# Transcriptomic and molecular investigations into the estrogenic activity of enterolactone in MCF-7 breast cancer cells

**A thesis submitted for the degree of**

Doctor of Philosophy

By

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# DECLARATION

The thesis titled **"Transcriptomic and molecular investigations into the estrogenic activity of enterolactone in MCF-7 breast cancer cells''** represents my original research work carried out in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, India, under the supervision of Dr. Anil Mukund Limaye. Sincere efforts have been made to acknowledge contributions from other investigators who helped in conceptualizing and executing the research work. Those who have provided suggestions and technical help have been duly acknowledged. All the research articles and resources used have been cited in the reference section.

27th September 2025

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# CERTIFICATE

This is to certify that the work described in the thesis titled, **"Transcriptomic and molecular investigations into the estrogenic activity of enterolactone in MCF-7 breast cancer cells"** submitted by **Juana Hatwik** (Roll No. 196106032) to the Indian Institute of Technology Guwahati, India, for the award of the degree of Doctor of Philosophy is an authentic record of the research work carried out under my supervision in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, India.

This thesis or any part thereof has not been submitted elsewhere for the award of any other degree or diploma.

27th September 2025

Dr. Anil Mukund Limaye
Thesis Supervisor


*To all who illuminated my path with knowledge*

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# Chapter 1

## Introduction

## 1.1. Introduction

Breast cancer is the most diagnosed cancer, and one of the leading-causes of cancer-associated mortality among women worldwide[1]. Breast carcinogenesis results from a complex interplay between genetic and environmental factors. Numerous genetic alterations or mutations are involved in breast cancer development and progression[2]. Observational and experimental evidences substantiate the importance of nutritional factors in cancer etiology and prevention[3]. Nutrigenomic research demonstrated the ability of several nutrients to modulate gene expression at transcriptional and post-transcriptional levels[4]. Also, several nutritional molecules are involved in cancer prevention and amelioration[5].

Phytoestrogens are bioactive compounds, found in plants. They are classified into isoflavones, coumestans, and lignans[6]. Lignans have attracted considerable interest because they are common constituents of the human diet, which exhibit a wide range of properties including anti-inflammatory, antioxidant, and antitumor properties[7]. The mammalian lignan, enterolactone, is a metabolite produced in the intestine upon microbial metabolism of dietary lignans. Enterolactone is detected in the human serum in nanomolar range[8,9]. The association between lignans intake (or enterolactone levels), and breast cancer is reported in the literature. In cell cultures and animal models, researchers demonstrated that enterolactone may have anti-cancer activity by interfering with tumor invasion, metastasis, angiogenesis, and survival[10].

## 1.2. Aim and scope

Many populations incorporate lignan-rich foods into their regular diets[11]. Based on published data, researchers suggested possible link between lignan intake, or enterolactone concentration and breast cancer risk in numerous cohorts[12–15]. It was found that higher intake of lignans, or higher concentrations of enterolactone in the plasma may be associated with a decrease in breast cancer risk among women. Although the data are inconsistent, six meta-analyses affirmed that the protective effect of enterolactone against breast cancer is more pronounced in post-menopausal women[16–21].

The prolonged exposure to estrogen is one of the most established risk factors of breast cancer[22]. Enterolactone is an estrogen-like compound. It binds to estrogen receptors alpha (ERα) and beta (ERβ)[23], and displays estrogenic or anti-estrogenic effect in breast cancer cells, depending on its concentration[24]. Further, studies have showed several cellular and molecular targets

modulated by enterolactone in breast cancer cells. For instance, enterolactone reduces MDA-MB-231 cell growth by downregulating cell proliferation-related genes, such as Ki67, Proliferating Cell Nuclear Antigen (PCNA), forkhead box protein M1 (FoxM1), Cyclins E1, A2, B1, and B2 ($IC_{50}$ = 261.9 ± 10.5 μM)[25]. Also, 25, 50, and 75 μM enterolactone downregulate N-cadherin and vimentin, and block ERK/NF-κB/Snail signaling pathway exhibiting an anti-metastatic activity in MDA-MB-231 cells[26]. Further, 100 μM enterolactone inhibits cell growth, and suppresses the expression and activity of telomerase in MCF-7 breast cancer cells[27]. At lower concentrations, enterolactone enhances the growth of ERα-positive cells, and stimulates the expression of estrogen target genes[28,29]. Although several molecular targets of enterolactone are known[30], the global transcriptomic effect in breast cancer cells is unaddressed. Furthermore, in the context of breast cancer, the molecular basis of the significant beneficial effect of enterolactone in post-menopausal women is not mechanistically understood.

## 1.3. Objectives

The work presented in this thesis was carried out with the following objectives:

1. To assess the impact of enterolactone on the viability of breast cancer cells.
2. To generate next generation sequencing-based transcriptomic (RNA-seq) data in MCF-7 breast cancer cells.
3. To correlate transcriptomic data with enterolactone's effect on viability.
4. To investigate the mechanism of enterolactone's action in MCF-7 cells, that can explain its protective effect against breast cancer.

