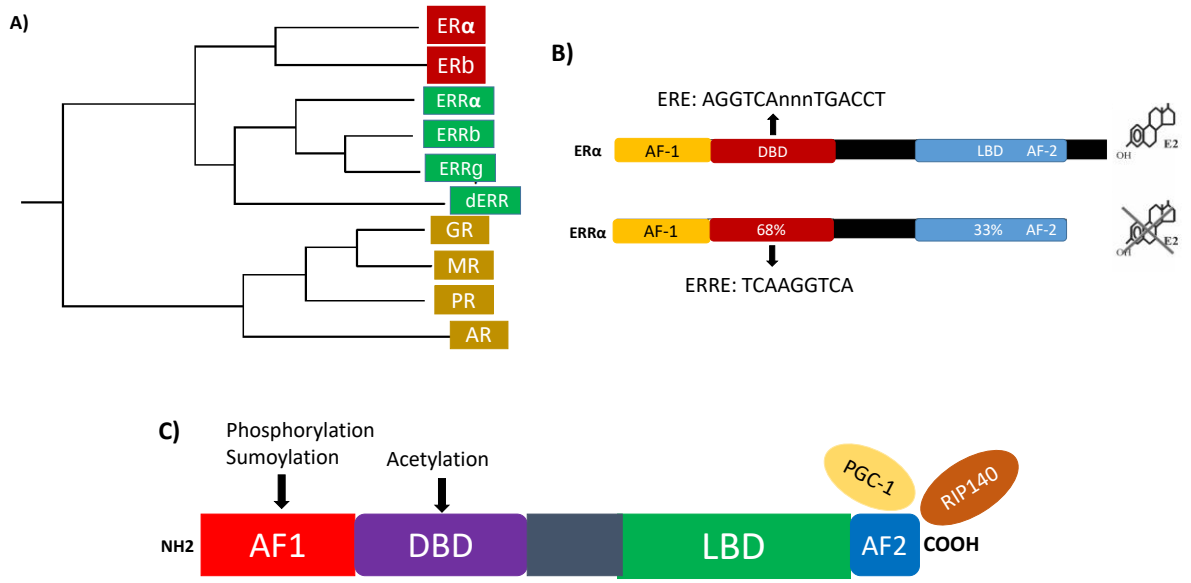



**Figure 1. Structural and functional similarities between ERα and ERRα. A)** Evolutionary tree of group III nuclear receptors. **B)** ERα and ERRα display high similarity in their DNA-binding domain (DBD), but they share only 33% kinship in their Ligand-binding domain (LBD). Therefore, estradiol (E2) does not show affinity to ERRα. **C)** Schematic representation of ERRα structure. NH2-terminal region contains a ligand-independent transcriptional activation function (AF-1), and is subject to post-translational modification; DNA-binding domain (DBD) with highly conserved zinc finger motifs; a linking region that helps the protein flexibility; Ligand-binding domain (LBD), which contains a conserved activation function 2 (AF-2) helix motif. LBD of ERR facilitates the recruitment of its coactivators such as PGC-1 or corepressors like RIP140.

### 1.2.3. Estrogen-related-receptors (ERRs) ligands

#### 1.2.3.1. Endogenous ligands of ERRs

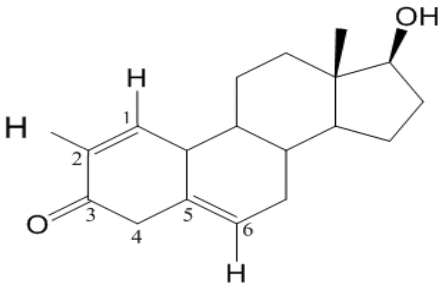
It has been shown that the LBDs of ERRs show only 36% similarity to ERs. Thus, the natural ligands of ERs such as 17β-estradiol (E2), estrone, and estriol do not impact ERRs' transcriptional activity [23, 24]. Also, the identification of the crystal structure of the ERRα-LBD exhibited that in the absence of a ligand, helix 12 of the AF-2 domain is located in the active conformation to fill the ligand-binding pocket by the bulky side-chains [25]. It is interesting to note that since the LBD of ERRs is able to adopt a conformation that can favor the recruitment of nuclear receptor coactivators, such as PGC-1α containing hydrophobic LxxLL motif, ERRs are constitutively active and demonstrate transcriptional activity in the

absence of the exogenous ligands [20, 21]. For example, the crystal structure of LBD of ERRα displayed that phenylalanine Phe328 is responsible for the binding of ERRα's LBD to PPARγ coactivator-1α (PGC-1α) [22]. Notably, it has been reported that there is an open ligand-binding pocket in ERRγ (220 °A) and in ERRα (100 °A), which enable them to accommodate small molecules to modulate their activity [26-30]. These observations indicate the potential "druggability" of ERRs. Many attempts were made to identify the endogenous ligands of ERRs due to the clinical importance of ERRs. However, no endogenous ligands have been identified for ERRs; until recently, cholesterol was identified as an endogenous agonist of ERRα [31]. The identification of a natural nuclear receptor ligand is crucial in order to regulate the receptor activity. This information is useful to develop therapeutics by manipulating the level of ligands. For instance, an aromatase inhibitor was developed to treat ERα-driven cancer, as we gained the knowledge that estrogen, which activates the estrogen receptors, is a product of the aromatase enzyme [32]. It has recently been demonstrated that cholesterol isolated from the brain and kidney of mice acts as an endogenous ligand of ERRα [31]. The computational docking of cholesterol into the LBD of ERRα demonstrated that hydroxyl group of cholesterol make hydrogen bond to E235 of ERRα's LBD. Also, it has been shown that F232 and L228 possibly make important hydrophobic bound with cholesterol. In this paper, the authors demonstrated that in bone tissue the impact of cholesterol, statins, and bisphosphonate on osteoclastogenesis is ERRα-dependent. Moreover, statin-induced muscle toxicity and cholesterol-suppressed macrophage cytokine secretion are abrogated by inhibition of ERRα [31].

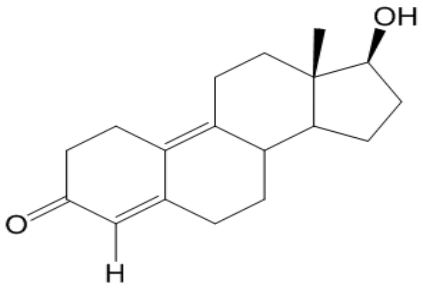
#### 1.2.3.2. Discovery of Estradienolone (ED)

The endogenous steroid ED obtained from human pregnancy urine and blood was initially reported by Philip and Murphy in a study of steroids binding the sex hormone-binding globulin (SHBG) [33, 34]. It was demonstrated that this endogenous steroid bound strongly to

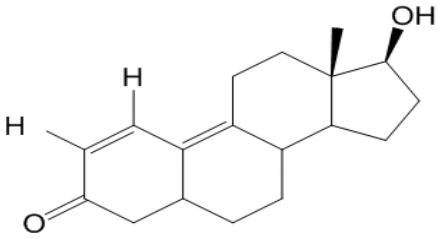
SHBG with an approximately similar affinity of testosterone. It is well-known that the steroids which bind strongly to SHBG are typically bioactive androgens and estrogens, such as testosterone, dihydrotestosterone, and estrogen [35]. Based on the elution pattern obtained from Sephadex LH-20 columns of SHBG-bound material, ED was eluted with other non-polar steroids, immediately following elution of androstenedione. It has been shown that ED levels decrease significantly in association with human labour [34]. ED's maternal serum levels at 30-38 gestation weeks were found to be 3.6±1.5 ng/mL, while ED's maternal serum levels in women who underwent spontaneous labour at the same gestational age were found to be 0.8±0.5 ng/mL [34]. It has been suggested that the placenta is the site for ED synthesis because the highest levels of ED were found in the placenta, although the exact precursor or enzyme involved in ED synthesis is not known [33]. A 17β-hydroxy-(1,5)-estradien-3-one structure has been hypothesized as a putative structure for ED based on the GC-MS data with the mass 273 m/z. In addition, it has been demonstrated that ED could isomerize to estradiol under alkaline conditions [34]. Based on these properties, four possible structures for ED were hypothesized (Figure 2), and compounds 1 and 2 were chemically synthesized [36, 37]. Among these four possible structures, 17β-hydroxy-(1,5)-estradien-3-one (compound 1) was tested and was reported to show lower affinity to SHBG compared to ED, and its elution pattern obtained from Sephadex LH-20 column does not match with the one from ED [37]. Moreover, it has been shown that ED shows a low affinity to estrogen receptors and a high affinity to the progesterone receptor, suggesting that ED may function as a pregestational agent (Philip, unpublished observation). Furthermore, our group's previous studies suggested that ED acts as an inverse agonist of ERRα and ERRγ [36, 37] and decreases cell proliferation of breast cancer cells [37]. However, given the unstable structure of ED, the fine structural features of ED remained to be determined. In addition, whether ED directly binds to ERRs and alters its transcriptional activity will be answered in this thesis.



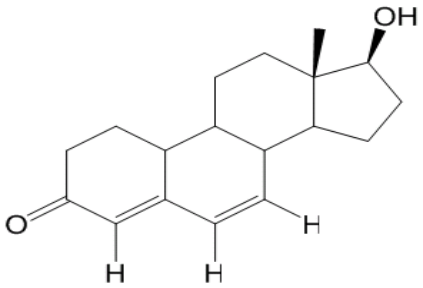

1. 17b-hydroxy-19-norandrosta-1,5 –dien-3-one




2. 17b-hydroxy-19-norandrosta-4,9 –dien-3-one




3. 17b-hydroxy-19-norandrosta-1,9 –dien-3-one




4. 17b-hydroxy-19-norandrosta-4,6 –dien-3-one

**Figure 2. Four possible structures of the endogenous estradienolone (ED). 1)** Compound 1 was chemically synthesized and did not correspond to the endogenous ED. **2)** Compound 2 (C2) was chemically synthesized. **3)** Compound 3 was not synthesized due to extreme structural instability. **4)** Compound 4 is commercially available.

#### 1.2.3.3. Synthetic ligands of ERRs

Knowing that there is a small ligand-binding pocket in ERRs' LBD, researchers synthesized several agonists and inverse agonists for ERRs. The inverse agonist of ERRs binds its LBD and changes the structural conformation in the ERR-LBD that interferes with the co-activator binding. This leads to the inhibition of ERRs' transcriptional activity. For instance, the specific inverse agonists of ERRα, such as compound A, XCT790, and compound 29 [38-40] have been shown to induce cancer cell death [41], inhibit tumor growth [42, 43], and improve diabetes type 2 [44]. 4-hydroxytamoxifen (4-OHT) and its analogs (e.g. GSK5182), have been found as inverse agonists of ERRγ; however, some phenolic acyl hydrazones are shown as a weak selective agonist for ERRγ [45, 46]. Surprisingly, bisphenol A (BPA), a significant environmental contaminant and endocrine disruptor, is an agonist of ERRγ and

binds to this receptor with higher affinity compared to ERs [47, 48]. Diethylstilbestrol (DES) has been identified as an inverse agonist of all three isoforms of ERRs, the ERRα, ERRβ and ERRγ [46, 49]. In addition, some phytoestrogens have been found as agonists or inverse agonists of ERRα, ERRγ and ERRβ. For instance, 4-methylenesterols from theonella swinhoei sponge has been shown to be the natural marine antagonist of ERRβ [50]. Kaempferol, a dietary flavonoid, has been demonstrated as an inverse agonist for ERRα and ERRγ [51]. Moreover, isoflavone has been identified as an agonist of ERRα [52].

### 1.3. Regulation of ERRs

#### 1.3.1. Coactivators and corepressors of ERRs

The activity of ERRs is regulated partially by their interaction with various co-regulator proteins, involving the nuclear receptor coactivator (NCOA) family, also known as the steroid receptor coactivator (SRC) family, and less well understood proline-rich nuclear coactivator 1 and 2, which act as coactivators of ERRs [13, 21, 53]. However, the preferred coactivators of ERRs are Peroxisome Proliferator-Activated Receptor gamma coactivator-1 alpha and beta (PGC-1α and PGC-1β) [54, 55]. It is important to note that PGC-1α also interacts with many other transcription factors, including peroxisome proliferator-activated receptor γ (PPARγ), myocyte enhancer factor (MEF), nuclear respiratory factors (NRFs), and forkhead box O1 (FOXO1) to regulate its effects on mitochondrial biogenesis, mitochondrial oxidative pathways, gluconeogenesis, glycolysis, and glucose and fatty acid transport [56]. However, it has been reported that unlike other transcriptional factors, ERRα capacity to activate the metabolic gene transcription is mostly reliant on its interaction with PGC-1 coactivators, and for this reason, the PGC-1s are often referred to as "protein ligands for ERRs" [57]. In addition, ERRα's metabolism activation occurs most strongly when it is co-expressed with PGC-1. For instance, ERRα directs the activation of PGC-1α-dependent enzymes associated with most of the mitochondria oxidative pathways through ERRα, directly targeting those genes [58].

Intriguingly, PGC-1α mediates ERRα expression via an autoregulatory mechanism, which includes the polymorphic ERR responsive region in the ESRRA gene promoter [59]. Similarly, ERRα controls PGC-1α expression levels in skeletal myocytes by directly binding the ERRE site of PPARGC-1α gene to the promoter, and activating it [60, 61]. Interestingly, The PGC-1s display the same tissue distribution compared to the ERRs, and their expression levels are induced in tissues with high metabolic demands [62]. They regulate energy homeostasis by controlling mitochondrial functions like fatty acid oxidation (FAO), reactive oxygen species (ROS) production, and oxidative phosphorylation (OXPHOS) [57, 63-68]. The prospero homeobox protein-1 (PROX1) inhibits the induced-PGC-1α effect on activation of ERRα and acts as a transcriptional repressor of ERRs/PGC-1 axis[69].

The nuclear receptor cofactor receptor-interacting protein-140 (RIP140) has been demonstrated to act as a corepressor of ERRs in skeletal muscle, adipose tissues, and the heart. In adipocytes, RIP140 suppresses the genes involved in glucose uptake, tricarboxylic acid cycle (TCA cycle), and respiratory chain acting via ERRα pathway [70]. A similar repression pattern was observed in the transgenic mice, overexpressing RIP140 in the heart, which causes downregulation of FAO and mitochondrial metabolism by inhibiting ERRα or ERRγ transcriptional activity [70-73].

### 1.3.2. Post-translational regulation of ERRs

Nuclear receptors are modulated by various posttranslational modifications, including phosphorylation, acetylation, sumoylation, methylation, and ubiquitination. Similarly, for ERRs, posttranslational modification plays a significant role in the control of their transcriptional activity. It has been shown that ERRα is a phosphoprotein that is phosphorylated on multiple sites within its amino-terminal region and DBD [74-77]. For instance, ERRα is phosphorylated in response to epidermal growth factor (EGF) signaling [78, 79]. It has been demonstrated that protein kinase Cδ (PKCδ) phosphorylates ERRα on its DBD.

This process enhances ERRα's DNA binding and induces its transactivation, which in turn attributes to the selective activation of ERRα's target genes in breast cancer cells [74]. Moreover, it has been reported that ERRα and -γ are sumoylated within their amino-terminal regions, and this modification is phosphorylation-dependent. Sumoylation negatively impacts ERRα and -γ transcriptional activity without changing their interaction with the coactivator PGC-1α, the receptors' localization, or their DNA binding characteristics [16]. Furthermore, there is increasing evidence that the DBD of ERRα is acetylated by p300 coactivator associated factor (PCAF) on four highly-conserved lysines and that this modification represses ERRα transactivation by decreasing its DNA binding activity. In contrast, histone deacetylase 8 (HDAC8) and sirtuin 1 homolog (Sirt1) directly interact with ERRα and deacetylate the DBD of ERRα, which in turn increases its DNA binding affinity and its transcriptional activity [19].

### 1.3.3. Modulation of activity of ERRs via nutrient, energetic, and growth signals

Consistent with ERRs' role in metabolism, ERRα and -γ expression and activity are heavily regulated by alteration in energy and nutrient demands. For instance, it has been shown that in rodent models, short-term and endurance exercise leads to induction in ERRα and -γ transactivation and an increase in ERRα expression in skeletal muscles[80, 81]. Similarly, cold exposure augments ERRα expression levels in mice's skeletal muscle and brown adipose tissue [82]. Moreover, the ERR-PGC-1 complex response to nutrient and energetic changes are controlled via several growth signaling pathways. For instance, it has been reported that activation of human epidermal growth factor receptor2 (HER2) and insulin-like growth factor 1 receptor (IGF-1R) pathway augments the expression of PGC-1β via induction of c-MYC (MYC). Similarly, the expression of PGC-1α is induced by the activation of mammalian target of rapamycin (mTOR)/ Yin Yang-1 (YY-1), the downstream pathway of phosphoinositide3-kinase (PI3K)/protein kinase B (Akt) signaling. Interestingly, hypoxia and nutrient stress increase PGC-1α expression, while saturated fatty acids and cytokines induce PGC-1β under

physiological and pathological conditions. Moreover, ERRα expression is upregulated by mTOR activation. The resulting ERR/PGC-1 complex induces the genes involved in metabolism. Notably, it has been reported that ERRα interacts with the β-catenin/ T-cell factor (TCF) complex and with hypoxia-inducible factor 1 (HIF-1) and reciprocally regulate each other's transcriptional activity to the effect of cell migration and angiogenesis.

#### 1.3.4. Degradation of ERRs

ERRα controls its transcriptional activity via autoregulation [59]. ERRα and ERRγ sumoylation in a phosphorylation-dependent manner result in ubiquitin-mediated proteasomal degradation of the receptors, decreasing their transcriptional activity [15, 62]. It has been shown that several ERRα's synthetic inverse agonists, including XCT-790 [83], and compound A [38, 39] increase proteasomal degradation of the receptor. In addition, mTOR regulates ERRα protein degradation through the ubiquitin–proteasomal pathway. It has been reported that in hepatocytes, inhibition of mTOR signaling increases the total ubiquitinated protein levels as well as specific ubiquitination of ERRα through a mechanism involving the ubiquitin ligases Stub1 and Ubb [84]. The ubiquitin-protein ligase Parkin (Park2) increases the degradation of all 3 ERR isoforms in the mouse brain and human fibroblasts [85].

### 1.4. Expression pattern of ERRs in human tissue

The ERRα are widely expressed in most of the adult tissues with the highest levels of mitochondrial oxidative metabolism for producing adenosine triphosphates (ATP), such as kidney, heart, skeletal muscle, brown adipose tissue, cerebellum, and intestine [55, 75, 86-88]. Moreover, ERRα has been detected in adrenal cortex tissues, and its expression is related to adrenal development and possibly fetal adrenal development. For example, ERRα is involved in activating steroid sulfotransferase (SULT2A1) that works to maintain a high level of peripheral dehydroepiandrosterone sulfate (DHEAS) production in adult life [89]. ERRβ plays

a crucial role in the placenta's development and function, as evidenced by studies in mouse embryos lacking ERRβ [90]. In these ERRβ knock-out embryos, placental development is severely impaired with abnormal trophoblast proliferation and differentiation. Postnatally, ERRβ's expression is low in the kidney, heart, stomach, skeletal muscle, and liver [91-93]. ERRγ expression has been detected in numerous tissues, including the thyroid glands, brain, lung, and fetal heart and skeletal muscle of mice and humans [86, 94]. Recent studies in the human placenta have shown that ERRs are rapidly up-regulated from the second trimester until the expected term delivery, indicating their possible involvement in placental growth regulation [95].

### 1.5. Function of ERRs in normal cell physiology

The high expression of ERRs in tissues with high energy demands suggests a significant involvement of ERRs in regulating cellular energy homeostasis in the cellular processes, including differentiation, proliferation, and development. Moreover, identifying the first target gene of ERRs, medium-chain acyl-coenzyme A dehydrogenase (MCAD), which is involved in the initial step of fatty acid beta-oxidation, suggests that ERRs play an essential role in cellular energy metabolism [75, 88]. This notion was supported by the identification of other ERRα's target genes, including pyruvate dehydrogenase kinase isoenzyme 4 (PDK4) [80] and phosphoenolpyruvate carboxykinase (PEPCK) [96], which are involved in cellular metabolism. Furthermore, it has been shown that ERRs modulate the PGC-1α and β–induced mitochondrial biogenesis in tissues with high metabolic demands [54, 55, 66].

Several ERRs' global and tissue-specific knockout mice studies further confirmed the role of ERRs as master regulators of energy metabolism. It has been reported that ERRα-/- mice are resistant to diet-induced obesity and have decreased adiposity [92]. It has also been shown that these mice demonstrate a downregulation in enzymes associated with lipid

metabolism and a decrease in lipid handling in the intestine [97]. The physiological effect of ERRα on normal tissues was better understood when ERRα was absent in the high energy demanding tissues, such as the heart, skeletal muscle, activated macrophages, and brown adipose tissue (BAT). BAT is a specialized tissue involved in thermogenesis. ERRα-/- mice demonstrate defects in mediating the genes involved in adaptive thermogenesis in BAT. Therefore, these mice are not able to survive in the cold [98]. ERRα deficiency reduces mitochondria biogenesis and oxidative capacities to produce heat in these mice. In addition, ERRα is highly active in the heart and skeletal muscles to derive oxidative metabolism. ERRα knock-out heart shows a reduced energetic reserve and impaired mitochondrial function, resulting in rapid depletion of phosphocreatine and delayed ATP recovery [99].

The global deletion of ERRγ in mice has negatively affected post-natal survival, and this ERRγ deletion is connected to a defective heart and central nervous system [94]. Several studies reveal a pivotal role for ERRγ in post-natal survival. ERRγ is involved in reprogramming metabolism from glycolysis to fatty acid oxidation and ion transport in the myocardium. In ERRγ deficient mice, this metabolic reprogramming and ion transport in the myocardium are impaired, leading to a decrease in myocardial mitochondrial function and a downregulation of voltage-gated potassium transporters, which are the direct target genes of ERRγ [100].

Regarding ERRβ's role in cellular physiology, homozygous deletion of the ESRRB gene causes death at 9.5–10.5 d.p.c. due to the impaired placental formation [90]. ERRβ knocked out in the developing embryo has been shown to be lean with increased activity and basal metabolic rate [101].

### 1.6. ERRs in pregnancy

Some endocrine studies have revealed that steroid hormones are necessary for the placental viability and maintenance of pregnancy [90]. It has been reported ERRβ expressed

during embryogenesis may be involved in early placental development. In addition, mice treated with DES (a synthetic inverse agonist of ERRβ) exhibit abnormal early placenta development associated with an overabundance of trophoblast giant cells and an absence of diploid trophoblast. Thus, ERRβ is essential for normal placental formation, and pharmacological modulation of its activity could influence reproductive function [102, 103]. Moreover, ERRγ expression peaks during vascularization of the placenta [104, 105], and it plays a major role in trophoblast differentiation by mediating oxygen-dependent induction of aromatase (CYP19) and of the voltage-gated potassium channel, both of which are implicated in human trophoblast differentiation [104, 106]. In addition, ERRγ regulates mitochondrial biogenesis, expression of energy metabolism target genes, and 17β-hydroxysteroid dehydrogenase type 1 (HSD17B1), an enzyme essential in estradiol production and prevention of fetal loss, in trophoblasts [105, 107]. ERRα is mainly expressed in the placental syncytial trophoblast (ST) and is important for the proliferation and migration of trophoblasts [108]. ERRα is strongly implicated in the development of preeclampsia in pregnancy, a major cause of maternal and fetal morbidity and mortality [109]. It is interesting to speculate that given that the placenta can produce steroids, there may be an endogenous agonist or inverse agonist that creates a balance between giant cell formation and stem-cell proliferation for the normal development of the early placenta [110].

### 1.7. ERRs in metabolic diseases

There is some evidence showing that ERRs are involved in the onset of obesity. ERRα is overexpressed in white adipose tissue (WAT), where it regulates body fat metabolism in humans [92]. In addition, it has been shown that ERRs are also involved in insulin resistance and type 2 diabetes[100]. As previously was mentioned, ERRα knockout mice are resistant to a high-fat-induced-obesity diet, and despite ERRα suppression of gluconeogenic genes in hepatic tissue, the glucose level does not increase in these mice [92, 111]. Moreover, treating

mice on a high-fat diet with compound 29 (Cpd29) as an inverse agonist of ERRα prevents hyperlipidemia and increases insulin sensitivity [44]. Interestingly, the effect of Cpd29 on insulin sensitivity and glucose tolerance is as efficient as the anti-diabetic drug rosiglitazone in Zucker diabetic fatty rats [84]. Moreover, overexpression of hepatic ERRγ was reported in a diabetic mouse model. Based on previous studies, increased ERRγ activity in diabetic mice may cause hyperglycemia, hence, using ERRγ inverse agonist GSK5182 significantly decreases fasting serum glucose levels in diabetic mice, similarly to the anti-diabetic drug metformin [112]. Based on the current evidence, ERRγ plays an important role in glucose homeostasis in normal and diabetic states and can be considered an attractive target to develop an anti-diabetes drug.

Moreover, it has been demonstrated that ERRs are involved in heart failure. In the heart, the ERRα expression level is high because it facilitates ATP production, which is required for its high metabolic activity. There is some evidence to reveal that ERRα controls a wide range of cardiac metabolic activities; therefore, by inhibiting ERRα activity, significant changes occur in the metabolic adaptation of the heart. For instance, it has been reported that ERRα knockout mice develop heart failure more rapidly than the wild-type one [99]. Furthermore, many studies have supported the role of ERRα in heart failure progression [99, 113].

ERRs involvement in osteoporosis was also shown in several studies [114]. ERRα is involved in osteoblast through regulation of the genes encoding bone matrix protein, for example, osteopontin and bone sialoprotein. It has been demonstrated that ERRα overexpression in primary rat calvarial cells leads to the expression and differentiation of these bone matrix proteins [114, 115]. In addition, in ERRα knockout mice, the isolated bone marrow from mesenchymal stem cells (MSC) showed defected osteoblast differentiation and mineralization [115]. However, in the whole body ERRα -/- female mice, they became resistant

to bone loss from aging and ovariectomy. Therefore, in the case of estrogen deficiency, ERRα induces bone loss. On the other hand, osteoclasts have abundant mitochondria in order to supply their high energy demand. There is some evidence based on knockout models that ERRα and PGC-1β has a vital role in normal osteoclastogenesis by regulating mitochondrial biogenesis and oxidation [116]. In ERRα-/- mice, impaired osteoclastogenesis in bone marrow-derived MSCs, leads to reduced fatty acid oxidation, TCA cycle, and electron transport chain activity. These findings show a reduced number of osteoclasts in bone tissue, resulting in reduced bone density due to the impaired bone resorption [116]. In conclusion, the above-mentioned evidence suggests that targeting ERRs may increase osteoblast activities, resulting in induced bone formation.

### 1.8. ERRs in cancers

ERRs are known as a master regulator of metabolism and are shown to play essential roles in various cancers. ERRs are linked to ovarian cancer where the overexpression of ERRα is correlated with higher grades of ovarian tumors and, therefore, is associated with low survival rates [117, 118]. Similarly, the expression of ERRγ is also shown to be high in ovarian tumors. However, the patients with overexpressed ERRγ demonstrated a better survival rate. It has been demonstrated that the low expression levels of ERRβ is not related to the disease status or survival rates [117]. Moreover, it has been reported that overexpression of ERRα in prostate cancer is correlated with low survival rates [119]. However, the expression level of ERRβ and ERRγ have been shown to be low in prostate tumors, indicating they act as a favorable marker in prostate cancer patients [120]. ERRs are also highly expressed in endometrial tumors, and this overexpression of ERRα is linked to the high clinical stage of this cancer and is correlated to the unfavorable clinical outcome for endometrial cancer patients.

In contrast, in uterine cancer, there is no consistent evidence about the expression level of ERRα. While the expression of ERRγ has been indicated to be high in uterine tumors

compared to the normal tissue, and also correlated with myometrial invasion [118]. In colorectal tumor cells, there is an increase in ERRα expression, which means that ERRα is involved in human colorectal carcinoma. ERRα is emerging as a biomarker of colorectal cancer because of its messenger expression level changes with tumor progression. Although ERRγ is expressed in colorectal cancer, its expression level is weak in both cancer and adjacent tissue [121]. Surprisingly, ERRβ mRNA was not detected in human colorectal tumors and adjacent tissue compartments [122]. In addition, ERRs have been shown to be overexpressed in breast cancer, and their impact on breast cancer progression will be discussed in detail in the sections below.

## 1.9. Breast cancer

### 1.9.1. Breast cancer subtypes

Breast cancer (BC) is the most diagnosed disease among women and has the second-highest incidence among all cancer types worldwide [123]. It has been reported that breast cancer is a heterogeneous disease and differs significantly among patients (intertumor heterogeneity) and even within each tumor (intratumor heterogeneity). Genome expression analysis categorizes breast cancer into four main intrinsic molecular subtypes with prognostic and therapeutic implications: luminal A, luminal B, HER2-enriched, and basal-like [124]. The luminal A and luminal B subtypes illustrate tumor heterogeneity within ER-positive breast carcinomas and have better survival than HER2-enriched and basal-like subtypes. Luminal A subtype expresses ERα and progesterone receptor (PR). The luminal B subtype also is positive for ERα expression but is negative for PR. Luminal B tumors are characterized by increased expression of the genes associated with proliferation and have a worse prognosis than luminal A tumors [82]. The HER2-enriched subtype is identified by its high expression in the ERBB2 gene (HER2) on human chromosome 17, associated with aggressive phenotype and poorer survival to breast tumors [125]. HER2, as a member of the HER/EGFR/ERBB group, belongs

to a receptor tyrosine kinase (RTK) superfamily. This subtype of breast cancer comprises ER-/PR-/HER2+ and ER+/PR+/HER2+ tumors. Interestingly, luminal and HER2 subtypes derive from epithelial cells of the lumen.

Finally, the triple negative (TN) subtype of breast cancer, which includes basal-like subtype is enriched with genes expressed in basal myoepithelial cells and is negative for the three above-mentioned markers [124]. This subtype shows aggressive phenotype and adverse clinical outcome [126]. In addition to the subtypes mentioned above, recent sub-classifications were defined based on the newly discovered breast cancer markers. These recent subtypes incorporate claudin-low tumors with stem-like signature [127] and AR-positive molecular apocrine tumors [128]. The claudin-low subtype is defined as the triple-negative tumors expressing low levels of specific cell junction proteins, and this subtype is linked to poor prognosis [129]. Similarly, it has been shown that the nuclear expression of the AR can be detected in 12–55% of triple-negative (ER-/PR-/HER2-) breast cancer [130]. However, AR's role in TN breast cancer remains controversial. Some studies reported that AR expression is associated with improved survival in other tumor subtypes [131]. However, there is a study that revealed that using AR antagonists shows promising results in AR+ triple-negative breast carcinomas [132].

### 1.9.2. Therapeutic approaches

Defining the heterogeneity via the expression levels of the established predictive biomarkers, hormone receptors, and HER2 oncoprotein is the foundation for targeted therapy. Gene expression profiling and distinguishing the cancer-related gene expression profile from the normal one allow us to develop personalized therapy [133]. Based on this information, there are distinct therapies to treat various subtypes of breast cancer. For instance, patients with ERα-positive breast tumors often respond to endocrine therapy (ET) alone or in combination with targeted agents that reduce the tumor burden. Some of the current ETs include using specific

inhibitors of aromatase to deplete estrogen levels, estrogen receptor modulators (SERMs) or selective estrogen down-regulators (SERDs) to directly target ER, and inhibition of other targets (e.g., cyclin-dependent kinase 4/6 (CDK 4/6), PI3K, etc.) [134, 135]. Moreover, there are several approaches to treat patients with HER2-amplified tumors. One approach uses antibodies against the HER2 protein [136], such as trastuzumab and pertuzumab, to block the tyrosine kinase activity by preventing dimerization [137]. Other strategies to target ERBB2-overexpressed breast tumor is using small molecules that inhibit intracellular ERBB2 kinase activity like lapatinib [138].

In contrast to the above-mentioned subtypes of breast cancer, the treatment options for triple-negative breast cancer (TNBC) patients are limited, and chemotherapy is the main systematic treatment option. Standard chemotherapeutic agents used are taxanes, and doxorubicin. [139, 140]. For TNBC patients with BRCA mutation, some chemotherapeutic drugs may be considered to treat these patients, such as platinum drugs (like cisplatin) or the targeted PARP inhibitor, talazoparib (Talzenna)[141]. Advanced TNBC that expresses the PD-L1 protein may be treated first with the immunotherapy drugs like atezolizumab and Abraxane (albumin-bound paclitaxel) [142]. Although several targeted therapies for breast cancer were developed to improve their survival rate, resistance to these therapies can cause tumor relapse. Therefore, there is a need to develop novel drug-targeted therapy to treat these patients.

### 1.10. Breast cancer and pregnancy

Early childbirth is one of the factors that consistently decreases the lifetime risk of breast cancer regardless of ethnicity. Before the age of 20, women who have undergone a first/full-term pregnancy/birth (FFTB) have a 50% reduced risk of developing breast cancer [143, 144]. However, women over 35 years of age who have undergone a first/full-term pregnancy/birth have shown an increased risk of developing breast cancer [144]. It is important to note that the protective pregnancy impact is only limited to ER+ breast cancer [145]. The

other factor associated with a decrease in breast cancer risk is the increasing number of births. Women who have undergone five or more full-term births have half the breast cancer risk of women who have not undergone birth [146]. However, the underlying mechanisms on how pregnancy-dependent breast cancer protection occurs are not well understood [147].

## 1.11. Breast cancer and obesity

### 1.11.1. Cholesterol impact on breast cancer progression

#### 1.11.1.1. Clinical and animal studies

Obesity and metabolic syndrome have emerged as significant risk factors for breast cancer [148, 149]. The initial evidence associating cholesterol to cancer was identified in 1909 when crystals of a 'fatty nature' were observed in tumor sections [150]. However, after several decades, the mechanism underlying cholesterol's impact on increased cancer risk remains unclear. There is growing evidence that obesity and cholesterol increase breast cancer risk and are linked to a shorter term to disease recurrence and higher mortality rate [151, 152]. Recently, an epidemiological study revealed that patients with developed breast cancer showed higher low-density lipoprotein-cholesterol (LDL-cholesterol) and very-low-density lipoprotein-cholesterol (VLDL-cholesterol). In contrast, no correlation between high-density lipoprotein (HDL) or total cholesterol and breast cancer was observed [153]. Another study examined the correlation between pre-diagnostic serum lipid concentrations and breast cancer risk and survival. It is noteworthy that dietary saturated fat intake is a synonym of dietary cholesterol intake and that saturated fat increases LDL-cholesterol [154], which is a common comorbidity in obesity [155]. This study demonstrated that serum lipids are linked to breast cancer risk [156]. Moreover, Li and colleagues conducted a meta-analysis investigating the correlation between dietary cholesterol and breast cancer. They demonstrated that the link between dietary cholesterol and breast cancer recurrence was significant when cholesterol consumption was higher than 370 mg/day [157]. These observations have been confirmed by other

epidemiological studies that suggest an association between dietary cholesterol consumption and the increase in the risk of both ER+ and TN breast cancer [101, 157-162].

Furthermore, cholesterol has been correlated with increased breast cancer risk in animal models. It has been shown that a high-fat, high-cholesterol (HFHC, Western) diet promotes tumor progression in the murine MMTV-PyMT model of breast cancer [163, 164]. In addition, another study reported that MDA-MB-231 cell-derived xenografts demonstrated significantly greater tumor proliferation and angiogenesis when fed with an HF/HC diet [163, 165, 166]. The role of dyslipidemia in breast tumor progression has also been investigated in highly hypercholesterolemic mice that lack apolipoprotein E ($ApoE^{-/-}$), an important protein for the lipoprotein particles' transportation; thus, it helps for lipid homeostasis. This study also demonstrated that a high cholesterol diet (HCD) with a normal fat content significantly promoted tumor growth and metastasis in a transgenic mouse model with a replaced mouse Apoe gene with a human homolog, APOE3 [167]. Moreover, it has been reported that the growth of breast cancer xenografts in mice fed with HFHC was abolished using Ezetimibe, an inhibitor of intestinal cholesterol uptake [166]. This finding supports the notion that cholesterol itself can impact tumor pathophysiology, and some of the effects of HFD on breast cancer risk can be associated with cholesterol.

#### 1.11.1.2. Molecular mechanistic studies

Cholesterol is a necessary element for the plasma membrane's stability and architecture and is involved in the production of bile acid and steroid hormone in mammals [168]. Under normal conditions, the circulating cholesterol levels are balanced by cellular cholesterol biosynthesis, dietary cholesterol, and removal of the excess cholesterol from tissues [169]. It is well understood that the level of free cholesterol in most cells is continuously regulated by a series of homeostatic processes involved in efflux, uptake, de novo synthesis, and plasma and endoplasmic membrane partitioning [170]. The most important regulators of intercellular

cholesterol level are transcription factors, including sterol regulatory element-binding protein 1 and 2 (SREBP1 and 2) and liver X receptors (LXRs) [171-173]. SREBPs, specially SREBP2, play an essential role in the control of cholesterol biosynthesis. For instance, in the case of low cholesterol levels in the endoplasmic reticulum, SREBP2 is cleaved and translocated to the nucleus where binds sterol response elements (SREs) to activate the expression of cholesterol-biosynthesis enzymes, like HMG-CoA reductase (HMGCR) and for cholesterol influx, such as the low-density lipoprotein receptor (LDL-R) [174]. These SREBP2-mediated cholesterol levels are believed to be critical in cholesterol-producing normal tissues and are assumed to function similarly in tumors of most cancer types [174]. Another key player in regulating cholesterol levels is the LXRs. The LXRs are the ligand-regulated transcription factors whose activity increases by oxysterol ligands derived from cholesterol within cells [175]. LXR returns cells to normal cholesterol conditions by increasing the gene expression levels involved in mediating cholesterol uptakes, such as inducible degrader of LDLR (IDOL), an E3 ubiquitin ligase that activates the lysosomal degradation of LDLR and regulating cholesterol reverse transport – ATP-binding cassette subfamily A1 (ABCA1) and ATP-binding cassette subfamily G member 1 (ABCG1) [176]. Given the complexity involving the mechanisms that control intracellular cholesterol homeostasis, it has been hard to understand how increases in circulating cholesterol can impact cancer pathogenesis. However, it is clear that the transformed cells have the ability not to follow the processes that maintain cholesterol homeostasis during rapid proliferation when they require high cholesterol levels to grow faster. Accumulating evidence suggests that alteration in cholesterol metabolism may also play a significant role in carcinogenesis and malignant progression [177]. Carcinogenesis is a complicated process that involves massive reprogramming of genetic information, signaling mechanisms, structural components, and energy metabolism [178] of the transformed cells. It can be assumed that this significant transformation process is the consequence of the cancer

cells' induced metabolic requirements [179] to sustain tumor proliferation, migration, and metastatic activities. Given the importance of cholesterol metabolism and transport in cells, these well-regulated mechanisms will likely become altered during rapid cell division and membrane synthesis required for tumor development [180]. Several studies of cancer cells reported that cholesterol synthesis in the transformed cells is enhanced compared to the untransformed cells [181, 182], and possibly cancer cells promote the upregulation of cellular cholesterol synthesis via the abundant availability of precursors (acetyl-CoA) through glycolysis pathway that also initiates de novo fatty acid synthesis [179].

Cholesterol plays an intricate role in the plasma membrane, as it accumulates in specific regions of the membrane and combines with sphingolipids creating highly stable micro-domains known as lipid-rafts. It is well-known that lipid-rafts act as signaling platforms, and depending on their lipid composition and their target proteins, they have a specific structure and function [183]. It has been demonstrated that several proteins that are associated with lipid-rafts have been implicated in key signaling pathways linked to tumor development. Accumulating evidence demonstrated that an increase in cholesterol content in the plasma membrane facilitates lipid raft formation and induces signaling activity at the membrane.

Notably, the underlying mechanism of enhanced tumor growth in the above-mentioned ApoE/mice, which were fed with an HFHC diet, is due to enhanced PI3K activation and AKT phosphorylation [164]. Interestingly, when these mice were treated with a PI3K inhibitor, the tumor growth was inhibited. This finding suggested that the PI3K/AKT signaling pathway is involved in cholesterol's pathological actions in tumors. However, this finding has been criticized because the plasma cholesterol levels in this mouse model exceed 2000 mg/dL, which is greater than what would be relevant to hypercholesterolemia in humans (240 mg/dL). It has also been shown that the required concentration of exogenous cholesterol to promote cellular growth in breast cancer cells is much lower than that needed for lipid raft formation and AKT

phosphorylation [164]. This piece of evidence would argue against the idea that cholesterol applies its pathological impact by enhancing lipid raft formation and membrane signaling and would support the notion that cholesterol, or its derivatives, affects the tumor pathology as a signaling molecule. Consistently, the cholesterol metabolite 27-hydroxycholesterol (27HC) has been demonstrated to function as an endogenous ER modulator (SERM) and an LXR agonist [184, 185]. It augments ER-positive breast cancer cell growth *in vitro* [184, 186], and acts as an endogenous agonist of ER in the breast cancer cells. It has also been shown that activated ER is able to inhibit LXR signaling, reducing its protective effect. However, the underlying mechanism of this process remained to be elucidated [187]. The pro-tumor impacts of 27HC have been attributed to several breast tumors, including MCF7 cell-derived xenografts and MMTV-PyMT transgenic mice [187]. Intriguingly, accumulating evidence demonstrated that the cholesterol metabolite (27HC) acts as a signaling molecule through ER and LXR in ER+ breast cancer cells, which may explain how hypercholesteremia increases breast cancer risk in ER+ patients [187-189]. However, several studies have demonstrated that obesity and high cholesterol intake increase the risk not only in ER+ breast cancer but also in triple-negative breast cancer [190-192]. This supports the notion that cholesterol itself functions as a signaling molecule and that such signaling may involve pathways other than the ER pathway. Here we show that ERR is involved in cholesterol pathology in breast cancer.

### 1.11.2. Cholesterol-lowering drugs on breast cancer survival rate

#### 1.11.2.1. Clinical studies

Inhibition of the mevalonate pathway is commonly used during the treatment of hypercholesterolemia. Statins, known as cholesterol-lowering drugs, are competitive inhibitors of HMG-CoA reductase. Thus, they block the mevalonate pathway and limit the downstream reactions that produce the final products, such as cholesterol, isoprenoids, dolichol, ubiquinone, and isopentenyl adenine [193].

Evidence strongly suggests that the mevalonate pathway's inhibition using statin drugs influences oncogenic processes such as tumor growth and metastasis [193-195]. Statins use impacts the outcome of various type of cancer, including breast [196, 197], prostate [198], ovarian [199], lymphoma [200], renal cell carcinoma [201], and colorectal [202] cancer. Some of these studies reported that statins use is correlated to longer disease-free survival, while others suggest no benefits. A recent meta-analysis demonstrated that the average effect of statin use is beneficial for overall survival and cancer-specific survival [203]. Remarkably, another study reported that colorectal, prostate, and breast cancers, the three most common cancer-type, demonstrated a benefit from statins use [203]. Notably, these three tumors have demonstrated high expression levels of ERRα correlated to tumor progression and poor survival [204].

Moreover, there is growing clinical evidence supporting the protective effect of statins on reducing recurrence in breast cancer [205]. For instance, a study obtained from a population of all stage I–III breast cancer patients in Denmark [206] reported that there is a 10% decrease in breast cancer recurrence among women prescribed a lipophilic statin (simvastatin), but this reduction was not observed among women who were prescribed a hydrophilic statin [206]. Clinical data suggested that statins decrease breast cancer recurrence and increase survival rates in breast cancer patients [207].

Bisphosphonates, in particular, nitrogen-containing bisphosphonates (N-BPs), are primarily used to improve bone health in cancer patients, as they are able to negatively regulate osteoclast-mediated bone resorption that is extensively associated with metastasis in various cancer types [208]. However, NBPs are also involved in inhibiting the cholesterol biosynthesis pathway. They function by blocking two enzymes involved in the mevalonate pathway called farnesyl pyrophosphate synthase (FPPS) and geranylgeranyl pyrophosphate synthase (GGPPS). Based on clinical studies, the anticancer effect of bisphosphonates is still contradictory. However, some studies reported that bisphosphonates decrease metastatic cancer

in non-solid and solid tumors [209]. In addition, apart from N-BPs' impact on protecting bone tissue, several in vitro and clinical studies demonstrated that N-BSs directly affect cancer cells by reducing tumorigenicity, inducing apoptosis, and inhibiting cell proliferation, invasion, and angiogenesis [209].

#### 1.11.2.2. Molecular mechanistic studies

Blocking the mevalonate pathway inhibits the synthesis of isoprenoid molecules (farnesyl pyrophosphate and geranylgeranyl pyrophosphate) that are required for post-translational modification and activation of Ras, Rac, and Rho GTPases molecules. It has been shown that isoprenoid molecules are involved in tumor proliferation [210, 211]. While statin therapy inhibits the intracellular synthesis of cholesterol, it may alter the cholesterol content of tumor cell membranes, interfering with crucial signaling pathways [210, 212].

Statins may adopt several molecular mechanisms to exert their anticancer effect: 1) through inhibiting protein prenylation, leading to reducing tumor progression, and 2) via suppressing rat sarcoma (Ras) signaling, resulting in increased apoptosis by downregulation of Akt phosphorylation and mTOR [213, 214]. Since statins impact the early phase of the mevalonate pathway, it may demonstrate potential therapeutic benefits of statins to hinder cancer development by inhibiting cholesterol synthesis or preventing isoprenoids (non-steroid branch) production. Moreover, statins are known to have anti-inflammatory effects that may reduce tumor growth and development [168].

Several preclinical studies reported that lipophilic statins, such as atorvastatin, simvastatin, and lovastatin, might have anti-cancer effects mainly on HR-negative breast cancer[215, 216]. This class of statins may demonstrate some anti-cancer effects, as they can freely diffuse across cell membranes. This characteristic provides them with a greater bioavailability in peripheral tissues, such as the breast. Some studies have shown that lipophilic statins result in significant growth inhibition in HR-negative breast cancer cell lines, but only

limited effects in HR-positive cell lines *in vitro* [217-219]. Notably, the human breast cancer cell line MDA-MB-231, which corresponds to the triple-negative breast cancer phenotype, is particularly responsive to lipophilic statins [218].

## 1.12. ERRs and breast cancer

### 1.12.1. Nuclear receptors and breast cancer

From a clinical perspective, the link between NR function and breast cancer biology was traced to Sir Thomas Beatson's report in 1896 that removing ovaries from young women with advanced breast cancer could lead to tumor regression [220]. Several epidemiological studies reported that exposure to estrogen has been associated with increased breast cancer risk [221]. In addition, a requirement for ERα in normal mammary gland development is supported by knockout mouse models [222]. ERα is expressed in as many as 70% of breast cancers and is a very effective biological target for breast cancer treatment [223]. Moreover, it has been demonstrated that ERα protein levels are the key markers for a potential response to hormone therapy. Progesterone receptor (PR), an ERα target gene expression, is an additional marker for responsiveness. However, not all tumors that are classified as ERα-positive respond to treatments, and in 40% of cases, resistance to endocrine therapy has been reported [224].

Clinically, PR expression is commonly assessed in breast cancer tissue samples by immunohistochemistry (IHC) using an antibody that recognizes both PR-A and PR-B. It has been reported that PR expression is frequently positively correlated with ERα expression. However, recent studies reported that in the absence of PR expression in ER-positive tumors, an increased growth factor signaling was observed. For instance, PR-negative tumors have been demonstrated an approximately 25% increase in HER-2 expression compared to ER/PR-positive tumors (10%) [225].

The AR is necessary to develop male reproductive organs and is also expressed in both ERα-positive and triple-negative breast cancer subtypes. AR expression has been recently

investigated in several subtypes of breast cancer. In these cases, AR expression was correlated with a good prognosis in ER/PR-negative cancers [226]; consistently, loss of AR was linked to a poor prognosis in lymph node-positive ER/PR/HER2-negative breast cancers [227]. Although the exact role of AR in TNBC remained to be determined, several studies demonstrated that AR agonists, such as 5α-dihydrotestosterone [228] or DHEAS, inhibit cell growth in AR-positive breast cancer cell lines. This suggests that AR induces an inhibitory growth signal in breast cancer [229].

It has been reported that glucocorticoid receptors are expressed in 50% of invasive breast cancers and many breast cancer cell lines [230]. Understanding the molecular mechanisms behind GR's antitumor effect on breast cancer has potential physiological relevance, as it has been shown that stress increases endogenous cortisol responses, which have been associated with breast cancer progression [231]. In addition, the routine administration of high doses of synthetic glucocorticoids as premedication for chemotherapy treatment has the potential to activate GR-mediated tumor cell survival pathways, hence, diminishing chemotherapy's efficiency [232]. The current pipeline of selective GR modulators under development may introduce new options for preventing chemotherapy-associated side effects without inducing cell survival mechanisms in breast cancer cells [233].

All PPAR isoforms play an important role in regulating cellular metabolism, including lipid biosynthesis and glucose metabolism in breast cancer cells. Interestingly, PPARγ synthetic ligands inhibit cellular growth in human breast cancer cell lines *in vitro* [234]. For example, a specific PPARγ agonist (Rosiglitazone) induces apoptosis growth arrest in MCF7 cells via activation of cross-talk between p53 and PPARγ [235]. Similarly, it has been reported that PPARγ expression levels are positively correlated with an improved clinical outcome for breast cancer patients [236]. Together, these studies demonstrate that PPARγ plays a key role

in anti-tumorigenicity in human breast cancer, and pharmacological activation of these receptors is likely to have significant consequences in breast cancer therapy.

In addition, several studies demonstrated that the retinoic acid receptor (RAR) is involved in breast cancer suppression, and its agonist inhibits the growth of monolayer cultures of several ERα-positive (but not ER-negative) cell lines in a dose-dependent manner [237]. The next section will discuss the involvement of ERRα in the development of breast cancer.

### 1.12.2. ERRs and Breast cancer

There is increasing evidence to show that ERRα's expression level is high in human breast tumors [238, 239]. Based on a cohort of breast cancer patients, there is a positive correlation between the expression of ERRα and the expression of ERBB2 and ERRα' coactivators, including SRC-3 and PGC-1 with the induced risk of recurrence, and poorer survival in these patients. In addition, ERR γ overexpression has been associated with resistance to anti-estrogenic drugs, such as tamoxifen (TAM) therapy in ER+ breast cancer cells [240]. Interestingly, it has been demonstrated that ERRα is overexpressed in ER-negative and HER2 positive, or in the ones with p53 mutation, which are the markers of adverse clinical outcome and recurrence [239, 241, 242]. Thus, the ERRα's expression levels could be used as markers of poor prognosis and response to therapy. ERRα expression has been reported in approximately 55% of breast tumors where it was detected in the nucleus, perinucleus, and cytoplasm of cancer cells while normal cells express ERRα only in the nucleus [239]. By contrast, overexpression of ERRβ is associated with inhibition of breast cancer cell proliferation, and it is associated with a favorable outcome for breast cancer patients [243]. Moreover, overexpression of ERRγ has been demonstrated in some breast cancer tumors, and its expression is positively correlated with ERBB4 expression, lymph node-negative status, as well as ERα and PR expression. [238, 244]. Also, as was mentioned above, ERRγ overexpression induces resistance to TAM in ER+ breast cancer models [240].

### 1.12.3. Crosstalk between ERRs and ERs

Some studies indicated that overexpression of ERRα is inversely linked to the expression level of ERα and PR, which are known as positive prognostic markers in breast cancer [241]. Initially, it was assumed that ERRα and ERα may show similar transcriptional activity and hence, play the same roles in breast cancer. However, it has been reported that only a few genes are regulated via both ERα and ERRα in MCF7 breast cancer cell lines [43]. This evidence was in line with other studies; ChIP-on-chip techniques were used in order to determine ERα and ERRα binding sites in MCF7 cells and only approximately 18% of ERα target genes has been shown to be co-regulated by ERRα [245]. Interestingly, based on Gene Ontology analysis, ERα is primarily involved in regulating breast cancer tissue development and cell proliferation. However, ERRα plays a key role in the expression of genes related to cellular metabolism, involving those enzymes encoded in the OXPHOS and TCA cycle [246]. Several studies demonstrated that knockdown of ERRα *in vitro* and *in vivo* dramatically inhibited the cell growth of MDA-MB-231 triple-negative breast cancer cells [38, 39, 43, 241, 247]. Collectively, this evidence confirms the ER-independent activities of this receptor in breast tumors. Although a high degree of amino acid similarity was found in the DBDs of ERRs and ERα (e.g., hERRα, 68% identity), ERRs do not bind tightly to the exact palindromic sequence of ER response elements. However, it has been reported that ERRs share some target genes with ERα [14, 248-250]. Most importantly, ERRs bind only a part of a multi-site module that regulates estrogenic responses, and based on the gene context, ERR and ER may support each other's full activation (e.g., lactoferrin) or may block one another's activity (e.g., monoamine oxidase B(MAO-B)) [249, 250]. However, several studies demonstrated that ERRα and ERα both are involved in the regulation of the expression levels of the gene Trefoil Factor-1 (TFF1), but they do it independently through binding their own response elements in close proximity with the target promoter [251]. All the above-mentioned evidence alongside

the identification of a specific binding site for the ERRs do not support the idea that the two receptors largely co-regulate most of the estrogen-mediated genes [75].

### 1.12.4. Mechanism of action of ERRs in breast cancer

It has been shown that ERRα is overexpressed in breast cancer cells, and its overexpression is correlated with poor prognosis and high recurrence in breast cancer patients [43, 241]. The mechanism of action by which ERRα exerts its effect on breast cancer cells is commonly dependent on the expression of its coactivator PGC-1α/β. There is an overexpression of PGC-1α in the hypoxic region of the tumor, which results in VEGF ERRα-dependent expression, leading to angiogenesis [252-254]. Another study revealed that in breast cancer that metastasized to the brain, there is overexpression of PGC-1α/β and ERRα, resulting in the upregulation of the corresponding target genes such as the TCA cycle, OXPHOS, and glycolysis. These changes in gene expression of metabolic events help tumor cells in the metastatic stage survive in a low glucose environment of the brain [91]. In HER2 positive breast cancer, HER2 activation initiates a signaling cascade resulting in phosphorylation of several serine residues at the N-terminus of ERRα and induces the expression of ERRα and its target genes [16, 75, 255] as a downstream pathway in breast cancer cells [241]. This stimulation of ERRs activity by the HER2 receptor induces metabolic target genes of ERRα to rewire breast cancer metabolic programming [256]. It has been demonstrated that ERRα's response to the growth factors, such as HER2 and EGF, is target gene-specific [74].

Furthermore, ERRα promotes growth in breast cancer xenografts via increasing the vascular endothelial growth factor (VEGF), which is involved in angiogenesis [257]. In addition to the involvement of ERRα in the proliferation of breast cancer cells, it also regulates migration in these cells. It has been demonstrated that lack of ERRα expression inhibits cellular migration in ER+ and TN breast cancer cells *in vitro* by reducing ERRα-induced target genes, including WNT11 and VEGF [253, 258]. Similarly, another study indicated that ERRα

deletion reduces breast cancer cell growth and migration by repressing the gene expression levels of WNT11 and CCNE1(Cyclin E1), both target genes of ERRα [259]. Based on all the above-mentioned evidence, ERRα is an unfavorable marker in breast cancer [241]. Thus, ERRα may be considered as a promising therapeutic target for the treatment of breast cancer.

However, regarding ERRγ's role in breast cancer, there are several contradictory studies. As ERRγ gene expression levels are positively correlated with ER+/PR+ breast cancer, it was assumed that ERRγ is a marker of a favorable outcome in breast cancer [238]. It has been demonstrated that ERRγ downregulation increases the cellular growth in ER+ breast cancer cells [260]. Consistently, it has been shown that ERRγ induces mesenchymal-to-epithelial transition (MET) markers involved in reduced cell invasion via increasing E-cadherin [261]. In contrast, several studies demonstrated that ERRγ activation has been linked to TAM resistance via AP-1 induction in several ER+ breast cancer models *in vitro* and *in vivo* [262, 263]. Interestingly, it has been reported that ERRγ expression levels are high during TAM resistance in ER+ breast cancer cells, and that ERRγ-mediated AP1 activation could be a major player in TAM resistance [264, 265].

### 1.12.5. ERRs and metabolic reprogramming

#### 1.12.5.1. Cancer cell metabolism

One of the important hallmarks of cancer is the ability of the transformed cells to rewire cellular metabolism to satisfy the demands of rapid cellular growth. Cancer cells display differential aspects of cellular metabolism compared to normal differentiated cells. The idea that the metabolic features of the malignant cells differ from the normal cells can be first traced to Otto Warburg [179, 266, 267]. Most normal tissues, in the presence of oxygen, metabolize glucose to pyruvate via glycolysis and then oxidize most of the generated pyruvate to carbon dioxide in the mitochondria through OXPHOS. However, in the absence of oxygen, normal cells convert a large fraction of the glycolytic pyruvate to lactate [268]. In Warburg's studies,

the fundamental paradigm was that in contrast to normal cells, rapidly proliferating tumors metabolize glucose to lactate under aerobic conditions. Although this process does not generate a higher amount of ATP per molecule of glucose compared to OXPHOS [268], it generates the intermediate metabolites, which are needed for nucleic acid synthesis. This phenomenon is referred to as the Warburg effect or aerobic glycolysis, and it has been shown within various tumor types with a remarkable increase in glucose consumption. For instance, in the clinical setting, glucose consumption is measured using 18F-deoxyglucose-positron emission tomography (FDG-PET) [269]. Although glucose catabolism through aerobic glycolysis has been significantly attributed to the altered cancer metabolism, it alone cannot explain all the metabolic changes that are possibly essential to satisfy cancer cell growth requirements [270]. For example, for cancer cells to survive in a hypoxic environment, the TCA cycle remains active to produce essential amino acids and fatty acids. In addition, in order for cancer cells to proliferate, they use glutaminolysis and the pentose phosphate pathway (PPP) to produce abundant reductive power, such as nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) for fatty acid synthesis and also cellular detoxification [271]. Moreover, since intratumoral glucose availability is limited and oxygen tension is dynamic, mitochondria would play an essential role in producing energy and biomass in tumors [272]. Furthermore, in order for cancer cells to survive harsh metabolic conditions, they must develop the ability to sense and respond to the nutrient level alterations in their proximal environment [273]. Mitochondria are a vital component of such adaptive activities as they participate not only in the oxidation of glucose but can also oxidize fatty acids, glutamine, and lactate to satisfy the bioenergetic and/or biosynthetic needs of cancer cells [272, 274, 275]. In addition, growing evidence reported that the transformed cells utilize both glycolysis and mitochondrial oxidative metabolism to support their metabolic demands [267, 276]. It is important to note that although it has been demonstrated that intratumoral regions are mainly hypoxic where oxygen-dependent OXPHOS

was assumed to be inactive, mitochondrial oxidative phosphorylation is active within cells located in the region with low oxygen levels (0.5%) [277-279]. This information demonstrates that even within hypoxic regions of tumors, complete oxidation of glucose is possible and is likely to be essential for tumor cell viability. Importantly, it has been shown that while using mitochondrial metabolism, cancer cells exhibit decreased sensitivity to chemotherapeutics and some targeted therapies [272, 280-283]. Hence, in addition to targeting glycolysis, inhibiting selective mitochondrial metabolism will also be necessary to improve the therapeutic strategies in the malignant cells. Hence, it is interesting to assume that ERRα, a druggable transcription factor that mediates mitochondrial biogenesis and function, would be a beneficial therapeutic target.

#### 1.12.5.2. Metabolic reprogramming of ERRs in breast cancer

It is well-documented that ERRs have an important role in regulating cellular energy metabolism in normal tissues. Thus, it is interesting to suggest that they also participate in breast cancer cells' metabolic reprogramming. Several studies support that ERRα is involved in the regulation of genes controlling mitochondrial oxidative profiles [66, 284, 285]. In addition, another study in breast cancer cells that metastasize to the brain displayed a profile with increased expression of ERRα, PGC-1α, and PGC-1β, as well as known ERRα target genes involved in glycolysis, the TCA cycle, and OXPHOS pathways, implying that brain metastatic breast cancer cells derive energy from glucose oxidation in an ERRα-dependent manner [91]. Similarly, induction of ERRα activity via overexpression of PGC-1α in breast cancer cells increases oxidative metabolism [261]. These findings suggest that in breast cancer cells with overexpressed ERRα, ERRα promotes the glycolytic profile and supports oxidative mitochondrial respiration to sustain the essential building blocks for cancer cells' growth.

In addition, ERRα controls its target genes associated with anabolic biosynthesis pathways, such as nucleic acid, amino-acid, and lipid biosynthesis. It also involves the

regulation of the expression levels of enzymes linked to glutaminolysis in breast cancer cells. This indicates that ERRα is possibly involved in maintaining an active TCA cycle via glutamine-derived anaplerosis [39]. It is noteworthy to mention that there are some contradictory studies regarding the regulation of ERRα in glutaminolysis. Some studies showed that overexpression of ERRα induces the glutaminolysis genes in breast cancer cells [286, 287]. However, a recent study demonstrated that lack of ERRα increases the genes involved in glutaminolysis [273]. Moreover, it has been reported that ERRα is also involved in the regulation of the PPP and one-carbon metabolism (OCM), in the generation of NADPH, and in protecting the cells against oxidative damage and ROS production produced by oxidative metabolism. This process is reflected via the induction of detoxifying enzymes like glutathione S-transferase MU-1 (GSTM1), liver glutaminase (GLS2), and superoxide dismutase 2 (SOD2), which form the glutathione (GSH)-dependent detoxifying system [39, 43]. Thus, the role of ERRs in mediating the reprogramming of breast cancer cell metabolism likely extends beyond the induction of the Warburg effect and implies that ERRs act as central regulators of the metabolic reprogramming in breast cancers.

Furthermore, it is well-established that the RTKs' signaling has an impact on the metabolic reprogramming of breast cancer cells. Moreover, inhibiting glycolysis sensitizes HER2-positive cells to Herceptin treatment [288]. Hence, modulation of ERRα's transcriptional activity via RTK signaling could further impact cancer cells' metabolic reprogramming.

In addition, MYC can enhance metabolic reprogramming by mediating HIF-induced glucose consumption and glycolysis in breast cancer cells [289-291]. HIF-1α interacts with ERRα and enhances glycolysis and detoxification pathways in breast cancer cells [292]. ERRα binds MYC's promoter, and its expression levels are positively correlated to MYC expression levels in breast tumor specimens [242]. Furthermore, MYC mediates ERRα activity via

induction of PGC-1β expression through the HER2 pathway [241], and it also increases aerobic glycolysis in breast cancer cells [292].

## 1.13. The clinical implication of ERRs in breast cancer

### 1.13.1. ERRs emerge as a predictive marker in breast cancer

Identifying markers to predict prognosis and response to breast cancer therapy is crucial as breast cancer is a heterogeneous disease. Currently, there are several established biological markers with predictive powers, including ER, PR, and HER2 for breast cancer treatment. However, there is still an urgent need to identify novel prognostic markers to improve breast cancer patients' management. ERRα has emerged as an exciting candidate, as it is expressed in several subtypes of breast cancer, and its expression is positively correlated with the high clinical stage of breast tumors. In addition, it has been reported that ERRα activation is involved in a subset of genes that are able to predict breast cancer patients' outcomes regardless of the subtype [241]. This finding suggests that ERRα metabolic signature expression levels might be a better predictive marker than ERRα expression levels on their own [293].

### 1.13.2. ERRs emerge as potential druggable targets in breast cancer

Considering ERRα as a druggable target in breast cancer cells is due to its involvement in regulating mitochondrial biogenesis and oxidative respiration in the transformed cells [69, 99, 241]. As it was mentioned earlier, mitochondria play a crucial role in tumor progression via the upregulation of OXPHOS. This process may cause resistance in response to chemotherapy as observed in several cancer patients [276-278, 280, 283]. Notably, there is increasing interest in using metformin, a common anti-diabetic drug that can inhibit mitochondria's complex I, as an anti-cancer drug. However, metformin may cause undesirable side-effects on normal cells [294, 295]. Therefore, there is a need to introduce ERRα inhibitors that interfere with mitochondrial function and minimize the side-effect on the normal cells. ERRα expression levels are high in most cancers, and its induced activity is correlated with

poor clinical outcome in several cancers [118, 239, 241]. In addition, ERRα's expression pattern in human tissues is restricted and has shown subtle phenotypes in ERRα-/- animal models [272]. Hence, it is interesting to propose that inhibiting ERRα using its small molecule inhibitor would selectively disrupt mitochondrial activity in the transformed cells. Currently, several small-molecule inhibitors are targeting different metabolic steps in tumor cells [291, 296]. Notably, some of these small molecule inhibitors directly target the genes that are regulated by ERRs [273]. This may suggest that targeting ERRs could be a promising therapeutic strategy to disrupt cancer cells' metabolism. Moreover, identification of the ligand-binding pocket of ERRα and ERRγ [26, 28] exhibits that the ligand-binding pocket of ERRs is able to accommodate a small molecule [29]. Therefore, several ligands were synthesized for ERRs in order to regulate their transcriptional activity. This suggests that the endogenous ligands of ERRs possibly exist and ERRs could represent potential druggable targets in breast cancer therapy.

### 1.14. Rationale and objectives for the current study

Breast cancer is a common cancer with a high mortality rate among women [297]. The ER-positive subtype of breast cancer belongs to the largest breast cancer subgroup. Even though this subtype has several therapeutic strategies, many challenges, such as drug resistance, still persist [298]. In addition, the triple-negative breast cancer (TNBC) subtype, which is defined by lack of ER, PR, and HER2, accounts for about 15~20% of all newly diagnosed breast cancer cases. TNBC is considered more metastatic and has a poorer prognosis and higher risk of recurrence than other breast cancer subtypes. The death rate in patients with TNBC is twice that of ERα positive tumors, mainly because there are fewer targeted therapies that treat TNBC patients [299]. Therefore, it would be crucial to discover novel drug-targeted therapy for breast cancer patients. Several lines of evidence indicate that estrogen-related receptors (ERRs), which belong to the orphan nuclear receptor superfamily, play a crucial role in breast

cancer, with ERRα overexpression reportedly leading to adverse clinical outcomes in breast cancer patients [43]. Despite many attempts made by researchers, no endogenous ligand, except cholesterol, has been identified for ERRs thus far [31]. The discovery of ligands that bind these receptors will allow manipulating their pathways and may result in promising therapeutic strategies for breast cancer treatment, particularly TNBC. Our group previously reported identifying a novel endogenous estradienolone-like steroid (ED) in pregnant women [34]. In addition, our team's previous data suggested that ED does not show affinity to the ER, but acts as an endogenous inverse agonist of ERRα and inhibit breast cancer cell proliferation [37]. These findings raise the exciting possibility that ED-ERR interaction represents a novel druggable pathway in breast cancer. Therefore, in chapter 2 (manuscript 1), I **hypothesized** that ED binds directly to the ERR ligand-binding domain and that ED inhibits breast cancer cellular growth in an ERRα-dependent manner.

To address this hypothesis, I have established the following **three objectives,** which will be applied to chapter 2:

**(1)** To develop an improved method for the purification of ED using GST-ERR-LBD affinity-based column and liquid chromatography-tandem mass spectrometry, and confirm the fine structural features of ED.

**(2)** To establish that ED directly binds ERRs-LBD and regulates its transcriptional activity.

**(3)** To determine whether ED inhibits breast cancer cell growth in an ERRα-dependent manner.

The studies presented in chapters 3 and 4 were initiated when another group and I demonstrated that cholesterol acts as an endogenous ligand of ERRα [31, 300]. Interestingly, it has been shown that obesity and high dietary cholesterol intake are associated with increased risk of breast cancer recurrence and higher mortality in both ER-positive and TNBC patients [191, 192, 301-305], and cholesterol-lowering drugs such as statins have been shown to

improve survival rates in breast cancer patients [304, 306-309]. However, the underlying mechanisms by which elevated cholesterol levels reprogram the metabolism of the transformed cells to increase breast cancer recurrence risk and mortality rate are not well-understood [304, 309-311]. The nuclear receptor, estrogen-related receptor alpha (ERRα), plays an essential role in cancer cell metabolism, and its levels are upregulated in breast cancer cells, with its overexpression linked to poor survival [43, 312, 313]. It has been shown that ERRα is involved in cellular energy metabolism and regulation of mitochondrial metabolic pathways [245, 273, 314, 315]. Therefore, I **hypothesized** that the pathological impact of cholesterol on increasing breast cancer risk is mediated via cholesterol-ERRα/PGC-1α pathway in breast cancer cells.

In order to test this hypothesis, I have established **four objectives** indicated below**,** which will be applied to chapters 3 and 4:

**(1)** To demonstrate whether cholesterol enhances the interaction between ERRα and its coactivator PGC-1α.

**(2)** To determine whether cholesterol modulates ERRα activity in breast cancer cells.

**(3)** To elucidate whether cholesterol alters metabolic pathways via ERRα axis.

**(4)** To demonstrate whether the impact of cholesterol on breast cancer cell growth is ERRα dependent.

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**Connecting Text 1:**

Estrogen-related receptors (ERRs) belong to the orphan nuclear receptor subfamily and play a critical role in breast cancer. It has been shown that ERRα is overexpressed in breast cancer cells, and its overexpression is associated with adverse clinical outcomes in breast cancer patients. Thus, there is an increasing interest in identifying ERRs' endogenous ligands to manipulate their transcriptional activity in cancer cells. Interestingly, despite many efforts to identify ERRs' endogenous ligands, no ligands have been identified for ERRs, aside from cholesterol recently being identified as an agonist of ERRα. My group previously demonstrated a novel endogenous steroid with estradienolone like-structure (ED) that binds strongly to SHBG protein. Interestingly, our team's primary data demonstrated that ED does not show affinity to the ER, but acts as an inverse agonist of ERRs. Therefore, we hypothesized that ED directly binds to ERRs and inhibits breast cancer cell growth in an ERRα-dependent manner. To test this hypothesis, I established these two objectives: first, to demonstrate whether the endogenous ED directly binds to ERRs and regulates their transcriptional activity; and second, to show whether ED inhibits breast cancer cell proliferation via the ERRα axis. This chapter is a manuscript that has been published in the Journal of Steroid Biochemistry and Molecular Biology.

# CHAPTER 2 – MANUSCRIPT 1

## Isolation and functional characterization of a novel endogenous inverse agonist of estrogen-related receptors (ERRs) from human pregnancy urine

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## 2.1 ABSTRACT

Estrogen-receptor related receptors (ERRs), which consists of ERRα, ERRβ, and ERRγ belong to the orphan nuclear receptor subfamily 3, group B (NR3B) subfamily, and are constitutively active. ERRs have been shown to actively modulate estrogenic responses, and to play an essential role in pregnancy, and are implicated in breast cancer progression. Despite intensive efforts, no endogenous ligand other than the ubiquitous sterol, cholesterol, which binds ERRα, has been identified for ERRs so far. The discovery of ligands that bind these orphan receptors will allow the manipulation of this pathway and may lead to novel strategies for the treatment of cancer and other diseases. We previously reported the identification of a novel endogenous estradienolone-like steroid (ED) that is strongly bound to sex hormone-binding globulin in pregnant women. Our recent results show that ED acts as an inverse agonist of ERRα and ERRγ by directly interacting with these receptors and inhibiting their transcriptional activity. We also demonstrate that ED inhibits the growth of both estrogen receptor-positive (MCF-7) and estrogen receptor-negative (MDA-MB-231) breast cancer cells in a dose-dependent manner while of displaying a little effect on normal epithelial breast cells. Furthermore, the anti-mitogenic effect of ED in breast cancer cells is ERRα-dependent. These data suggest that ED-ERR interaction may represent a novel physiologically relevant hormone response pathway in the human. The finding that ED inhibits both ER-negative and ER-positive breast cancer cell growth may have important implications in the pathophysiology of breast cancer.



**KEYWORDS:**

Estrogen receptor-related receptors, Endogenous inverse agonist, Steroids, Pregnancy, Breast cancer

## 2.2 INTRODUCTION

Estrogen-receptor related receptors (ERRs) belong to the orphan nuclear receptor NR3B subfamily and consists of 3 isoforms: ERRα, ERRβ, and ERRγ [1,2]. ERRs are known to be critically involved in fetoplacental development and in the regulation of estrogen receptor (ER) signaling [3–5]. In addition, ERRs play a pathophysiological role in insulin resistance, heart failure [6] and osteoporosis [7] and Obesity [8] in the human. Furthermore, recent studies suggest that ERRs may represent potential therapeutic targets for several types of cancers [9–13], including breast cancer [14–16].

Several lines of evidence indicate that ERRs play a crucial role in breast cancer. Overexpression of ERRα has been reported to cause adverse clinical outcomes in breast cancer patients in an ER status independent manner [16–19]. Furthermore, ChIP-chip analyses of breast cancer cells reveal that most of the genes regulated by ERRα are distinct from the ones regulated by ERα, and only a few genes are regulated by both [20,21]. In addition, it has been suggested that, as overexpression of ERRα is associated with angiogenesis and hypoxia in solid tumors, it may act as a transcriptional metabolic regulator, which may also promote cancer development [22]. Also, while ERRγ overexpression induces resistance to tamoxifen in ER+breast cancer models [23], ERRβ overexpression is associated with inhibition of breast cancer cell proliferation and correlates with favorable outcomes in breast cancer patients [24].

Due to ERRs' clinical importance, there have been intensive research efforts to identify natural ligands for ERRs. Study into the crystal structure of the ligand-binding domain of ERRs has led researchers to suggest that the ligand-binding pocket of ERR may be too small to accommodate any potent natural agonist ligands and that the activation function 2 (AF2) helix (structure responsible for ligand-induced activation in other nuclear receptors) of ERRs is in the active configuration in the absence of a ligand, indicating that ERRs are constitutively active [25]. However, the crystal studies have also shown that the ligand-binding pocket

although small in the unliganded state can be rearranged to accommodate synthetic molecules that occupy larger volumes [26]. Consistent with this notion, diethylstilboestrol (DES), 4-hydroxytamoxifen (4OHT), and XCT790 [27,28] have been identified as inverse agonists of ERRs, thus inhibiting ERR transcriptional activity. Also, ERR transcriptional activity can be stimulated by synthetic phenolic acyl hydrazone compounds, DY131 and GSK4716 [29,30]. Furthermore, the ligand-binding domain of ERRs is functionally conserved, suggesting that ERR activity in vivo is likely controlled by a natural ligand, possibly by an inverse agonist [31,32]. Recently the ubiquitous sterol, cholesterol was identified as an endogenous agonist of ERRα, as cholesterol binds ERRα and further augments its activity [33]. To date, no endogenous inverse agonist of ERRs, although predicted [6,19,34], has been reported. Identification of a natural ligand of ERRs is of fundamental importance, as it will allow regulation of ERR activity by manipulation of the level of ligands which in turn may lead to novel avenues for the treatment of diseases where ERRs play a pathophysiological role. Such a notion is based on successful precedence set by the development of numerous drugs such as the one for ERα-driven breast cancer which antagonizes estrogen-ER interaction [35].

We have previously reported the identification of a novel 19-nor steroid with an estradienolone structure (ED), which binds strongly to sex hormone-binding globulin (SHBG), in human pregnancy serum [36,37]. A chromatographic analysis of SHBG-bound substances in pregnancy serum showed that ED accounted for approximately 50% of the total material bound to SHBG. Gas chromatography-mass spectrometric analysis of this substance suggested that it represents a 19-nor androgen, an estradienolone [36,37]. However, the position of double bonds and fine structure of this molecule remains unknown. Importantly, the serum levels of ED in pregnant women were found to decrease close to term and in association with premature spontaneous labour [36,37], suggesting that ED may play an important role in fetal development and/or in the maintenance of pregnancy.

The mechanism by which ED exerts its effects remains to be determined. While it is identified by its strong binding to SHBG, the cellular receptors through which ED transduces its signals are not known. In the current study, we examined whether ED interacts with the classical ER or the orphan receptors ERRα and ERRγ and modulates their activity and whether ED regulates cellular responses in breast cancer cells with different hormone receptor status.

## 2.3 MATERIALS AND METHODS

### Chemicals and reagents

Tritiated testosterone, androstenedione, 17β-estradiol, and thymidine (S.A. 50–100 Ci/mMol) were obtained from the Perkin Elmer/New England Nuclear Corp. (Boston, MA). Non-radioactive 17β-estradiol (E2), XCT-790, 4-hydroxytamoxifen (4-OHT), diethylstilbestrol (DES), hydrocortisone (HCT), and dihydrotestosterone (DHT) were purchased from Sigma Chemical co. (St. Louis, MO), or from Steraloids Inc. (Wilton, NH). Dichloromethane, Heptane, and anhydrous ethanol were purchased from Sigma Aldrich (Mississauga, ON). Analytical grade ethyl acetate, nanopore water, and methanol were supplied by Millipore sigma (Mississauga, ON). Enzyme Glusulase (a preparation containing 10,000 units of sulfatase and 90,000 units of β-D-glucuronidase per mL (Dupont Pharmaceuticals)) was purchased from Perkin Elmer/New England Nuclear Corp. (Boston, MA). Human tissues were obtained, with the patient's consent from the institutional review board (IRB), at delivery or hysterectomy from the Department of Obstetrics & Gynecology, Royal Victoria Hospital, Montreal.

### Purification of estradienolone (ED)

Urine samples (24 h) were collected from healthy pregnant women at 28–38 weeks' gestation and were stored at −80 °C until processed as previously described [36,37]. As it is shown in Fig. 1A, this involved digestion of urine with the enzyme Glusulase to prepare unconjugated ED. The digested urine was then extracted with 3 times volume of ethyl acetate

and purified initially on a Sephadex LH-20 (Pharmacia, Piscataway, NJ) column (15 cm x 1 cm) using a mobile phase of dichloromethane, heptane, and methanol in the ratio of 50:50:1, and a spike of 3H-androstenedione as a marker. Fractions containing 3H-androstenedione were pooled and fractionated two times on (60 cm x 1 cm) Sephadex LH-20 columns using the same mobile phase as above. Based on the sex hormone-binding globulin (SHBG) assay, the peak which elutes a few fractions after 3H-androstenedione (marker) which contains ED was dried down under nitrogen and reconstituted in methanol with a spike of 3H-androstenedione (marker) and was injected in HPLC (Waters model 2487). A mobile phase consisting of a mixture of dichloromethane, heptane, and methanol in the ratio of 30:70:0.1 was used. The separation was performed on a Nova Pak normal phase silica column (4 μm, 3.9 x 150 mm, Waters), a 600S model controller, 626 model pump, and 2487 model UV detector. After injection of the sample, 1 mL fractions were collected at the flow rate of 1 mL/min with 100% isocratic mobile phase for 48 min. SHBG assay was performed in order to detect the fraction which contains ED. The fraction which contains ED was analyzed by LC-MS/MS (Agilent technologies, model 1260 infinity with 1260 Infinity Diode Array Detector HS, coupled with an MS detector, Bruker, model impact HD, and NMR-MS-bridge pump, Bruker, model 9999). The separation was performed on an Agilent zorbax eclipse plus C18 column (4.6 x 10 mm, 3.5 μm). Mobile phase B was acetonitrile/ 0.05% acetic acid, and mobile phase A was water/ 0.05% acetic acid. The elution gradient was held at 40% B for the first 0.5 min, 40–100% B from 0.5 to 30 min, held at 100% B from 30 to 36 min, 100%-40% B from 36 to 37 min, held at 40% B from 37 to 42 min. The flow rate of the mobile phase was 1 mL/min, and the injection volume was 20 μL. LC coupled with a mass spectrometry instrument with (high-resolution quadrupole time-of-flight (Bruker, model Impact HD)), using NMR-MS-bridge. The electrospray ionization was operated in positive and total scan mode. For mass spectrometry, capillary voltage was 4000 V, fragmentor voltage was 500 V; nebulizer gas was 73 psi; drying

gas temp was 350 °C with the flow of 12 L/min; m/z range was from 150 to 800 Daltons. For comparison, 17β-estradiol was analyzed by the same method.

**Sex hormone-binding globulin (SHBG) assay**

The detection of ED and the determination of its levels were as previously described [37]. The procedure was based on the competition of ED with 3H-testosterone for binding to sites on human sex hormone-binding globulin (SHBG). Levels of ED were expressed as testosterone equivalents. Since we have previously shown that ED is bound to SHBG 95% as strongly as testosterone under physiological conditions [36], the concentration of ED expressed in testosterone equivalents approximates the true concentration of ED.

**Uterine cytosol assay to determined binding of ED to estrogen receptors**

Human proliferative endometrium was collected at hysterectomy and stored frozen in liquid nitrogen. The tissue was pulverized in an acetone bath. The powdered tissue was homogenized in 7 volumes of ice-cold phosphate-glycerol buffer (5 mM sodium phosphate, 12 mM monothioglycerol, 10.1% glycerol, pH 7.4) containing 20 mM sodium molybdate. The homogenate was centrifuged at 105 000 x g for 1 h at 4 °C. The supernatant (cytosol) was aliquoted, quickly frozen in liquid nitrogen, and stored at −80 °C until analysis. The binding of ED to estrogen receptors was determined as described previously [38]. Briefly, endometrial cytosol was incubated for 18 h at 4 °C with 3H-estradiol (10 nM) alone or in the presence of increasing concentrations of non-radioactive estradiol or ED. Non-specific binding was evaluated by the addition of 1 μM non-radioactive estradiol in parallel incubations. The protein-bound fraction was separated using a Sephadex LH-20 column (5 × 0.4 cm). The elution solvent was the same phosphate-glycerol-molybdate buffer used for cytosol preparation. The eluate containing the receptor-bound hormone was counted in a liquid scintillation counter to an error of 1%.

**Bioluminescence resonance energy transfer (BRET) assay**

HEK293T cells were transfected with expression vectors for ERα, glucocorticoid receptors (GR) or androgen receptor (AR) conjugated to Renilla Luciferase II (ERα-RLucII)/(GR-RLucII)/(AR-RLucII) as the donors, and an LXXLL coactivator motif (CoA) fused to venus (CoA-venus) was used as the acceptor for ERα-RLucII/GR-RLucII, and RNF14 FXXLF motif fused to YFP (YFP-FXXLF-NLS-YFP) was used as the acceptor for AR-RLucII, as previously described [39,40]. Cells were harvested at 48 h post-transfection in PBS 1X and treated with 10 nM E2 or/and 10 nM ED for ERα-RLucII, and for GR-RLucII, the cells were supplemented with 10 nM hydrocortisone (HCT) or/and 10 nM ED, and for AR RLucII, the cells were treated with 2 nM dihydrotestosterone (DHT) or/and 2 nM ED. The treated cells were incubated at 37 °C for 1 h. The BRET signal was measured using FlexStation II microplate reader and was calculated as (emission 550 nm)/(emission 485 nm).