# Chapter 2

## Review of literature

## 2.1. Dietary lignans

Dietary lignans are phytoestrogenic, phenolic compounds found in many plants. The main sources of dietary lignans are fiber-rich foods[31], such as cereals, fruits, vegetables, and nuts. Flaxseed is one of the richest sources of lignans[32]. Secoisolariciresinol (SECO) is one of the most abundant dietary lignans[33]. It occurs in a glycosylated form called secoisolariciresinol diglucoside (SDG). SDG content in whole flaxseed ranges between 6.1 and 13.3 mg/g[34]. It is considered as one of the main precursors of mammalian enterolignans namely enterolactone [2,3-bis(3'-hydroxybenzyl) butyrolactone] (EL), and enterodiol [(2R,3R)-2,3-bis(3-hydroxyphenyl) methyl] butane-1,4-diol] (ED). Matairesinol (MAT), pinoresinol, lariciresinol, and isolariciresinol are other lignans found in flaxseed in small quantities[33].

## 2.2. EL production in the human gut

Dietary lignans undergo microbial metabolism in the colon to produce the mammalian enterolignans via several enzymatic reactions (Figure 2.1). These reactions occur in a sequence. First, after SDG intake, sugar groups in SDG are hydrolyzed, converting SDG to SECO[35–37]. The microflora in the colon then converts SECO to ED by dehydroxylation and demethylation. ED thereafter is converted by the microflora to EL. EL is also derived directly from MAT[37]. EL was first isolated and reported in humans by Setchell *et al.* in the early 1980s[38]. It was so named because it is synthesized in the intestine, and possesses γ-butyrolactone moiety in its structure[38,39].

In humans, there are inter- and intra-individual variations in EL levels in the serum. Typically, serum concentrations are in the lower nanomolar range (0–95.6 nmol/L in men, and 0–182.6 nmol/L in women)[40]. EL appears in the blood 8-10 h, and reaches its maximum levels 19.7 ± 6.2 h after lignan intake. Elimination of the conjugated metabolites occurs through either fecal or renal excretion. EL is eliminated in urine after 5 days. *In vivo* studies showed that after lignan consumption, EL is detected in in the liver. Also, it is found in traceable levels in the intestine (especially in the caecum), kidneys, uterus, and prostate[41]. The level of plasma EL is determined by several factors. Plasma EL concentrations are positively associated with alcohol consumption, fiber-rich diet, caffeine intake, and constipation[40,42]. However, the major determinant of enterolignan concentration in plasma is the gut microflora[40]. Antibiotics negatively impact the formation of EL in the intestine[43,44].

## 2.3. Dietary lignans as nutraceuticals

Dietary lignans are bioactive agents, that exhibit a spectrum of health benefits. Dietary lignans and their metabolites are antioxidants, which protect against DNA damage[45]. They are hypoglycemic agents which can lower the incidence of type 2 diabetes[46]. Other biological effects of dietary lignans include anti-fungal, anti-bacterial[47], and cardio-protective[48] properties.

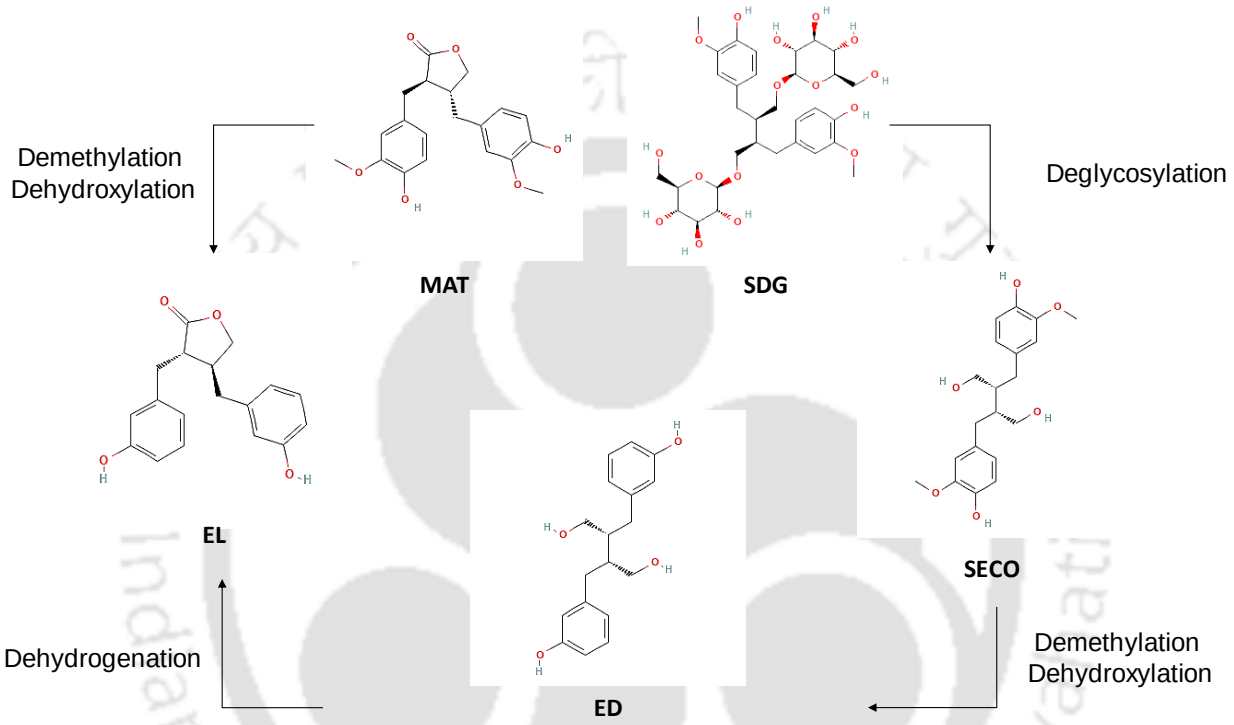


**Figure 2.1. Gut-microbial metabolism of EL.** MAT, matairesinol SECO, secoisolariciresinol, SDG, secoisolariciresinol diglucoside, ED, enterodiol, EL, enterolactone.

Also, lignan may alleviate the conditions associated with menopausal symptoms[49]. Importantly, on their relationship with cancer, dietary lignans were found to prevent tumorigenesis via many mechanisms including antioxidant, anti-inflammation, anti-metastasis, and anti-estrogenic mechanisms[49–51].

## 2.4. Breast cancer

Breast cancer is the second most common cancer among women worldwide. Globally, 2.3 million women were diagnosed with breast cancer in 2022[52]. Breast cancer is classified into four types (Table 2.1) depending on the presence or absence of estrogen receptor (ER), progesterone

receptor (PR), or human epidermal growth factor receptor 2 (HER2)[53]. Molecular classification is useful in prognosis, and for targeted therapy. Luminal A tumors have the best prognosis, and respond to hormonal therapy (tamoxifen or aromatase inhibitor). Luminal B tumors have a poor prognosis compared to luminal A tumors. These tumors benefit from hormone therapy as well as chemotherapy. The HER2-positive subtype is more aggressive than the luminal types, and responds to chemotherapy. Basal-like (triple negative breast cancer) is characterized by its aggressiveness, early relapse, and poor survival. It responds to chemotherapy in neoadjuvant or adjuvant settings[54].

**Table 2.1. Breast cancer classification***

| Molecular subtype | Receptor status | | |
|---|---|---|---|
| | ER | PR | HER2 |
| **Luminal A** | + | + | - |
| **Luminal B** | + | + | ± |
| **HER2-positive** | - | - | + |
| **Basal-like** | - | - | - |

*A Goldhirsch et al[53].

Many factors influence breast cancer development, but estrogen exposure, endogenously or exogenously, is considered the primary determinant of risk[22,55]. The exposure of endogenous estrogen increases in women with early menarche, late menopause, nulliparity or low parity, and late age at first birth. The exogenous exposure to estrogen is due to contraceptives or hormone replacement therapy. Further, the diet as a lifestyle factor, is linked to breast cancer risk[56]. The data suggest that diets rich in fruits and vegetables may reduce the risk of carcinogenesis, likely because of the actions of fibers and polyphenols, which can help in lowering estrogen levels in the bloodstream[57].

## 2.5. Estrogens

Estrogens are endogenous steroid sex hormones. They include estrone (E1), 17β-estradiol (E2), and estriol (E3)[58]. E2 is the most potent estrogen, which regulates the growth and development of the reproductive system, and influences bone growth, protein synthesis, and fat disposition[59]. E2 is produced in the ovaries (Figure 2.2). Androstenedione derived from cholesterol is then converted to testosterone by 17β-hydroxysteroid dehydrogenase. Testosterone, thereafter,

is converted to E2 by the action of aromatase. Estrogen metabolism occurs in the liver where cytochrome P450 family 1 subfamily A member 1 (CYP1A1) and cytochrome P450 family 1 subfamily B member 1 (CYP1B1) convert E2 to 2-hydroxyestradiol (2-OHE2) and 4-hydroxyestradiol (4-OHE2), respectively. Catechol-O-methyltransferase (COMT) converts 2- and 4-hydroxyestradiol to 2- or 4-methoxyestradiol, respectively[60]. Hydroxyestradiol metabolites have genotoxic potential[61], because of their ability to form DNA adducts. Thus, estrogen metabolites may be associated with breast carcinogenesis.