**Equilibrium dialysis assay to determine the binding of ED to ERRs**

To determine whether ED binds to ERR, we developed an equilibrium dialysis assay, using purified ligand-binding domain (LBD) of ERRγ (kindly provided by Dr. R. Moaddel, National Institute of Health, USA). 7.5 × 10−4 μM ED or 2.5 μM 4-OHT was dissolved in PBS and added inside the dialysis tubing, while 8.5 nM purified ligand-binding domain of human ERRγ was dissolved in PBS and added outside of the dialysis tubing. As a negative control, $7.5 \times 10^{-4}$ μM ED was placed inside as above, and 3 nM BSA [without ERRγ] was placed outside of dialysis tubing. As an additional control, 3.6 μM E2 [instead of ED] was placed inside the dialysis tubing with 8.5 nM ERRγ outside of the dialysis tubing. The vials were incubated at 4 °C for 24 h with shaking. Then the steroids [38] inside and outside of the tubing were extracted separately using ethyl acetate for ED and E2, and hexane and 2-propanol (95:5) for 4-OHT. The samples were evaporated under nitrogen gas and reconstituted in methanol. The amount of ED inside and outside of the tubing was compared using SHBG assay, while

the concentrations of 4-OHT and E2 were measured using UV–vis spectrophotometer (Agilent technologies, Cary Series UV–vis-NIR spectrophotometer) at the maximum wavelength of 245 nm and 281 nm, respectively.

**GST-ERR pull-down assay to determine binding of ED to ERRs**

To confirm that ED directly binds to ERRα and ERRγ, GST-ERRα and GST-ERRγ pull-down assays were performed. GST-ERRα-LBD and GST-ERRγ-LBD plasmids were constructed using pGEX-6P-1 vector (GE Healthcare), as previously described [41]. The chemically competent DH-5α cells were transformed with the plasmids (GST, GST-ERRα-LBD or GST-ERRγ-LBD), and the expression of GST and GST- fusion proteins were induced by 1 mM isopropyl-β-D- thiogalactopyranoside (IPTG). The bacteria were disrupted by sonication, and the proteins were isolated with a 50% slurry of glutathione-Sepharose 4B beads, GE Healthcare [42]. Equal amounts of GST-ERRα-beads and GST-ERRγ-beads were incubated with $7.5 \times 10{-}4$ μM ED for 24 h at 4 °C. The supernatant which contains the unbound steroids were collected, and the protein-ED complex was eluted using 10 mM L-glutathione reduced solution. Both supernatant and pulldown were extracted using ethyl acetate, then dried down under nitrogen and reconstituted in methanol. The concentration of ED in both supernatant and pulldown fractions were measured using SHBG assay. As the positive control, 4-OHT (2.5 μM) instead of ED was used for GST-ERRγ, and XCT790 (2 μM) instead of ED was used for GST-ERRα. The concentration of 4-OHT and XCT-790 were measured using a UV–vis spectrophotometer at the maximum wavelength of 245 nm and 368 nm, respectively. To exclude false positive data, the same test was done using the beads-GST, and the supernatant and pulldown fractions were assayed by SHBG.

**Tryptophan fluorescence quenching assay to determine the relative affinity of ED for ERRs**

Ligand binding domain of ERRγ (400 nM) was incubated with varying concentrations of ED, 4-OHT, or E2 in binding buffer (20 mM Tris (pH 8.0), 150 mM NaCl, and 5% ethanol (v/v)) for 30 min in room temperature. The incubations were performed in a 96 well plate (Corning, black with clear half bottom, 3881). Fluorescence emission was measured at 310 nm following excitation at 295 nm using a microplate reader (Infinite M200PRO, TECAN). The dissociation constant ($K_d$) was determined using Graph Pad software.

**GST pull-down assay to determine whether ED modulates ERR-coactivator interaction**

The GST, GST-PGC-1α, or GST-GRIP1 RID (glucocorticoid receptor-interacting protein-1 receptor-interacting domain) expressing plasmids were transformed into E. coli and the expression of GST and GST- fusion proteins were induced by isopropyl-1-thio-ß-D-galactopyranoside. The bacteria were disrupted by sonication, and the proteins were isolated with a 50% slurry of glutathione-Sepharose beads [42]. Equal amounts of GST, GST-PGC-1α proteins were incubated with the in vitro-translated 35S-labeled ERRα in the presence of estradiol (E2), 4-hydroxy-tamoxifen (4-OHT), DES, or ED for 24 h at 4 °C. This was also done with GST, GST- GRIP1 RID proteins incubated with the in vitro-translated 35S-labeled ERRγ. The amount of 35S-labeled ERRα or 35S-labeled ERRγ pulled down with GST-PGC-1α, or GST-GRIP1 RID was examined with SDS-PAGE and visualized by autoradiography [43].

**Transient transfection assays to determine whether ED regulates ERR transcriptional activity**

To determine whether ED regulates transcriptional activity of ERRα or ERRγ, the human embryonic kidney 293 (HEK-293) were transfected with the pS2Luc reporter plasmid (400 ng), ERRα or ERRγ expression plasmids (300 ng) with or without the proliferator-

activated receptor-gamma coactivator-1 (PGC-1α) co-activator expression plasmid (300 ng), and Renilla luciferase (20 ng) [43,44]. The pS2Luc reporter plasmid contains the promoter of the estrogen-responsive gene pS2 linked to the luciferase reporter gene. 16 h after transfection, cells were treated with ED, XCT-790, 4-OHT, or vehicle control for 24 h and harvested. Luciferase activity was measured, and values were normalized using the Renilla luciferase transfection efficiency control. Each experiment was performed in triplicates, and the values shown are representative of three independent experiments.

**Cell proliferation assays to determine the effect of ED on breast cancer cell growth**

**Cell culture**

The MCF-7 and MDA-MB-231 cells were cultured in DMEM with 10% FBS and 1% penicillin/streptomycin. The human non-tumorigenic epithelial breast MCF-10 A cell line (American Type Culture Collection (ATCC), Manassas, VA) was maintained in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) (Wisent) supplemented with 100 nM cholera toxin, 0.01 mg/mL insulin, 0.5 μg/mL hydro-cortisone, 0.01 μg/mL epidermal growth factor, 10% FBS and 1% pe-nicillin/streptomycin (all obtained from Sigma).

**3H-thymidine incorporation assay**

MCF-7 and MDA-MB-231 Cells were serum-starved 48 h prior to the start of the experiment. $1 \times 10^5$ cells were plated and cultured for 24 h and then treated with ED and/or estradiol at varying concentrations (1–100 nM) in phenol red-free medium containing 5% charcoal-stripped serum for 24 h. 3H-thymidine (1 μCi/mL, Amersham) was added to each well for the final 6 h of ED or estradiol treatments. The cells were washed three times with phosphate-buffered saline and once with 10%trichloroacetic acid. The cells were then solubilized in 1% SDS, and the incorporated radioactivity was determined by liquid scintillation counting.

**Cellular growth assay**

The cell proliferation assay was performed using MTS Cell Proliferation Assay Kit (G3582, Promega) according to the manufacturer's instructions. MCF-7, MDA-MB-231, and MCF-10 A cells were plated at a density of 104 cells per well in 96 well plates. Cells were supplemented with fresh phenol red-free medium containing 5%charcoal stripped serum medium containing indicated concentrations of ligands. The medium was changed every 48 h with a fresh medium containing the ligands as above throughout the course of the 5 days experiment. 20 μL of MTS reagent per well was then added to each well and was incubated for 2 h at 37 °C under standard culture conditions. The optical density (OD) value was measured at 490 nm using a microplate reader (Infinite M200PRO, TECAN).

**siRNA transfection**

siRNAs directed against ERRα (Invitrogen, AM16708 / 289481) with the sense sequence 5′-CCGCUUUUGGUUUUAACC-3′ and antisense sequence 5′-GGUUUAAAACCAAAAGCGG-3′ and control scrambled siRNAs (Invitrogen, AM4611, negative control) were transfected into MCF-7 and MDA-MB-231 using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen, Canada) according to manufacturer's instructions. At 48 h post-transfection, fresh phenol red-free medium containing 5%charcoal stripped serum medium was added, and cells were treated with ED (5 nM) and used for cell growth assay and immunoblot analysis.

**Immunoblotting**

Cell lysates were analyzed by SDS-PAGE under reducing conditions and transferred to a nitrocellulose membrane. ERRα was detected using a rabbit monoclonal anti-ERRα antibody (ab76228, Abcam). A mouse monoclonal anti-alpha tubulin antibody (ab7291, Abcam) was used to detect alpha-tubulin (loading control). Densitometric analysis of immunoblots was performed using Image-J software.

### Statistical analysis

Data are expressed as mean ± standard deviation (SD). The statistical significance of differences between samples was determined by a two-tailed Student t-test for all experiments. *$P < 0.05$ was considered to be statistically significant.

## 2. 4. RESULTS

### ED purification and characterization

The purification scheme and typical elution patterns of ED and 3H-androstenedione tracer at the Sephadex LH20 (60 cm column) purification stage in the urine of pregnant women are shown (Fig. 1A and B). As reported previously, ED elutes in a low polarity region, a few fractions after the tracer 3H-androstenedione [36,37]. The yield of ED in the urine of pregnant women varied between 1–3 μg/L, without accounting for procedural losses, which may represent approximately 40%. ED containing fractions from the 60 cm column were then further purified by HPLC. Typical chromatograms of SHBG-bound material obtained by HPLC separation for pregnant urine are shown in Fig. 1C. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of ED and estradiol indicate that the mass corresponding to ED is the same as that of 17β-estradiol (272.18 m/z). However, the fragmentation pattern of ED is distinctly different from that of 17β-estradiol. ED's fragmentation pattern is consistent with having an estradienolone structure (ED, 17-hydroxy-estradien-3-one) (Fig. 1D). Indeed, ED mass analysis is consistent with our previous finding obtained by GC–MS after conversion of the samples to their methyloxime, trimethylsilyl (TMS) ether derivatives [36]. Together these findings indicated that ED has an estradienolone structure, and it was therefore of interest to test whether ED binds to the estrogen receptor.

### ED binds poorly to estrogen receptors (ER) and glucocorticoid receptor (GR)

Studies of the binding of ED to human endometrial ER using an 3H-estradiol competition assay show that ED even at 1000-fold excess concentrations (1000 nM) displaced

less than 0.2% of 3H-estradiol (1 nM), while 1 nM non-radioactive estradiol displaced 70% of 3H-estradiol, demonstrating that ED has little affinity for ER (Fig. 2A). Non-specific binding measured with a 100-fold excess of non-radioactive estradiol was 2–3% of the total bound.

We next performed a BRET assay to determine whether ED binds to ER, GR, or/and AR. As shown in Fig. 2B, for ERα, E2 induces a sig-nificant increase in the net BRET value, while ED exhibits no effect with the net BRET value remaining at the basal level. Furthermore, ED is ineffective in altering the E2-induced activation of ERα. For GR, hydrocortisone (HCT) induces a significant increase as shown in Fig. 2B, while ED does not change the basal activity of GR-RLucII/CoA-Venus. In addition, in the presence of both ED and HCT, the activity level of GR remains the same as HCT alone (Fig. 2B). The results obtained with AR-RLucII/RNF14 FXXLF-YFP in the BRET assay were inconclusive (data not shown).

**ED binds directly to ERRα and ERRγ orphan receptors**

We next considered the possibility that ED is a ligand of ERR orphan receptors and examined whether ED binds to ERRγ or ERRα. In an equilibrium dialysis experiment, where ED was placed inside a dialysis tubing and ERRγ protein (ligand-binding domain) was placed outside, the concentration of ED at equilibrium is 11-fold higher outside com-pared to ED concentration inside ($P < 0.05$) (Fig. 3A and B). When BSA instead of ERRγ was used, and ED was dialyzed against BSA (Fig. 3A), there is no significant difference between the concentration of ED inside and outside of the dialysis tubing at equilibrium, demonstrating negligible binding of ED to BSA. As an additional negative control, when estradiol instead of ED was dialyzed against ERRγ, there is no significant difference between the concentration of E2 inside and outside of the dialysis tubing at equilibrium ($p < 0.05$) (Fig. 3A). As a positive control, 4-OHT, which is a synthetic inverse agonist of ERRγ, was dialyzed against ERRγ, and the result shows that the concentration of 4-OHT outside of dialysis tubing is 3 times higher than inside at equilibrium ($p < 0.05$) (Fig. 3A).

We next performed a pull-down assay using GST-ERRα-LBD and GST-ERRγ-LBD (Figs. 3C and 3D). As shown in Fig. 3C, when the interaction of ED with GST-ERRγ-LBD was examined, the concentration of ED in the pulldown fraction is 10 times higher than in the supernatant fraction, which is consistent with the result of the equilibrium dialysis experiment above. The concentration of 4-OHT, the synthetic inverse agonist of ERRγ, in pulldown fraction is approximately 4 times higher than its concentration in supernatant. This indicates that ED shows higher affinity to ERRγ compared to its synthetic ligand 4-OHT. We next tested ED interaction with ERRα using GST-ERRα-LBD, and show that the concentration of ED in the pulldown is approximately 12 times higher than the supernatant, while the concentration of XCT-790, selective inverse agonist of ERRα, in the pull-down is 5 times higher than in the supernatant. This suggests that ED binds to ERRα more strongly than XCT-790 (Fig. 3C). To demonstrate that ED binding to ERRα and ERRγ is specific in the above experiments, we performed the same experiment using GST alone, which shows that the concentration of ED in the supernatant is 20 fold higher than its concentration in the pulldown (Fig. 3C). Together these results indicate that ED interacts directly with ERRα and ERRγ orphan receptors.

To quantify ED's relative binding affinity to ERRγ, we performed a tryptophan fluorescence quenching assay using the ligand-binding domain of ERRγ. As shown in Fig. 3E, in the presence of ED and ERRγ's synthetic ligand (4-OHT), the percent change of quenching of tryptophan fluorescence increase in a dose-dependent manner, suggesting that ED and 4-OHT bind to the ligand-binding domain of the receptor and change its conformation, resulting in the quenching of fluorescence emission of the receptor. Importantly, in the presence of E2, the fluorescence quenching remained unchanged at all concentrations of E2 tested. The $K_d$ for 4-OHT and ED were determined at 74.98 and 11.81 nM, respectively, showing that ED binds to the LBD of ERRγ with higher affinity than its synthetic ligand, 4-OHT.

**ED disrupts the interaction of ERRα and ERRγ with their coactivators**

To determine whether ED modulates the interaction of ERRα and ERRγ, with their coactivator PGC-1 and GRIP1 RID, a GST-pull down assay using PGC-1α and GRIP1 RID was performed. As shown in Figs. 4A and 4B, ED disrupted the interaction between ERRα and ERRγ with their coactivators PGC-1α and GRIP1 RID, respectively. In addition, it shows that ED is a more potent inverse agonist than DES for ERRα and ERRγ. These observations cannot be explained by the possible binding of ERRα or ERRγ to other steroids, which may have co-eluted with ED since no endogenous steroids have been shown to bind the ERRs. Similarly, the potential intracellular conversion of ED to estradiol does not account for this inhibition since natural estrogens (including estradiol) are not ERR ligands [2].

**ED negatively regulates ERRα and ERRγ transcriptional activity**

We next determined, ED's ability to regulate ERRα and ERRγ responses. When cells transfected with a reporter plasmid (pS2Luc), co-activator PGC-1 and the full length of ERRα or ERRγ, were treated with ED, there was a significant reduction in the pS2Luc reporter activity as compared to cells not treated with ED (Fig. 4C). This suggests that ED is interfering with the interaction of ERRα/ERRγ with its co-activator (PGC-1α) to inhibit the receptors' constitutive transcriptional activity and function as an inverse agonist. It is interesting to note that ED at lower concentration inhibits ERRα/ERRγ interaction with PGC-1α coactivator more strongly than XCT-790 and 4-OHT (Fig. 4C), which are known as a potent inverse agonist of ERRα and ERRγ, respectively [41].

**ED inhibits proliferation of breast cancer cells of distinct hormone receptors status**

We next examined whether ED regulates cellular proliferation at ER-negative (MDA-MB-231) and ER-positive (MCF-7) breast cancer cells using a thymidine incorporation assay. As shown in Fig. 5A, only the fraction that contains ED has the ability to inhibit MDA-MB-231 cell proliferation, while adjacent fractions not containing ED have no effect. Fig. 5B

confirms that ED inhibits the basal and estradiol-induced proliferation of ER-positive MCF7 cells and the proliferation of ER-negative MDA-MB-231 cells. Interestingly, ED is able to inhibit both MCF-7 and MDA-MB-231 cells, even in the presence of estradiol. As expected, estradiol has no effects on MDA-MB-231 cells (Fig. 5B). We then examined whether the inhibition of ED is dose-dependent in these cells (Fig. 6). Our results demonstrate that ED inhibits cellular proliferation of MCF-7 and MDA-MB-231 in a dose-dependent manner. The concentration of ED required for 50% inhibition of cell growth (IC50) was determined using a 5-day growth curve with both MCF-7 and MDA-MB-231 (Fig. 6). ED inhibited both breast cancer cell types with an approximately similar IC50 (3.2 nM and 4.3 nM for MCF-7 and MDA-MB-231 cells, respectively) (Fig. 6D). Thus ED is a highly potent inhibitor of breast cancer cell growth at low doses. Anti-estrogens such as XCT-790 are used in the μM range for the treatment of hormonal breast cancer. ED is unique in that it is the first endogenous steroid which inhibits cell proliferation of both ER-positive and ER-negative breast cancers (Figs. 6 A and 6B). It's interesting to note that ED's inhibitory effect on cell proliferation is limited to tumorigenic breast cells. ED has no effect on the non-cancerous immortalized epithelial breast cells (MCF-10 A) proliferation (Fig. 6C).

To determine whether ED's inhibitory effect on breast cancer cells is mediated through the ERRα pathway, we performed the cell proliferation assay using breast cancer cells in which ERRα was knocked down. As shown in Fig. 6E and F, ED decreases the proliferation of MCF-7 and MDA-MB-231 cells treated with vehicle or scrambled siRNA, while ED shows no anti-proliferative effect on siRNA-ERRα treated breast cancer cells. This finding is consistent with the report that XCT-790, a synthetic ERRα inverse agonist, displays an anti-proliferative effect in breast cancer cells [45]. Interestingly, despite the fact that ERRα protein level is significantly decreased in siRNA-ERRα treated cells compared to the control siRNA treated cells (Fig. 6G, H, I and J), knocking down of ERRα in MCF-7 and MDA-MB-231 does not change the basal

cell growth in these cells. This result although unexpected, is in agreement with that reported by others [46,47].

### 2. 5. DISCUSSION

ED is the first endogenous inverse agonist shown to interact with ERR orphan receptors and regulates their activities. Initially, ED came to our attention not only because of its high affinity for SHBG, but also because ED is the most prominent SHBG-bound ligand in late pregnancy serum [36,37]. The fact that all steroids that bind strongly to SHBG (testosterone, dihydrotestosterone, androstanediols, estradiol, 2-methoxyestrone, and 2-methoxyestradiol) exhibit potent biological activity implied that ED is physiologically important. Our findings showing that, ED elutes in the low polar region of the chromatogram of sepharose LH-20 column, suggesting that it is a non-polar steroid. LC-MS/MS analysis showing that ED and estradiol has a similar mass (272.18 m/z), but a distinct fragmentation pattern indicates a non-aromatic, likely an estradienolone structure [36] (Fig. 1D). Due to the lack of sufficient quantity of ED available for nuclear magnetic resonance (NMR) analysis, the exact structure of ED (positioning of double bounds) remained to be elucidated. Previously reported that ED is likely produced in the placenta, as its concentration in that tissue is higher than that in other tissues. However, ED's biosynthetic pathway and the enzyme(s) responsible for its synthesis are unknown. In the current study, we show that despite having estradienolone structure, ED does not bind to the estrogen receptor (ER). In addition, our finding suggests that ED does not show affinity to the glucocorticoid receptor (GR) (Fig. 2B).

In the current study, we demonstrate that ED binds directly to purified human ERRγ ligand-binding domain and that it does so with a higher affinity than its synthetic ligand 4-OHT, as detected by a tryptophan quenching fluorescence assay. Our results indicate that the dissociation constant ($K_d$) of ED-ERRγ interaction is approximately 7 times smaller than that of 4-OHT-ERR interaction. The above findings are confirmed using an equilibrium dialysis

assay and a GST pull-down assay with GST-ERRα and GST-ERRγ. In addition, our results demonstrate that ED interacts with ERRα and ERRγ and inhibits their transcriptional activity. Together, these results suggest that ED directly binds to ERRγ and ERRα and that ED represents the first reported endogenous inverse agonist of these orphan receptors.

Since the discovery of the ERR orphan receptors, intensive efforts have been focused on the identification of possible ligands for the ERRs in order to control the expression of their target genes and manipulate their physiological functions. Although synthetic compounds such as DES, 4-OHT, and XCT790 have been shown to bind to these receptors, they do so at micromolar concentrations [43,48]. In the current study, we demonstrated that unlike DES and 4-OHT which are known to interact with both ER and ERRs, ED selectively interacts with ERRs but not ER. In addition, ED is able to inhibit the interaction of the PGC-1α and/or GRIP1co-activators with the ERRα and ERRγ receptors and block their constitutive transcriptional activity. This inhibitory effect of ED cannot be attributed to other steroid contaminants or the potential conversion to estradiol, as all the endogenous steroids, including estradiol, failed to bind to the ERRs [2]. Although the significance of ED binding to ERRs remains to be determined, identification of ED as their inverse agonist will greatly facilitate delineation of the role and mechanism of action of these important orphan receptors in development, regulation of estrogen responses, energy metabolism, and cancer progression.

In the current study, we present evidence to show that ED inhibits the growth of both ER-positive MCF-7 and ER-negative MDA-MB-231 breast cancer cells (which are known to overexpress ERRα), while ED does not alter the growth of MCF-10 A cells (which express low levels of ERRα). Furthermore, we demonstrate that the anti-mitogenic effects of ED on MCF-7 and MDA-MB-231 breast cancer cells are ERRα-dependent. These results are consistent with other studies which demonstrated that ERRα expression is correlated to an adverse outcome for breast cancer patients [48]. Therefore, treating breast cancer cells by an inverse agonist of

ERRα may result in inhibiting breast cancer cell growth [14]. Interestingly, based on Gene Ontology analysis, ERRα controls the expression of genes related to cellular energy metabolism, including those encoding enzymes in the oxidative phosphorylation and tricarboxylic acid cycle [49]. Several studies demonstrated that knockdown of ERRα dramatically inhibited the tumor growth of triple-negative breast cancer [14,46,50]. Furthermore, it has been demonstrated that in breast cancer metastasized to the brain, PGC-1α/β and ERRα are overexpressed as-sociated with an upregulation of the corresponding target genes involved in the TCA cycle, oxidative phosphorylation and glycolysis. This implicates that these changes in gene expression of metabolic events help the tumor cells in the metastatic stage survive in a low glucose environment. Moreover, it is well documented that in breast cancer, activation of ERRα/PGC-1 complex upregulates the expression of VEGF and WNT11, which are positively associated with angiogenesis in breast cancer [51]. In the current study, although the exact mechanism by which ED inhibits breast cancer cell growth is not known, it is likely that it involves ED-ERR interaction. Whether the ED-ERR pathway can be therapeutically targeted to suppress the expression of genes involved in the TCA cycle, oxidative phosphorylation, energy metabolism or angiogenesis and thus to inhibit cancer progression in breast cancer patients [49,51] remains to be determined.

A major finding in the current study is that the fraction corresponding to purified ED, but not the fractions adjacent to the ED peak (fractions before and after ED peak) have no effect on cell proliferation (Fig. 5A). Our finding that the effective dose (IC50) of ED to inhibit breast cancer growth is in the low nanomolar range (approximately 3.2 nM and 4.3 nM for MCF-7 and MDA-MB-231 cells, respectively) (Fig. 6D), and ED does not inhibit the cellular growth in knockdown ERRα breast cancer cell lines (Fig. 6E,F), reveal that ED is a potent inhibitor of breast cancer cell growth and that ERRα mediates this effect. The anti-estrogens ICI 164,384 (ICI) and 4-OHT are effective only in the μM ranges for the treatment of hormone-sensitive

breast cancer [43,48]. Interestingly, estradiol is not able to rescue the anti-proliferative effect of ED when both steroids are present in similar concentrations, suggesting that the ED pathway may dominate over the estradiol pathway (Fig. 5B). It has been shown that ERRα exhibits both ER-dependent and independent activities in breast tumors. A recent report indicates only 18% of ERRα target genes are co-regulated by ERα in MCF7 breast cancer cells [20,46]. Therefore, this raises the possibility that ERR pathway is dominant over the ER pathway in MCF7 cells. Interestingly, our results demonstrate that ED has no effect on the non-cancerous epithelial breast cells (MCF-10 A) proliferation possibly because these cells express a low level of ERRα (Fig. 6C).

Our findings showing that ED inhibits cellular proliferation of breast cancer cells and that this anti-mitogenic effect of ED is ERR-dependent is highly intriguing, although the precise mechanisms are remained to be elucidated. The finding that ED inhibits both ER-negative and ER-positive breast cancer cells may have an important ramifications in breast cancer therapy.

### 2. 6. CONCLUSIONS

In the current study, we identified ED as the first endogenous inverse agonist of ERRα and ERRγ orphan nuclear receptors in the human. ED directly interacts with ERRα and ERRγ with high affinity and disrupting the ERR-co-activator complexes and inhibits ERR transcriptional activity. These findings suggest that ED binds to ERRs, which may change its structural conformation, preventing PGC-1 binding to ERRs, leading to a significant decrease in ERR transcriptional activity. This in turn may result in downregulation of the TCA cycle, oxidative phosphorylation, and glycolysis in the breast cancer cell (Fig. 7), leading to inhibition of the breast cancer cell growth. The biological properties of ED, such as its regulation of ERR transcriptional activity and inhibition of breast cancer cell proliferation, make ED a unique molecule worthy of future investigation.

## DECLARATION OF INTEREST

None.

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## 2. 8. FIGURES

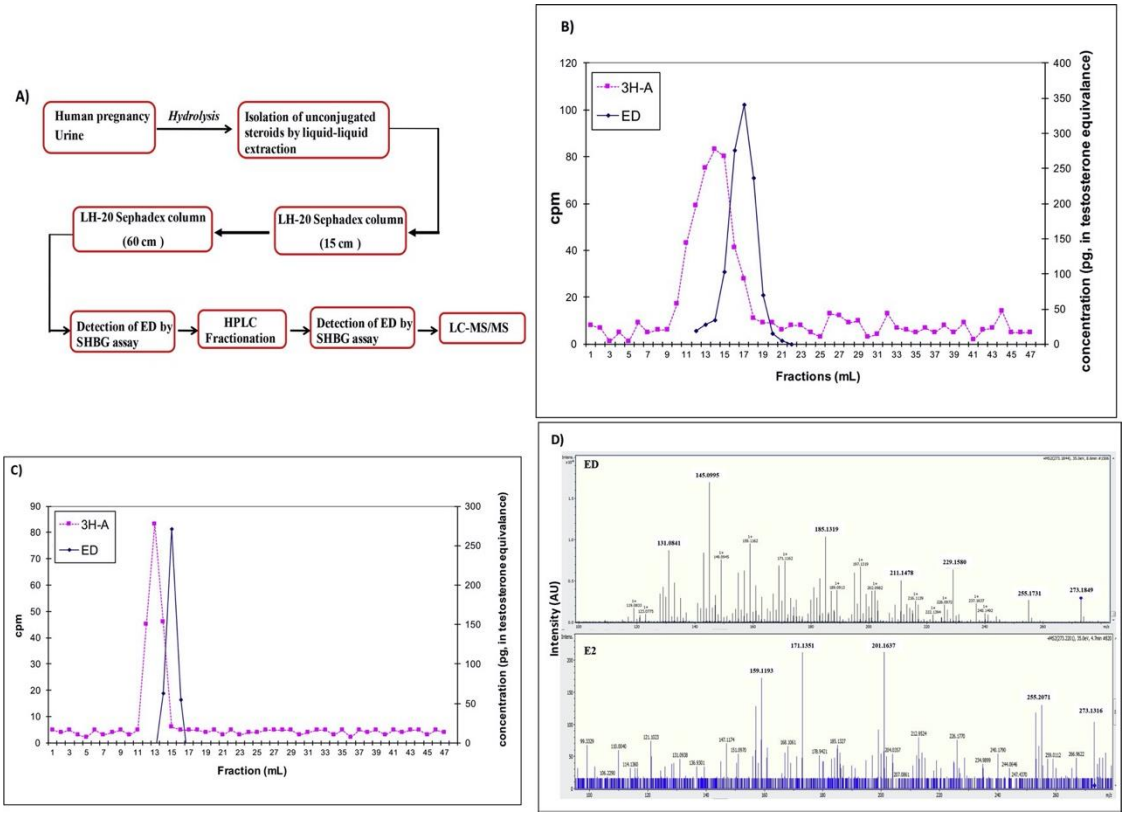


**Fig. 1. Purification and analysis of ED using column chromatography, HPLC and LC-MS/MS.** (A) Schematic diagram of ED purification method, (B) Elution pattern of SHBG-bound material in the region of low polarity from urine of pregnant women: 24-hour urine samples were chromatographed on a Sephadex LH-20 column (60 × 1 cm) with a mobile phase consisting of dichloromethane, heptane and methanol (50:50:1). 48 fractions of 1 mL were collected and an aliquot of each fraction was assayed for SHBG binding. Pink line represents the elution pattern of $^{3}$H-androstenedione (3H-A) tracer, and blue line represents the peak corresponding to ED. A typical chromatogram representative of 4 different experiments is shown. (C): HPLC chromatogram of ED peak: Fractions containing ED from 60 cm Sephadex LH-20 columns were pooled and further separated by HPLC. 1 mL fractions were collected during HPLC using a mobile phase of dichloromethane: heptane: methanol (30:70:0.01) at a flow rate of 1 mL/min. An aliquot from each fraction was used to determine $^{3}$H–androstenedine (3H-A) marker and for SHBG assay. (D) Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of purified ED: The data from MS/MS analysis of purified ED obtained after HPLC purification(up panel) compared to that of 17β-estradiol (E2) (down panel).

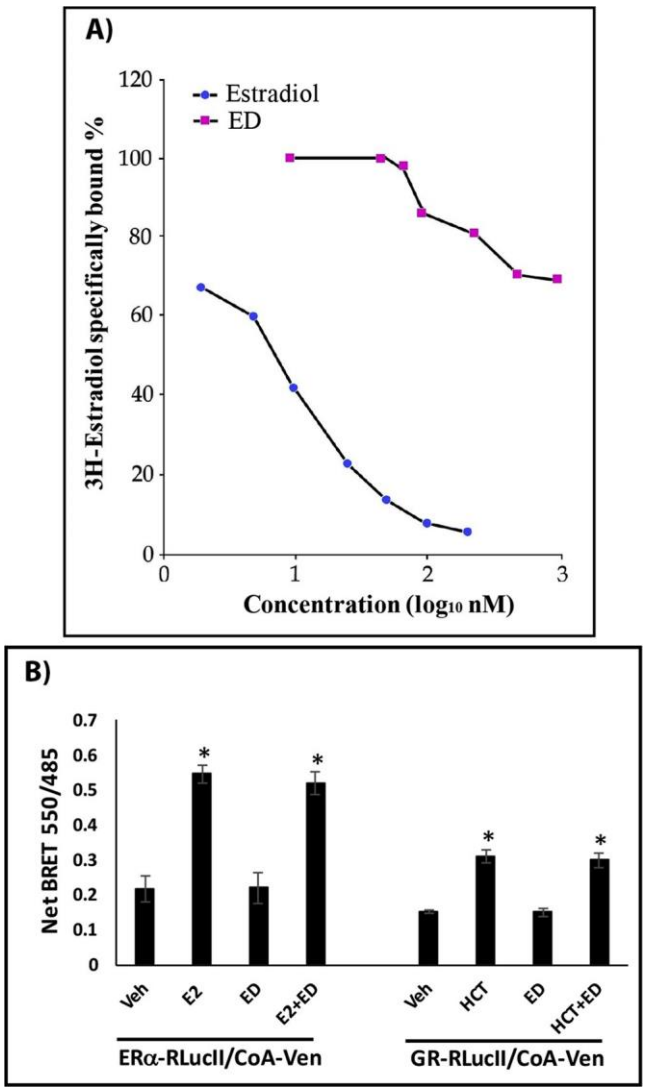


**Fig. 2. Relative affinity of ED for ERα and GR**: (A) ED binds poorly to human uterine estrogen receptor: Human endometrial cytosol was incubated for 18 h at 4 °C with 3H-estradiol (10 nM) alone or in the presence of increasing concentrations of non-radioactive estradiol or ED. The protein-bound fraction was separated using a Sephadex LH-20 column (5 × 0.4 cm). The eluate containing the receptor-bound hormone was counted in a liquid scintillation counter to an error of 1%. The data is representative of 3 different experiments. (B) ED does not interact with ER and GR, as indicated by a BRET assay: Net BRET signals (550/485) were determined (i) in HEK293T cells expressing the ERα-RLucII and CoA-Venus (CoA-Ven) after treatment with 10 nM E2 and/or 10 nM ED, for 1 h (ii) in HEK293T cells expressing GR-RLucII and CoA-Ven after treatment with 10 nM hydrocortisone (HCT) and/or 10 nM ED, for 1 h. The data are re- presentative of 3 independent experiments. A value of $p < 0.05$ was considered significant (*) and compared with the control group.

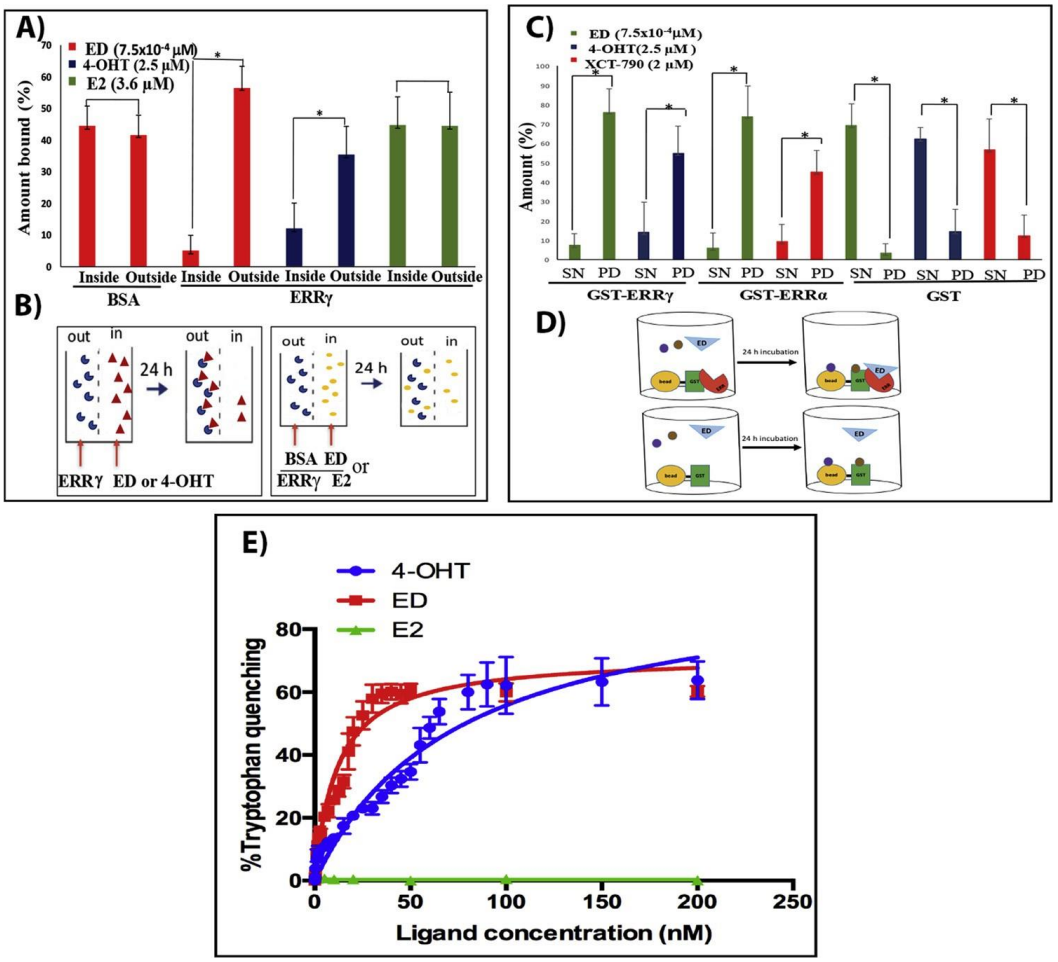


**Fig. 3. ED directly binds to ERRα and ERRγ**. (A)ED directly binds to purified ligand-binding domain of ERRγ, as evaluated by dialysis equilibrium: Purified ligand-binding domain of ERRγ or BSA was placed outside the dialysis tubing (Outside), and ED or 4-OHT or E2 was placed inside the dialysis tubing (Inside). After 24 h of dialysis to reach equilibrium, the amount of ED inside and outside was measured using SHBG assay, and the concentration of 4-OHT and E2 were determined using spectrophotometer at the max wavelength of 245 nm and 281 nm, respectively. A minimum of three independent experiments was done. A value of $p < 0.05$ was considered significant (*). (B) Schematic figure depicting the dialysis method used. Right Panel: When LBD of ERRγ is placed outside and ED or 4-OHT is placed inside and dialyzed to reach equilibrium, ED and 4-OHT get concentrated in the outside compartment containing the ERRγ-LBD Left panel: When BSA is placed outside and ED is placed inside, or when LBD of ERRγ is placed outside and E2 is placed inside, and dialyzed to reach equilibrium, ED and E2 will display equal concentrations in both compartments. (C)ED directly interacts with ERRα and ERRγ with a higher relative binding than 4-OHT and XCT790 as detected by GST-pulldown assay. (D)Schematic diagram of GST-ERRs-beads vs. GST-beads pulldown assay: Equal amounts of GST-ERRα-beads, GST-ERRγ-beads, or GST- beads were incubated with ED or 4-OHT or XCT790 for 24 h. The supernatant (SN) which contains the unbound ligands [ED, 4-OHT and XCT-790], and the pull-down (PN), which contains the bound ligands to ERRs ligand-binding domain [ERR-ED/4-OHT/XCT-790] were separated by centrifugation. ED concentration in supernatant and pulldown fractions was then determined using SHBG assay. However, for 4-OHT and XCT-790, UV–vis spectrophotometer was used to measure their concentration at the max wavelength of 245 nm and 368 nm, respectively. The figure

represents a minimum of three independent experiments. *$P < 0.05$ compared with control group. (E) ED binds directly to LBD of ERRγ with high affinity, as determined by a tryptophan quenching fluorescence assay. The LBD ERRγ was incubated with varying concentrations of ED, 4-OHT, or E2 and the fluorescence emission was measured at 310 nm following excitation at 295 nm. The dissociation constant ($K_d$) was determined using the Graph Pad software.

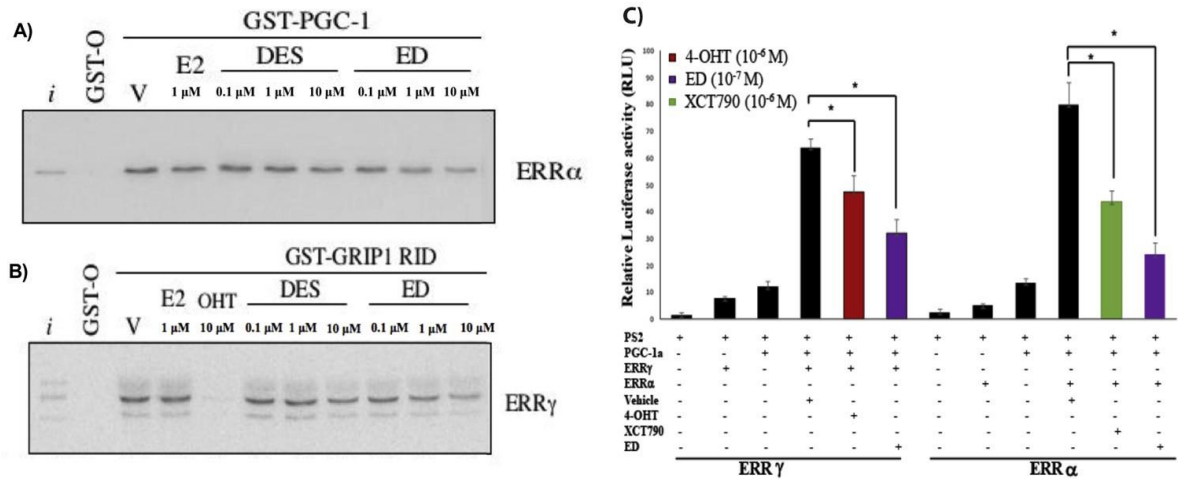


**Fig. 4. ED is an inverse agonist of ERRα and ERRγ** (A): ED disrupts the interaction between ERRα and the coactivator GST-PGC-1. Purified GST–PGC-1 was incubated with [$^{35}$S] methionine-labeled ERRα in the presence of vehicle (V; lane 3), 17β-estradiol (E2; lane 4), DES (lane 5–7), or ED (lane 8–10); or (GST-O; lane2) negative control containing an equal amount of GST protein alone. (B): GST-pull down experiment confirms that ED interrupts the in- interaction between ERRγ and coactivator GST- GRIP1 receptor-interacting domain (GST–GRIP1 RID). Purified GST–GRIP1 RID was incubated with [$^{35}$S] methionine-labeled ERRγ in the presence of vehicle (V; lane 3), Estradiol (E2; lane 4), 4-hyroxytamoxifen (OHT; lane 5), DES (lane 6–8), or ED (lane 9–11); or (GST-O; lane 2) negative control containing an equal amount of GST protein alone. (C): ED inhibits ERRα and ERRγ transcriptional activity. HEK 293 cells were transiently transfected with pS2-LUC promoter reporter plasmid and co-transfected with expression vectors for ERRα or ERRγ plasmid, with or without the PGC-1α co-activator plasmid. Cells were treated with 100 nM ED, 1 μM 4-OHT, or 1 μM XCT790. Luciferase activity was measured and values normalized using Renilla transfection efficiency control. *P $<$ 0.05 considered as significant and compared with the control group.

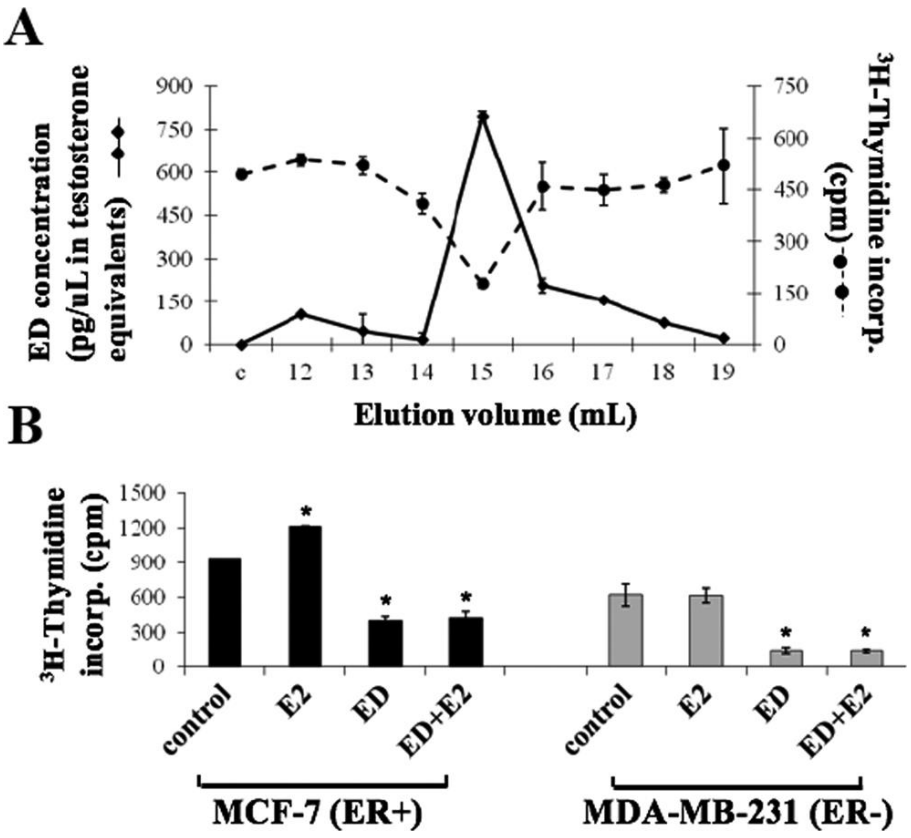


**Fig. 5. The purified ED fraction but not adjacent fractions inhibits the proliferation of ER-negative MDA-MB-231 and ER-positive MCF-7 breast cancer cells.** (A) ED extracted from pregnancy urine was chromatographed, as described in the Methods. MDA-MB-231 cells were then treated for 24 h with aliquots from fractions corresponding to the ED peak from the 60 cm column. Cell growth was then determined using a $^{3}$H-thymidine incorporation assay, (B) MCF-7 and MDA-MB-231 cells were treated with E2 (25 nM) or ED (25 nM), both ED and E2 or vehicle control for 24 h. Cell growth was then determined using a $^{3}$H-thymidine incorporation assay. *$P < 0.05$ compared with control group.

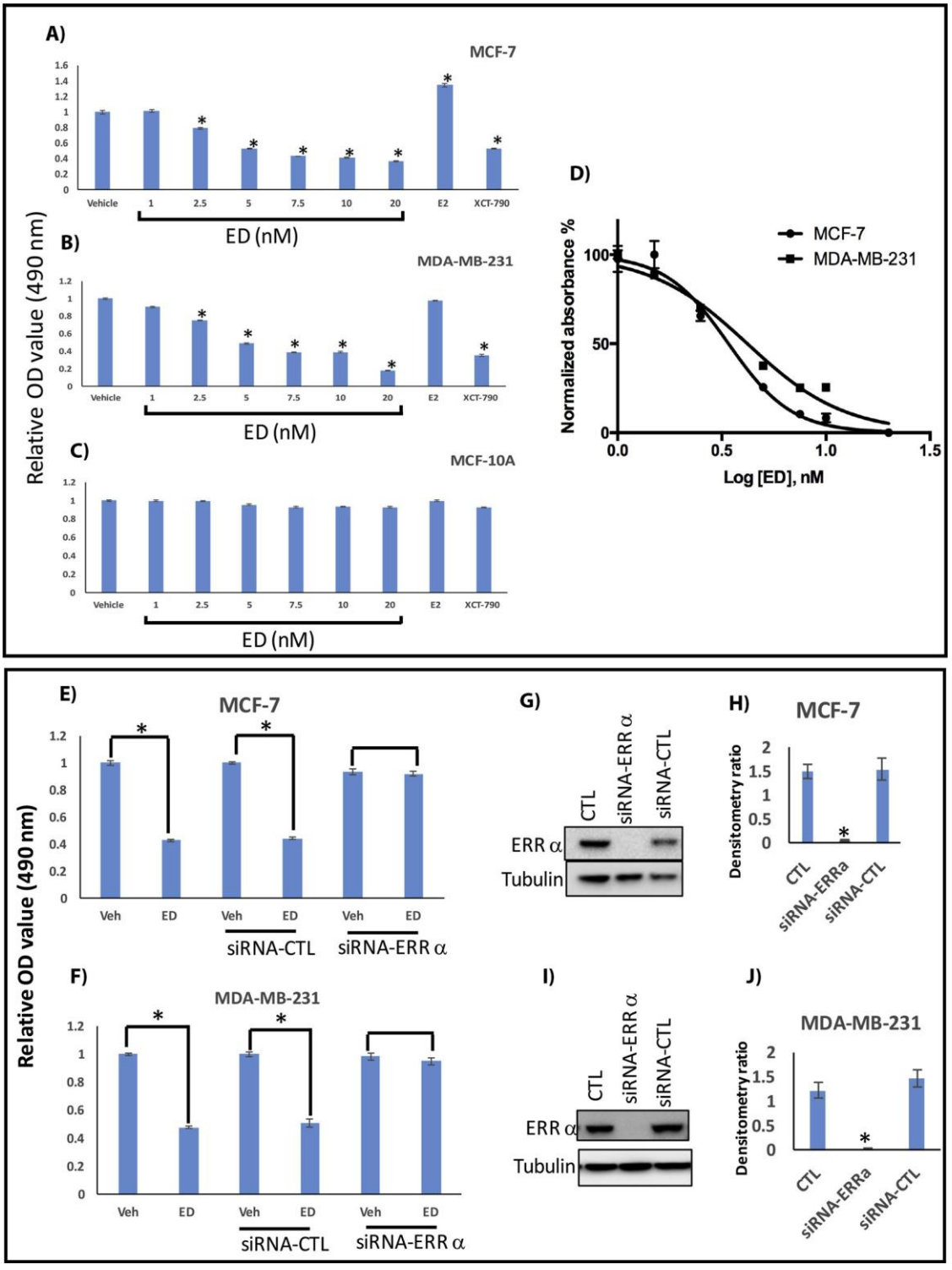
A)
MCF-7
B)
MDA-MB-231
C)
MCF-10A
Relative OD value (490 nm)
Vehicle
1
2.5
5
7.5
10
20
E2
XCT-790
ED (nM)
D)
MCF-7
MDA-MB-231
Normalized absorbance %
Log [ED], nM
E)
MCF-7
F)
MDA-MB-231
Veh
ED
siRNA-CTL
siRNA-ERR α
G)
CTL
siRNA-ERR α
siRNA-CTL
ERR α
Tubulin
H)
MCF-7
Densitometry ratio
CTL
siRNA-ERRa
siRNA-CTL
I)
J)
MDA-MB-231

**Fig. 6. ED inhibits proliferation of ER-negative (MDA-MB-231) and ER-positive (MCF-7) breast cancer cells in a dose-dependent manner while not altering the proliferation of the non-cancerous immortalized breast epithelial cells (MCF-10 A).** (A)MCF-7, (B) MDA-MB-231 and (C) MCF-10 were treated with ethanol (vehicle), ED at the indicated concentrations, estradiol (E2, 25 nM), XCT-790 (1 μM). Medium was changed at 48 h intervals and replenished with fresh medium containing the same concentrations of compounds as above, for a total of 5 days. Cells were harvested on the 5th day and cellular growth was quantified using MTS cell proliferation kit, according to manufacturer's instructions. The data shown are representative of at least 3 independent experiments. *P $<$ 0.05 compared with control group. (D) Analysis of the data demonstrating the dose-dependent inhibition of proliferation by ED indicates that 50% inhibition of growth ($IC_{50}$) occurs at an ED concentration of approximately 3.2 nM and 4.3 nM for MCF-7 and MDA-MB-231 cells, respectively. E–J) The anti-proliferative effect of ED on breast cancer cells is ERRα- dependent: MCF-7 and MDA-MB-231 cells were transfected with siRNA-ERRα or scrambled control siRNA (siRNA-CTL), and were treated with 5 nM ED, and cultured as above for 5 days, and cellular proliferation was measured by MTS cell proliferation kit (E, F). Immunoblotting for ERRα levels (G, I) and the densitometric analysis of the data (H, J) show that knocking down ERRα with siRNA is effective. All data shown are representative of a minimum of 3 independent experiments *P $<$ 0.05 compared with control group.

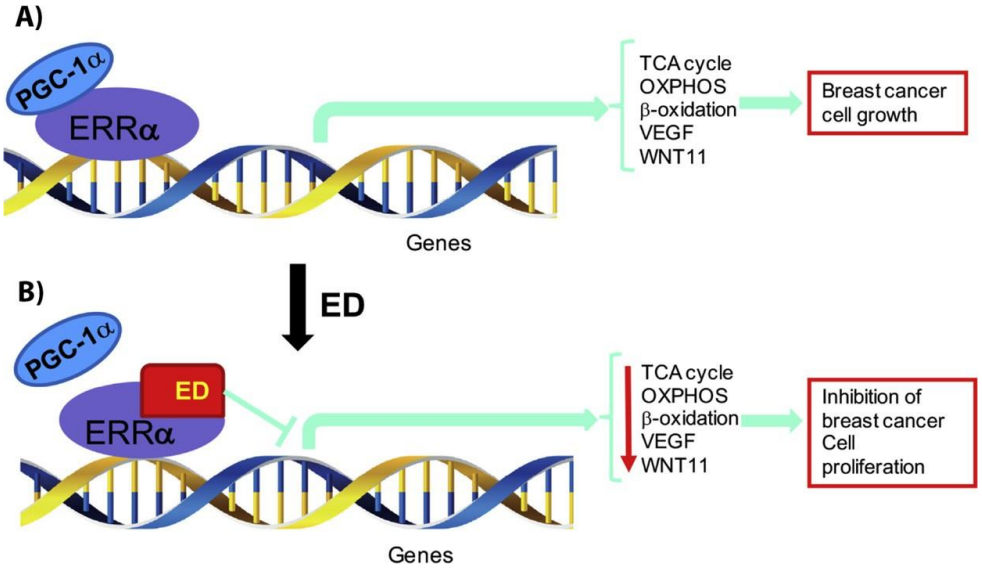


**Fig. 7. Schematic figure depicts the possible mechanism by which ED may inhibit breast cancer cell growth.** (A) ERRα /PGC-1 complex upregulates the genes involved in the TCA cycle, (oxidative phosphorylation) OXPHOS and other processes involved in energy metabolism, angiogenesis (VEGF) and oncogenesis (WNT11) [49,51], which will result in breast cancer cell growth.(B) In the presence of ED, ED directly binds to ERRα, changes its conformation, therefore its coactivator, PGC-1 cannot bind to it, resulting in a decrease in the expression of the above-mentioned genes, therefore inhibit cell proliferation of breast cancer cells.

**Connecting text 2:**

In the previous chapter, I showed that endogenous ED acts as an inverse agonist of ERRα and ERRγ and inhibits breast cancer cell proliferation in an ERRα-dependent manner. However, the ED's chemical structure remained to be elucidated. In order to determine the exact structure of ED, a large quantity of ED, in the range of milligrams, needs to be isolated and purified from human pregnancy urine and blood. However, ED's yield is approximately one microgram or lower per one liter of human pregnancy urine, using the Sephadex LH-20 chromatography method (data not shown). In order to increase ED's yield, I investigated several isolations and purification methods, such as solid-phase extraction using oasis HLB cartridges or the extraction of ED using JEG3 human trophoblast cells' secreted steroids, however, these methods do not demonstrate an increased purified ED yield compared to the previous method (data not shown). Therefore, I used affinity-based method using GST-ERR-LBD to enrich the endogenous ligand of ERRs and I developed a LC-MS/MS method to identify the enriched endogenous ligand of ERRs and analyzed their possible structures using their fragmentation pattern. Interestingly, I demonstrated that two distinct compounds with the mass 273 m/z (consistent with the previous MS data of purified ED) and 333 m/z were enriched in GST-ERRγ-LBD, and I further analyzed the affinity of the possible structures of these compounds to SHBG and ERRγ-LBD. My data indicates that a possible structure of the compound with the mass 273 m/z (predicted and chemically synthesized previously by our team, and also predicted based on my MS data) show high affinity to SHBG and human purified ERRγ-LBD using SHBG and dialysis equilibrium assays, respectively (data not shown).

In this chapter, I demonstrated that cholesterol isolated from human pregnancy serum is also enriched in a GST-ERRα-LBD affinity column. These data are in line with another group that recently reported that cholesterol acts as an endogenous agonist of ERRα. Interestingly, it has been shown that obesity and high cholesterol blood levels increase the risk

of breast cancer recurrence, and cholesterol-lowering drugs, notably statins, improve breast cancer patients' survival. However, the underlying mechanism by which cholesterol exerts its pathological effect on breast cancer is not well-understood yet. Importantly, there is accumulating evidence that ERRα is overexpressed in breast cancer cells, and this overexpression is linked to unfavorable outcomes in breast cancer patients. Therefore, in this chapter, I hypothesized that the pathological effect of cholesterol on increasing breast cancer risk is mediated via the cholesterol-ERRα/PGC-1α pathway in breast cancer cells. To verify this hypothesis, I established three objectives: 1) To demonstrate whether cholesterol enhances the interaction of ERRα with its coactivator, PGC-1α; 2) To determine whether cholesterol modulates ERRα activity in breast cancer cells; and 3) To demonstrate whether the impact of cholesterol on breast cancer cell growth is ERRα-dependent. The content of this chapter has been published in the Journal, Cells.

## CHAPTER 3 – MANUSCRIPT 2

# Cholesterol as an Endogenous Ligand of ERRα Promotes ERRα-Mediated Cellular Proliferation and Metabolic Target Gene Expression in Breast Cancer Cells

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### 3.1. ABSTRACT

Breast cancer is the 2nd leading cause of cancer-related death among women. Increased risk of breast cancer has been associated with high dietary cholesterol intake. However, the underlying mechanisms are not known. The nuclear receptor, estrogen-related receptor alpha (ERRα), plays an important role in breast cancer cell metabolism, and its overexpression has been linked to poor survival. Here we identified cholesterol as an endogenous ligand of ERRα by purification from human pregnancy serum using a GST-ERRα affinity column and liquid chromatography-tandem mass spectrometry (LC-MS/MS). We show that cholesterol interacts with ERRα and induces its transcriptional activity in estrogen receptor-positive (ER+) and triple-negative breast cancer (TNBC) cells. In addition, we show that cholesterol enhances ERRα-PGC-1α interaction, induces ERRα expression itself, augments expression of several metabolic target genes of ERRα, and increases cell proliferation and migration in both ER+ and TNBC cells. Furthermore, the stimulatory effect of cholesterol on metabolic gene expression, cell proliferation, and migration requires the ERRα pathway. These findings provide a mechanistic explanation for the increased breast cancer risk associated with high dietary cholesterol and possibly the pro-survival effect of statins in breast cancer patients, highlighting the clinical relevance of lowering cholesterol levels in breast cancer patients overexpressing ERRα.



**KEYWORDS:** breast cancer; cholesterol; estrogen-related receptor α; statins; human pregnancy serum

## 3.2. INTRODUCTION

Breast cancer is the most frequently diagnosed, and second deadliest cancer in women, with more than 200,000 new patients, and approximately 40,000 estimated deaths per year only in the United States [1]. Therefore, it is necessary to reduce the incidence and improve outcomes by therapeutic approaches addressing known breast cancer risk factors. Obesity, dyslipidemia, and high dietary cholesterol intake are critical risk factors for breast cancer in pre- and post-menopausal women [2]. Several studies have indicated that obesity is associated with a higher risk of breast cancer in both triple-negative breast cancer (TNBC) [3,4], and in ER-positive women [5,6]. Indeed, post-menopausal women with high dietary cholesterol intake have been reported to have a ~50% increase in the risk of breast cancer [2,7]. In addition, in several different mouse models of breast cancer, high dietary cholesterol alone resulted in a significant decrease in tumor latency, and an increase in tumor volume and total tumor burden [8–12]. Interestingly, it was shown that established breast cancer is associated with higher low-density lipoprotein (LDL), and very-low-density lipoprotein (VLDL)-cholesterol; however, no link was identified with total cholesterol or high-density lipoprotein (HDL) [13].

There is some evidence that a high-cholesterol diet affects the biophysical properties of lipid raft microdomains of the plasma membrane and enhances signaling activity via phosphoinositide 3-kinase (PI3K) and AKT/protein kinase B in breast cancer cells [9]. However, blood cholesterol levels in the mouse model used above were far higher than in human hypercholesterolemia. In addition, in the above study, exogenous cholesterol concentrations required for cancer cell proliferation were much lower (nanomolar range) than those required for lipid raft formation. Thus, it is unlikely that the pathological effects of cholesterol in breast cancer progression occur via alterations in lipid raft structure and associated

signaling pathways [14], raising the possibility that cholesterol functions as a signaling molecule in breast cancer cells.

Interestingly, some studies have shown that the cholesterol metabolite 27-hydroxycholesterol acts as a signaling molecule through ER and liver X receptor (LXR) in ER+ breast cancer cells, which may explain how hypercholesteremia increases the risk in ER+ breast cancer cells [2,11,15]. However, several studies have reported that obesity and high cholesterol intake increase the risk not only in ER+ breast cancer but also in triple-negative breast cancer [3–5,16], supporting the notion that cholesterol itself acts as a signaling molecule and that such signaling may involve pathways other than the ER pathway.

The nuclear receptor estrogen-related receptor alpha (ERRα; NR3B1) plays important roles in energy metabolism by regulating the expression of genes involved in cellular energy metabolism, including those encoding enzymes in the oxidative phosphorylation (OXPHOS), tricarboxylic acid (TCA) cycle, glycolysis, and in anabolic biosynthesis pathways like lipid, amino-acid and nucleic acid biosynthesis [17–19]. Importantly, ERRα adapts the metabolic pathways to fuel tumor growth via its interaction with the peroxisome proliferator-activated receptor coactivator-1α (PGC-1α) [20,21].

ERRα belongs to the family of orphan nuclear receptors for which no endogenous ligands have been identified [22]. ERRα levels have been shown to be upregulated in ER+ and, in particular, in TNBC cells and its overexpression is linked to poor survival in those patients [17,18,23,24]. Following the identification of ERRα, it was initially thought that ERRα and ERα may have a large overlap in target genes and activity and therefore, play similar roles in breast cancer. However, it was subsequently shown that only few genes that are commonly regulated by both ERRα and ERα in MCF7 breast cancer cells [18]. Consistently, ChIP-on-chip studies demonstrated that only approximately 18% of ERα target genes are co-regulated by ERRα in MCF7 cells [21]. Furthermore, despite a high degree of amino acid similarity

(68%) in the DNA binding domains (DBDs) of ERRs and ERα, ERRs do not bind strongly to perfect palindromic ER response elements [19,21,25]. In addition, several studies have shown that pharmacological modulation of ERRα activity with inverse agonists decreases the proliferation of both ER-positive and -negative breast cancer cells in vitro as well as tumorigenicity in nude mice [17,26–28]. Taken together, the above findings indicate that ERRα exhibits ER-independent pro-tumorigenic activities in breast cancer cells.

Recently, we showed that estradienolone (ED), an endogenous steroid from human pregnancy, acts as an inverse agonist of ERRs [28], and during ED's characterization, we identified cholesterol as an agonist of ERRα, a finding consistent with a recent report that demonstrated that cholesterol isolated from mouse brain or kidney acts as an endogenous agonist of ERRα [29]. To better understand the mechanism by which high cholesterol levels increase breast cancer risk, we examined in the present study whether cholesterol acts through the ERRα pathway in TNBC and ER+ breast cancer cells. We show that the potent effects of cholesterol on cellular responses, gene expression in TNBC and ER+ cells are mediated via the ERRα pathway.

### 3. 3. METHODS

#### Extraction and Identification of ERRα Endogenous Ligands

Human pregnancy blood samples were collected from healthy pregnant women at 28–38 weeks of gestation using informed consent. The samples were centrifuged at 3000 rpm for 10 min at 4 °C and the plasma samples were pooled and stored at −20 °C for further analysis. In order to identify endogenous ligand(s) of ERRα, a GST-ERRα pull-down assay was performed. GST-ERRα-LBD (ligand-binding domain) plasmid was constructed and GST-ERRα-beads were produced as previously described [28]. Sample preparation involves removing proteins from human pregnancy serum using methanol (Sigma Aldrich, Mississauga, ON, Canada). The precipitated proteins were centrifuged, and the supernatant

was dried down under nitrogen and reconstituted in methanol (Sigma Aldrich). Equal amounts of GST-ERRα-beads and GST-beads were incubated with extracted samples in PBS for 24 h at 4 °C with continuous shaking. The supernatants, which contain the unbound steroids and lipids, were collected and the ERRα-ligand complexes were eluted using 10 mM L-glutathione reduced solution (Sigma Aldrich). The proteins were removed from the eluted samples as described above. The eluted samples were subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis (model 1260 Infinity with 1260 Infinity Diode Array Detector HS, Agilent Technologies, Santa Clara, CA, USA, coupled with an Impact HD MS detector, Bruker, Milton, ON, Canada). An Agilent Zorbax Eclipse Plus C18 column (4.6 × 10 mm, 3.5 µm) was used for separation. Mobile phase A was methanol/water/0.1% formic acid (3:1, *v*/*v*), and mobile phase B was 100% isopropanol/0.1% formic acid (Millipore, Sigma-Aldrich). The elution gradient was held at 40% B for the first 0.5 min, 40–90% B from 0.5 to 4.5 min, held at 90% B from 4.5 to 6.5 min, 90–40% B from 6.5 to 6.6 min, held at 40% B from 6.6 to 7 min. The flow rate of the mobile phase was 1 mL/min, and the injection volume was 20 µL. For mass spectrometry, the electrospray ionization was operated in positive and total scan mode. For mass spectrometry, capillary voltage was 4000 V, fragmentor voltage was 500 V; nebulizer gas was 73 psi; drying gas temp was 350 °C with the flow of 12 L/min; *m*/*z* range was from 150 to 800 Daltons.

**GST-ERRα Pull-Down Assay**

To confirm that cholesterol directly binds to ERRα-LBD, a GST-ERRα pull-down assay was used as described above. Briefly, 2 µM of cholesterol, XCT-790 or estradiol (E2) (Sigma Aldrich) were incubated with beads-GST-ERRα-LBD and beads-GST. The pull-down and supernatants were dissolved in methanol, as described earlier. Cholesterol concentrations were measured using a multiple reaction monitoring (MRM) mode by LC-MS/MS as above.

However, XCT-790 and E2 concentrations were determined using a UV–vis spectrophotometer (Cary Series UV–vis-NIR spectrophotometer, Agilent Technologies) at the maximum wavelength of 368 nm and 281 nm, respectively.

**Tryptophan Fluorescence Quenching Assay**

GST-ERRα-LBD (PV4665) was purchased from Life Technologies (Grand Island, NY, USA). Four hundred nM of GST-ERRα-LBD was incubated with varying concentrations of cholesterol, XCT-790, and E2 as previously described [28]. Fluorescence excitation was at 295 nm and the florescent emission was measured at 310 nm using a microplate reader (Infinite M200PRO, TECAN, Männedorf, Switzerland). The dissociation constant ($K_d$) was determined using Graph Pad software (San Diego, CA, USA).

**Cell Culture**

Human embryonic kidney 293 (HEK-293) cells were purchased from Sigma. The MDA-MB-231 and MCF-7 cell lines were purchased from ATCC (Manassas, Virginia, USA). All the above-mentioned cell lines were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. For all experiments, cells were switched 24 h before cell treatments to fresh phenol red-free medium (21063029, Thermo Fisher Scientific, Grand Island, NY, USA) supplemented with 2% lipoprotein depleted and charcoal-stripped FBS. Lipoprotein depleted FBS was purchased from Kalen Biomedical LLC (Germantown, MD, USA) and was charcoal-stripped in order to remove steroid hormones as described previously [30]. Lovastatin (sc-200850A, Santa Cruz Biotechnology, Santa Cruz, CA, USA), a known cholesterol-lowering drug, was used to decrease cholesterol intracellular level. XCT-790 and compound 29 (cpd29), known synthetic inverse agonists of ERRα, were used to decrease ERRα transcriptional activity. XCT-790 (X4753-5MG) was purchased from Sigma Aldrich,

and cpd29 was a generous gift from Dr. Donald McDonnell (Duke University, Durham, NC, USA).

### Antibodies

Rabbit monoclonal anti-ERRα antibody (ab76228), mouse monoclonal anti-VEGF antibody [VG-1] (ab1316), and mouse monoclonal anti-alpha tubulin antibody (ab7291) were from Abcam (Cambridge, MA, USA). Anti-PGC-1α mouse (4C1.3. mAb) antibody and mouse monoclonal anti-ERRα antibody (sc-65715) were from Millipore Sigma and Santa Cruz Biotechnology, respectively. Anti-GAPDH rabbit (mAB#2118) was purchased from Cell Signaling Technology (Danvers, MA, USA).

### Luciferase Reporter Assay to Determine Cholesterol's Effect on ERRα's Transcriptional Activity

To determine whether cholesterol regulates transcriptional activity of ERRα, HEK-293 were transfected with the pS2-Luc reporter plasmid (400 ng), with or without ERRα expression plasmid (300 ng), with or without the proliferator-activated receptor-gamma coactivator-1 (PGC-1α) co-activator expression plasmid (300 ng), and a Renilla luciferase expression vector (20 ng) as previously described [31]. 48 h after transfection, cells were treated with varying concentrations of cholesterol and XCT-790 (5 μM) as a positive control. Luciferase activity was measured after 24 h and values were normalized to Renilla. The values shown are representative of three independent experiments.

### Immunoblotting and Immunoprecipitation

MDA-MB-231 cells were seeded in 10 cm plates and were treated with vehicle (veh), lovastatin (lova), cholesterol + lovastatin (chol + lova) or cholesterol (chol), all at 5 μM. After 24 h the cells were harvested and lysed with non-denaturing lysis buffer (20 mM Tris-HCl, pH 8, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 2 mM EDTA, and protease inhibitors

(Sigma Aldrich)). Co-immunoprecipitation (co-IP) was carried out as described previously [32]. The above-mentioned cell lysates (500 µg of total protein) were immunoprecipitated with rabbit anti-ERRα antibody (10 µg) or control rabbit immunoglobulin G (IgG, 10 µg) (12-370(CH), Millipore) overnight at 4 ˚C with end over end shaking, followed by a 2 h incubation with Protein A Sepharose 4B (20 µL) (10-1141, Invitrogen) at 4 ˚C. Supernatants were removed after sample centrifugation. The pellets containing beads were washed three times with ice-cold lysis buffer and bead-bound proteins were eluted, denatured and immunoblotted using mouse anti-ERRα antibody or mouse anti-PGC-1α antibody. 2% of the total cell lysates (TCL) were used to detect endogenous levels of ERRα and PGC-1α in cells treated with vehicle, lovastatin, cholesterol + lovastatin or cholesterol.

For MCF-7 cells, the immunoprecipitation procedure was slightly different. Cells were seeded in 10 cm plate and treated with vehicle or 10 µM cholesterol. After 24 h of treatment, a Pierce co-immunoprecipitation (Co-IP) kit (26149, Thermo Fisher Scientific) was used for the preparation of whole-cell lysates and immunoprecipitation using an anti-ERRα antibody, according to the manufacturer's instructions. Briefly, the anti-ERRα antibody was first covalently immobilized to AminoLink Plus coupling resin for 2 h. The resin was then washed and incubated with 500 µg of the above-mentioned cell lysates overnight. After incubation, the resin was washed and the protein complexes were eluted. A negative control (Pierce Control Agarose Resin), provided with the IP kit to determine nonspecific binding, received the same treatment as the co-IP samples, including the anti-ERRα antibody. In this case, the coupling resin is not amine-reactive, therefore, it prevents the antibody from covalent immobilization onto the resin. The eluted co-IP proteins were analyzed by SDS-PAGE and immunoblotted (IB) with a PGC-1α or ERRα antibody. 2% of the total cell lysates (TCL) were used to detect endogenous levels of ERRα and PGC-1α in cells treated with vehicle or cholesterol.

**siRNA Transfection**

siRNAs directed against ERRα (AM16708/289481, Invitrogen, Carlsbad, CA, USA) with the sense sequence 5′-CCGCUUUUGGUUUUAACC-3′ and antisense sequence 5′-GGUUUAAAACCAAAAGCGG-3′ or control scrambled siRNAs (AM4611, negative control, Invitrogen) were transfected into MCF-7 and MDA-MB-231 cells using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) according to manufacturer's instructions. At 48 h post-transfection, fresh phenol red-free medium containing 2% lipoprotein-depleted serum was added and cells were treated with cholesterol and/or lovastatin (5 μM for MDA-MB-231, 10 μM for MCF-7 cells). The knock-down breast cancer cells were used for cell growth assays, immunoblotting and qPCR analyses.

**RNA Preparation and Analysis**

Total RNA was extracted using an RNeasy mini kit (74104, Qiagen, Germantown, MD, USA). One microgram of total RNA was used for the first-strand synthesis with high-capacity cDNA reverse transcription kit (4368814, Life Technologies, Grand Island, NY, US). Real-time PCR was performed using EvaGreen qPCR master mix (Applied Biological Materials Inc., Mastermix-R, Diamond, Richmond, BC, Canada) with gene-specific primers. The targets included in this study are: isocitrate dehydrogenase 3A (IDH3A), pyruvate dehydrogenase kinase 4 (PDK4), vascular endothelial growth factor (VEGF), glutathione S-transferase M1 (GSTM1), superoxide dismutase 2 (SOD2) and secreted phosphoprotein 1(SPP1). The sequences of the above-mentioned primers are indicated in Supplementary Table S1. Real-time PCR was performed on the 7500 real-time PCR system (Applied Biosystems, Woburn, MA, USA). Data analysis was performed using real-time PCR software 7500, version 2.1 (Applied Biosystems). The relative RNA concentrations of the genes of interest were

determined using the comparative threshold cycle ($\Delta C_T$) method after normalization to the endogenous control glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

**Immunocytochemistry (ICC)**

MCF-7 and MDA-MB-231 cells were cultured on cover slips (170-C12MM, Ultident Scientific, Saint-Laurent, QC, Canada). The cells were treated with vehicle or cholesterol (5 µM) for 24 h, and then washed with PBS twice and fixed with 4% paraformaldehyde in PBS for 10 min, followed by three PBS washes. The cells were blocked using 3% BSA for 30 min, and then incubated with the VEGF primary antibody overnight at 4 °C. Following three washes the cover slips were incubated with the secondary antibody (Alexa Fluor 488 anti-mouse, A11029, Molecular Probes, Eugene, OR, USA) for 1 h. The cover slips were washed again, and the nuclei were counterstained with DAPI, and cover slips were attached. The images were obtained using a florescent microscopy (IX71, Olympus, Richmond Hill, ON, Canada) with a 20× objective and a LSM780 laser scanning confocal microscope (Zeiss, White Plains, NY, USA) with a 20×/0.4 LD "Plan-Neofluar" objective.

**Cell Proliferation Assay**

In order to obtain half-maximal effective concentrations ($EC_{50s}$) of cholesterol in MDA-MB-231 and MCF-7 cells, and determine the half-maximal inhibitory concentration (IC50) of lovastatin on MDA-MB-231 and MCF-7 cells, proliferation assays with an MTS Cell Proliferation Assay kit (G3582, Promega, Madison, WI, USA) were performed. The MTS assay is based on the reduction of the MTS tetrazolium compound by viable cells to generate a colored formazan dye in metabolically active cells. According to the manufacturer's instructions, MCF-7 and MDA-MB-231 cells were plated at a density of $l0^4$ cells per well in 96 well plates. Cells were treated with varying concentrations of cholesterol, lovastatin, lovastatin (5 µM) + cholesterol, and the treatment medium was replaced every 48 h throughout the 5 days'

duration of the experiment. Also, MDA-MB-231 cells were treated with varying concentrations of compound 29, in the absence or presence of cholesterol (5 μM), and with medium replacement every 72 h throughout the 6 day duration of the experiment. MTS reagent (20 μL of per well) was then added to and was incubated for 2 h at 37 °C under standard culture conditions. The optical density (OD) value was measured at 490 nm using a microplate reader (Infinite M200PRO, TECAN).

**Migration Assay**

Scratch-wound migration assays were performed on MDA-MB-231 cells. Confluent monolayer cells were pre-incubated with serum-free and phenol red-free medium for 24 h to inhibit cell proliferation. Then monolayers of MDA-MB-231 cells were scratched using a 1mL pipet tip to create a cell-free line and were washed three times to remove cellular debris. The culture plates were replenished with fresh red phenol-free media containing vehicle, cholesterol, lovastatin and/or XCT-790 (all at 5 μM). Wound closure was monitored at times 0 and 24 h, and representative images were photographed using a bright-field microscope (Evos XL core, Life Technologies). Wound width for each treatment was calculated based on averaging six individual measurements at time points 0 and 24 h using ImageJ software. Cell migration was expressed as a percentage of the scratch area filled by migrating cells at 24 h post scratch: migration % = (scratch width at *T* 0 h − scratch width at *T* 24 h/width at *T* 0 h) × 100.

**Statistical Analysis**

All values are expressed as means of at least three independent experiments ± SEM. The statistical significance of differences between two experimental groups was analyzed by a two-tailed Student *t*-test, and comparisons between more than two groups were analyzed by one-way

ANOVA. The experiments were repeated at least three times to obtain *p* values. * represents $p < 0.05$, and was considered to be statistically significant.

### 3.4. RESULTS

#### Identification of Cholesterol as a Candidate Endogenous Ligand of ERRα

To identify endogenous ligands of ERRα from human pregnancy serum, steroids and lipids were extracted from samples in methanol and incubated with beads-GST-ERRα-LBD and beads-GST (as a negative control). The eluted samples were analyzed using LC-MS/MS in full scan mode with a mass range of 200–500 m/z. In order to identify specific binders of ERRα-LBD, the mass spectra obtained with beads-GST-ERRα-LBD and beads-GST were compared. As shown in Figure 1A, a fragment of 369.3 m/z was detected, which represents a daughter ion of cholesterol in the ESI system at the elution time of 3.2 min. The intensity of the cholesterol daughter ion (369.3 m/z) in beads-GST-ERRα-LBD is 5-fold higher than the one in beads-GST. This result suggests that cholesterol acts as an endogenous ligand of ERRα-LBD.

#### Cholesterol Directly Binds to ERRα and Increases Its Transcriptional Activity

In order to verify whether cholesterol directly binds to ERRα-LBD, we performed a GST-ERRα-LBD pull-down assay (Figure 1B). As shown in Figure 1B, the concentration of cholesterol in the pull-down fraction is approximately three times higher than in the supernatant fraction. As a reference, the concentration of XCT-790, a synthetic inverse agonist of ERRα, in the pull-down fraction is approximately 4 times higher than its concentration in supernatant. To demonstrate that cholesterol binding to ERRα-LBD is specific, we performed the same experiment using GST alone. In this negative control, cholesterol concentration is about 2.5 times lower in the pull-down than in the supernatant

(Figure 1B). Together, these results suggest that cholesterol directly interacts with ERRα-LBD.

To quantify the relative affinity of cholesterol for ERRα, we performed tryptophan fluorescent quenching assays using GST-ERRα (Figure 1C). As indicated in Figure 1C, quenching of fluorescence increases in the presence of either cholesterol or XCT-790 (a positive control) in a dose-dependent manner, suggesting that cholesterol and XCT-790 bind to ERRα and change its conformation, resulting in changes in the fluorescent emission of the receptor in the presence of varying concentrations of cholesterol or XCT-790. However, in the presence of estradiol (E2) as a negative control, fluorescence quenching of the GST-ERRα protein remained unchanged. The $K_d$ values for cholesterol and XCT-790 were determined at 213.4 and 49.94 nM, respectively.

We next determined the impact of cholesterol on the transcriptional activity of ERRα. HEK-293 cells were transiently transfected with a reporter plasmid (pS2Luc) and expression vectors for full length ERRα and/or the PGC-1α co-activator (Figure 1D). Cells were transfected with ERRα, PGC-1α or both, and then treated with varying concentrations of cholesterol; XCT-790 was used as a positive control. As demonstrated in Figure 1D, in the presence of both ERRα and PGC-1α, increasing concentrations of cholesterol significantly enhances ERRα transcriptional activity. In contrast, transfection with ERRα or PGC-1α alone does not significantly increase transcriptional activity. This indicates that the effects of cholesterol on ERRα transcriptional activity require both ERRα and PGC-1α. Taken together, these data demonstrate that cholesterol, as an endogenous ligand of ERRα, binds to ERRα-LBD with a relative affinity of 213.4 nM, and increases the transcriptional activity of ERRα in a PGC-1α dependent manner.

**Cholesterol Enhances ERRα-PGC-1α Interaction in Breast Cancer Cells**

To determine whether cholesterol regulates ERRα-PGC-1α interaction in triple-negative (MDA-MB-231) and estrogen receptor-positive (MCF-7) breast cancer cells, co-immunoprecipitation experiments were performed. For MDA-MB-231 cells, the procedure involved treating the cells with vehicle, cholesterol, lovastatin, or lovastatin + cholesterol, and immunoprecipitating the cell lysates using an anti-ERRα antibody or control IgG. All samples were then subjected to immunoblotting with an anti-PGC-1α antibody. As demonstrated in Figure 2A, cholesterol significantly enhances the association of PGC-1α to ERRα compared to the vehicle. No significant decrease in ERRα-PGC-1α association was detectable in the presence of lovastatin, a cholesterol-lowering drug. This result is possibly due to technical error, as lovastatin is expected to decrease endogenous cholesterol synthesis. Significantly, cholesterol was able to enhance ERRα-PGC-1α association even in the presence of lovastatin. Importantly, this association was not detectable when control IgG was used instead of ERRα antibody. Together, these data suggest that exogenous cholesterol increases ERRα and PGC-1α interaction in MDA-MB-231 cells.

The co-immunoprecipitation procedure for MCF-7- cells involved treating the cells with vehicle or cholesterol, and incubating the cell lysates with AminoLink Plus Coupling Resin and immunoprecipitating using anti-ERRα antibody. As shown in Figure 2B, cholesterol significantly increases the interaction of PGC-1α to ERRα. As expected, this interaction was not detectable in the negative control experiment using uncoupled Pierce Control Agarose Resin and anti-ERRα antibody (-ve ctl). Together, these results show that cholesterol treatment enhances the interaction of ERRα and PGC-1α in both MDA-MB-231 and MCF-7 cells.

### Cholesterol Increases ERRα Levels in Breast Cancer Cells

To assess whether cholesterol regulates ERRα expression levels, we treated cells with varying concentrations of cholesterol, and ERRα protein and mRNA levels were measured. As shown in Figure 3A, in the presence of increasing concentrations of cholesterol in MDA-MB-231 cells, a significant increase in ERRα protein levels was observed. To determine whether cholesterol increases ERRα protein levels in the presence of lovastatin (a known cholesterol lowering drug), MDA-MB-231 cells were treated with vehicle, lovastatin, or lovastatin + cholesterol and subjected to immunoblotting (Figure 3B). As shown in Figure 3B, lovastatin does not alter ERRα protein levels. However, adding cholesterol in the presence of lovastatin significantly increases ERRα protein levels. Consistent with these results, we observed a significant induction in ERRα's mRNA levels in MDA-MB-231 cells upon cholesterol treatment (Figure 3C). Moreover, as shown in Figure 3D, when MCF-7 cells were treated with increasing concentrations of cholesterol, ERRα protein levels were significantly increased. In agreement with these results, we observed a significant induction in ERRα mRNA level in MCF-7 cells upon cholesterol treatment (Figure 3E). Altogether, these data demonstrate that exogenous cholesterol significantly enhances the mRNA and protein levels of ERRα in MDA-MB-231 and MCF-7 cells.