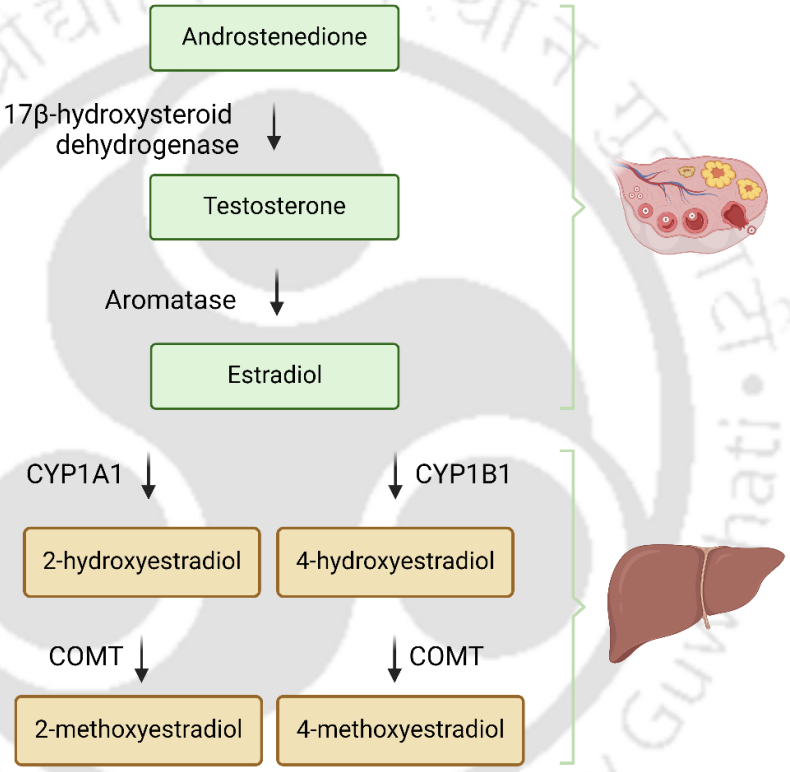


**Figure 2.2. Estrogen biosynthesis and metabolism.**

Although E2 plays important roles in several biological functions, its prolonged exposure is linked to increased breast cancer risk. Estrogens bind to the nuclear estrogen receptors (ERs), which operate as transcriptional factors in the genomic pathway of estrogen signaling (Figure 2.3). Upon ligand binding, ER dimerizes, translocates to the nucleus, and binds to DNA at transcriptional regulatory sequences; the estrogen-responsive elements (EREs), and modulates the expression of target genes[62]. Estrogen also signals via G protein-coupled estrogen receptor 1

(GPER1). Binding of estrogen to GPER leads to cyclic adenosine monophosphate (cAMP) production, intracellular calcium mobilization, and mitogen-activated protein kinase (MAPK) activation. E2-activated GPER1 facilitates matrix metalloprotease (MMP) secretion to activate epidermal growth factor (EGF). EGF then binds Epidermal growth factor receptor (EGFR) resulting in extracellular signal-regulated kinase (ERK) pathway activation[62].

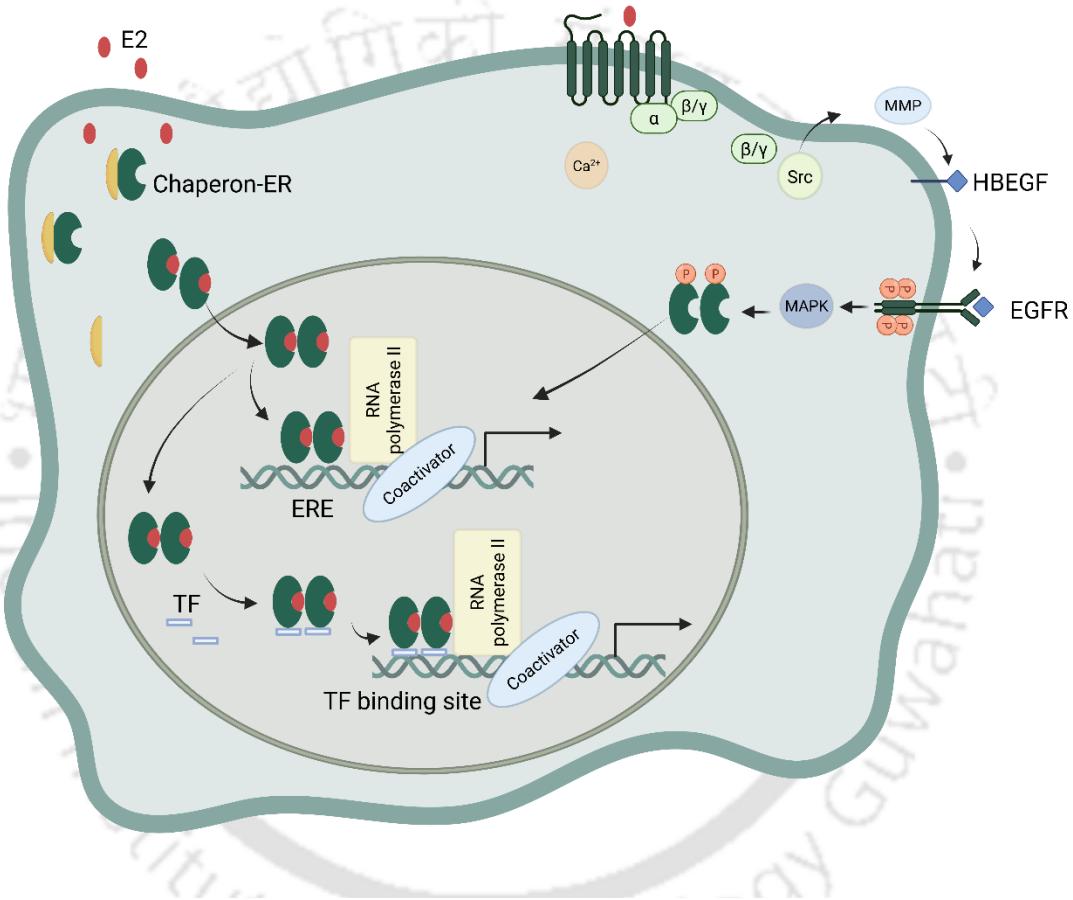


**Figure 2.3. Estrogen signaling**

## 2.6. ER modulatory activity of EL

EL binds ERs, albeit with lower affinity than estradiol[23]. Estrogen responsive reporter constructs revealed that EL, dose-dependently, induces the transcriptional activity of ERα, and to a lesser extent ERβ[63]. EL is less efficient in activating transactivation function (AF-1), and primarily exerts its effects through the transactivation function (AF-2)[64].Additionally, Penttinen et al. demonstrated that ligand binding domain (LBD) is sufficient for EL activity[23]. The required EL's concentration to activate ERα is lower than that required to activate ERβ[23], suggesting EL's

binding preference to ERα. EL is a weak partial agonist/antagonist of ER[65]. Compared to E2 or other phytoestrogens such as coumestrol and zearalenone, EL shows lower efficacy to activate ERα or ERβ[65]. Based on its concentration, EL impacts breast cancer cell proliferation, and induces ERα target genes progesterone (*PR*) and trefoil factor 1 (*TFF1*) in ERα-positive cell lines[28,29]. Those findings were further supported via microarray-based gene expression profiling[29], revealing the estrogenicity of EL. The interaction between EL and ERs has significant implications in the physiological processes, incidence, and progression of breast cancer.