### Cholesterol Enhances ERRα-Induced Metabolic Target Genes Through ERRα Pathway

Next, we determined whether cholesterol regulates ERRα metabolic target genes in MDA-MB-231 and MCF-7 cells. The cells were first transfected with siRNA-control or siRNA-ERRα, followed by treatment with cholesterol. As shown in Figures 4A,B, upon

cholesterol treatment, there is a significant increase in the expression of metabolic target genes of ERRα, including IDH3A, VEGF, PDK4, SOD2, GSTM1, and SPP1, in breast cancer cells. However, in ERRα knockdown breast cancer cells, cholesterol does not enhance the expression of ERRα target genes in either MDA-MB-231 or MCF-7 cells. As shown in Figures 4A,B, the efficiency of knockdown-ERRα was 89.9% and 82.7% for MDA-MB-231 and MCF-7 cells, respectively.

These data indicate that the induction of ERRα metabolic target gene expression by cholesterol is ERRα-dependent. To confirm that cholesterol increases expression of ERRα target genes in breast cancer cells via the ERRα pathway, levels of the VEGF protein were assessed in cells treated with siRNA-ERRα or siRNA-control in the presence or absence of cholesterol. As shown in Figure 4C–F, cholesterol increases VEGF protein expression in MDA-MB-231 and MCF-7 cells, as detected by immunoblotting and immunocytochemistry using anti-VEGF antibody. In the absence of ERRα, the stimulatory effects of cholesterol were abolished in both types of breast cancer cells. These data suggest that cholesterol enhances VEGF protein expression through ERRα.

**Cholesterol Enhances Cellular Proliferation of Breast Cancer Cells via the ERRα Pathway**

To determine whether cholesterol regulates cellular proliferation in MDA-MB-231 and MCF-7 cells, cells were treated with varying concentrations of cholesterol. As shown in Figure 5A–C, cholesterol enhances cellular proliferation of these cells in a dose-dependent manner, and the EC50s of cholesterol for MDA-MB-231 and MCF-7 cells are approximately 70 and 110 nM, respectively. These results showing that cholesterol enhances cell proliferation at low nano-molar concentrations support the notion that cholesterol may act as a signaling molecule in these cells. Interestingly, lovastatin decreases cell proliferation of

breast cancer cells in a dose-dependent manner and cholesterol inhibits this effect (Figure 5D,E). As demonstrated in Figure 5F, the IC50 of lovastatin in MDA-MB-231 cells is 1.81 μM, which is slightly lower than the one in MCF-7 cells (5.34 μM), possibly due to the higher expression of ERRα in MDA-MB-231 compared to MCF-7 cells.

To demonstrate that the effect of cholesterol on breast cancer cell proliferation is mediated via ERRα, the expression of ERRα was knocked down by siRNA and cells were treated with lovastatin or cholesterol (Figure 5G,H). As shown in Figure 5G,H, ERRα was successfully knocked down in MDA-MB-231 and MCF-7 cells and cholesterol-induced cell proliferation is abrogated when ERRα expression is suppressed. Similarly, lovastatin-induced inhibition of cell proliferation is abolished in ERRα-deficient cells. These results suggest that both cholesterol-induced cell proliferation, and lovastatin-induced inhibition of cell proliferation are mediated via ERRα. Consistent with the result shown in Figure 5D,E, cholesterol is able to rescue the lovastatin-induced inhibition of cell proliferation. The lovastatin inhibitory effect on cellular proliferation is likely due to lowering intercellular cholesterol level, although cholesterol-independent effects of lovastatin cannot be ruled out. In order to confirm that cholesterol mediates cell proliferation in an ERRα-dependent manner, we performed dose-competition assays between the ERRα antagonist cpd29 and cholesterol. As shown in Figure 5I (black bars), cpd29 decreases cell proliferation in a dose-dependent manner in MDA-MB-231cells. Importantly, cpd29 also decreases cholesterol-induced cell proliferation in a dose-dependent manner in those cells (Figure 5I gray bars). Collectively, these data show that cholesterol increases cell proliferation of both MDA-MB-231 and MCF-7 cells, acting via ERRα.

### Cholesterol Rescues the Inhibitory Effect of Lovastatin on Cellular Migration, but not that of XCT-790 in MDA-MB-231 Cells

To verify whether the effects of cholesterol on breast cancer cell migration are mediated through ERRα, we performed a scratch assay. Cells were treated with lovastatin, cholesterol, and/or XCT-790. As shown in Figure 6A,B, adding exogenous cholesterol does not significantly increase cellular migration although a trend in that direction is observed upon cholesterol treatment for 24 h. It is possible that a significant increase in cell migration requires a cholesterol treatment duration of more than the 24 hours used in the current study (i.e., 48 h or 72 h). In order to further probe whether cholesterol displays any effect on breast cancer cellular migration within 24 h, we used lovastatin to decrease intracellular cholesterol levels. Interestingly, cholesterol is able to rescue the lovastatin-induced inhibition of cellular migration in a significant manner. Next, to verify whether ERRα mediates the stimulatory effect of cholesterol on breast cancer cellular migration in the presence of lovastatin, XCT-790, a small molecule inhibitor of ERRα activity, was used. As shown in Figure 6A, XCT-790 decreases MDA-MB-231 cellular migration. However, adding exogenous cholesterol was unable to rescue the migration inhibitory effect of XCT-790 even in the presence of lovastatin. Together, these results suggest that the inhibition of cell migration induced by the cholesterol-lowering agent lovastatin is rescued by cholesterol. However, when ERRα is inhibited by XCT-790, cholesterol does not increase cell migration, nor does it restore the effect of lovastatin.

## 3. 5. DISCUSSION

There is accumulating evidence that obesity and high blood cholesterol increase the risk of breast cancer recurrence [33–35], while the use of statins, known cholesterol-lowering drugs, is linked to increased disease-free survival in breast cancer patients [11,35–38]. However, the underlying mechanisms by which high cholesterol levels increase breast cancer

recurrence risk and mortality are poorly understood [35,38]. As ERRα orphan receptor is a master regulator of energy metabolism, and its levels are upregulated in breast cancer with overexpression associated with poor survival, we pursued identification of its endogenous ligands. We recently reported the identification of an estradienolone-like molecule (ED) from human pregnancy urine, as an endogenous inverse agonist of ERRα [28]. In the current study, we demonstrate that cholesterol isolated from human pregnancy blood acts as an endogenous agonist of ERRα. We show that cholesterol binds ERRα and enhances its transcriptional activity in ER-positive and triple-negative breast cancer cells, which overexpress ERRα. Furthermore, we demonstrate that cholesterol enhances the interaction of ERRα with its transcriptional co-activator, PGC-1α, resulting in the activation of several ERRα's target genes (including VEGF and ERRα itself), and in promoting cellular proliferation and migration in an ERRα-dependent manner, in breast cancer cells. Importantly, lovastatin inhibits cell proliferation and migration in both ER-positive and triple-negative breast cancer cells, possibly due to a decrease in intracellular cholesterol levels [39], and cholesterol is able to rescue these effects of lovastatin. The anticancer effects of statins have been shown to involve multiple molecular pathways, including inhibition of protein kinase B (AKT)/mammalian target of rapamycin (mTOR) [40,41]. Nevertheless, the current study demonstrates that in the presence of lovastatin, addition of cholesterol is able to reverse the inhibitory effects of lovastatin on cell proliferation and migration of MDA-MB-231 cells via the ERRα pathway, while ERRα protein levels remain unchanged. This, together with our results showing that a well-characterized ERRα antagonist, cpd29 [42–44], is able to inhibit cholesterol-induced cellular proliferation supports the premise that cholesterol-induced cellular proliferation is mediated via ERRα. Our finding that cholesterol isolated from human pregnancy blood is an endogenous agonist of ERRα is in agreement with the findings from another group using cholesterol isolated from mouse brain and kidney [29]. Our results

showing that cholesterol binds directly and specifically to the purified ligand-binding domain of ERRα, with a dissociation constant of approximately 210 nM, and increases the transcriptional activity of ERRα in a PGC-1α-dependent manner in both ER-positive and triple-negative breast cancer cells, indicate that cholesterol acts as an endogenous agonist of ERRα-PGC-1α signaling in these cells. In addition, our findings suggest that the mechanism by which cholesterol enhances ERRα transcriptional activity involves increasing the recruitment of PGC-1α to ERRα, as detected by enhanced interaction between ERRα and PGC-1α in the presence of cholesterol, whether in the presence or absence of lovastatin. It is possible that cholesterol acts as an allosteric activator by binding to the ERRα protein and changes its conformation, leading to enhanced interaction with its coactivator PGC-1α, and thus promoting ERRα transcriptional activity.

The ERRα/PGC-1α/β complex is the main regulator of genes involved in energy metabolism and mitochondrial biogenesis and directs metabolic reprogramming in cancer cells. It has been reported that this complex controls the expression of genes involved in the TCA cycle, OXPHOS, lipid metabolism, and glycolysis in breast cancer cells [18,21]. It is thus significant that cholesterol binding to ERRα and cholesterol-mediated increase in ERRα-PGC-1α interaction results in increased expression of ERRα itself and its metabolic target genes including IDH3A, VEGF, SOD2, GSTM1, PDK4, SPP1 in breast cancer cells. The finding that the cholesterol-mediated increase in the expression of these genes requires ERRα in both ER-positive and triple-negative breast cancer cells provides a mechanistic explanation for the adverse effect of high circulating cholesterol levels and may explain the beneficial effect of statins on breast cancer outcome.

Cholesterol's ability to increase ERRα mRNA and protein levels in a dose-dependent manner can be explained by ERRα specific auto-induction, as ERRα activates the promoter of its own gene, ESRRA, thus providing positive regulation of its own expression [18,21,27].

It is possible that when cholesterol binds to ERRα and enhances its interaction to PGC-1α, this leads to the binding of the ERRα/PGC-1α complex to the ESRRA promoter and induction of ERRα expression itself as well as that of the metabolic target genes of ERRα. IDH3A, a major metabolic target gene of ERRα, is a key enzyme in the TCA cycle and is known to stimulate angiogenesis and metabolic reprogramming of cancer cells to provide the necessary nutrients for cancer cell growth [45,46]. Similarly, cholesterol-mediated increase in PDK4 is of significant interest in this regard, as it is also a key enzyme in glucose and fatty acid metabolism, and its expression is upregulated in breast cancer and correlates with poor patient outcomes [47]. Together, these findings suggest that cholesterol induces metabolic gene expression via its modulation of ERRα activity.

SOD2 and GSTM1 are responsible for the detoxification of reactive oxygen species (ROS) and electrophilic compounds, which are produced mainly by mitochondria in cancer cells [20,48]. The elevation of ROS was shown to be essential for the metabolic reprogramming toward glycolysis [49]. Based on our findings, we suggest that high cholesterol levels resulting in increased interaction of ERRα with PGC-1α and ERRα-PGC1α signaling provide protection against the production of ROS from oxidative stress by increasing cell detoxification enzymes like SOD2 and GSTM1 and thus help avoid irreversible damage on mitochondria and other organelles of cancer cells. In addition, as SPP1 is known to be a direct target gene of ERRα and has been shown to be overexpressed in breast cancer cells and functionally contribute to cancer progression [48,49], our finding that the cholesterol-induced increase in its expression requires ERRα in breast cancer cells is consistent with cholesterol's adverse effects on breast cancer outcome.

Vascularization is an important process in metastatic progression. ERRα and its coactivator PGC-1α have been reported to bind to the promoter of VEGF (known to be involved in tumor invasion and angiogenesis), and enhance its expression [18,50–52]. In the

current study, we showed that the expression of VEGF is significantly increased in the presence of cholesterol in ER-positive and triple-negative breast cancer cells and that this cholesterol effect requires ERRα, strongly suggesting that cholesterol enhances ERRα-induced VEGF expression in breast cancer cells. The ability to proliferate and migrate are two metastatic hallmarks of cancer cells. Cholesterol promotes cell proliferation and migration in ER+ and triple-negative breast cancer cells in an ERRα dependent manner, whereas statin shows the opposite effect. Importantly, cholesterol also rescues the effect of statin on proliferation and migration in an ERRα-dependent manner. Importantly, our results show that cholesterol increases cell proliferation of triple-negative and ER+ breast cancer cells in a dose-dependent manner within a nanomolar range, implicating cholesterol as a signaling molecule. Lovastatin displays the opposite effect, decreasing both cell proliferation and migration of both cell types, and cholesterol rescues lovastatin's effect. However, cholesterol is unable to rescue the inhibitory effect of XCT-790 (a known inverse agonist of ERRα) on breast cancer cell migration, presumably because XCT-90 mechanism of action involves degradation of ERRα [53,54]. Our finding that cholesterol mediates cell proliferation in an ERRα-dependent manner, was further confirmed using dose-competition assays between cholesterol and another well-characterized ERRα antagonist cpd29 [42–44] in MDA-MB-231 cells. Together, these results demonstrate that the knockdown of ERRα or inhibition of ERRα activity using XCT-790 or cpd29, results in abrogation of the enhancing effect of cholesterol on breast cancer cell proliferation and migration. These findings suggest that the stimulatory effects of cholesterol on cell proliferation and migration are mediated via ERRα.

Based on our findings, we propose that the mechanism by which cholesterol may exert its effects on ER+ and TNBC cells involves cholesterol binding to ERRα and changing its conformation, thereby enhancing ERRα interaction with its coactivator PGC-1α, with the

increased ERRα-PGC-1α interaction resulting in augmented expression of ERRα itself (auto-induction) and of its target genes implicated in cellular metabolism, including IDH3A, PDK4, SOD2, GSTM1, and VEGF. We further propose that together, this may result in the reprogramming of tumor metabolism to provide sufficient biomass and detoxification against oxidative stress for breast cancer cells to proliferate and migrate faster (Figure 7). In contrast, treatment with the lipid-soluble statin, lovastatin, an inhibitor of HMG-CoA reductase, the rate-limiting enzyme in the cholesterol biosynthetic pathway, results in reduced cell proliferation and migration in breast cancer cells (Figure 7), likely via reducing cholesterol intracellular levels [39], and its effects are reversed by exogenous cholesterol addition. Together, our findings provide insight into the potential mechanisms underlying the increased risk of breast cancer associated with elevated levels of circulating cholesterol and the protective effect of statins in improving breast cancer patients' survival.

ERRα/PGC-1α/β activity is under the regulation of several oncogenic signals, including the PI3K/AKT/mTOR pathway which plays a key role in activating SREBP, a critical transcription factor involved in intracellular cholesterol synthesis in cancer cells [55]. Thus, cholesterol may provide a link between the mTOR pathway and ERRα/PGC-1 complex activation in cancer cells.

In the present study, we have not performed a detailed analysis of the cholesterol-ERRα binding complex and kinetics, by methods such as nuclear magnetic resonance (NMR), or X-ray crystallography. However, a previous study by another group has reported the binding kinetics and structural basis of cholesterol-ERRα interaction [29]. Using computational docking of cholesterol into the LBD of ERRα they have demonstrated that the hydroxyl group of cholesterol makes a hydrogen bond to E235 of ERRα's LBD. Also, they have shown that F232 and L228 of ERRα possibly make important hydrophobic contacts with cholesterol [29]. A limitation of our study is that cholesterol-lowering drugs like statins have

been reported to have cholesterol-independent effects [40,41]. Unfortunately, there are no known cholesterol depleting agents that do not exhibit cholesterol-independent effects. While our findings in the current study show that cholesterol enhances cell proliferation and migration in an ERRα-dependent manner, the question as to whether other upstream targets are involved in this process remains to be determined. In the current study, we have used a cholesterol concentration of 5 μM for MDA-MB-231 and 10 μM for MCF-7 cells, because these doses showed the optimal response in the biological assays used. It is not possible to ascertain the physiologic relevance of the dose chosen as it is difficult to mimic in vivo concentrations of extracellular or intracellular cholesterol levels in vitro in the cell lines. In addition, it should be noted that the results presented in the present study are limited to a representative triple-negative (MDA-MB-231) and a representative ER+ (MCF-7) breast cancer cell line; using other breast cancer cells expressing varying levels of ERRα would strengthen the clinical implications of the current study.

### 3. 6. CONCLUSION

In the present study, we demonstrate that cholesterol binds ERRα, enhances its interaction with its transcriptional co-activator PGC-1α, and promotes ERRα transcriptional activity in ER-positive and in triple-negative breast cancer cells. Furthermore, we show that cholesterol activates several ERRα metabolic target genes and enhances cellular proliferation and migration, ERRα being required for these effects. Statins inhibit cell proliferation and migration in both ER-positive and triple-negative breast cancer cells, possibly by decreasing intracellular cholesterol levels [39]. Importantly, exogenous cholesterol is able to rescue these effects of statin.

There is increasing evidence that the expression levels of ERRα are higher in human breast tumors when compared to normal breast tissue [23], and that ERRα overexpression is associated with adverse clinical outcome and recurrence in breast cancer patients [52,56,57].

Thus, it has been suggested that the expression of ERRα could be used as a marker of unfavorable prognosis and response to therapy in breast cancer [56]. The interest in inhibiting ERRα activity in breast cancer patients is based on ERRα's strong involvement in regulating a vast array of oncogenic functions, including metabolic reprogramming of cancer cells [58,59]. Thus, the identification of cholesterol as an endogenous agonist of ERRα provides a potential avenue for targeting intracellular cholesterol action to globally impinge on the metabolic impairments in cancer cells. Further studies are warranted to explore the potential of drugs such as statins and SREBP inhibitors to prevent or treat breast cancer, in particular TNBC, which has a poor prognosis and no satisfactory treatment options. Furthermore, identification of cholesterol as an agonist of ERRα and a regulator of ERRα target gene expression, and proliferation in ER+ and TNBC cells, also has relevance to other subtypes of breast cancer, like the human epidermal growth factor receptor 2 positive (HER2+) subtype, and other cancer types such as prostate, ovary, and colorectal cancers, where ERRα is overexpressed and known to play a pathological role.

**Supplementary Materials:** The following are available online at http://www.mdpi.com/2073-4409/9/8/1765/s1, Table S1: The primer sequences used for qPCR.

**Author Contributions:** F.G.—designed, performed, analyzed, interpreted, and wrote the original draft of the manuscript. A.P.—supervised the project, wrote and reviewed the manuscript. S.M.—contributed to data interpretation and manuscript reviewing. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare that they have no competing interests.

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### 3. 8. Figures and Supplementary Material

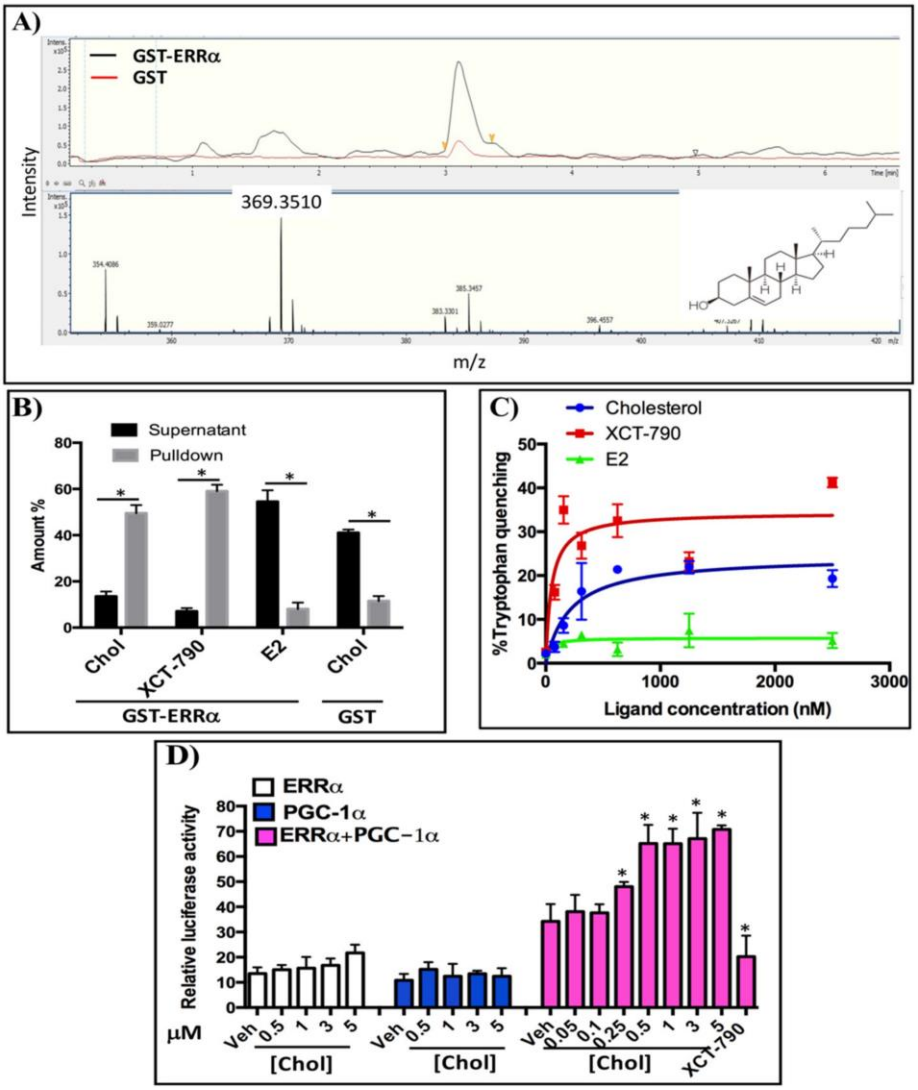


**Figure 1. Cholesterol binds to ERRα and increases its activity**: (A) Human pregnancy serum was incubated with Sepharose beads coupled to GST-ERRα-LBD and or unfused GST for 24 h at 4 ◦C and pull-down samples were analyzed using LC-MS. The upper panel shows the UV chromatogram and the lower panel displays the mass spectrum. The cholesterol structure is located at the right corner of this spectrum. (B) Cholesterol directly binds to ERRα-LBD. GST-ERRα-LBD pull down assays were performed and cholesterol concentrations were measured using LC-MS in MRM mode. XCT-790 and E2 concentrations were determined using a UV–vis spectrophotometer. Amounts are reported as % input. (C) Relative affinity of cholesterol for ERRα was assessed using a tryptophan quenching assay with fluorescence excitation at 295 nm and fluorescent emission at 310 nm. (D) Cholesterol increases the transcriptional activity of ERRα in a PGC-1α dependent manner in a luciferase reporter assay. HEK 293 cells were transiently co-transfected with a pS2-LUC reporter plasmid with or without expression vectors for ERRα and the PGC-1α co-activator. The data are representative of 3 independent experiments. A value of $p < 0.05$ compared with the control group was considered significant (*).

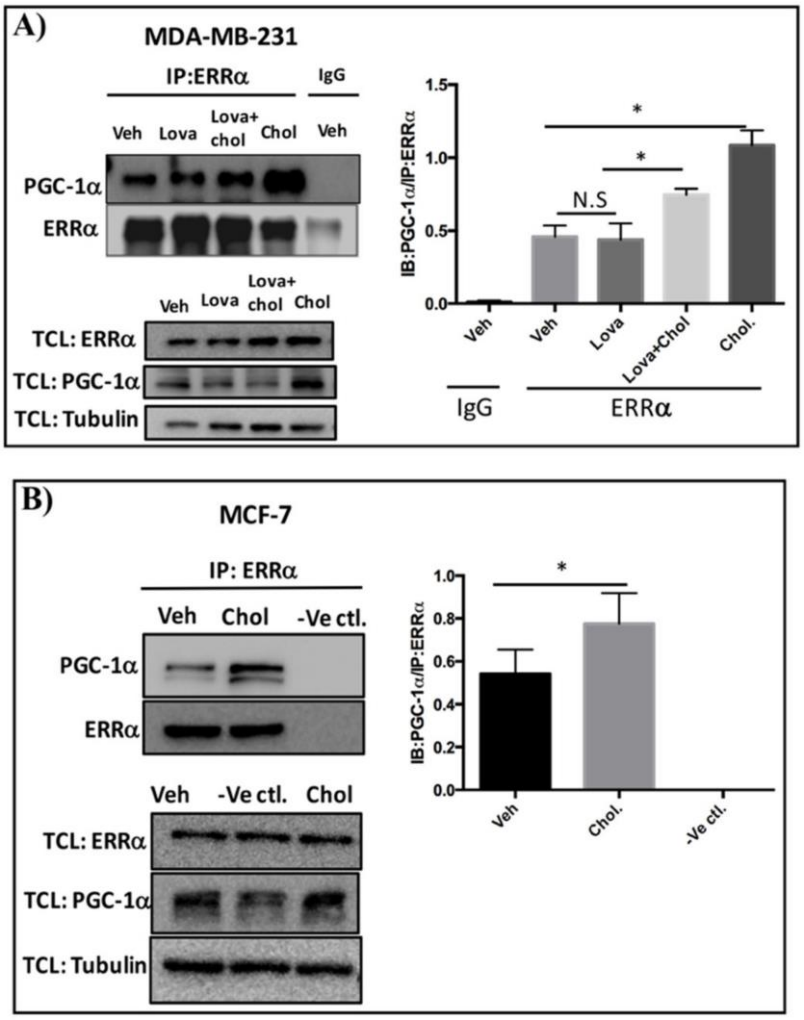


**Figure 2. Cholesterol enhances ERRα-PGC-1α interaction in breast cancer cells.** (A) MDA-MB-231 cells were treated with vehicle (Veh), lovastatin (Lova), lovastatin+cholesterol (Veh + Chol) or cholesterol (Chol) at 5 µM for 24 h. Cell lysates were subjected to immunoprecipitation (IP) with anti-ERRα or control IgG antibody. The protein complexes were separated by SDS-PAGE and immunoblotted (IB) with anti-PGC-1α or anti-ERRα antibodies to detect PGC-1α as a co-immunoprecipitated protein and ERRα as an immunoprecipitated protein. 2% of total cell lysate (TCL) were used to detect the endogenous levels of ERRα and PGC-1α. To quantify PGC-1α/ERRα ratio, densitometry analysis of PGC-1α and ERRα proteins derived from the same immunoblot was performed using ImageJ software. (B) MCF-7 cells were treated with vehicle (Veh) or cholesterol (Chol) at 10 µM for 24 h. Immunoprecipitation was performed using a Pierce co-immunoprecipitation (Co-IP) kit. The cell lysates were incubated with the AminoLink Plus Coupling Resin and immunoprecipitated with an ERRα antibody. Also, as a negative control (-Ve Ctl.), the vehicle-treated cell lysate received the same concentration of anti-ERRα antibody except that the AminoLink Plus Coupling Resin was replaced with a Pierce Control Agarose Resin that is not amine-reactive, preventing ERRα antibody from binding to the resin. The bound proteins were eluted and separated by SDS-PAGE and immunoblotted with anti-PGC-1α or anti-ERRα antibodies to

detect PGC-1α as a co-immunoprecipitated protein and ERRα as an immunoprecipitated protein, respectively. 2% of TCL were used to detect the endogenous protein levels of ERRα and PGC-1α. To quantify the PGC-1α/ERRα ratio, densitometry analysis of PGC-1α and ERRα proteins derived from the same immunoblot was measured using ImageJ software. A minimum of 3 independent experiments were performed. A value of $p < 0.05$ was considered significant (*).

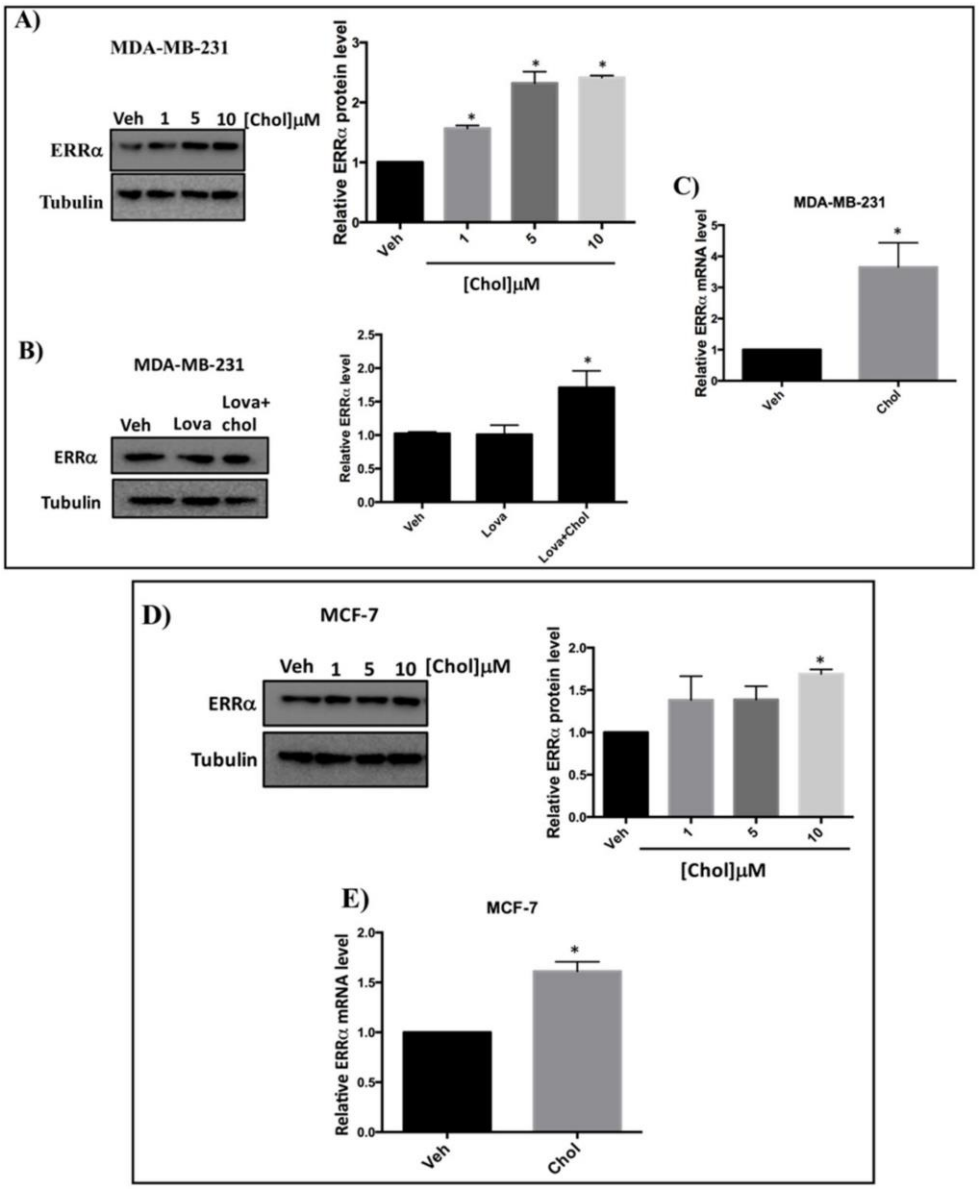


**Figure 3. Cholesterol increases ERRα protein and mRNA levels in MDA-MB-231 and MCF-7 cells in a dose-dependent manner.** (**A**) Cholesterol increases ERRα protein levels in MDA-MB-231 cells in a dose-dependent manner. MDA-MB-231 cells were treated with vehicle (Veh) and varying concentrations of cholesterol (1, 5, 10 μM) and subjected to western blotting. (**B**) Cholesterol increases ERRα protein levels even in the presence of lovastatin in MDA-MB-231 cells. MDA-MB-231 cells were treated with vehicle (Veh), lovastatin (Lova) or Lovastatin + cholesterol (Lova + Chol) at 5 μM for 24 h. Cell lysates were subjected to western blotting. Relative ERRα protein levels were assessed using ImageJ software. (**C**) Cholesterol induces ERRα mRNA levels in MDA-MB-231 cells. Cells were treated with 5 μM cholesterol for 24h and ERRα mRNA levels were assessed by RT-qPCR and were normalized to endogenous GAPDH. (**D**) Cholesterol increases ERRα protein levels in MCF-7 cells in a dose-dependent manner. MCF-7 cells were treated with varying concentrations of cholesterol (1, 5, 10 μM) or vehicle (Veh) and subjected to western blotting. Relative ERRα protein levels were calculated using ImageJ software. (**E**) Cholesterol induces ERRα mRNA levels in MCF-7 cells.

Cells were treated with 10 μM cholesterol for 24 h and ERRα mRNA levels were assessed by RT-qPCR, and were normalized to endogenous GAPDH. A minimum of three independent experiments were performed. A value of $p < 0.05$ was considered significant (*).

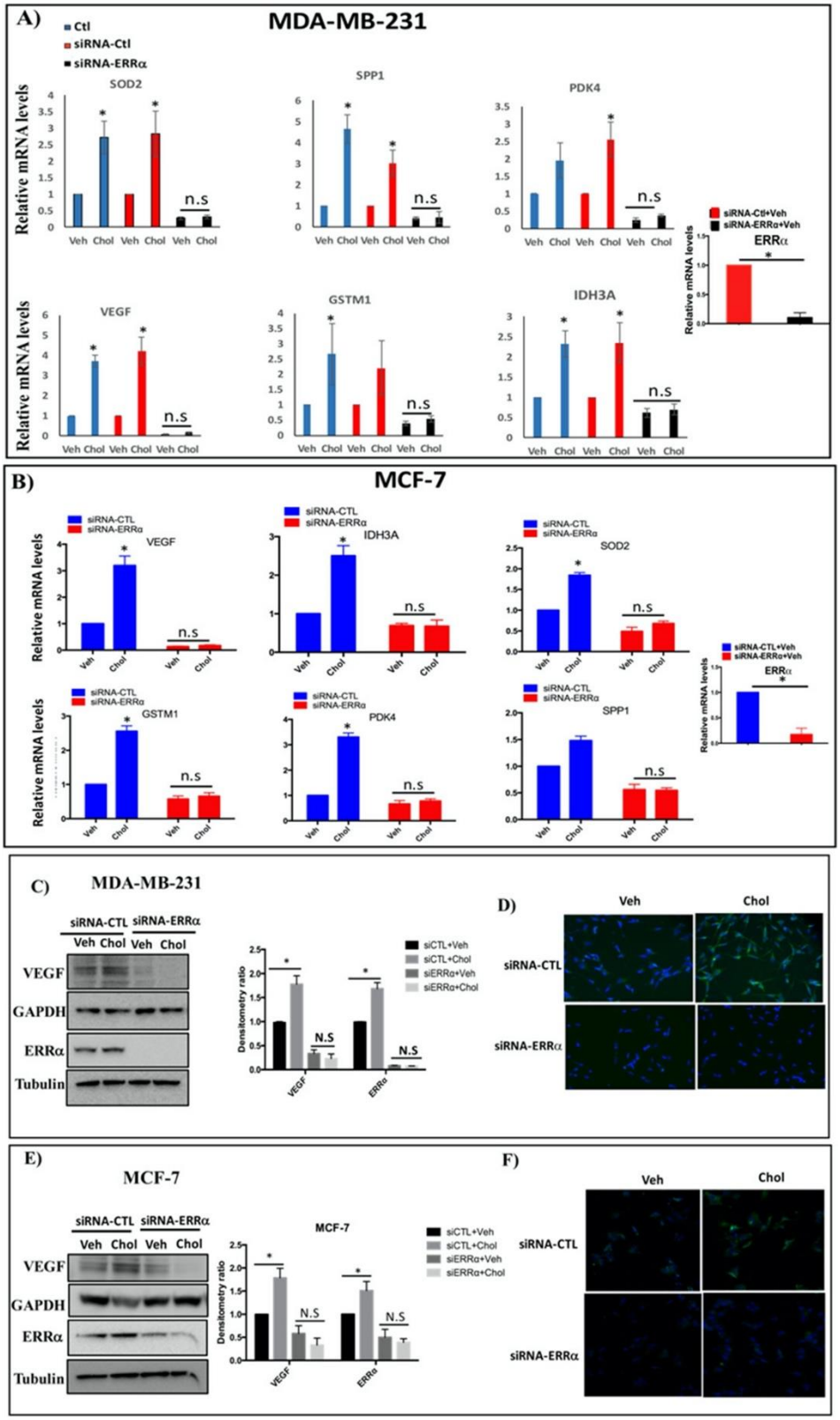


**Figure 4. Cholesterol enhances expression of ERRα metabolic target genes through ERRα in MDA-MB-231 and MCF-7 cells.** (**A**,**B**) Cells were transfected with siRNA-ERRα (siERRα) or siRNA-control (siRNA-CTL) for 48 h, and treated with vehicle (Veh) or cholesterol (Chol, 5 µM) for 24 h. Total RNA was extracted and analyzed by RT-qPCR. Genes detected included: isocitrate dehydrogenase 3A (IDH3A), pyruvate dehydrogenase kinase 4 (PDK4), vascular endothelial growth

factor (VEGF), glutathione s-transfrase M1(GSTM1), superoxide dismutase 2 (SOD2) and secreted phosphoprotein 1(SPP1). The mRNA data were normalized to endogenous GAPDH (**C**,**E**) Cholesterol increases ERRα-induced VEGF protein expression in MDA-MB-231 and MCF-7 cells using western blotting. The blots for ERRα and VEGF were generated from the same cell lysates loaded on 2 different gels due to the similar molecular weights of the two proteins. GAPDH was used as a loading control for VEGF and tubulin was used as a loading control for ERRα. The densitometry ratio was calculated using ImageJ software. (**D**,**F**) Immunocytochemistry (ICC) was performed to detect VEGF protein expression using anti-VEGF antibody. All cells were transfected with siRNA-ERRα or siRNA-control (siRNA-CTL), and were treated with cholesterol (5 µM for MDA-MB-231, 10 µM for MCF-7 cells) or with vehicle for 24 h. DAPI is shown in blue and VEGF in green. A minimum of three independent experiments were performed. A value of $p < 0.05$ was considered significant in comparison with the control group (*).

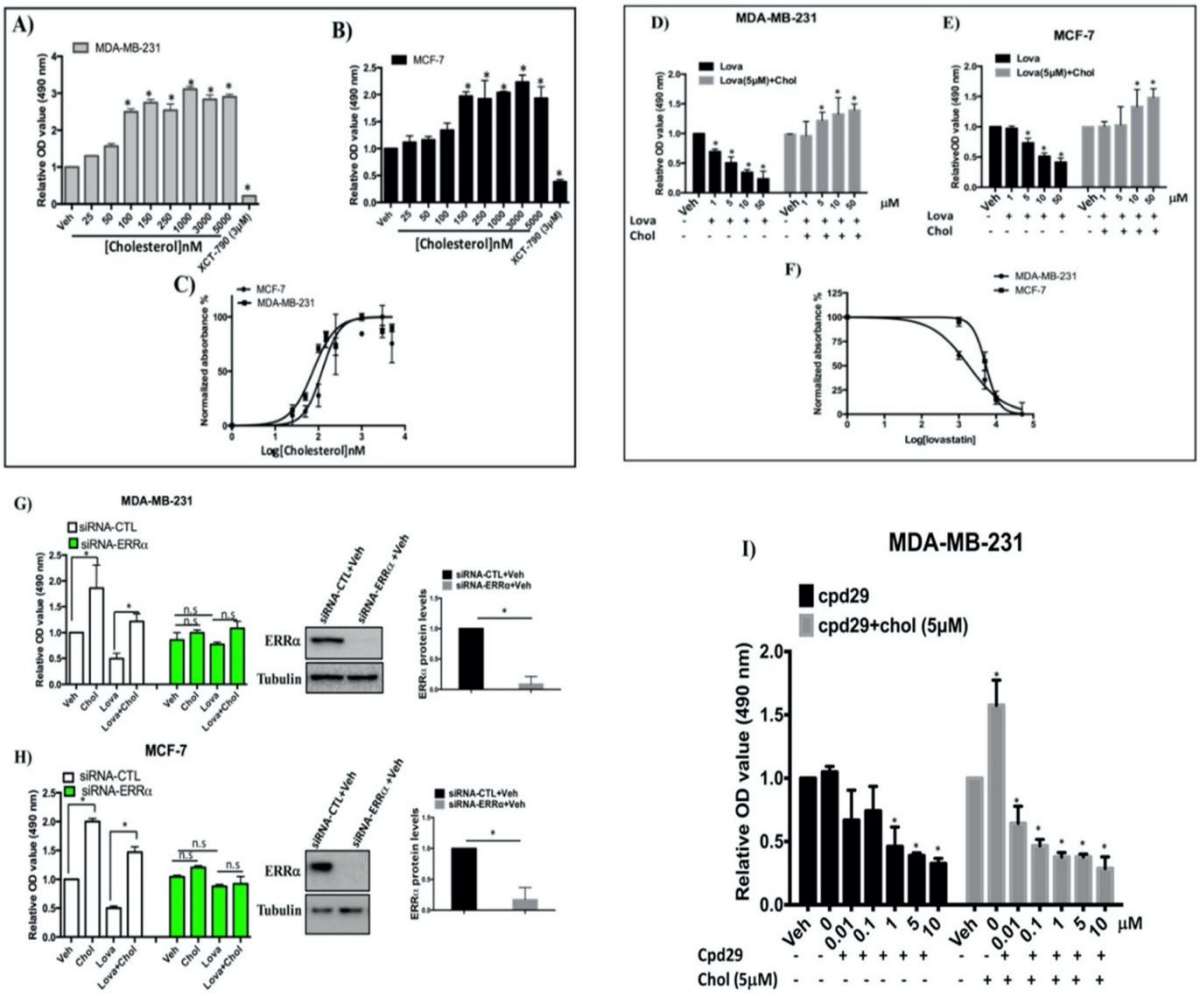


**Figure 5. Cholesterol increases cell proliferation of breast cancer cells in an ERRα-dependent manner.** (**A**,**B**) In order to obtain half-maximal effective concentrations ($EC_{50}$s) of cholesterol in MDA-MB-231 and MCF-7 cells, an MTS Cell Proliferation Assay kit was used to assay the cell proliferation of MDA-MB-231 and MCF-7 cells with varying concentrations of cholesterol (as indicated in the Figure) on day 5. (**C**) EC50s were calculated for both MDA-MB-231 and MCF-7 cells using the Prism software. (**D**,**E**) Cell proliferation in the presence of Lovastatin (Lova) and lovastatin (5 μM) + cholesterol (Chol), at varying concentrations indicated in the Figure, was measured using an MTS kit on day 5. (**F**) IC50s of lovastatin were calculated for both MDA-MB-231 and MCF-7 cells using Prism. (**G**,**H**) MDA-MB-231 and MCF-7 cells were transfected with siRNA-ERRα or siRNA-control (siRNA-CTL) and the cells were treated with vehicle, cholesterol (chol), lovastatin (lova) or lovastatin + cholesterol (lova + chol), all at 5 μM for MDA-MB-231 cells and at 10 μM for MCF-7 cells. Cell proliferation assays were performed using an MTS kit on day 5. Cell lysates were immunoblotted using an anti-ERRα antibody. A value of $p < 0.05$ was considered significant (*). (**I**) MDA-MB-231 cells were treated with compound 29 (cpd29) in varying concentrations as indicated in the figure (black bars). Also, cholesterol at a fixed concentration of 5 μM was co-administered with varying concentrations of cpd29 as indicated in the figure (gray bars); 0 indicates cholesterol (5 μM) alone, without cpd29 treatment.

Cell proliferation assays were performed using an MTS kit on day 6. A minimum of three independent experiments were performed. All treatments were compared to the respective vehicle group, and a value of $p < 0.05$ was considered significant (*).

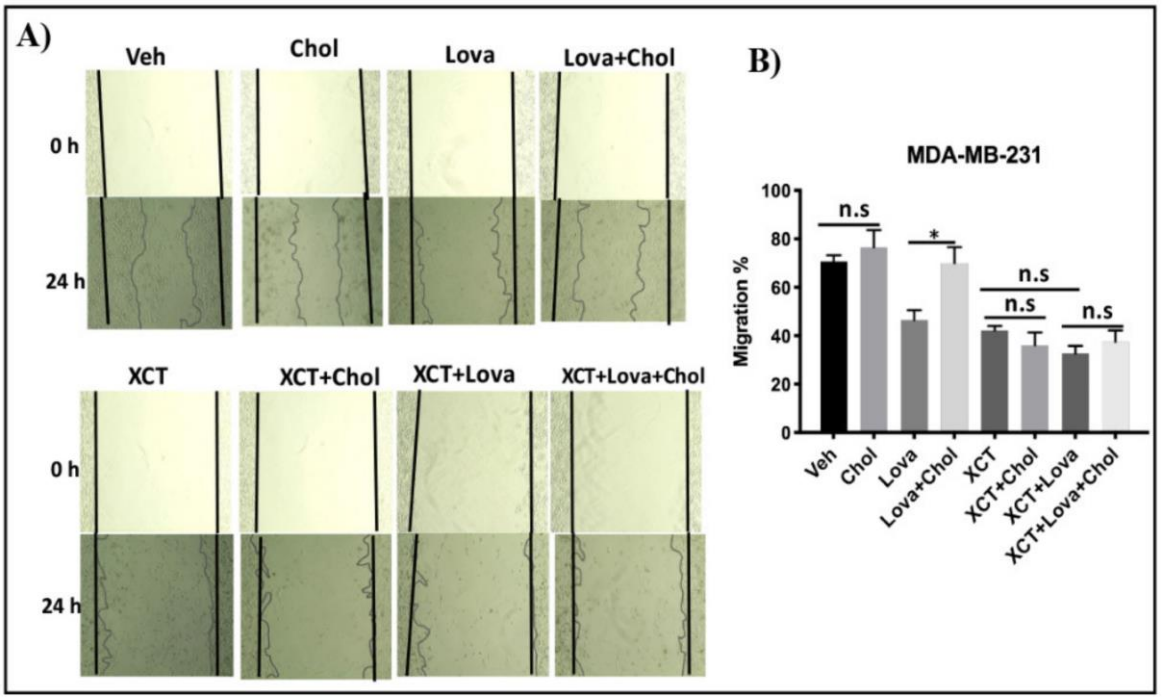


**Figure 6.** (**A**) **Lovastatin decreases the migration of MDA-MB-231 cells, while cholesterol rescues the effect of lovastatin, but not the effect of XCT-790 using a scratch-wound migration assay**, MDA-MB-231 cells were treated with vehicle(Veh), cholesterol (chol), lovastatin (Lova), Lovastatin + cholesterol (Lova + Chol), XCT-790 (XCT), XCT-790 + cholesterol (XCT + chol), XCT-790 + lovastatin (XCT + Lova) or XCT-790 + lovastatin + cholesterol (XCT + Lova + Chol), all at 5 µM. Wound closure was monitored at 0 and 24 h, and representative images are provided. (**B**) Migration percentages were calculated as follows: migration % = (*T* 0 h scratch width − *T* 24 h scratch width/*T* 0 h scratch width) × 100. The results represent 3 independent experiments. A value of $p < 0.05$ was considered significant (*).

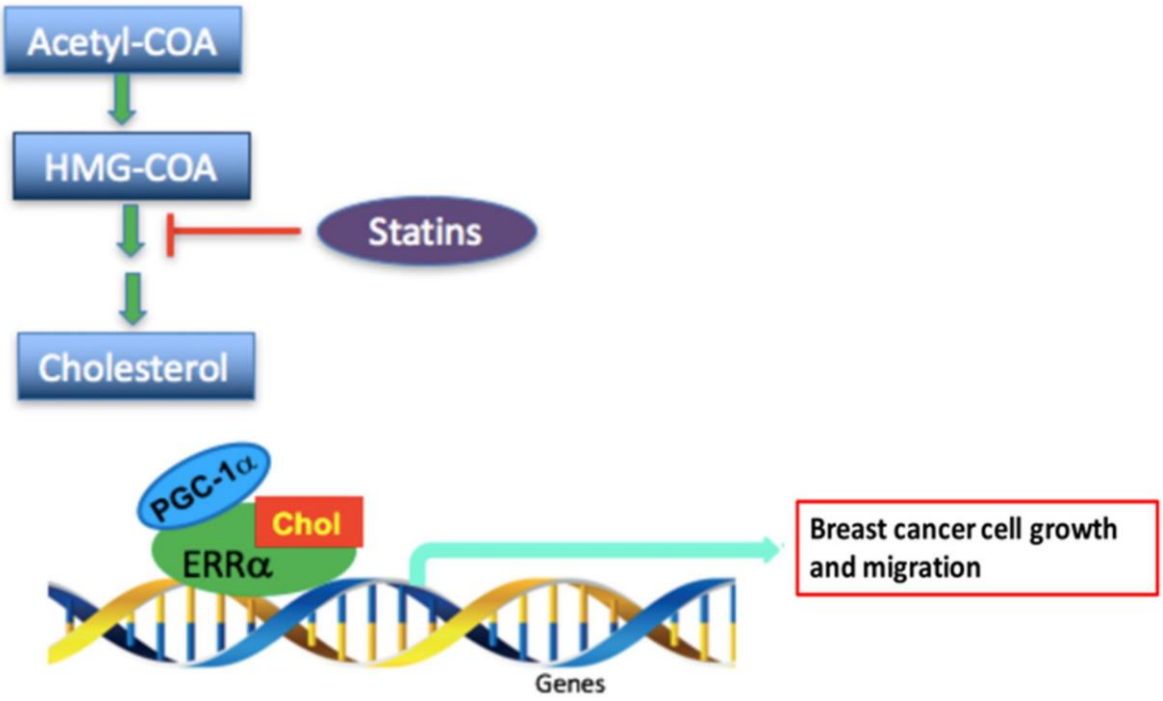


**Figure 7. Schematic representation of the proposed mechanism by which cholesterol promotes breast cancer cell growth and migration via the ERRα-PGC-1α pathway and how statins (cholesterol-lowering drug) may inhibit this effect.** The proposed scheme depicts that cholesterol binds to ERRα and changes its conformation, which causes an increase in recruitment of PGC-1α and as a result induces transcription of the metabolic target genes of ERRα and increases breast cancer cell growth and migration. However, statins, drugs that inhibit HMG-CoA reductase, possibly lower cholesterol levels and as a result decrease breast cancer cell progression.

**Supplementary material:**

**Table S1.** The primer sequences used for qPCR

| **Genes** | | **Sequence: 5′ to 3′** |
|---|---|---|
| SOD2 | F | CTGGACAAACCTCAGCCCTAAC |
| | R | AACCTGAGCCTTGGACACCAAC |
| IDH3A | F | TCGGTGTGACACCAAGTGGCAA |
| | R | TTCGCCATGTCCTTGCCTGCAA |
| VEGF | F | TTGCCTTGCTGCTCTACCTCCA |
| | R | GATGGCAGTAGCTGCGCTGATA |
| PDK4 | F | AGGTGGAGCATTTCTCGCGCTA |
| | R | GAATGTTGGCGAGTCTCACAGG |
| SPP1 | F | CGAGGTGATAGTGTGGTTTATGG |
| | R | GCACCATTCAACTCCTCGCTTTC |
| GSTM1 | F | TGATGTCCTTGACCTCCACCGT |
| | R | GCTGGACTTCATGTAGGCAGAG |
| ERR1 | F | CCACTATGGTGTGGCATCCTGT |
| | R | GGTGATCTCACACTCGTTGGAG |
| PGC-1 | F | CCAAAGGATGCGCTCTCGTTCA |
| | R | CGGTGTCTGTAGTGGCTTGACT |
| GAPDH | F | GTCTCCTCTGACTTCAACAGCG |
| | R | ACCACCCTGTTGCTGTAGCCAA |

**Connecting text 3:**

In the previous chapter, I reported that cholesterol modulates ERRα activity in breast cancer cells, thereby increasing breast cancer cell proliferation and migration via the ERRα axis. Given the growing evidence demonstrating that obesity and elevated blood cholesterol enact their pathological effect on breast cancer cells by altering the transformed cells' metabolomic pathways, and that ERRα is a central regulator of cellular metabolism, it was interesting to hypothesise that cholesterol alters metabolic pathways in breast cancer cells via the ERRα axis. In order to verify this hypothesis, we established three objectives; 1) to demonstrate whether exogenous cholesterol alters the metabolite levels in MDA-MB-231 cells via the ERRα pathway; 2) to assess whether cholesterol regulates the mRNA levels of the selected metabolic target genes of ERRα, that are involved in the oxidative phosphorylation (OXPHOS), TCA cycle, pentose phosphate pathway (PPP), and one-carbon metabolism (OCM) pathways using MDA-MB-231, MCF-7 and Triple Negative Breast Cancer-Patient-Derived Xenograft (TNBC-PDX) cells; and 3) to compare the findings from the above-mentioned studies with that obtained from obese versus non-obese breast cancer patients with basal-like breast tumors, using the Gene Expression Omnibus (GEO) database. For this chapter, the manuscript will be submitted shortly.

## CHAPTER 4 – MANUSCRIPT 3

# Cholesterol-induced metabolic reprogramming in breast cancer cells is mediated via ERRα pathway

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## 4.1. ABSTRACT

Obesity and a high cholesterol diet are associated with altered metabolic programming to fuel the uncontrolled growth of breast cancer cells. However, the molecular pathway underlying this process is not yet well-understood. We previously reported that cholesterol is an endogenous ligand of the estrogen-related receptor alpha (ERRα), and that it alters cellular proliferation and lovastatin-inhibitory effect in migration of breast cancer cells, in addition to ERRα metabolic target gene expression in those cells. Here, we show through functional assays, metabolomics, and genomics that exogenous cholesterol alters the metabolic pathways in triple-negative breast cancer (TNBC) cells via increasing oxidative phosphorylation (OXPHOS), the TCA cycle, aerobic glycolysis, and the expression of 6-phosphogluconate dehydrogenase (6PGD) involved in the pentose phosphate pathway (PPP); however, in ER positive (ER+) breast cancer cells, all of the above metabolic pathways are upregulated, except aerobic glycolysis. We further demonstrate that exogenous cholesterol does not alter the metabolite levels involved in glutaminolysis, one-carbon metabolism (OCM), or pentose phosphate pathway (PPP) in ER+ and TNBC cells, but increases NADPH levels in these cells and promotes TNBC-patient-derived xenograft (TNBC-PDX) cellular growth. Importantly, this cholesterol stimulatory effect on the above metabolic pathways, NADPH levels and cellular proliferation in breast cancer cells is ERRα-dependent. Given the close link between high cholesterol levels and obesity, we analyzed the ERRα metabolic gene signature profile of basal-like breast tumors obtained from obese patients versus the non-obese patients using Gene Expression Omnibus (GEO) database. Our subsequent findings are largely consistant with our *in vitro* findings obtained with exogenous cholesterol. Our findings, both *in vitro* and patients' data provide a mechanistic explanation underlying the association between cholesterol/obesity and

metabolic reprogramming in breast cancer patients, leading to the development of novel therapeutic strategies for these patients via targeting their metabolic vulnerabilities.

**Keywords:** Estrogen-related receptor alpha, Metabolism, Breast cancer, Cholesterol, Obesity

## 4.2. INTRODUCTION

Obesity and elevated blood cholesterol levels are associated with an increased risk and poor prognosis in breast cancer patients, and this has been linked to profound metabolic alterations that promote tumor growth, progression, and/or response to therapy [1, 2]. However, the underlying mechanism is not well-understood. Breast cancer is the second leading cause of death in the United States [3] and is associated with not only dysregulated cell growth but also altered cellular metabolism [4]. Sustained and rapid cell proliferation requires greater accessibility of building blocks to support cell growth and survival under oxidative stress conditions, and breast cancer cells acquire this support to grow faster through metabolic reprogramming [5-7]. Growing evidence shows that many cancer cells, compared to normal cells, mainly rely on glucose metabolism, using aerobic glycolysis (even in the presence of oxygen), in a process that is termed the Warburg effect [7, 8]. Even though in this process, ATP generation is less efficient than oxidative phosphorylation (OXPHOS), it invests the carbon skeletons of glucose in several biosynthetic pathways necessary to generate required molecular building blocks for cell proliferation [8]. Interestingly, it has been shown that intratumoral glucose concentration is minimal, and also, oxygen tension is dynamic within tumors [9]. Hence, cancer cells alter their metabolism according to their environment to align with their high-energy needs, and to produce the building blocks necessary for proliferation and survival. In fact, there is increasing evidence that tumor cells use both glycolysis and mitochondrial oxidative metabolism to satisfy the bioenergetic and/or biosynthetic needs of cancer cells [10-12]. The metabolic alteration that cancer cells adopt must be responsive to their environment; in solid breast tumors that experience bouts of hypoxia, glycolysis is often favored, however, it has been shown that mitochondrial OXPHOS levels can remain active even at low oxygen levels [13-15]. Moreover, in conditions in which

cancer cells no longer depend on OXPHOS for ATP production, mitochondrial metabolism can remain an important source of anabolic intermediates [16-18]. It has been demonstrated that breast cancer progression is associated with increased reliance on OXPHOS, as this can favor survival in circulating tumor cells, site-directed metastasis, and promote resistance to chemotherapy and targeted therapeutics [15, 19-21].

The estrogen-related receptor alpha (ERRα) is a transcription factor, which is well-known to regulate mitochondrial OXPHOS, the TCA cycle, and glycolysis [9, 22-24]. ERRα is expressed in most breast cancer cells, and its increased activity is correlated with unfavorable outcomes in breast cancer patients [9, 12, 25-30]. It has been reported that knockdown of ERRα *in vitro* and *in vivo* significantly inhibits the growth of estrogen receptor-positive (ER+) and triple-negative breast cancer (TNBC) cells [25, 31-34]. We and others have recently reported that cholesterol is an endogenous ligand of ERRα and that cholesterol increases ERRα's transcriptional activity [35, 36]. To better understand the mechanism by which obesity and high cholesterol intake alters metabolic pathways in breast tumors and promotes tumor growth, here, we investigated whether cholesterol, as an agonist of ERRα, promotes alteration in metabolic pathways in breast cancer cells via ERRα axis. We demonstrate that exogenous cholesterol alters breast cancer cell metabolism in an ERRα dependent manner. Specifically, cholesterol increases OXPHOS, the TCA cycle, glycolysis, and NADPH levels, while having no impact on the levels of metabolites involved in the glutaminolysis, pentose phosphate pathway (PPP), and one-carbon metabolism (OCM) pathway in breast cancer cells. The above findings are in line with the gene expression profiles in primary tumors of the obese breast cancer patients obtained from the Gene Expression Omnibus (GEO) database.

## 4.3. METHODS

### Cell Culture

The MDA-MB-231 and MCF-7 cell lines were obtained from Dr. Sylvie Mader (Université de Montréal). The above-mentioned cell lines were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The TNBC PDX cell line GCRC1887 was obtained from the breast tissue and data bank at the Goodman Cancer Research Centre-Research Institute of McGill University Health Centre (MUHC) supported by the Réseau de Recherche en Cancer of the Fonds de Recherche du Québec-Santé and the Quebec Breast Cancer Foundation. Banking of human specimens and associated clinical data is approved by MUHC research ethics board (study approval SUR-2000-966 and SUR-99-780). All patient data and biological samples were obtained from patients at the MUHC after obtaining informed consent. These cells were maintained in DMEM/F12 (3:1), 5% FBS, Hydrocortisone 0.4 μg/mL (Sigma, H0888-5G), recombinant human Epidermal Growth Factor (EGF) 10ng/mL(AF-100-15, PeproTech), Insulin 5 μg/mL (12585-014, Gibco), Y-27632 dihydrochloride, Rho inhibitor 10 μM (ab120129, Abcam), Prostaglandin E2 (PGE2) 1μM (S3003, Selleckchem), Gentamicin 50μg/mL (15710-072, Gibco), Pen/Strep 1x (Sigma, 15140-122), Fungizone 0.5x (15290-026, Invitrogen). For all experiments, cells were switched 24 h before cell treatments to their relatively basic medium with no phenol red supplemented with 2% lipoprotein depleted, and charcoal-stripped FBS. Lipoprotein depleted FBS was purchased from Kalen Biomedical LLC and charcoal-stripped to remove steroid hormones as described previously [37]. Cholesterol-water soluble (C4951-30MG) was purchased from Millipore Sigma. Lovastatin (sc-200850A, Santa Cruz Biotechnology), a known cholesterol-lowering drug, was used to decrease intracellular cholesterol levels. Compound 29 (cpd29), a known synthetic inverse agonist of ERRα, was used to decrease

ERRα transcriptional activity, and it was a generous gift from Dr. Donald McDonnell (Duke University).

**Real-time metabolic analysis:**

Multiparameter metabolic analysis of MDA-MB-231 cells was performed simultaneously in the Seahorse XF96 extracellular flux analyzer (Seahorse Bioscience, Agilent). Briefly, MDA-MB-231 cells were transfected with either siRNA-control (si-CTL) or siRNA-ERRα (si-ERRα) for 48 h, followed by treatment with vehicle or 5μM cholesterol for 24 h. On the day of the assay, the treated MDA-MB-231 cells were plated on XF96 (20,000 cells per well), and the culture medium was replaced with Seahorse base media DMEM (supplemented with 2mM glutamine, 2mM pyruvate, and 12.5 mM Glucose, pH 7.4) 1 hour before the assay and for the duration of the experiment. Mitochondrial complex inhibitors were prepared based on the mitochondria stress kit (Agilent, 103015-100) instructions. After establishing the baseline oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) readings, mitochondria inhibitors (oligomycin, FCCP, and Rotenone/ Antimycin) were injected accordingly, and OCR and ECAR were measured.

**Metabolites measurement:**

Cellular glucose, glutamine, lactate, glutamate, and ammonium were measured using the Bio- Profile 400 analyzer (Nova Biomedical Corp., Waltham, MA, USA). MDA-MB-231 and MCF-7 cells were transfected with either siRNA-control (si-CTL) or siRNA-ERRα (si-ERRα) for 48 h, followed by treatment with vehicle or 5μM or 10μM cholesterol for 24 h, respectively. In addition, for MCF-7 cells, the cells were treated with 10μM cholesterol and/or lovastatin and/or cpd29 for the duration of 48 h. TNBC-PDX cells were treated with vehicle, cholesterol (10 μM) and/or cpd29 (10 μM) for 48 h. The media were then removed and centrifuged at 15000 rpm for 10 minutes to remove the cell debris, and the media were maintained on ice until analysis. Glucose

or glutamine uptake was calculated as the differences in glucose or glutamine content between culture media and unseeded media incubated in parallel plates. Lactate, glutamate, and ammonium production were reported as measured using the instrument, and all the data were normalized for cell count and its respective vehicle.

**Metabolomics:**

Metabolic profiling was performed in a metabolomics core facility located at the Rosalind & Morris Goodman Cancer Research Centre at McGill University, using gas chromatography-mass spectrometry (GC/MS) and liquid chromatography-mass spectrometry (LC-MS). MDA-MB-231 cells were plated in 10 cm dishes and were treated with vehicle, cholesterol, and/or cpd29 with a concentration of 5 μM for 48 h. For GC/MS, the cells were rinsed in saline, quenched in 80% HPLC-grade methanol, sonicated, centrifuged, and the supernatants were dried in a cold trap (Labconco) overnight at -1 °C. Pellets were solubilized in methoxy-amine HCl, incubated at room temperature for one h, and derivatized with MTBSTFA at 70 °C for one h. Next, 1 μL was injected into an Agilent 5975C GC/MS in SCAN mode, and the data were analyzed using Masshunter software (Agilent Technologies) [38]. LC/MS sample preparation was performed according to the core facility protocol [30]. Briefly, cells were rinsed in 150 mM ammonium formate (Sigma) and extracted using 230 μL of LC/MS grade 50% methanol/ 50% water mixture and 220 μL of cold acetonitrile. Samples were then homogenized and centrifuged. The upper aqueous layer was dried by vacuum centrifugation (Labconco). Samples were separated by UHPLC (Ultra-High Performance Liquid Chromatography) (1290 Infinity, Agilent Technologies). Then, metabolites were eluted into an electrospray ionization source (ESI) and detected by Multiple Reaction Monitoring (MRM) using a triple quadrupole mass spectrometer (6430 QQQ, Agilent Technologies).

**siRNA transfection**

As it was previously described [39], siRNAs directed against ERRα (Invitrogen, AM16708 / 289481) with the sense sequence 5′-CCGCUUUUGGUUUUAACC-3′ and antisense sequence 5′-GGUUUAAAACCAAAAGCGG-3′ or control scrambled siRNAs (Invitrogen, AM4611, negative control) were transfected into MCF-7 and MDA-MB-231 cells using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen, Canada) following the manufacturer's instructions. After 48 h of post-transfection, fresh phenol red-free medium containing 2% lipoprotein-depleted and charcoal-stripped serum was added, and cells were treated with cholesterol (5 μM for MDA-MB-231, and 10 μM for MCF-7 cells). The knocked-down ERRα breast cancer cells were used for metabolic assays.

**Immunoblotting**

To determine whether ERRα was successfully knocked down in MCF-7 and MDA-MB-231 cells, the cell lysates were subjected to immunoblotting. ERRα levels were detected using the rabbit monoclonal anti-ERRα antibody (ab76228), and the mouse monoclonal anti-alpha tubulin antibody (ab7291) was used to detect alpha-tubulin as a loading control. These antibodies were purchased from Abcam. Image-J software was used for densitometric analysis of immunoblots.

**RNA Preparation and Analysis**

Total RNA was extracted using an RNeasy mini kit (74104, Qiagen). One microgram of total RNA was used for the first-strand synthesis with a high-capacity cDNA reverse transcription kit (4368814, Life Technologies). Real-time PCR was performed using the BrightGreen qPCR master mix (ABMMastermix-R, Diamond) with gene-specific primers. The sequences of the primers included in this study are included in Supplementary Table S1. Real-time PCR was performed on the 7500 real-time PCR system (Applied Biosystems). Results were quantified using

the $2^{-\Delta\Delta CT}$ method and were normalized to the endogenous control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

**NADPH quantification assay:**

The intracellular NADPH levels were measured using the $NADP^+$/NADPH assay kit (Abcam, ab65349). MDA-MB-231 and MCF-7 cells were transfected with either si-CTL or si-ERRα for 48 h, followed by treatment with vehicle or cholesterol (5 μM for MDA-MB-231 cells, and 10 μM for MCF-7 cells) for 24 h. The above-mentioned kit was used according to the manufacturer's protocol, and the NADPH concentration was determined colorimetrically based on the absorbance at 450 nm. All the data were normalized to the respective vehicle.

**Cell proliferation assay:**

To determine whether the impact of cholesterol on TNBC-PDX cell proliferation is ERRα-dependent, the MTS Cell Proliferation Assay kit (ab197010, Abcam) was utilized to assay the cell proliferation of TNBC-PDX cells. Based on the manufacturer's instructions, TNBC-PDX cells were plated at a density of $l0^4$ cells per well in 96 well plates. The cells were treated with a 10μM concentration of cholesterol and/or cpd29. The medium was changed with the fresh medium containing the treatment every 48 hours throughout the six days of the experiment. 20 μL of MTS reagent per well was then added to each well and was incubated for one h at 37 °C under standard culture conditions. The optical density (OD) value was determined at 490 nm using a microplate reader (Infinite M200PRO, TECAN).

**The Cancer Genome Atlas analyses (TCGA)**

The breast cancer gene expression data and their correlation to ERRα gene expression levels, which were obtained from TCGA datasets. Data were downloaded and visualized using UALCAN web-portal at http://ualcan.path.uab.edu [40].

**ERRα signature analysis in basal-like obese breast cancer patients' primary tumors vs. non-obese ones:**

To determine the ERRα gene expression signature profiles in basal-like obese breast cancer tumors in patients with BMI>25 compared to those in patients with BMI ≤ 25 (non-obese), we analyzed the gene expression datasets from GEO: GSE78958 using the R interface. According to the GSE78958 study description, the gene expression data were generated using Affymetrix U133 2.0 gene expression for primary breast tumors, whereby their RNA was isolated from laser microdissected tissues [41]. The basal-like primary breast tumors were categorized based on the patients' BMI (BMI>25 considered as obese and BMI ≤ 25 considered as non-obese). We further stratified the data based on their ERRα expression levels, for analyzing the gene expression profiles. The patients' ID used in this study is indicated in Supplementary Table S2 for the basal-like subtype of breast cancer.

**Statistical Analysis**

All values are expressed as means of at least three independent experiments ± SEM. A two-tailed Student t-test was used to analyze the statistical significance of differences between two experimental groups, and two-way ANOVA was used to analyze comparisons between more than two groups. The experiments were repeated at least three times to obtain p values. * represents $p < 0.05$ and was considered to be statistically significant. The data were plotted using GraphPad Prism 8 or R software.

## 4.4. RESULTS

### Cholesterol increases aerobic glycolytic rates in triple-negative breast cancer cells in an ERRα-dependent manner

To demonstrate whether cholesterol regulates aerobic glycolytic rates in breast cancer cells in an ERRα-dependent manner, we determined the levels of glycolytic metabolites in MDA-MB-231, and glucose uptake and lactate production levels in MDA-MB-231, TNBC-PDX, and MCF-7 cells. As shown in Figure 1A, cholesterol increases the extracellular acidification rate (ECAR), which was used to approximately measure the glycolysis capacity post drug injection in MDA-MB-231 cells. To verify whether this cholesterol-induced effect on extracellular acidification rate is mediated by ERRα, we knocked down ERRα in MDA-MB-231 and MCF-7 cells. As shown in Figures S1 A&B, ERRα was successfully knocked down in MDA-MB-231 and MCF-7 cells, and cholesterol-induced increase in extracellular acidification rate is abrogated when ERRα expression is suppressed (Figure 1A). This suggests that cholesterol increases the aerobic glycolytic capacity in an ERRα-dependent manner in these cells. Accordingly, cholesterol treatment is associated with greater levels of glucose consumption and lactate production, which is linked to glycolysis, in MDA-MB-231 cells. However, cholesterol-induced effects are impaired when ERRα expression is suppressed in these cells (Figure 1B). These results were further validated by measuring the glycolytic intermediate levels in MDA-MB-231 cells. As displayed in Figure 1C, cholesterol treatment leads to an increasing trend in the indicated glycolytic intermediate levels and significantly enhances the accumulation of Glyceraldehyde 3-Phosphate (GA3P), Fructose-1,6-bisphosphate (F1-.6 bis P), and 2-phosphoglycerate/3-phosphoglycerate (2PG/3PG) metabolite levels. However, when ERRα is inhibited using cpd29, the cholesterol-induced effect is largely abrogated. We also confirmed that upon treating MDA-MB-231 cells with

exogenous cholesterol, intracellular cholesterol levels increase to approximately 2-fold compared to the vehicle-treated controls (Figure 1D). Moreover, we demonstrate that cholesterol enhances glucose uptake and lactate production levels in TNBC-PDX cells via ERRα (Figure 1E). Together, the data showing that cholesterol increases glucose consumption and lactate production levels in TNBC cells implies that these cells are likely engaging glycolysis at a higher level in response to exogenous cholesterol. This further aligns with the above-mentioned result that the cells treated with cholesterol were able to increase their extracellular acidification rate to a higher level than cells treated with vehicle, when challenged with the mitochondrial ATP synthase inhibitor (Oligomycine). Importantly, our data also demonstrate that this cholesterol-induced effect is mediated via ERRα in MDA-MB-231 and TNBC-PDX cells.

Surprisingly, in MCF-7 cells, cholesterol does not similarly alter glucose uptake or lactate production levels (Figure 1F). Based on the TCGA database, the gene expression levels of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR), a rate-limiting enzyme involved in the cholesterol biosynthesis pathway, are significantly higher in luminal breast tumors compared to in normal tissues (Figure 1G). As MCF-7 breast cancer cells are considered as the luminal subtype, hence, it is possible that there are increased levels of intracellular cholesterol in MCF-7 cells and that it may mask the effect of exogenous cholesterol in increasing glucose uptake and lactate production levels in MCF-7 cells. Thus, to decrease intracellular cholesterol levels and sensitize cells to exogenous cholesterol, we used lovastatin (a known HMGCR inhibitor). As shown in Figure 1H, in the presence of lovastatin, cholesterol leads to an increasing trend in glucose consumption and lactate production levels as compared to the cells treated with vehicle or cholesterol alone in MCF-7 cells. Interestingly, the cells treated with cpd29 demonstrate a significant increase in glucose consumption and lactate production levels, and this suggests that

ERRα inhibition using cpd29 increases aerobic glycolysis. Importantly, adding cholesterol does not significantly alter the glucose uptake and lactate production levels. Although adding cholesterol and lovastatin significantly decreases lactate production levels in MCF-7 cells, it does not alter glucose uptake levels in these cells. The observed significant decrease in the presence of cpd29+cholesterol+lovastatin compared to the cpd29 alone could possibly be due to the impact of lovastatin on other pathways, including inhibition of protein kinase B (AKT)/ mammalian target of rapamycin (mTOR) [42, 43] in cancer cells. Collectively, these data suggest that cholesterol increases glycolysis rates in triple-negative breast cancer cells, such as MDA-MB-231 and TNBC-PDX cells; however, it does not alter this pathway in ER+ breast cancer cells, such as MCF-7 cells.

**Cholesterol increases OXPHOS rates in breast cancer cells via ERRα pathway**

To investigate whether cholesterol regulates the oxidative phosphorylation (OXPHOS) rates in breast cancer cells via ERRα, we transfected MDA-MB-231 cells with siRNA-control or siRNA-ERRα and then treated these cells with vehicle or cholesterol. In addition, MDA-MB-231, MCF-7, and TNBC-PDX cells were treated with vehicle, cholesterol, and/or cpd29. The data shown in Figure 2 A demonstrate that cholesterol significantly increases the maximal respiration, which is associated with a greater spare capacity compared to the vehicle-treated one in MDA-MB-231 cells, suggesting that cholesterol increases oxidative capacity and the ability of MDA-MB-231 cells to respond to increased energy demand or stress conditions. To verify whether this cholesterol-induced effect on OXPHOS rate is mediated by ERRα, we knocked down ERRα in MDA-MB-231 cells. As demonstrated in Figure 2A, cholesterol does not significantly alter the maximal respiration and spare capacity levels when ERRα is impaired. This suggests that this cholesterol stimulatory effect is ERRα-dependent. As shown in Figure 2B-D, cholesterol also significantly enhances the expression of ERRα-induced OXPHOS target genes (NDUFB7,

ATP5L, and COX5B) in MCF-7, and TNBC-PDX cells, and NDUFB7, ATP5L genes in MDA-MB-231 cells. However, in the presence of cpd29, which is a small molecule inhibitor of ERRα, the cholesterol-induced effect is not significant in these breast cancer cells, except for in MCF-7 cells, where the expression of COX5B is significantly higher in the presence of cholesterol while ERRα was inhibited. These data may suggest that cholesterol increases the expression of COX5B via other pathways than ERRα in MCF-7 cells. In addition, we demonstrate that upon cholesterol treatment, the expression of ERRα (encoded by the *ESRRA*) increases. However, in the presence of cpd29, ERRα mRNA levels significantly decrease, and cholesterol does not rescue cpd29's inhibitory effect on MDA-MB-231, MCF-7, and TNBC-PDX cells (Figure S2A). It is important to mention that based on TCGA datasets, there is a positive correlation between the expression of ERRα and that of NDUFB7 ($R^2$=0.32), COX5B ($R^2$=0.34) and ATP synthetase subunit delta, mitochondria (ATP5D) ($R^2$=0.31) (the enzymes involved in the electron transport chain (ETC)). This positive correlation is particularly significant in basal-like breast tumors (studied *in vitro* using MDA-MB-231 and TNBC-PDX cells) and luminal breast tumors (studied *in vitro* using MCF-7 cells) (Figure S2B). This supports the findings from other studies that ERRα modulates the enzymes involved in OXPHOS in breast tumors [9, 22]. Overall, these findings suggest that exogenous cholesterol induces OXPHOS rates in breast cancer cells, and that this effect is mediated via ERRα axis.

**Cholesterol augments TCA cycle metabolite abundances via the ERRα pathway**

We further determined whether cholesterol regulates TCA cycle intermediate levels in an ERRα-dependent manner in breast cancer cells. As shown in Figure 3A, cholesterol leads to an increasing trend in the indicated TCA cycle intermediate levels and it significantly augments aconitic acid and fumaric acid levels in MDA-MB-231 cells. However, when ERRα was inhibited

using cpd29, no significant differences are observed in the TCA cycle intermediate levels in the presence of cholesterol. Aligning with this result, cholesterol induces the expression of ACO2, CS, and FH, the ERRα metabolic target genes involved in the TCA cycle, in MDA-MB-231, MCF-7, and TNBC-PDX cells, and in the absence of ERRα, this cholesterol-induced effect is not significant. Overall, these findings suggest that cholesterol primes breast cancer cells for increased levels of mitochondrial oxidative metabolism, and that this is ERRα-dependent. This further aligns with the above-mentioned findings in increased oxidative capacity, as the TCA cycle is intrinsically linked to OXPHOS.

**Cholesterol does not alter the abundances of metabolites in the glutaminolysis pathway in breast cancer cells**

Next, we assessed the impact of cholesterol on the glutamine metabolism in MDA-MB-231, MCF-7, and TNBC-PDX cells. As shown in Figure 4A, no alterations are observed in the levels of glutamine uptake and glutamate excretion upon cholesterol treatment. Interestingly, when ERRα expression is suppressed, there is a significant increase in the above-mentioned metabolite levels; however, the presence of cholesterol does not alter their levels. These data are further confirmed by measuring the intracellular glutamine and glutamic acid levels in MDA-MB-231 cells (Figure 4B). As shown in Figure 4B, cholesterol does not alter intracellular glutamine and glutamic acid levels in these cells via the ERRα axis. Consistently, these findings that cholesterol does not alter glutamine uptake and glutamate excretion levels are observed in MCF-7 and TNBC-PDX cells (Figures 4C&D). Importantly, our result shows that in the absence of ERRα, we observe a significant increase in glutamine uptake and glutamate excretion levels. Together, these data suggest that although lack of ERRα increases the abundances of metabolites involved in the

glutaminolysis pathway, the exogenous cholesterol does not alter the glutaminolysis metabolite levels in breast cancer cells.

**Cholesterol does not alter the abundances of metabolites in the pentose phosphate pathway (PPP) and the one-carbon metabolism (OCM) pathway in TNBC cells**

We further analyzed the effect of cholesterol on metabolite accumulation and the expression of some key enzymes associated with the PPP and the OCM pathway. As shown in Figure 5A, although exogenous cholesterol stimulates an increasing trend in PPP metabolite levels, its stimulatory effect is not significant in MDA-MB-231 cells. In ERRα-inhibited breast cancer cells, a significant increase of Ribulose-5p was observed, and that adding exogenous cholesterol does not significantly alter the levels of metabolites. We further determine the relative gene expression levels of G6PD and 6PGD, two critical enzymes involved in NADPH production in the pentose phosphate pathway (Figure 5B). As shown in Figure 5B, cholesterol significantly decreases the expression of G6PD; however, it significantly increases the expression of 6PGD in TNBC-PDX cells. This may suggest that cholesterol increases the expression of 6PGD to maintain NADPH homeostasis in breast cancer cells. Interestingly, the expression of G6PD and 6PGD significantly increases when ERRα is inhibited using cpd29, and adding exogenous cholesterol does not significantly alter the expression levels of these enzymes in the presence of cpd29. These data suggest that the effect of cholesterol on the expression of these enzymes is ERRα dependent.

We also verified whether cholesterol modulates the OCM pathway via ERRα, as shown in Figure 5C, cholesterol does not significantly alter OCM metabolite levels. However, cpd29, an ERRα small molecule inhibitor, significantly increases the levels of several OCM intermediates, including phospho-Serine (p-Serine), Taurine, and Histidine, in MDA-MB-231 cells. However, exogenous cholesterol does not significantly alter the OCM metabolite levels, regardless of the

presence or absence of cpd29. Importantly, in the presence of cpd29, cholesterol demonstrates a decreasing pattern in p-Serine levels compared to the cpd29 alone; however, it is not statistically significant. We further verified the expression of MTR and GART, two key enzymes involved in the OCM pathway. As demonstrated in Figure 5D, exogenous cholesterol significantly decreases the expression of GART, while it does not significantly decrease the expression of MTR. Moreover, in the presence of cpd29, cholesterol does not significantly alter the expression levels of MTR and GART in TNBC-PDX cells. Overall, our findings align with other studies suggest that ERRα acts as a suppressor of the PPP and the OCM pathway [9, 44]. Consistently, cholesterol, as an agonist of ERRα, slightly decreases the expression of G6PD and GART in an ERRα-dependent manner and does not significantly alter the abundances of metabolites involved in the PPP and the OCM pathway.

**Cholesterol increases NADPH levels and cell proliferation in breast cancer cells**

As we observed that exogenous cholesterol increases mitochondrial metabolism, glycolysis rates, and the expression of 6PGD involved in NADPH production, it was interesting to assess the impact of cholesterol on intracellular NADPH levels in breast cancer cells. Our result demonstrates that cholesterol significantly augments NADPH levels compared to the vehicle-treated controls in the above-mentioned cell lines, however, cholesterol-induced NADPH levels are abrogated when ERRα expression is suppressed (Figures 6A&B). These results suggest that cholesterol-induced NADPH levels are mediated via ERRα.

In addition, to determine whether the observed alteration in metabolic pathways and NADPH levels upon cholesterol treatment impacts TNBC-PDX cell proliferation, these cells were treated with vehicle, cholesterol, and/or cpd29. As displayed in Figure 6C, cholesterol significantly increases cell proliferation in TNBC-PDX cells. However, when ERRα is inhibited using cpd29,

the enhancing effect of exogenous cholesterol on TNBC-PDX cell proliferation is not significant compared to the cells treated with cpd29 alone.