## 2.7. The association between lignan intake or EL and breast cancer

Being nutritional bioactive constituents, dietary lignans have garnered significant interest for their potential in health. The interest stems largely from the health benefits of the mammalian lignan, EL. As a result, EL levels in the plasma or serum are often used as a biomarker of dietary lignan intake[42], serving as a measure to evaluate its potential on health outcomes. From an epidemiological perspective, the association between lignan-rich diets (or EL exposure) and breast cancer has been a focal point of investigation (Table 2.2). Numerous study groups investigated the association between EL concentrations in the urine/serum/plasma and breast cancer (Table 2.2). The epidemiological studies have yielded conflicting results. While several studies supported the inverse association between dietary lignan intake (or EL exposure) and breast cancer, others found no link[66,67], or found negative effect of EL against breast cancer[15,68], highlighting the complexity of this relationship. The inconsistency in the findings may be due to the variations in study design, population characteristics, assessment methods, or other confounding factors that influence lignan metabolism and bioavailability.

## 2.8. Meta-analyses of EL-breast cancer relation

In the light of the inconclusive data on the association between lignan intake (or serum EL) and breast cancer, several research groups conducted meta-analyses to assess their relationship. Higher lignan intake, or serum EL reduced breast cancer risk among post-menopausal women according to Buck *et al.*[21] (risk estimate = 0.86, $P_{heterogenity} = 0.32$), Zaineddin *et al.*[20] (odds ratio = 0.65, $P_{trend} < 0.0001$), and Velentzis *et al.*[18] (odds ratio = 0.85, $P < 0.001$). On the other hand, Seibold *et al.*[17], Liu *et al.*[19], and Die *et al* [16] addressed the relation between lignan intake (or plasma EL) with breast cancer prognosis. The study by Die *et al.*[16] showed that increased pre-diagnostic plasma EL, but not lignan intake significantly reduced the risk of all cause- (hazard ratio = 0.69,

$P_{heterogenity}$ = 0.59) and breast cancer-specific mortalities (hazard ratio = 0.72, $P_{heterogenity}$ = 0.57), particularly among post-menopausal women. Seibold *et al.*[17] found that high EL concentrations were significantly associated with lower all-cause mortality (hazard ratio = 0.94, $p < 0.01$), and breast cancer-specific mortality (hazard ratio = 0.94, $p < 0.02$). Similarly, Liu *et al.* [19], found that higher lignan intake or serum EL had a significant reduction in all-cause (hazard ratio = 0.73, $P_{heterogenity}$ = 0.063), as well as breast cancer-specific mortality (hazard ratio = 0.72) among post-menopausal women. In pre-menopausal women, however, the association was opposite, where increased EL concentrations seemed to increase the risk of all-cause mortality (hazard ratio = 1.57)[19].

All meta-analyses conducted to date have iterated that the beneficial effects of lignans (or EL) against breast cancer are primarily observed in post-menopausal women. This might be attributed to the difference in hormonal profiles between pre- and post-menopausal women. In pre-menopausal women, the production of endogenous estrogen is high, whereas post-menopausal women experience a substantial decline in estrogen levels, with a greater reliance on peripheral estrogen synthesis. The authors suggested that lignans or EL may be more effective in a low milieu of estrogen[18,21].

**Table 2.2. Epidemiologic studies investigating lignan intake or plasma/serum/urine EL concentrations in association with breast cancer risk**

| Study design | Participants | Country | Method of estimating lignan intake or measuring EL | Daily lignan intake or plasma/serum/ urine EL | Findings | Reference |
|---|---|---|---|---|---|---|
| Prospective | Pre- and postmenopausal women without breast cancer | Netherlands | Dietary assessment using FFQ<br>Phytoestrogen intake based on literature* | Median of daily lignan intake (mg/day): 0.67 | Enterolignans did not have a significant impact on breast cancer risk. | 69 |
| Case-control | Pre- and postmenopausal women diagnosed with breast cancer | Canada | FFQ interrogating the diet in the 2 years before recruitment<br><br>Phytoestrogen intake was assessed based on Ontario database | Median of daily lignan intake (µg/day): 857 | Among all women, lignan intake associated inversely and significantly with breast cancer risk.<br>Stratification by BMI showed significant association was observed only among overweight women. | 70 |
| Prospective | Premenopausal women | Sweden | Dietary assessment using FFQ during the 6 months preceding enrollment | Median of daily lignan intake (µg/day): Cohort without cancer: 1632 Cohort with cancer: 1639 | Phytoestrogen intake did not differ between women who developed breast cancer and who did not.<br>Lignan intake was not associated with breast cancer risk. | 71 |
| Case-control | Pre- and postmenopausal women with breast cancer | USA | FFQ about the diet the year prior to diagnosis | Quartiles of daily lignan intake (µg/day): <104<br>104–158<br>159–223<br>≥224 | Phytoestrogen intake was not associated with breast cancer risk. | 72 |
| Case-control | Premenopausal women | Germany | FFQ about the diet in the year prior to diagnosis | Median of daily lignan intake (µg/day) Cases: 570.3 | There was inverse association between matairesinol and premenopausal breast cancer risk. | 73 |

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| | | | Phytoestrogen intake was assessed based on published databases | Controls: 563.1 | High enterolignans were associated with lower risk.<br>The effect was independent of ER status. | |
| Case-control | Pre- and postmenopausal women diagnosed with breast cancer | Canada | FFQ to determine flaxseed intake in last 2 years before recruitment | - | Flaxseed consumption was inversely associated with risk reduction of breast cancer.<br>Based on menopausal status, the effect was observed among postmenopausal but not premenopausal women.<br>The effect was independent of hormone receptors. | 74 |
| Case-control | Pre- and postmenopausal women with breast cancer | Canada | FFQ about the diet 1 year before diagnosis<br><br>Lignan intake was calculated from FFQ based on method by Thompson et al[75] | Mean ± SD of daily lignan intake (µg/day):<br>Premenopausal:<br>Cases:<br>136 ±78<br>Controls:<br>156 ± 84<br><br>Postmenopausal:<br>Cases:<br>157 ± 100<br>Controls:<br>173 ± 109 | Higher lignan intake was associated with substantially reduced odds of having an invasive tumor.<br>Lignan intake was not associated with in situ breast cancer.<br>Higher lignan intake was inversely associated with risk of ER-negative breast cancer among premenopausal women, but was inversely related to ER-positive breast cancer among postmenopausal women.<br>Higher lignan intake was inversely associated with odds of HER2-positive tumors in pre- but not in post-menopausal women.<br>Lignan intake was inversely associated with the risk of triple negative tumors in premenopausal women. | 76 |
| Case-control | Pre- and postmenopausal women with breast cancer | USA | Assessment of dietary intake in 12 -24 months prior to the interviews | Quartiles of daily lignan intake (µg/day):<br>Premenopausal women:<br><329<br>329-472<br>472-673 | Lignan intake was not associated with risk of ER-positive tumors.<br>Among pre- but not postmenopausal women, lignan intake was associated with decreased risk of ER-negative tumors. | 77 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | >673<br>Postmenopausal women:<br><337<br>337–504<br>504-713<br>>713 | | |
| Case-control | Pre- and postmenopausal women with breast cancer, and known *CYP17* genotype | USA | FFQ about the diet 2-years prior to the interview | Range of daily lignan intake (mg/day):<br>0.06 - 2.48 | Significant decrease of breast cancer risk among premenopausal women with at least one *A2* allele for *CYP17* and higher dietary lignan intake. | 78 |
| Case-control | South Asian migrant women newly diagnosed with breast cancer | UK | FFQ about diet 2- 3 years prior to the diagnosis | Quartiles of daily lignan intake (µg/day)<br><85<br>85-128<br>129-235<br>≥236 | There was an inverse trend in breast cancer odds with lignan intake, with moderate evidence of a linear dose-effect response | 79 |
| Prospective | Postmenopausal women | Sweden | FFQ about the diet at recruitment. | Quartiles of daily lignan intake (µg/day)<br><712<br>712-866<br>867-1035<br>≥1036 | There was significant inverse association between lignan intake and breast cancer risk among postmenopausal hormone users | 80 |
| Prospective | Postmenopausal women | France | Pre-diagnosis diet questionnaire | Median (range) of daily lignan intake<br>1112 (0– 5701) µg/day | Lignan intake was inversely associated with breast cancer risk.<br>There was significant inverse association between lignan intake and ER+/PR+ but not for ER-/PR- tumors. | 81 |
| Case-control | Postmenopausal women with primary | Germany | FFQ about nutritional habits one year prior to diagnosis. | Median of EL (µg/day):<br>Cases: 276 | Sesame or flaxseed, were inversely but not significantly associated with breast cancer risk. | 83 |