**Expression of the metabolic target genes of ERRα is increased in obese breast cancer patients compared to those in the non-obese patients**

Next, we analyzed whether the ERRα metabolic gene signature profile in basal-like primary breast tumors obtained from obese patients is higher versus the non-obese patients using data obtained from the GEO database. As shown in Figure 7A, we observed that the expression of ERRα is higher in basal-like breast tumors in obese patients compared to the non-obese patients' tumors. Interestingly, the expression of several ERRα metabolic target genes involved in OXPHOS are significantly elevated in basal-like breast tumors in obese patients compared to those in the non-obese patients (Figure 7B). In addition, we observed a significant increase in the expression of FH, and an increasing trend in the expression of isocitrate dehydrogenase 2 (IDH2), CS, and ACO2 involved in the TCA cycle (Figure 7C). Our result also shows that even though there is an increasing trend in the expression of several ERRα target genes involved in the glycolytic pathway, such as lactate dehydrogenase B (LDHB) and hexokinase-2 (HK2); no significant alteration is seen in their gene expression levels in obese patients compared to the non-obese patients (Figure 7D). In addition, we demonstrate that in obese breast cancer patients, the expression levels of ERRα target genes involved in glutaminolysis remained unchanged compared to levels in the non-obese patients (Figure 7E). However, the expression of G6PD and GART, enzymes associated with the PPP and the OCM pathway (respectively) show a significant increase in obese breast cancer patients compared to the non-obese patients (Figures 7F&G). Furthermore, we observed a significant increase in the expression of GSTM1 and SOD2, two key enzymes related to reactive oxygen species (ROS) detoxification, in obese patients compared to the non-

obese patients (Figure 7H). Together, these data suggest that the ERRα metabolic target genes, such as genes involved in OXPHOS, TCA cycle, ROS detoxification, and key enzymes involved in PPP and purine biosynthesis pathways, such as G6PD and GART, respectively, are higher in obese breast cancer patients compared to the non-obese patients. These data were validated in *in vitro* breast cancer cell lines, such as MDA-MB-231, MCF-7, and/or TNBC-PDX cell lines as shown above and previously published [36].

### 4.5. DISCUSSION

Obesity and high dietary cholesterol intake have been associated with an increased likelihood of recurrence and a higher mortality rate in breast cancer patients [45-50] by altering the metabolic pathways in breast cancer cells [1, 2]. However, the underlying mechanism by which elevated cholesterol levels alter the metabolic pathways in breast cancer cells is not well-understood. Given that we and another group have shown that cholesterol acts as an endogenous ligand of ERRα and increases its transcriptional activity [35, 36], it was therefore of interest to elucidate the metabolic pathways by which the cholesterol-ERRα axis mediates its pathogenic effect in breast cancer cells. In the current study, we demonstrate that cholesterol enhances both mitochondrial oxidative metabolism (the expression of key enzymes and the abundances of metabolites involved in the TCA cycle, and OXPHOS) and the aerobic glycolysis in TNBC cells, and only mitochondrial oxidative metabolism in ER+ breast cancer cells, while the exogenous cholesterol does not significantly alter the abundances of metabolites involved in the glutaminolysis, PPP, and OCM pathway in these cells. This metabolic alteration possibly increases NADPH levels in these cells and promotes cellular growth in TNBC-PDX cells. These stimulatory effects of cholesterol on ER+ and TNBC cells are mediated via the ERRα pathway. Furthermore, since high blood cholesterol is a common comorbidity in obesity [51], the expression of ERRα

metabolic target genes were analyzed in basal-like breast tumors of obese versus non-obese patients. Basal-like breast cancer patients were chosen as a representative of the TNBC subtype of breast cancer cells, as it has been shown that ERRα is overexpressed in this subtype, and there is no satisfactory treatment for these patients [31, 52]. Our observation reveals an increase in the expression of ERRα metabolic target genes involved in OXPHOS, the TCA cycle, and detoxification enzymes in obese basal-like breast cancer patients compared to the non-obese patients.

Accumulating evidence demonstrates that most oncogenes enhance aerobic glycolysis and that this increased reliance on glycolytic metabolism is an inherent property of the transformed cells [53]. Interestingly, our finding that cholesterol enhances the levels of glycolytic metabolites in an ERRα-dependent manner in TNBC cells, but not in ER+ breast cancer cells, aligns with other studies reporting that enhanced aerobic glycolysis is positively correlated to the malignancy of tumor cells [53, 54]. Remarkably, our observation that HMGCR, a key enzyme involved in the cholesterol biosynthesis pathway [55], is overexpressed in the luminal subtype of breast tumors, is in line with our finding that using lovastatin (a known inhibitor of HMGCR) leads to a decreasing trend in lactate production levels, which is associated with glycolysis, in ER+ breast cancer cells. This decreasing trend in lactate production levels using lovastatin could possibly be due to a decrease in intracellular cholesterol levels in these cells [56]. In addition, adding exogenous cholesterol stimulates an increasing trend in lactate production levels in ER+ breast cancer cells. On the basis of this finding, we can speculate that decreasing intracellular cholesterol levels may decrease glycolysis in ER+ breast cancer cells.

There is growing evidence to demonstrate that mitochondria produce up to 90% of the generated ATP in some cancer cells and that OXPHOS is active even at 0.5% oxygen levels [11,

13, 14]. Several studies also reported that resistance to (1) Kras inhibitor in pancreatic cancer [21], and (2) BRAF inhibitors in melanoma [19] are associated with a shift to oxidative metabolism. Importantly, our finding demonstrates that exogenous cholesterol increases not only aerobic glycolysis rates, but also mitochondrial oxidative metabolism (TCA cycle intermediate accumulation and the OXPHOS rates) via the ERRα axis in breast cancer cells. Our data that cholesterol induces the expression of key enzymes involved in OXPHOS and the TCA cycle via ERRα are in line with other studies reporting that ERRα/PGC-1α is the master regulator of mitochondrial metabolism and is associated with the upregulation of enzymes involved in OXPHOS, the TCA cycle, and mitochondrial biogenesis [9, 57, 58]. These studies accord with our finding that cholesterol, as an ERRα agonist, enhances mitochondrial respiration and the accumulation of TCA cycle intermediates, however, whether cholesterol increases mitochondrial biogenesis has not been examined in this study. The upregulation in both glycolysis and oxidative metabolism levels has been shown to be associated with increased NADPH levels and metabolic flexibility, which cause breast cancer cells to proliferate faster and help them to survive oxidative stress conditions [59]. It has been also reported that enhanced OXPHOS is linked to the resistance to chemotherapeutics in certain cancers [13-15]. Notably, the data that cholesterol enhances oxidative metabolism are supported in the obese basal-like breast cancer patients.

It has been shown that the glutaminolysis, PPP, and OCM pathway are critical for cancer cells to generate nucleotides, nucleic acids, and NADPH, which are required for cancer cells to survive under stress conditions [9, 44, 60]. Interestingly, it has been reported that ERRα acts as a suppressor in glutamine oxidation, as well as the PPP, and OCM pathway [9, 44]. However, our data demonstrate that exogenous cholesterol, as an agonist of ERRα, does not significantly alter the abundances of metabolite corresponding to the glutaminolysis, PPP, and OCM pathway in

breast cancer cells. Our finding that ERRα inhibition using cpd29 increases glutamine uptake and glutamate excretion in breast cancer cells is consistent with a recent study reporting that this induction in the above-mentioned metabolite levels upon cpd29 treatment is linked to the reduced glutathione (GSH) production, which is involved in ROS elimination to help breast cancer cells survive the oxidative stress conditions [61]. In addition, our finding that cholesterol decreases the expression of G6PD, a key enzyme involved in NADPH synthesis in the PPP [62], and the expression of GART, an enzyme involved in the de novo purine synthesis (OCM-related) pathway [44] in an ERRα-dependent manner, is in agreement with other studies that demonstrate that ERRα downregulates the expression of enzymes involved in the OCM pathway [44]. Surprisingly, our data show that cholesterol significantly increases the expression of 6PGD, a third enzyme of the PPP involved in NADPH synthesis [63] in an ERRα-dependent manner. This cholesterol-induced increase in the expression of 6PGD could be an adaptive response to maintain NADPH homeostasis in breast cancer cells, which may be utilized for rapid tumor growth and survival under excessive oxidative stress conditions [63]. Interestingly, our *in vitro* finding with exogenous cholesterol contradicts the data for the expression of G6PD and GART in the basal-like breast tumors of obese breast cancer patients. In TNBC-PDX cells, exogenous cholesterol downregulates the expression of the above-mentioned enzymes in an ERRα-dependent manner. However, as high cholesterol levels are often linked to obesity, in obese basal-like breast cancer patients, obesity is associated with a higher expression of G6PD and GART enzymes than those of the non-obese patients. It is interesting to speculate that the discrepant expression levels of G6PD and GART between TNBC-PDX cells treated with cholesterol and obese basal-like breast cancer patients are possibly due to several factors, as obesity is a complex disease, and may upregulate several growth

factors that consequently activate other signaling pathways, such as the PI3K/Akt/mTOR pathway [64, 65].

In cancer cells, overcoming oxidative stress is a critical step for tumor progression. NADPH homeostasis plays a critical role in the ROS detoxification system by reducing oxidized glutathione (GSSG) to reduced glutathione (GSH), which is essential to mitigate ROS that is produced mainly during cell proliferation [62, 63, 66]. In addition, NADPH is a crucial electron source for reductive biomass synthesis, such as nucleotides, fatty acids, and amino acids, to sustain rapid tumor growth [67, 68]. One of our important findings demonstrates that exogenous cholesterol increases NADPH levels in breast cancer cells in an ERRα-dependent manner. It has been reported that ERRα is involved in NADPH generation for ROS elimination via the induction of key enzymes involved in ROS detoxification, such as SOD2 and GSTM1, which have been shown as target genes of ERRα [24]. As obesity is associated with high cholesterol levels, we observed a higher expression of SOD2 and GSTM1 in obese basal-like breast cancer patients compared to the non-obese patients. These data are in line with our previous finding that demonstrates that cholesterol induces the expression of these two key detoxification enzymes in breast cancer cells, and this effect is mediated via ERRα [36].

Furthermore, in alignment with our previous report that cholesterol promotes cellular growth in MDA-MB-231, triple-negative, and MCF-7, ER+ breast cancer cells [36], here, we have also demonstrated that exogenous cholesterol enhances the proliferation of TNBC-PDX cells, and that the stimulatory effect of cholesterol is ERRα-dependent in all three breast cancer cell lines. The mechanism by which cholesterol promotes cellular growth in breast cancer cells may involve cholesterol acting as an agonist of ERRα, and enhancing the interaction of ERRα with its coactivator PGC-1α, as we have previously demonstrated [36]. As a result, this enhanced

interaction induces ERRα's gene expression itself (specific auto-induction) [36, 69]. This process induces a cascade of metabolic pathways, such as aerobic glycolysis, oxidative metabolism (OXPHOS and TCA cycle), and the expression of 6PGD involved in the pentose phosphate pathway. These upregulated pathways have been shown to be involved in increased anabolic intermediates and in electron acceptors that are used in the electron transport chain (ETC) to provide the transformed cells with their bioenergetic and/or biosynthetic needs [10-12, 70]. The above-mentioned pathways are also involved in increasing NADPH levels possibly via malate-aspartate shuttle [9] and via upregulation of the 6PGD enzyme involved in the PPP [63] in the presence of exogenous cholesterol. These enhanced NADPH levels could potentially increase the biomass synthesis and ROS detoxification in breast cancer cells to promote cellular proliferation [71]. Importantly, these cholesterol-induced metabolic pathways and NADPH levels are mainly mediated via ERRα axis. Notably, as obesity has been linked to high blood cholesterol levels, using GEO databases, we observed a significant increase in the expression of ERRα target genes involved in OXPHOS, the TCA cycle, and the key enzymes associated with ROS detoxification in the obese basal-like breast cancer patients, who overexpress ERRα. Hence, it is interesting to speculate that the upregulated oxidative metabolism and the detoxification enzyme expression in obese basal-like breast cancer patients are possibly mediated via the cholesterol-ERRα axis, and this upregulation may cause resistance to chemotherapeutics and some targeted therapies [15, 19-21]. Interestingly, we observed that obesity is associated with an induction in the expression of G6PD, a key enzyme linked to NADPH production in the PPP, and the expression of GART, a key enzyme in purine biosynthesis correlated to the OCM pathway. Since it has been reported that ERRα suppresses the PPP and the OCM pathway in breast cancer cells [9, 44], this upregulation in the above-mentioned enzymes involved in the PPP and the OCM pathway possibly occurs via

activation of other signaling pathways, such as the PI3K/Akt/mTOR pathway in obese basal-like breast cancer patients.

Given that ERRα expression levels are high in primary breast tumors, particularly in TNBC, and its overexpression is associated with adverse clinical outcome [27, 53, 60, 64, 72], the above findings, both *in vitro* and patients' data, provide new insights into the molecular mechanism by which obesity and high cholesterol intake alter breast cancer cells' metabolism to exert its pathological effect partially via the ERRα pathway. This finding may lead to novel combinational therapy to target ERRα and intracellular cholesterol synthesis pathways to treat breast cancer.

**Author Contributions:** F.G.—designed, performed, analyzed, interpreted and wrote the manuscript. A.P.—supervised the project and revised the manuscript. M.P.— Developed the protocol and generated the TNBC PDX cell line GCRC1887 and established the culture conditions for those cells.

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**Conflicts of Interest:** The authors declare that they have no competing interests.

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## 4.7. FIGURES AND SUPPLEMENTARY MATERIAL

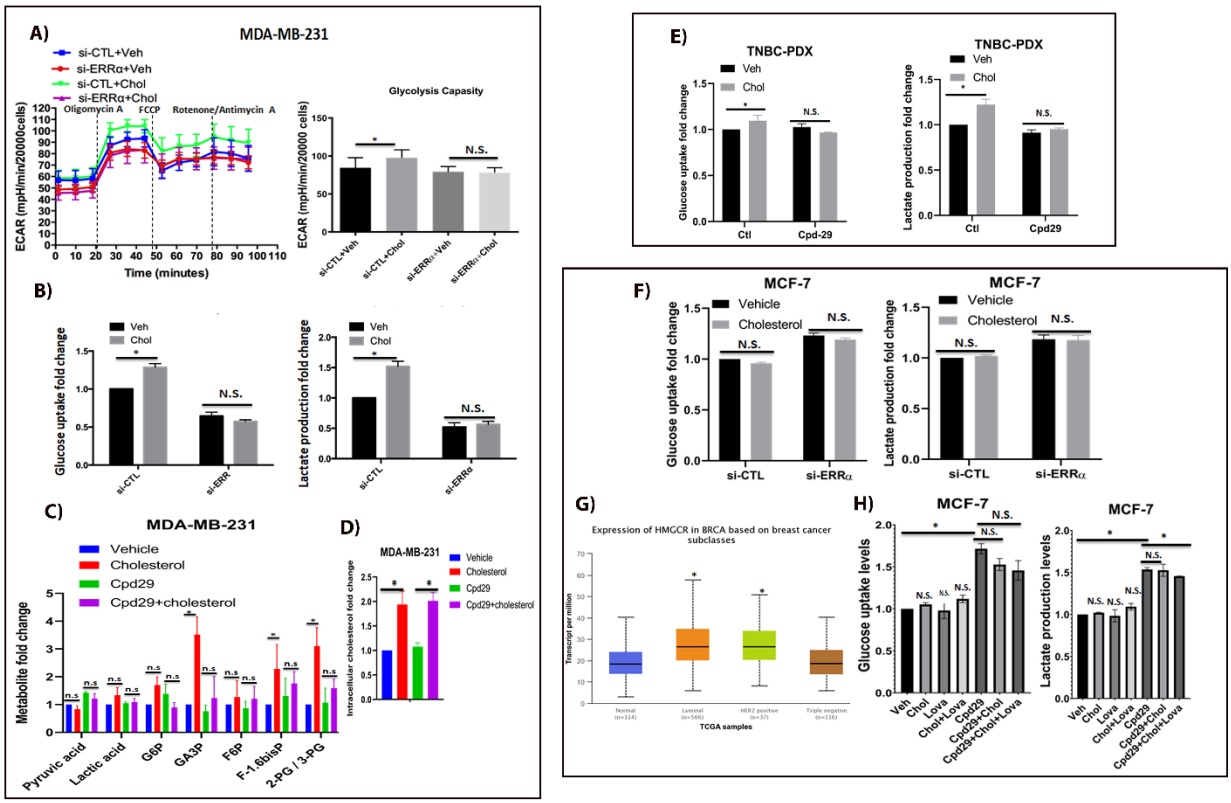
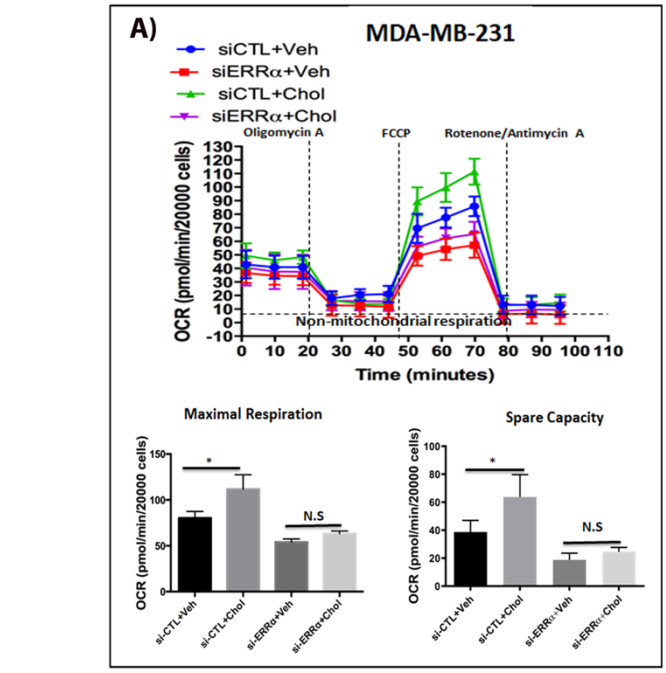



**Figure 1. Cholesterol enhances the abundances of glycolytic metabolites in TNBC cells, but not in ER+ breast cancer cells, in an ERRα-dependent manner. A)** MDA-MB-231 cells were transfected with siRNA-control (si-CTL) or siRNA-ERRα (si-ERRα) and then treated with vehicle (Veh) or cholesterol (Chol, 5 μM) for 24 h. The extracellular acidification rate (ECAR) was obtained using Seahorse XF96. Glycolysis capacity was measured after oligomycin drug injection. **B)** The glucose consumption and lactate production for MDA-MB-231 cells were obtained using Profile 400 analyzer. The cells were transfected and treated as above. **C)** The metabolic profiling on the glycolysis pathway was done in MDA-MB-231 cells using LC/MS. The data was normalized for cell count and the respective vehicle. The cells were treated with vehicle (Veh), cholesterol (Chol, 5 μM), and/or cpd29 (5 μM), which is an ERRα inhibitor, for 48 h. **D)** Intracellular cholesterol levels were measured in MDA-MB-231 cells using GC/MS. The data were normalized for cell count and the respective vehicle. MDA-MB-231 cells were treated with vehicle (Veh), cholesterol (Chol, 5 μM) and/or cpd29 (5 μM) for 48 h. **E)** Glucose uptake and lactate levels were measured in Triple-negative breast cancer patient-derived xenograft (TNBC-PDX) cells. The cells were treated with vehicle (Veh), cholesterol (Chol, 10 μM), and/or cpd29 (10 μM) for 48 h; Ctl (control) represents the breast cancer cells treated with cholesterol or vehicle. **F)** MCF-7 cells were transfected with siRNA-control (si-CTL) or siRNA-ERRα (si-ERRα), and then treated with vehicle or cholesterol (10 μM) for 24 h. **G)** Expression of HMGCR in various breast cancer subtypes, and represents the mRNA levels of HMGCR gene in breast tumors and corresponding normal tissue obtained from the TCGA database. The data were visualized using the UALCAN web-portal. The significance (*) was defined by comparing each subtype to the normal tissue. **H)**

MCF-7 cells were treated with vehicle (Veh) or 10μM of cholesterol (Chol) and/or lovastatin (Lova) and/or cpd29 for 48 h. The results represent three independent experiments. A value of $p < 0.05$ was considered significant (*).


A)
MDA-MB-231
siCTL+Veh
siERRα+Veh
siCTL+Chol
siERRα+Chol
Oligomycin A
FCCP
Rotenone/Antimycin A
OCR (pmol/min/20000 cells)
Non-mitochondrial respiration
Time (minutes)
Maximal Respiration
Spare Capacity
N.S
si-CTL+Veh
si-CTL+Chol
si-ERRα+Veh
si-ERRα+Chol

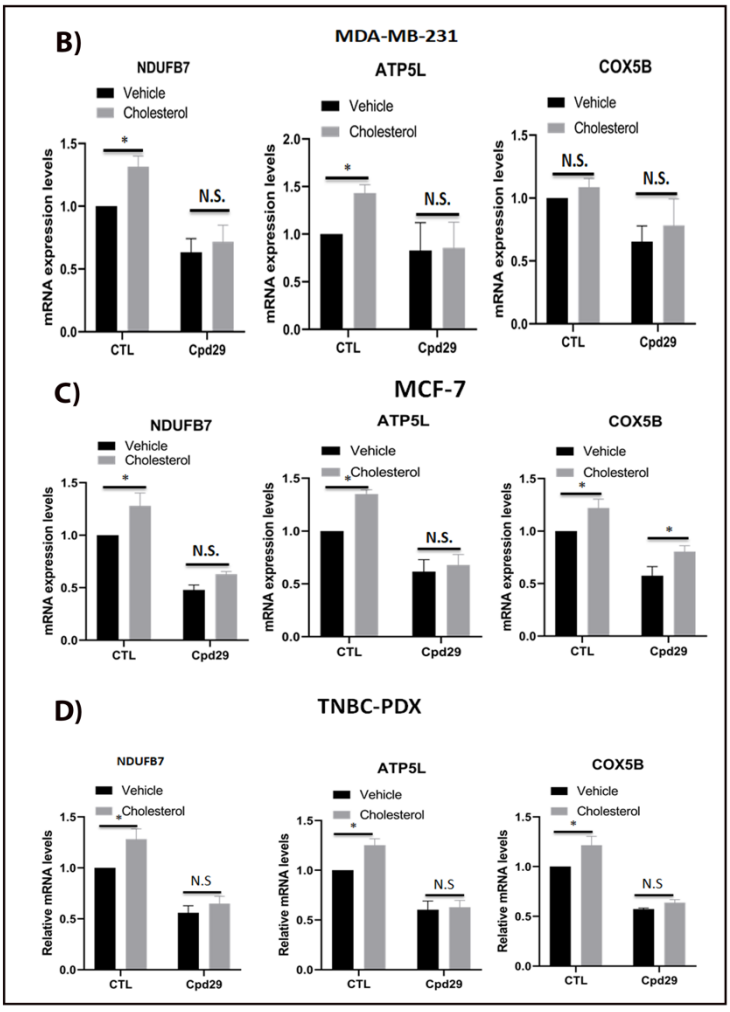

B)
MDA-MB-231
NDUFB7
ATP5L
COX5B
Vehicle
Cholesterol
mRNA expression levels
N.S.
CTL
Cpd29
C)
MCF-7
D)
TNBC-PDX
Relative mRNA levels
N.S

**Figure 2. Cholesterol induces cellular respiration in ER+ and TNBC cells via ERRα. A)** Oxygen consumption rate (OCR) was obtained by Seahorse XF96 in MDA-MB-231 cells. The cells were transfected with siRNA-control (si-CTL) or siRNA-ERRα (si-ERRα) and then treated with vehicle (Veh) or cholesterol (Chol, 5 μM) for 24 h. The maximal respiratory capacity (Maximal Resp.) represents the peak between FCCP and Rotenone/Antimycine A injection. Spare capacity was calculated by substracting the maximal respiration from the basal respiration, as indicated in the graph. **B-D)** MDA-MB-231 cells were treated with vehicle (Veh), cholesterol (Chol, 5 μM) and/or cpd29 (5 μM) for 48 h. Also, MCF-7 and TNBC-PDX cells were treated with vehicle (Veh), cholesterol (Chol, 10 μM), and/or cpd29 (10 μM) for 48 h. Total RNA was extracted and analyzed using RT-qPCR. Genes detected included: NDUFB7: NADH dehydrogenase (ubiquinone) 1 beta subcomplex subunit 7, ATP5L: ATP synthase subunit g, mitochondrial, and COX5B: Cytochrome *c* oxidase subunit 5B, mitochondrial. The mRNA data were normalized to endogenous GAPDH. Ctl (control) represents the breast cancer cells treated with cholesterol or vehicle. The data are expressed as means±SEM, and represent at least three independent experiments. p value $<0.05$ was considered as significant (*).

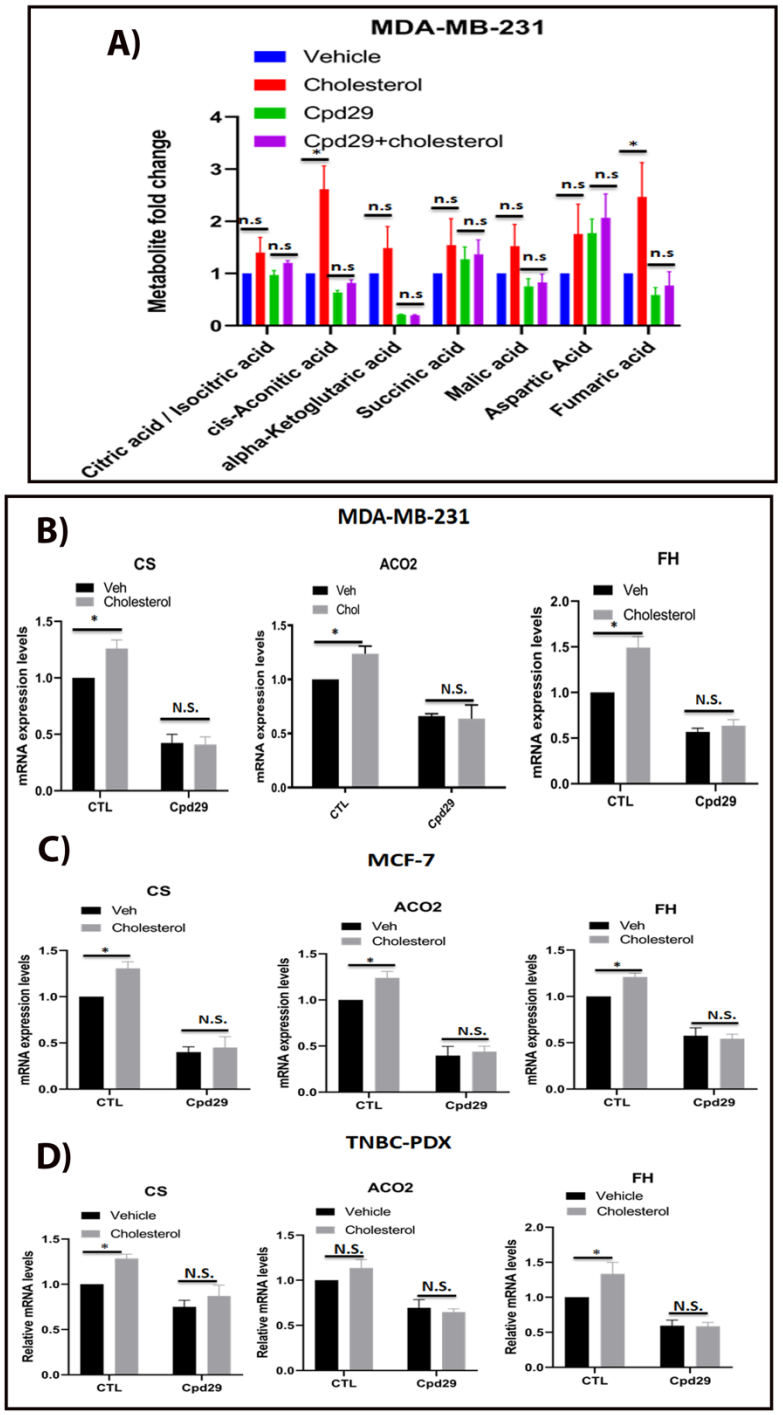



**Figure 3. Cholesterol increases TCA cycle in ER+ and TNBC cells in an ERRα-dependent manner. A)** The metabolic profiling on TCA cycle intermediates was done using LC/MS. The data were normalized for cell count and the respective vehicle. MDA-MB-231 cells were treated with vehicle (Veh), cholesterol (Chol, 5 μM) and/or cpd29 (5 μM), which is an ERRα inhibitor, for 48 h. **B-D)** MDA-MB-231 cells were treated with vehicle (Veh), cholesterol (Chol, 5 μM) and/or cpd29 (5 μM) for 48 h. Also, MCF-7 and TNBC-PDX cells were treated with vehicle (Veh),

cholesterol (Chol, 10 μM), and/or cpd29 (10 μM) for 48 h. Total RNA was extracted and analyzed using RT-qPCR. The genes included: CS: Citrate Synthase, mitochondria, ACO2: Aconitase 2, mitochondria, and FH: Fumarate Hydratase. The mRNA data were normalized to endogenous GAPDH. Ctl (control) represents the breast cancer cells treated with cholesterol or vehicle. The data are expressed as means±SEM and represent at least three independent experiments. p value <0.05 was considered as significant (*).

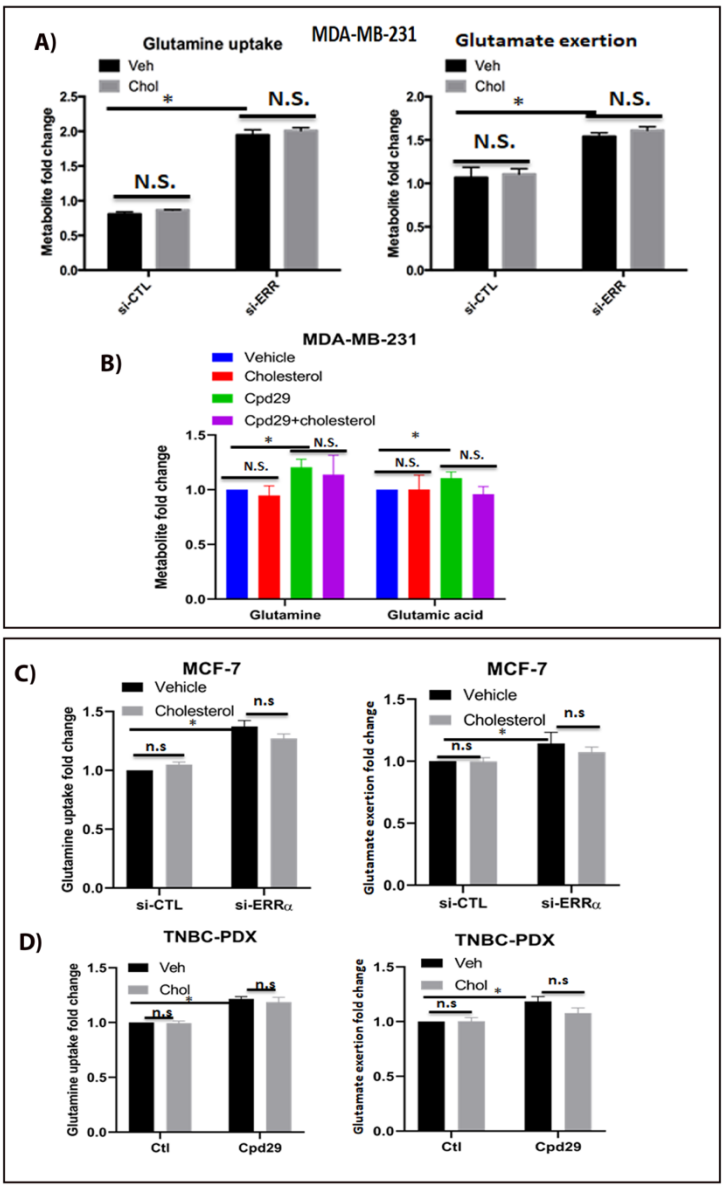



**Figure 4. Cholesterol does not alter the glutaminolysis metabolite levels in breast cancer cells.** **A)** The glutamine uptake and glutamate and ammonium production were measured using Profile 400 analyzer. MDA-MB-231 cells were transfected with siRNA-control (si-CTL) or siRNA-ERRα (si-ERRα) and then treated with vehicle (Veh) or cholesterol (Chol, 5 μM) for 24 h. **B)** The metabolite levels were measured using GC/MS. MDA-MB-231 cells were treated with vehicle (Veh), cholesterol (Chol, 5 μM) and/or cpd29 (5 μM) for 48 h. **C)** MCF-7 cells were transfected with siRNA-control (si-CTL) or siRNA-ERRα (si-ERRα) and then treated with vehicle (Veh) or cholesterol (Chol, 10 μM) for 24 h. **D)** TNBC-PDX cells were treated with vehicle (Veh), cholesterol (Chol, 10 μM) and/or cpd29 (10 μM) for 48 h. Ctl (control) represents the breast cancer cells treated with cholesterol or vehicle. The results represent three independent experiments. A value of $p < 0.05$ was considered significant (*).

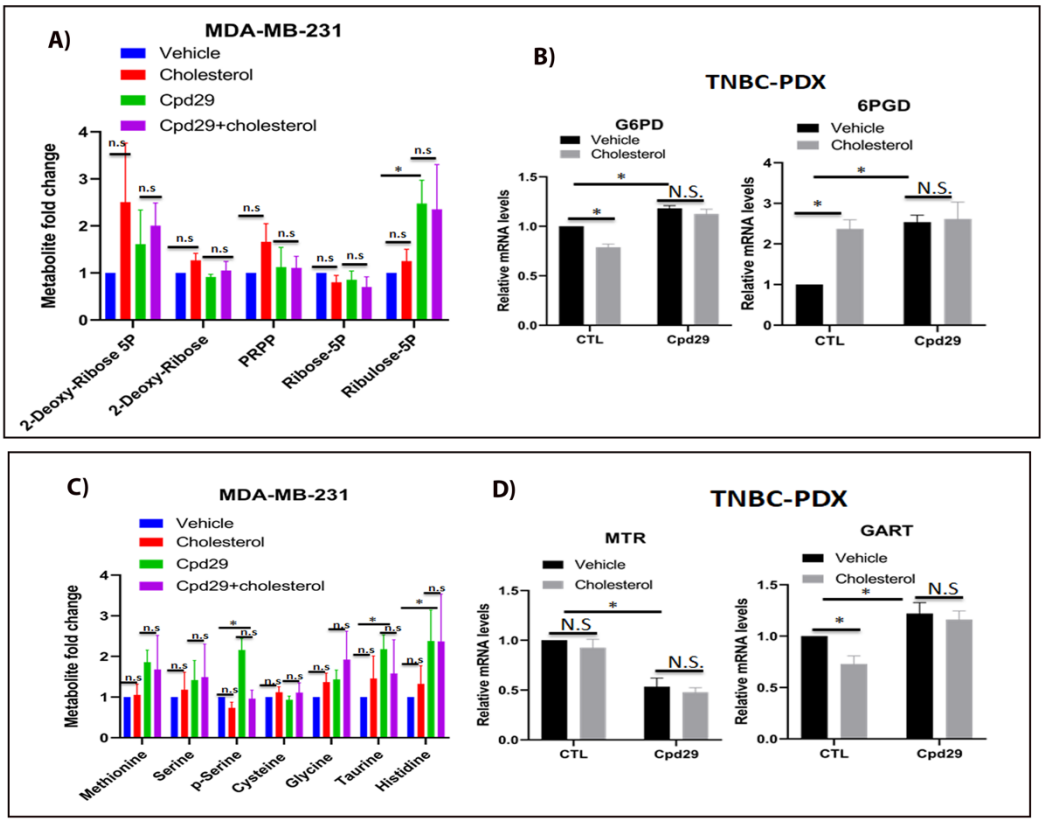



**Figure 5. Cholesterol does not alter the metabolite levels involved in PPP and OCM pathway in TNBC cells. A)** The metabolic profiling on PPP intermediates was done using LC/MS. The data were normalized for cell count and the respective vehicle. MDA-MB-231 cells were treated with vehicle (Veh), cholesterol (Chol, 5 μM) and/or cpd29 (5 μM), which is an ERRα inhibitor, for 48 h. **B)** TNBC-PDX cells were treated with vehicle (Veh), cholesterol (Chol, 10 μM) and/or cpd29 (10 μM) for 48 h. Total RNA was extracted and analyzed using RT-qPCR. The genes included: G6PD: Glucose-6-phosphate 1-dehydrogenase, 6PGD: 6-Phosphogluconate dehydrogenase. **C)** The metabolic profiling on OCM intermediates were done using GC/MS. The data were normalized for cell count and the respective vehicle. MDA-MB-231 cells were treated with vehicle (Veh), cholesterol (Chol, 5 μM) and/or cpd29 (5 μM) for 48 h. **D)** TNBC-PDX cells were treated with vehicle (Veh), cholesterol (Chol, 10 μM) and/or cpd29 (10 μM) for 48 h. Total RNA was extracted and analyzed using RT-qPCR. The genes included: MTR: 5-methyltetrahydrofolate-homocysteine methyltransferase, GART: Trifunctional purine biosynthetic protein adenosine-3. The mRNA data were normalized to endogenous GAPDH. CTL (control) represents the breast cancer cells treated with cholesterol or vehicle. The data are represented as means±SEM, and at least three independent experiments. p-value <0.05 was considered as significant (*).

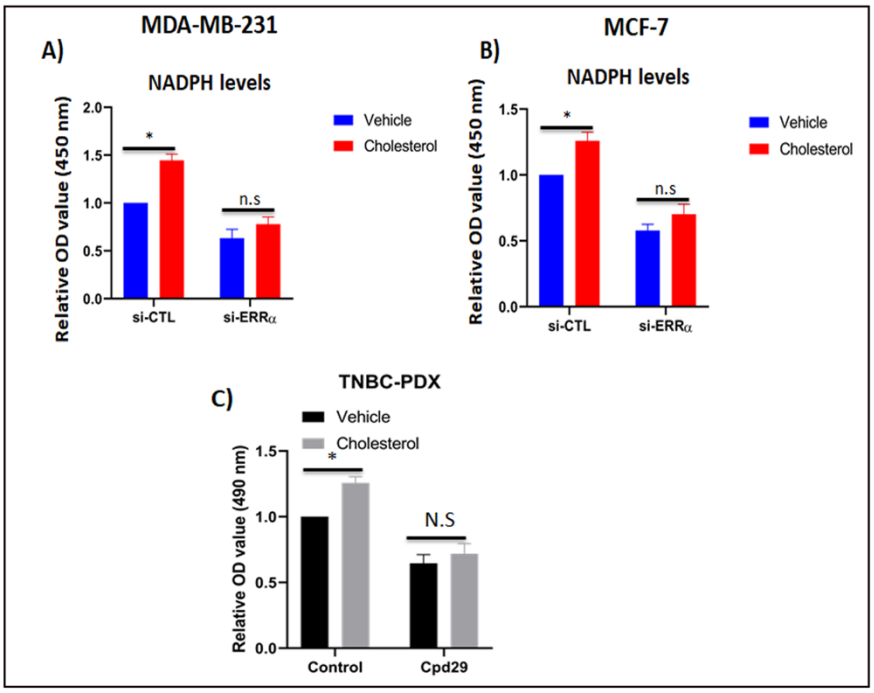



**Figure 6. Cholesterol induces NADPH levels and cellular growth in breast cancer cells via ERRα pathway. A&B)** The intracellular NADPH levels were quantified using the $NADP^+$/NADPH assay kit. MDA-MB-231 and MCF-7 cells were transfected with either siRNA-control (si-CTL) or siRNA-ERRα (si-ERRα) for 48 h, following by treatment with vehicle or cholesterol (5 μM for MDA-MB-231 cells, and 10 μM for MCF-7 cells) for 24 h. **C)** TNBC-PDX cell proliferation was measured using an MTS kit in the presence of vehicle (Veh), cholesterol (Chol, 10 μM) and/or cpd29 (10 μM) on day 6. Ctl (control) represents the breast cancer cells treated with cholesterol or vehicle.