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|---|---|---|---|---|---|---|
| | invasive or in situ breast cancer | | Lignan intake was calculated using database on phytoestrogen-containing foods. EL, ED were estimated from diet as per Thompson et al[82#] | Controls: 272<br><br>Median of Sesame/flaxseeds (g/day):<br>Cases: 1.42<br>Controls :1.42 | None of the individual plant lignans and enterolignans or overall fiber intake was significantly associated with breast cancer risk.<br>Inverse association between high consumption of sunflower/ pumpkin seeds and breast cancer risk. | |
| Prospective | Postmenopausal women with primary invasive or in situ breast cancer | Germany | Dietary assessment using FFQ after diagnosis. Enterolignans were estimated from diet as per Thompson et al[82#] | Median of EL:(ug/day):<br>Alive: 279.4<br>Deceased: 246.7 | High enterolignans were associated with significantly lower overall mortality independent of ER status. | 84 |
| Prospective | Pre- and postmenopausal women with invasive breast cancer | USA | FFQ one year before the interview | Daily lignan intake<br>0.2 to ≥9 mg/day | There was modest non-significant reduction in all-cause mortality for lignans.<br><br>Dose-response trends were not observed. | 85 |
| Prospective | Pre- and postmenopausal women with breast cancer | Denmark, France, Greece, Germany, Italy, the Netherlands, Norway, Spain, Sweden, and the United Kingdom | Assessment of pre-diagnostic usual diet. Polyphenols were estimated based on Phenol-explorer database | Median of daily lignan intake (mg/day):<br>1.4 | Higher lignan intake was associated with lower risk of breast cancer-specific mortality in postmenopausal women.<br><br>For premenopausal women, higher intake of lignans was significantly associated with higher risk of all-cause mortality and non-significantly with higher risk of breast cancer-specific mortality. | 86 |
| Prospective | Pre- and postmenopausal women with primary | USA | FFQ 12 -24 months before diagnosis. Lignan intake was calculated from a comprehensive | Average ± SD of daily lignan intake (µg/day):<br>Deceased:<br>244.2 ± 134.8 | Among postmenopausal women, lignan intake was inversely associated with all-cause mortality (50% lower HR), and breast cancer mortality (70% lower HR). | 87 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | incident breast cancer | | phytoestrogens database | Alive: 245.1 ± 133.3 | In premenopausal women, no association was observed. | |
| - | Postmenopausal women (omnivorous/ vegetarian/ healthy women with surgically removed breast tumor) | USA | EL was measured in 72 h-urine by capillary gas chromatographic procedure | Geometric mean of EL urinary excretion (µmol/24 h) Omnivores: 2.30 Vegetarians: 3.18 Breast cancer: 1.04 | Fiber intake was associated significantly with enterolignans excretion. Urinary excretion of EL in the breast-cancer group was significantly lower than in both the other groups. | 88 |
| Case-control | Pre- and postmenopausal women newly diagnosed breast cancer | China | Urinary excretion of phytoestrogen before any therapy LC/MS | Median of EL urinary excretion (nmol/mg): Cases: 1.34 Controls: 3.4 | Urinary excretion of lignans or enterolignans were lower in cases compared to controls. Breast cancer risk decreased with the increasing levels of lignans or enterolignans. The inverse association was more pronounced in premenopausal women. | 89 |
| Nested case-control | Postmenopausal women with invasive breast cancer | USA | Urinary phytoestrogen excretion. Pre-diagnostic overnight or a first morning urine specimen. HPLC with electrospray ionization tandem mass spectrometry | Median (mean) of EL urinary excretion (nmol/mg): Cases: 1.32 (2.48) Controls: 1.42 (3.63) | Phytoestrogen excretion was lower among breast cancer cases compared to controls. There was no relation between EL excretion and breast cancer risk. | 90 |
| Case-control | Pre- and postmenopausal women newly diagnosed with breast cancer | Australia | GC-MS of 72 h-urine collection before admission for surgery | Median of EL excretion rates (nmol/24 h) Cases: 1973·4 Controls: 3097·7 | EL excretion was inversely associated with breast cancer risk in both pre- and postmenopausal women. | 91 |
| Nested case-control | Postmenopausal women | Denmark | Urinary phytoestrogen excretion urine was collected 1 year at least before diagnosis. | Median of EL/creatinine excretion (µmol/mol) | Higher urinary EL excretion was weakly and non-significantly associated with an increased breast cancer risk. | 92 |

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|---|---|---|---|---|---|---|
| | | | TR-FIA | Cases: 516.6<br>Controls: 483.9 | | |
| Case-control | Pre- and postmenopausal women who suspected a breast cancer symptoms and community-based controls | Finland | Serum EL before examination<br>TR-FIA | Mean of serum EL (nmol/L):<br>Cases: 20<br>Controls: 26 | Serum EL was associated with breast cancer risk reduction.<br>Higher serum EL was linked with increased consumption of rye products, tea, fibers, and vitamin E. | 12 |
| Prospective | Pre- and postmenopausal women with palpable cysts | Italy | Serum EL on the time of first cyst aspiration, measured by TR-FIA | Mean± SD (median) of serum EL (nmol/L):<br>Women who developed breast cancer: 14.7 ± 4.25 (8.5)<br>Women who did not: 19.8 ± 1.01 (16) | Serum EL levels differed significantly among women who developed breast cancer and women who did not.<br>Higher serum EL was associated with lower breast cancer risk. | 93 |
| Prospective | Pre- and postmenopausal women with palpable cysts | Italy | Serum and intracystic EL measured by TR-FIA | Median (range) of serum EL (nmol/L):<br>17 (0 - 140)<br>Intracystic:<br>63 (0 - 872) | Intracystic EL did not correlate with breast cancer risk.<br>In women with lower intracystic EGF, EL was positively associated with increased breast cancer risk. Whereas, in women with higher intracystic EGF, inverse association was found between EL and risk. | 94 |
| Nested case-control | Pre- and postmenopausal women with invasive breast cancer | USA | Serum EL at enrolment (at least 6 months before diagnosis, measured by TR-FIA | Mean (median) ) of serum EL (nmol/L):<br>Premenopausal women: Cases: 18.3 (13.9), | Premenopausal cases had higher levels of EL than their matched controls.<br>There was a trend of increasing risk with increasing EL levels in premenopausal women, but no association among postmenopausal women. | 15 |