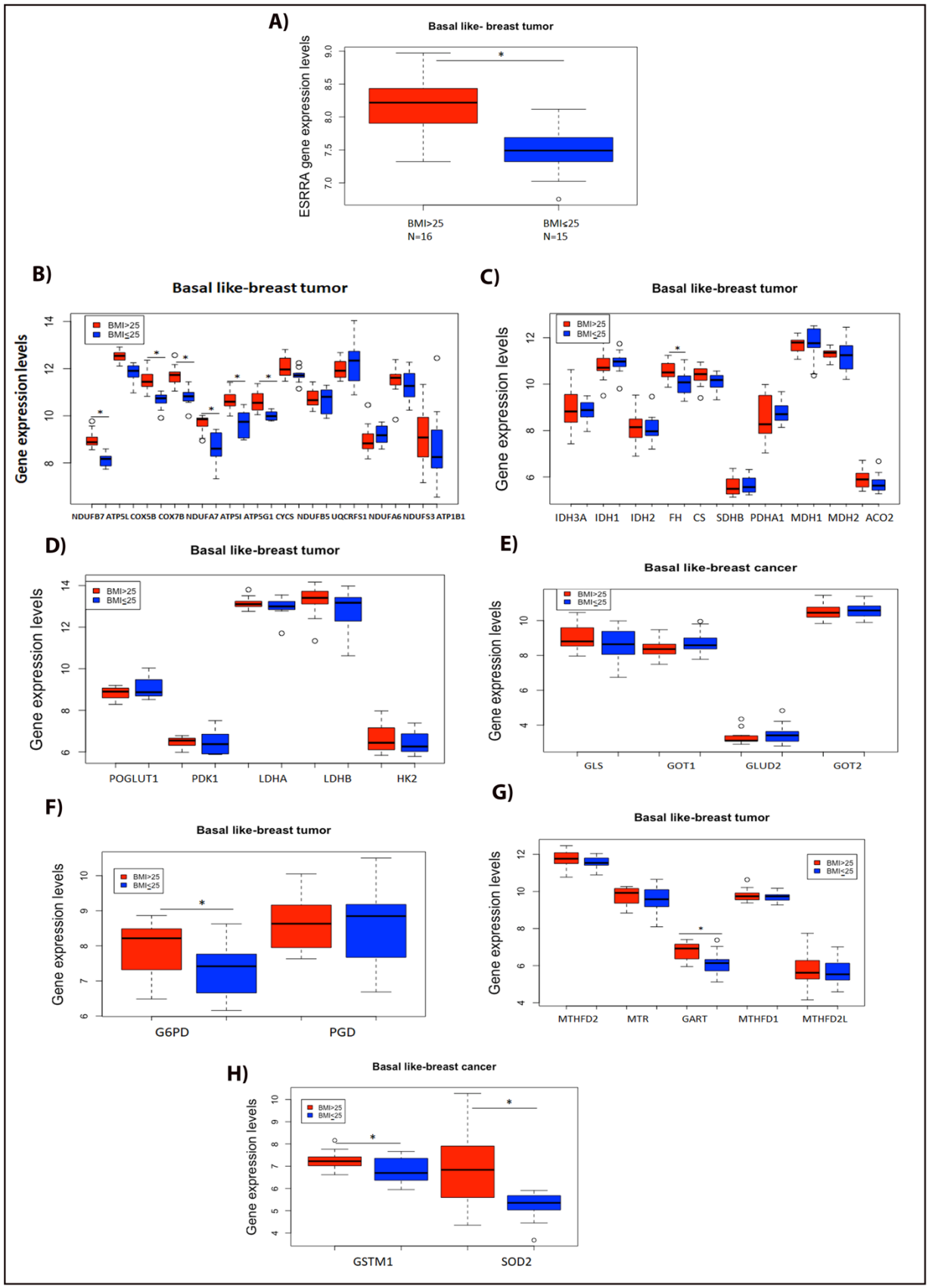

A)
Basal like- breast tumor
ESRRA gene expression levels
BMI>25
N=16
BMI≤25
N=15
B)
Basal like-breast tumor
Gene expression levels
BMI>25
BMI≤25
NDUFB7 ATP5L COX5B COX7B NDUFA7 ATP5I ATP5G1 CYCS NDUFB5 UQCRFS1 NDUFA6 NDUFS3 ATP1B1
C)
Basal like-breast tumor
Gene expression levels
IDH3A IDH1 IDH2 FH CS SDHB PDHA1 MDH1 MDH2 ACO2
D)
Basal like-breast tumor
Gene expression levels
POGLUT1 PDK1 LDHA LDHB HK2
E)
Basal like-breast cancer
Gene expression levels
GLS GOT1 GLUD2 GOT2
F)
Basal like-breast tumor
Gene expression levels
G6PD PGD
G)
Basal like-breast tumor
Gene expression levels
MTHFD2 MTR GART MTHFD1 MTHFD2L
H)
Basal like-breast cancer
Gene expression levels
GSTM1 SOD2

**Figure 7. ERRα metabolic gene signature profile increases in basal-like breast tumors of obese vs. non-obese patients. A)** ESRRA gene expression levels in obese breast cancer patients with BMI>25 vs. non-obese patients with BMI ≤ 25. **B)** ERRα metabolic target genes involved in the OXPHOS pathway. **C)** ERRα metabolic target genes associated with the TCA cycle. **D)** ERRα metabolic target genes linked to the glycolytic pathway. **E)** ERRα metabolic target genes related to the glutaminolysis pathway. **F)** Gene expression levels involved in PPP intermediates. **G)** ERRα metabolic target genes associated with the OCM pathway**. H)** ERRα metabolic target genes involved in detoxifying enzymes. The data were analyzed using R software.

**Supplementary material:**

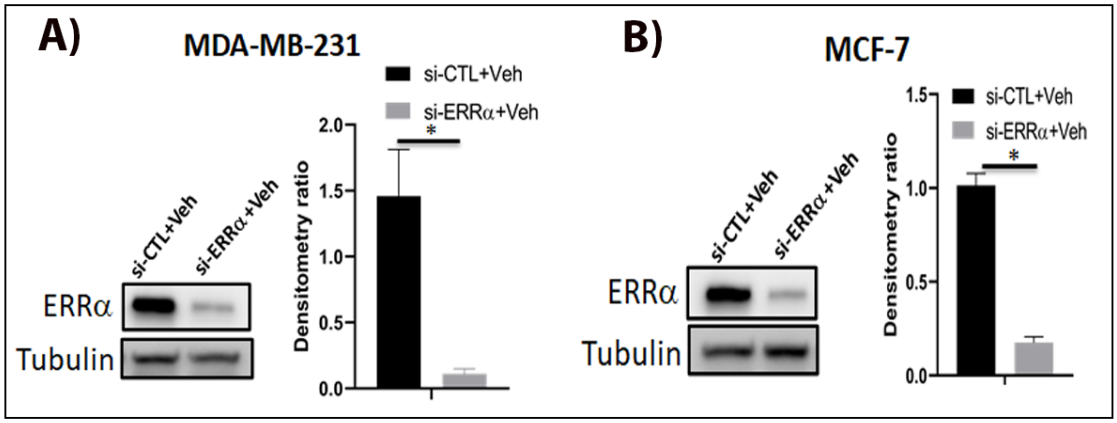



**Figure S1:** ERRα knock-down was successfully performed in MDA-MB-231 and MCF-7 cells. **A&B)** MDA-MB-231 and MCF-7 cells were transfected with either siRNA-control (si-CTL) or siRNA-ERRα (si-ERRα) for 48 h, following by treatment with vehicle or cholesterol (5 μM for MDA-MB-231 cells, and 10 μM for MCF-7 cells) for 24 h, respectively. Cell lysates were immunoblotted using an anti-ERRα antibody. The densitometry ratio was measured using Image-J software.

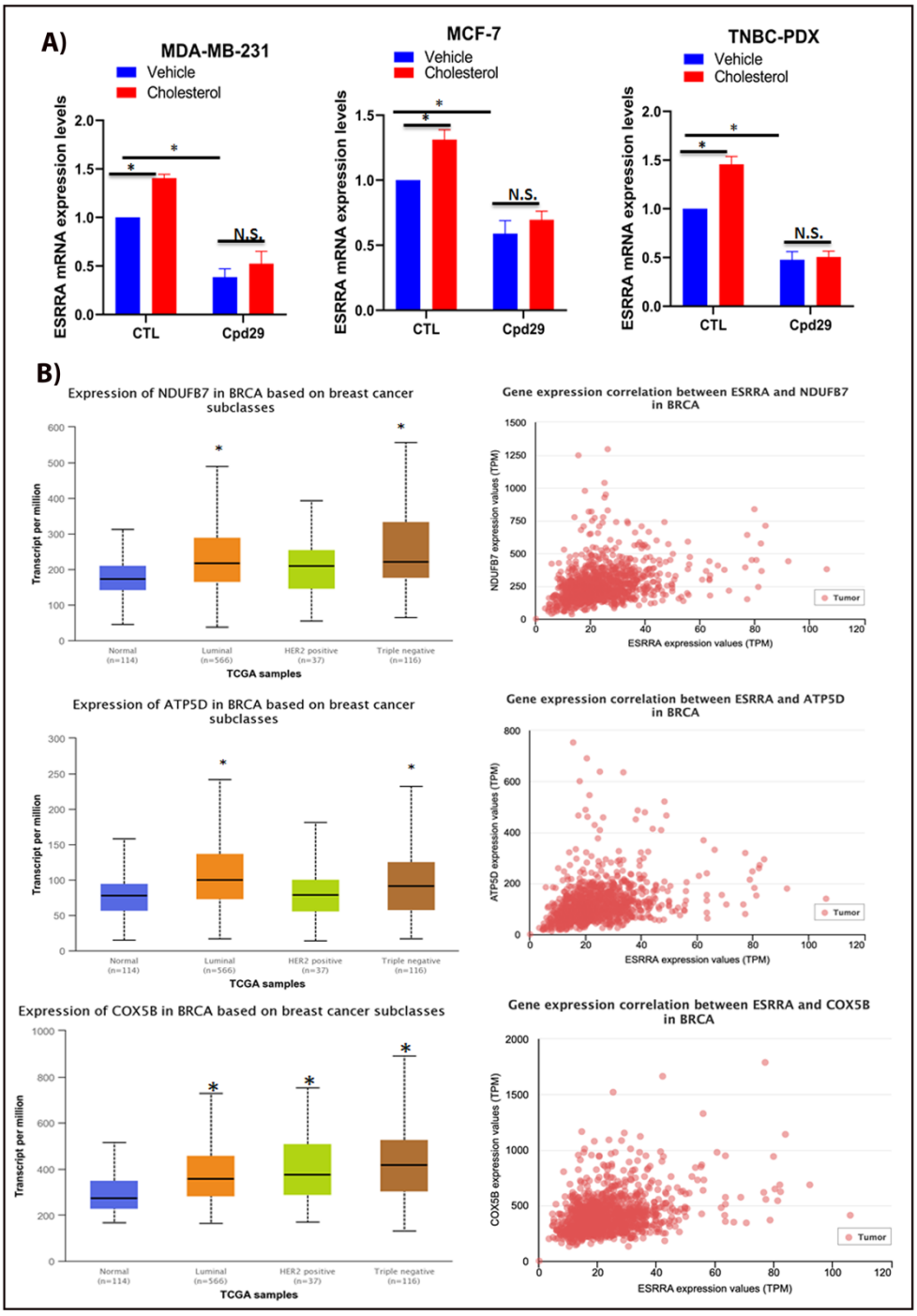



**Figure S2. ERRα gene expression levels are positively correlated with the key enzymes involved in OXPHOS using TCGA databases. A)** The relative mRNA levels of ESRRA in MDA-MB-231, MCF-7, and TNBC-PDX cells. Total RNA was extracted and analyzed using RT-qPCR. MDA-MB-231 cells were treated with vehicle (Veh), cholesterol (Chol, 5 μM) and/or cpd29 (5 μM) for 48 h. MCF-7 and TNBC-PDX cells were treated with vehicle (Veh), cholesterol

(Chol, 10 μM) and/or cpd29 (10 μM) for 48 h. **B)** The data represents the mRNA levels of the indicated genes in breast tumors and corresponding normal tissue obtained from the TCGA database. The graphs were visualized using the UALCAN web-portal. Expression of NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit **7 (**NDUFB7) and ATP synthase subunit delta, mitochondrial (ATP5D) and COX5B: Cytochrome *c* oxidase subunit 5B in different subtypes of breast cancer and their correlation to the ESRRA gene expression in breast tumors. The significance (*) was defined by comparing each subtype to the normal tissue.

Table S1: The human primers used for qPCR

| Name | Oligo Name | Sequence (5’ to 3’) |
|---|---|---|
| Glyceraldehyde 3-phosphate dehydrogenase | GAPDH Forward(F) | GTCTCCTCTGACTTCAACAGCG |
| | GAPDH Reverse (R) | ACCACCCTGTTGCTGTAGCCAA |
| NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 7 | NDUFB7 F | CTGCTCAAGTGCAAGCGTGACA |
| | NDUFB7 R | CGCTCAAACTCCTTCATGCGCA |
| Cytochrome *c* oxidase subunit 5B, mitochondrial | COX5B F | GGAGATCATGCTGGCTGCAAAG |
| | COX5B R | GCAGCCTACTATTCTCTTGTTGG |
| ATP synthase subunit g, mitochondrial | ATP5L F | CCTGCTGAGATCCCTAGAGCTA |
| | ATP5L R | CCGACATAAAACCACATCAACACC |
| Methionine synthase | MTR F | CCAACTTGTCCTTCTCCTTCCG |
| | MTR R | CATACACAGGGAGGTTTCCAGC |
| Trifunctional purine biosynthetic protein adenosine-3 | GART F | GCACATCTCTGCCTGTTTGGCT |
| | GART R | CATGGAACACCTCCAGTCCTAG |
| Glucose-6-phosphate dehydrogenase | G6PD F | CTGTTCCGTGAGGACCAGATCT |
| | G6PD R | TGAAGGTGAGGATAACGCAGGC |
| 6-Phosphogluconate dehydrogenase | 6PGD F | GTTCCAAGACACCGATGGCAAAC |
| | 6PGD R | CACCGAGCAAAGACAGCTTCTC |
| Estrogen-related receptor alpha | ESRRA F | CCACTATGGTGTGGCATCCTGT |
| | ESRRA R | GGTGATCTCACACTCGTTGGAG |
| Citrate synthetase | CS F | CACAGGGTATCAGCCGAACCAA |
| | CS R | CCAATACCGCTGCCTTCTCTGT |
| Fumarate hydratase | FH F | CCGCTGAAGTAAACCAGGATTATG |
| | FH R | ATCCAGTCTGCCATACCACGAG |
| Aconitase 2 | ACO2 F | CAATCGTCACCTCCTACAACAGG |
| | ACO2 R | GTCTCTGGGTTGAACTTGAGGG |

Tables S2: Basal-like breast tumor patient identification obtained from GEO: GSE78958 study.

| BMI status | Patient ID |
|---|---|
| BMI>25<br>N=16 | GSM2082151 |
| | GSM2082188 |
| | GSM2082294 |
| | GSM2082303 |
| | GSM2082370 |
| | GSM2082160 |
| | GSM2082163 |
| | GSM2082205 |
| | GSM2082300 |
| | GSM2082310 |
| | GSM2082440 |
| | GSM2082325 |
| | GSM2082454 |
| | GSM2082382 |
| | GSM2082340 |
| | GSM2082298 |
| BMI $\leq$ 25<br>N=15 | GSM2082095 |
| | GSM2082109 |
| | GSM2082169 |
| | GSM2082177 |
| | GSM2082274 |
| | GSM2082352 |
| | GSM2082384 |
| | GSM2082400 |
| | GSM2082385 |
| | GSM2082297 |
| | GSM2082234 |
| | GSM2082341 |
| | GSM2082379 |
| | GSM2082270 |
| | GSM2082423 |

# CHAPTER 5

# GENERAL DISCUSSION

## 5.1. General Discussion

### 5.1.1. Background

Estrogen-related receptors (ERRs) belong to the orphan nuclear receptor subfamily [1, 2] and play critical roles in feto-placental development [3-5] and human diseases, including breast, ovarian, and prostate cancers [6, 7]. It has been shown that ERRs promote human diseases by reprogramming cellular metabolism [8, 9]. Given ERRs are emerging as potential therapeutic targets in several human diseases, intensive efforts were made to discover their endogenous ligands in order to regulate their transcriptional activity. However, no endogenous ligand was identified for ERRs, except for cholesterol, which was recently identified as an agonist of ERRα [10]. In this thesis, I aimed to identify the endogenous ligands of ERRs using affinity-based chromatography and to determine the biological effects of the identified endogenous ligands on breast cancer cells.

Previously, our group demonstrated that a novel endogenous steroid purified from human pregnancy urine and serum, named ED, showed high affinity for the SHBG protein, with the putative structure, 17β-hydroxy-19-norandrosta-1,5 –dien-3-one, and a mass of 273 m/z [11, 12]. Furthermore, our group reported preliminary data that ED does not show an affinity for ER, but acts as an inverse agonist for ERRα and ERRγ and decreases breast cancer cell proliferation, with an affinity higher than that of the synthetic ligand diethylstilbestrol (DES) [13]

### 5.1.2. ED as an endogenous inverse agonist of ERRα/γ:

In this thesis, I have confirmed that ED isolated and purified from human pregnancy urine showed the mass of 273 m/z using a new method that I developed by LC-MS/MS and based on my MS data exhibited a distinct fragmentation pattern compared to the commercially available E2 compound. The fragmentation pattern of ED suggests that unlike the E2 compound, the novel endogenous ED's structure does not contain an aromatic ring. In addition, I demonstrated that the purified ED from human pregnancy urine does not show an affinity for ER, consistent with our

team's previous report [14]. In addition, I showed that ED has no affinity for GR. Our group previously demonstrated that ED disturbs the interaction of PGC-1 or GRIP1 with its corresponding receptor, ERRα or ERRγ [13]. I have further demonstrated that the endogenous ED, purified from human pregnancy urine, directly binds the human ERRγ-LBD protein, and exhibited a higher affinity to ERRγ-LBD than its synthetic ligand 4-OHT. Furthermore, I confirmed that ED interacts with ERRα and ERRγ and decreases their transcriptional activity. Remarkably, the endogenous ED shows an affinity for ERRα and -γ in nanomolar range concentration with higher affinity than the synthetic ligands [15, 16] whose affinity for ERRs is in the micromolar range. Together, our findings suggest that ED directly binds to ERRα and ERRγ in a nanomolar range, inhibits its interaction with its coactivator PGC-1 or GRIP1, and acts as the first known endogenous inverse agonist of ERRα and ERRγ.

### 5.1.3. Biological significance of ED-ERR pathway in breast cancer cells

Interestingly, our group's previous finding that ED inhibits cell proliferation of ER+ breast cancer cells, even in the presence of estradiol, could be explained by the possibility that the ED-ERR pathway may dominate over the estradiol-ER pathway in MCF-7 breast cancer cells. Several studies have reported that ERRα shows both ER-dependent and independent activity in breast tumors, and that depending on the gene context, the ER or ERR pathways may support or antagonize each other [17-21]. Notably, accumulating evidence demonstrated that ERRα co-regulates only 18% of ERα target genes, even though they share high similarity in their DBDs [17, 18, 22-24]. Together, the above finding indicates that the ED-ERRα pathway exhibits ER-independent inhibitory activity in breast cancer cells.

My results demonstrating that ED inhibits cell proliferation of MCF-7 and MDA-MB-231 cells in a dose-dependent manner with the effective inhibitory dose ($IC_{50}$) at the very low

nanomolar range while having no significant effect on MCF-10A cells is in agreement with our team's previous findings using another assay, and another batch of the purified ED [13]. That the inhibitory effect of ED is unlikely to be due to contaminants in the purified ED preparation, as it has previously been demonstrated by our team that only the purified fraction corresponding to ED but not any other fraction shows an effect on cell proliferation. Importantly, my finding that the inhibitory effect of ED is ERRα-dependent suggests that the inhibitory effect of ED on breast cancer cell growth is mediated via ERRα. This finding is consistent with the previous reports that ERRα expression levels are high in ER+ and, particularly, in TNBC cells, and that ERRα overexpression is linked to adverse clinical outcomes in those patients [17, 19-21]. The absence of a significant inhibitory effect on MCF-10A cells, a non-tumorigenic breast cancer cell line, is possibly due to the low levels of ERRα expression in these cells and is consistent with the notion that ED's anti-proliferative effect on breast cancer cell proliferation is mediated via ERRα. These findings are in line with the studies that demonstrated that inhibition of ERRα, using its synthetic inverse agonists, decreases ER+ and triple-negative breast cancer cell growth *in vitro* and *in vivo* [19, 25-27]. On the other hand, the impact of ED-ERRγ on breast cancer-cellular growth remains to be determined. Available reports on ERRγ's regulatory effect on breast cancer cell proliferation are contradictory. Several studies have shown that ERRγ overexpression promotes breast cancer cell growth and that it is involved in TAM resistance in ER+ breast cancer cells [28-30], while another study demonstrated that ERRγ expression activates the genetic program of MET, which upregulates E-cadherin and decreases breast cancer cell growth [31]. Whether ERRγ mediates ED's effects on breast cancer cell proliferation warrants further studies.

Based on our above-mentioned findings, we proposed a mechanism by which the ED-ERR pathway may regulate breast cancer cell proliferation: ED as a novel endogenous inverse agonist

of ERR, directly binds ERR with high affinity and possibly changes ERR's structural conformation so that PGC-1, its coactivator, does not interact with it, thereby inhibiting ERR's transcriptional activity. As ERRα is known to exert its pathogenic effects on breast cancer cells by inducing its metabolic target genes involved in OXPHOS, TCA cycle, and glycolysis to satisfy the metabolic demands of the metastatic tumor cells under stress conditions [17, 19, 24], ED's inhibition on breast cancer cell proliferation might lead to downregulation of the TCA cycle, OXPHOS, and glycolysis in breast cancer cells, resulting in inhibition of breast cancer cell growth. Although the exact mechanism of the ED-ERR pathway in breast cancer remains to be elucidated, this finding that ED, as the first endogenous inverse agonist of ERRα and ERRγ, inhibits both TNBC and ER+ breast cancer cells, with no effect on normal epithelial breast cancer cells, may have important ramifications in breast cancer therapy.

It is important to mention that despite all findings mentioned above, ED's exact structure remains to be elucidated. Determining the exact structure of ED is necessary to chemically synthesize and utilize it for further investigations. To elucidate the exact structure of ED, a large quantity of ED needs to be purified from human pregnancy urine and serum and requires analysis by Nuclear Magnetic Resonance (NMR). However, purifying a large quantity of ED (in the range of milligrams) is very challenging, as one liter of pregnancy urine contains around one microgram or less of ED (data not shown). In the current thesis, I used the affinity-based chromatography methodology using beads-GST-ERR-LBD and LC-MS/MS techniques to enrich the endogenous ligands of ERRs. My findings demonstrated that a compound with the mass 273 m/z (consistent with the previous MS data of purified ED), and another distinct compound with the mass 333 m/z were enriched in beads-GST-ERRγ.

### 5.1.4. Cholesterol as an endogenous agonist of ERRα

Importantly, I have demonstrated another molecule enriched in beads-GST-ERRα-LBD, which the LC-MS analysis revealed to have a mass of 369 m/z, a daughter ion corresponding to cholesterol. In further analysis, I demonstrated that cholesterol binds directly and specifically to ERRα-LBD and increases ERRα's transcriptional activity in a PGC-1α-dependent manner. This finding indicates that cholesterol acts as an endogenous agonist of ERRα/PGC-1α signaling. These data are in agreement with another group that recently published that cholesterol isolated from mouse brain and kidneys acts as an endogenous agonist of ERRα [10]. Interestingly, they demonstrated that the hydroxyl group of cholesterol makes a hydrogen bond to E235 of ERRα's LBD, using computational docking and that F232 and L228 of ERRα probably make hydrophobic bonds with cholesterol [10], providing a structural basis to the cholesterol-ERRα interaction.

### 5.1.5. Cholesterol-ERRα pathway in breast cancer metabolic reprogramming

My finding showing that cholesterol enhances ERRα's interaction with its coactivator PGC-1α suggests that cholesterol increases ERRα's transcriptional activity possibly by increasing the recruitment of PGC-1α to the ERRα protein. It is interesting to speculate that cholesterol may act as an allosteric activator by binding to ERRα and modifying its conformation, resulting in increased interaction between ERRα and its coactivator, PGC-1α, and inducing ERRα's transcriptional activity.

There is increasing evidence that the ERRα/PGC-1α axis is the master controller of cellular energy metabolism and metabolic reprogramming in cancer cells. This axis is involved in regulating genes associated with the TCA cycle, OXPHOS, lipid metabolism, and glycolysis [17, 18]. As such, I examined whether cholesterol-ERRα/PGC-1α alters the metabolic programming in breast cancer cells. My finding demonstrating that cholesterol increases ERRα mRNA and protein

levels in a dose-dependent manner suggests that cholesterol promotes ERRα's known ability [17, 18, 25] to activate its own gene (*ESRRA*) expression, and thereby this positive feedback loop. [17, 18, 25]. Thus, cholesterol binding to ERRα may enhance the interaction of ERRα with PGC-1α, leading to enhanced binding of the ERRα/PGC-1α complex to the ERRα gene promotor, resulting in an induction of its own expression levels and its target metabolic pathways.

My finding that cholesterol induces the abundances of metabolites involved in the glycolytic pathway in TNBC cells, but not in ER+ cells, is in line with other studies that have reported that enhanced aerobic glycolysis is positively correlated with the malignancy of tumor cells [32, 33]. My observation that cholesterol does not enhance lactate production, which is associated with the glycolysis pathway, in ER+ breast cancer cells, could possibly be explained by the high expression of HMGCR (a key enzyme in the cholesterol biosynthesis pathway) in this subtype of breast cancer, which may mask the effect of exogenous cholesterol.

An important finding in the current study is that exogenous cholesterol enhances the OXPHOS rates in ER+ and TNBC cells, and was associated with an enhanced oxygen consumption rate and increased expression of ERRα-induced metabolic target genes involved in the OXPHOS pathway, such as NDUFB7, ATP5L, and COX5B. Interestingly, the cholesterol's effects on the OXPHOS pathway are ERRα-dependent. These findings are supported by my observation that the expression of ERRα is positively correlated with that of key enzymes involved in the OXPHOS pathway. This was observed using data obtained from TCGA databases. These findings are in agreement with several studies that reported that ERRα modulates the expression of OXPHOS genes [27, 34, 35]. Furthermore, as high cholesterol levels are often associated with obesity [36], using GEO databases, I observed that the expression levels of the above-mentioned enzymes are higher in obese-basal-like breast tumors compared to the non-obese patients. This

observation is important, as OXPHOS is active in the tumor cells even at low oxygen levels [37-39], and this activation has been associated with causing resistance to chemotherapeutics [37-41].

Another important finding in the present study is that exogenous cholesterol increases TCA cycle metabolite levels in ER+ and TNBC cells and is associated with induced expression of ERRα-mediated enzymes involved in the TCA cycle, such as CS, FH, ACO2, and IDH3A in ER+ and TNBC cells in an ERRα-dependent manner. Based on GEO databases, several of these enzymes showed higher expression in obese breast cancer patients compared to the non-obese patients. This finding is consistent with other reports demonstrating that ERRα modulates the expression of enzymes involved in the TCA cycle [27, 34, 35], and that TCA cycle activation stimulates cancer cell metabolic reprogramming, and provides necessary molecular building blocks, energy, and NADPH needed for breast cancer cells to sustain rapid tumor growth and survive under oxidative stress conditions [42, 43].

Moreover, I found that exogenous cholesterol does not alter the levels of metabolic intermediates in the glutaminolysis, PPP, and OCM pathway in breast cancer cells. Notably, it has been reported that the above-mentioned pathways are upregulated in the transformed cells to help cancer cells survive oxidative stress conditions by generating NADPH to produce the reduced glutathione (GSH), which is essential to eliminate reactive oxygen specious (ROS) [27, 44, 45]. Interestingly, other groups recently reported that in the absence of ERRα, breast cancer cells rely on the glutaminolysis, PPP, and OCM pathway to survive and they suggested that ERRα acts as a suppressor of these pathways [27, 44]. This finding is consistent with my data demonstrating that exogenous cholesterol, as an agonist of ERRα, significantly decreases the expression of two key enzymes (G6PD and GART) involved in the PPP and de novo purine biosynthesis (OCM-related) pathway, respectively, in TNBC-PDX cells, via the ERRα axis. As high cholesterol levels are

often linked to obesity [36], using GEO databases, in obese basal-like breast cancer patients, I observed induction in the expression of G6PD and GART involved in the PPP and OCM pathway, respectively. The upregulation in the expression of these enzymes, for which ERRα acts as a suppressor, is possibly due to the activation of other signaling pathways, including PI3K/Akt/mTOR pathway in obese breast cancer patients [46, 47].

In addition to the findings that exogenous cholesterol enhances both mitochondrial oxidative metabolism (OXPHOS, and TCA cycle), and aerobic glycolysis levels in breast cancer cells, I also observed an induction in NADPH levels in these cells, which was mediated via the ERRα axis. NADPH homeostasis is crucial in ROS detoxification and reductive synthesis reactions for the transformed cells to proliferate rapidly and survive under excessive oxidative stress conditions [48, 49]. Using GEO databases, I observed that the gene expression levels of GSTM1 and SOD2, the two key enzymes involved in ROS detoxification [50-52], are significantly higher in obese breast cancer patients compared to the non-obese patients. This finding is in line with the studies that showed that ERRα is involved in NADPH generation and ROS detoxification via induction of the GSTM1 and SOD2 detoxifying enzymes [17, 53]. Furthermore, this evidence is in agreement with my data that exogenous cholesterol induces the gene expression levels of the GSTM1 and SOD2 detoxifying enzymes in an ERRα-dependent manner in breast cancer cells. These findings suggest that the cholesterol-ERRα pathway protects cancer cells from ROS produced by oxidative stress, helping cancer cells survive oxidative stress conditions.

Two of the critical hallmarks of cancer are cellular proliferation and migration. In this thesis, I demonstrated that the cholesterol-mediated increase in the expression of VEGF and SPP1 is of significant interest in this regard, as they are the direct target genes of ERRα and are involved in angiogenesis and cancer progression, respectively [51, 54-57]. Interestingly, it has been shown

that VEGF is involved in cellular proliferation and migration [54, 55, 58]. Hence, my data demonstrating that cholesterol induces ERRα-mediated VEGF levels is in line with my finding that cholesterol promotes cell proliferation and migration in ER+ and TNBC cells via the ERRα axis. Importantly, my finding further demonstrates that cholesterol increases cell proliferation of both ER+ and TNBC cells in a dose-dependent manner within a nanomolar range, indicating that cholesterol possibly acts as a signaling molecule in breast cancer cells. However, lovastatin, a known cholesterol-lowering drug, shows the opposite effect, and cholesterol rescues the effect of lovastatin, but XCT-790 or cpd29, as small molecule inhibitors of ERRα, do not. It is important to mention that in this thesis, lovastatin was used to decrease the intracellular cholesterol levels [59]. However, it has been reported that the anticancer effects of statins involve multiple molecular pathways, such as inhibition of the AKT/mTOR pathway [60, 61]. However, my results displayed that in the presence of lovastatin, cholesterol is able to rescue the inhibitory effect of lovastatin in breast cancer cell proliferation and migration, while the ERRα protein levels remained unchanged.

Based on the findings mentioned above, the proposed mechanism by which cholesterol promotes cell proliferation in ER+ and TNBC cells may involve cholesterol binding to ERRα, which may change the ERRα structural conformation to enhance the interaction between ERRα and PGC-1α, thereby increasing ERRα transcriptional activity. This process causes an induction in the ERRα expression level itself via specific autoinduction. Additionally, it increases VEGF levels and ERRα's target metabolic pathways, such as some metabolites and certain key enzymes involved in OXPHOS and the TCA cycle, as well as some metabolites involved in aerobic glycolysis, and the expression of 6PGD involved in the pentose phosphate pathway. These above-mentioned pathways are involved in increasing NADPH levels possibly via the malate-aspartate shuttle [27], and induced 6PGD expression levels in the presence of exogenous cholesterol. We

further propose that together, this alteration in breast cancer cells' metabolic programming and enhanced NADPH levels may result in an increase in anabolic biomass generation and detoxification against ROS in breast cancer cells, promoting cellular growth and migration [35, 39, 62-64].

As high blood cholesterol is a common comorbidity in obesity [36], it is important to mention that in obese-basal-like breast cancer patients, obesity is associated with the induced expression of enzymes involved in OXPHOS, the TCA cycle, and ROS detoxification, for which ERRα plays as a modulator in breast cancer cells [17, 52]. However, obesity is also associated with upregulation in key enzymes (G6PD and GART) involved in the PPP and the purine biosynthesis (OCM-related) pathway, respectively, for which ERRα acts as a repressor [27, 44]. Based on this observation, it is interesting to speculate that obesity may only partially exert its pathological outcome in breast cancer patients via the ERRα axis. Since obesity is a complex disease and may elevate several growth factors, the upregulation observed in the expression of the enzymes involved in the PPP and OCM metabolic pathway could occur via other activated signaling pathways, such as the PI3K/Akt/mTOR pathway [46, 47].

### 5.2. Concluding Remarks

Several studies demonstrated that ERRα is overexpressed in several subtypes of breast tumors compared to normal breast tissue [65]. This overexpression is associated with poor survival and increased recurrence in breast cancer patients [32, 41, 45, 46]. ERRα exerts its pathological effects on breast cancer cells by reprogramming cancer cells' metabolism [47, 48]. Hence, it is interesting to propose that ERRα could represent a potential druggable target in breast cancer therapy, and that the identification of endogenous ED as an inverse agonist of ERRα, and cholesterol as an endogenous agonist of ERRα provides new insight into the ERRα pathological

pathway in breast cancer patients and opens a potential avenue for targeting the ERRα pathway and intracellular cholesterol action to reverse dysregulated cancer cell metabolism. However, further investigations are required to explore the potential of drugs, such as statins and SREBP inhibitors, combined with ERRα inhibitors, such as ED, to prevent or treat breast cancer, notably TNBC, which has an adverse clinical outcome and no adequate treatment options [66]. Importantly, identification of ED as an inverse agonist of ERRα, and cholesterol as an agonist of ERRα and a regulator of ERRα metabolic pathways, which both affect cellular proliferation in ER+ and TNBC cells, can also be relevant to other subtypes of breast cancer, such as the human epidermal growth factor receptor 2 positive (HER2+) subtype, and other cancer types, which overexpress ERRα, such as prostate, ovary, and colorectal cancers. Moreover, ERRα is also over-expressed in white adipose tissue [67], and also it is associated with diabetic type 2 and insulin resistance [67, 68]. Interestingly, it has been shown that ERRα knock out mice on high fat/high cholesterol diet (HF/HC) show reduced adiposity, resistance to diet-induced obesity [67], and improved insulin resistance, when compared to wild-type mice [68]. These phenotypes observed in ERRα-/- mice even in the presence of HF/HC diet suggest that when ERRα is absent, the pathogenic effect of HF/HC diet (and thus the adverse effect of the cholesterol-ERRα pathway) on obesity and insulin resistance are mitigated in these mice.

The possible mechanism that protects ERRα-/- mice against obesity and insulin resistance while they are in HF/HC diet may involve deficit of ERRα-mediated lipid metabolism and intestinal apolipoprotein A-IV expression [67,69]. Thus, it is possible that when ERRα is absent, even in the presence of HF/HC diet, these mice display decreased lipid synthesis, and intestinal apolipoprotein A-IV expression and as a result reduced dietary lipid absorption. This may in turn lead to decreased adiposity and improved insulin resistance in these mice, even though they are on

a diet expected to induce obesity. Thus, targeting ERRα using its endogenous inverse agonist (ED) and inhibiting cholesterol synthesis pathway (using statins) could be beneficial in obese patients with insulin resistance.

### 5.3. Limitations and Future directions

One of the limitations in this thesis is that cholesterol and ED may be metabolized or converted to other metabolites that may have other biological effects. This can be addressed by isotope labeling ED and cholesterol and tracing these molecules using the LC-MS/MS technique. In addition, ED and cholesterol may have biological effects that are ERRα-independent. These concerns will be addressed in future studies using RNA-seq and CHIP-seq for ERRα and validating the results using the qPCR technique. Furthermore, the alteration in the relative usage of glutamine or glucose by breast cancer cells in the presence of exogenous cholesterol could be further investigated using stable isotope tracer techniques. In addition, the effect of exogenous cholesterol on lipid metabolism, fatty acid oxidation, and generation of lipid droplets needs furture investigation as these cholesterol effects might be involved in drug resistance in cancer patients [70]. Moreover, despite intensive efforts to elucidate the exact chemical structure of ED, its exact structure remains unknown. Once the exact structure of ED is determined, it can be chemically synthesized and can be used for further biological validation using patient-dervied xenogaft mice models to verify its biological significance and to determine its potentional side-effects using appropriate animal models.

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70. Cruz, A.L.S., et al., *Lipid droplets: platforms with multiple functions in cancer hallmarks.* Cell Death Dis, 2020. **11**(2): p. 105.

# APPENDIX

## Research Compliance Certificates

## Ethics approval for human pregnancy samples

Centre universitaire de santé McGill — McGill University Health Centre

### Annual renewal submission

Submit date: **2020-05-25 12:28**
Project's REB approbation date: **1999-12-09**
Project number(s): **2000-824, 99-038, eReviews_1076**
Form status: **Approved**

Submitted by: **Philip, Anie**
Nagano identifier: **99-038 REC**
Form: **F9-58279**

### Administration

1. **MUHC REB Panel & Co-chair(s):**
   Clinical Trials 2 (CT2)
   **Co-chairs: Bertrand Lebouché,Thomas Maniatis, Sonya Page**

2. **REB Decision:**
   Approved - REB delegated review

3. **Renewal Period Granted:**
   From 2020-06-16 Until 2021-06-15

4. **Date of the REB final decision & signature**
   2020-06-04
   **Signature**
   
   Sheldon Levy
   MUHC REB Coordinator
   for MUHC REB Co-chair mentioned above

5. FWA 00000840 - FWA 00004545

### A. General information

1. **Indicate the full title of the research study**

   Endocriine control of gestation in animals: Structural and Functional Characterization of a novel steroid, Estradioenolone (ED).

2. **If relevant, indicate the full study title in French**

   Not applicable

3. **Indicate the name of the Principal Investigator in our institution (MUHC)**

   Philip, Anie

4. **Are there local co-investigators & collaborators involved in this project?**

   No

5. **Indicate the name and the affiliation of the external collaborator(s),(if any)**

   Not applicable

6. **Identify the study coordinator(s)**

   Finnson, Kenneth

## B. Project development

1. **Study start date:**

   2006-01-01

2. **Expected ending date of the study:**

   ☐ Determined date
   ☑ Undetermined date

3. **Date of recruitment of the first participant?**

   ☑ 1st enrollment date is...
   ☐ No participant enrolled

   **1st participant enrollment date:**

   2006-05-02

4. **Add a brief statement on the study status**

   Ongoing study in progress

5. **Information about the participants at this institution, since the beginning of the project**

**Number of participants to be recruited according to protocol**

500

**Number of participants who have been recruited**

80

**Number of minors**

0

**Number of incompetent adults**

0

**Number of participants who have not yet completed the study (still in progress)**

0

**Number of participants who've completed the study**

80

**Number of participants who were recruited to the study, but who were then excluded or withdrawn:**

0

**Number of participants who dropped out (voluntary withdrawal):**

0

**Number of participants who died during the study**

0

6. **Information about the participants at this institution (MUHC) since the previous REB approval**

**Number of participants who have been recruited**

0

**Number of minors**

0

**Number of incompetent adults**

0

**Number of participants who have not yet completed the study (still in progress)**

0

**Number of participants who've completed the study**

80

**Number of participants who were recruited to the study, but who were then excluded or withdrawn:**

0

**Number of participants who dropped out (voluntary withdrawal):**

0

**Number of participants who died during the study**

0

7. **Since the previous REB approval (annual renewal or initial approval):**

**Were there any changes to the protocol (or to the databank management framework) ?**

No

**Specify the current version/date:**

2019

**Date approved by the REB:**

2019-06-15

**Were there any changes to the information and consent form?**

No

**Specify the current version/date:**

2018

**REB approval date:**

2019-06-15

**Were there any reportable adverse events at this site (or, for multi-center projects, at an institution under the jurisdiction of our REB) that should be reported to the REB under section 5.2.1 of " SOP- REB-404001 " ?**

https://muhc.ca/cae/page/standard-operating-procedures-sops

No

**Has there has been any new information likely to affect the ethics of the project or influence the decision of a participant as to their continued participation in the project ?**

No

**Were there any deviations / major violations protocol (life -threatening or not meeting the inclusion / exclusion criteria)?**

No

**Was there a temporary interruption of the project?**

No

**Have the project results been submitted for publication, presented or published?**

No

**Has the REB been notified of a conflict of interest - (apparent , potential or actual), of one or more members of the research team - that was not known when it was last approved project?**

No

**Do you want to bring any other info to the REB's attention?**

No

8. **Is there a data safety monitoring committee analyzing data on the safety and efficacy of the treatment?**

No

## C. Signature

1. **I confirm that all information is complete & accurate.**

**First & last name of person who completed the submission**

Anie Philip
2020-05-25 12:28

# Internal radioactive user permit

Centre universitaire de santé McGill — McGill University Health Centre

Service de Radioprotection
Radiation Protection Service

## Internal Radioisotope User Permit

CNSC License 01185-13-23.x (consolidated activities)

**Permit Number**
**MGH #0007**

*Any change to the information on this license must be reported to and approved by the Radiation Protection Service.*

### Permit Information

| **Version #** | 2020-01 | **Issued on** (yyyy/mm/dd) | 2020-09-11 | **Expiry** * (yyyy/mm/dd) | 2022-08-31 |
|---|---|---|---|---|---|

### Contacts (PI & Lab manager)

| Name | Email | Office # | Phone # |
|---|---|---|---|
| Dr. Anie PHILIP | Anie.philip@mcgill.ca | C10-148.4 | Ext 44533 |
| Meryem BLATI | bl_mery@hotmail.com | C10-160 | Ext 44535 |

### Authorized Users

See reverse for list of individuals authorized to work under this permit.

### Authorized locations

| Lab Room # | Classification | Use |
|---|---|---|
| C10.160 | Exempted | Laboratory - in vitro |

### Approved Radioisotopes

| Isotope | Max per vial (MBq) | Poss. Limit (MBq) |
|---|---|---|
| H-3 | 37 | 37 |

### Method of disposal

- All radioactive waste (solid and liquid) must be disposed of using the containers provided by McGill Waste Management and brought to the central waste storage room RS1.129
- MyLab inventory procedures must be followed at all times.

### Permit conditions

1. General conditions, see poster RPS-INFO-001

**Radiation Safety Officer:**
**Camille Pacher, Tel: (514) 934-1934 x 43866** **24H Emergency: x 53333**

** This permit is valid until date indicated unless otherwise suspended, amended, revoked or replaced.*

## Authorized Users List – Permit #MGH-0007

Advise RPS of any changes to this list. Up-to-date 2020-09-11.

- ✓ Faegheh GHANBARI
- ✓ Kenneth FINNSON

**Radiation Safety Officer:**
**Camille Pacher, Tel: (514) 934-1934 x 43866** **24H Emergency: x 53333**

** This permit is valid until date indicated unless otherwise suspended, amended, revoked or replaced.*