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| | | | | controls: 15.1 (10.9)<br>Postmenopausal women: Cases: 18.6 (14.5), controls 18.9 (14.3) | There was a moderate positive correlation between EL and SHBG which was stronger in controls.<br>There was no correlation between EL and estrogens. | |
| Nested case-control | Postmenopausal women | Denmark | Serum EL at recruitment, measured by TR-FIA | Quartiles of serum EL (nmol/L):<br>0.1-14.4<br>14.5-28.1<br>28.2-47.9<br>48-454.6 | There was breast cancer risk reduction with higher serum EL, restricted to ERα-negative breast cancer. | [95] |
| Nested case-control from 3 cohorts, VIP, MONICA, and MSP | Pre- and postmenopausal women | Sweden | Plasma EL at baseline, measured by TR-FIA | Mean of serum EL (nmol/L):<br>VIP&MONICA cases: 26.8, controls: 22.9<br>MSP cases: 19.3, controls: 20.4 | Low levels of EL were associated with increased breast cancer risk. Also, high EL concentrations in 2 cohorts were associated with increased risk. | [68] |
| Case-control | Premenopausal women | Germany | Plasma EL and genistein after diagnosis, measured by TR-FIA | Median (range) of plasma EL (nmol/L)<br>Cases: 6.3 (0-225.7)<br>Controls: 9.7 (0.1-65.7) | EL levels differed significantly between cases and controls.<br>High plasma EL levels reduced the risk of breast cancer by up to 72%. The effect did not differ by estrogen or progesterone receptor status. Plasma genistein did not have any effect on cancer risk. | [96] |
| Nested case-control | Pre- and postmenopausal women | Finland | Serum EL at the survey examination prior to, measured by TR-FIA | Mean ± SD of serum EL (nmol/L):<br>Cases: 25.2 ±22.2<br>Controls: 24.0 ± 21.3 | No significant association between EL levels and breast cancer risk.<br>High EL non-significantly reduced risk in premenopausal, and increased the risk among postmenopausal women. | [66] |

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| Case-control | Pre- and postmenopausal women | UK | 7-day dietary diaries. Serum or urine phytoestrogens at recruitment, measured by isotope dilution LC/tandem MS | Range: Serum: ND to 388.1 ng/ml Urine: ND to 9790 µg/mmol creatinine | EL concentrations were not associated with differences in risk of breast cancer. Isoflavones, equol and daidzein were associated with increased breast cancer risk. | 67 |
| Case-control | Pre- and postmenopausal women | China | Serum isoflavones and lignans after hospital admission, measured by UHPLC-MS/MS | Mean (range) of serum EL (ng/ml): Cases: 0.73 (ND-46.63) Controls: 1.07 (ND-37.35) | Higher serum concentrations of lignans or EL were significantly associated with lower odds of breast cancer among premenopausal women. The inverse association between EL and odds of breast cancer seemed significant only among ER-or PR-positive women. | 97 |
| Case-control | Pre- and postmenopausal women | Europe | Serum and urine phytoestrogens at recruitment, measured by LC-MS. | Median of serum EL(ng/ml): Cases: 5 Control: 5.83 | EL levels were not associated with the risk of breast cancer. | 98 |
| Nested case-control | Pre- and postmenopausal women | Sweden | Serum EL at baseline measured by TR-FIA | Median (range) of serum EL (nmol/L): Cases:14.5 (0.3-334) Control:16.1 (0.3-115) | Non-smokers, alcohol and high fiber consumers had higher EL levels. High EL levels were associated with decreased breast cancer risk. The risk reduction was only observed for ERα-positive and ERβ-negative tumors. The risk differed significantly between ERβ-negative and ERβ-positive, but not ERα-positive and ERα-negative tumors. | 99 |
| Nested case-control | Pre-and postmenopausal women | USA | Plasma EL before diagnosis, measured by LC-ESI-MS/MS | Median of plasma EL (nmol/L): Cases:11 Controls:11 | EL levels were not associated with risk reduction. The effect did not change according to ER or PR status. There was inverse association between EL concentrations and breast cancer risk among women with low follicular estradiol levels. No interactions were observed by BMI, or menopausal status. | 100 |

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| Nested case-control | Pre-or perimenopausal and postmenopausal women | Denmark | Plasma phytoestrogens at recruitment, before diagnosis, measured by isotope dilution LC/tandem MS | Median (range) of plasma EL (nmol/L): Pre- and Perimenopausal cases 2.98 (0.15-36.77), controls 3.07 (0.09-37.57) Postmenopausal cases 2.95 (0-57.77), control 2.99 (0-52) | No association between EL and breast cancer occurrence. Non-significant trend of increasing breast cancer risk with the circulating EL in pre- and perimenopausal women. | 101 |
| Case-control | Postmenopausal women | Germany | Serum EL after diagnosis, measured by TR-FIA | Median of serum EL (nmol/L): Cases: 19.5 Control: 22.8 | EL levels were significantly associated with breast cancer risk reduction. This involved ER-positive as well as ER-negative tumors. The association was stronger in ER-negative tumors, and did not differ by PR or HER2 status. Strong inverse association for combined ER-/PR-negative tumors compared to ER-/PR-positive tumors. | 20 |
| Prospective | Postmenopausal women with invasive or *in situ* breast tumors | Germany | Serum EL post diagnosis, measured by TR-FIA | Median of serum EL (nmol/L): Deceased: 17.0 Alive: 21.4 | Higher serum EL levels significantly reduced hazard ratio for overall mortality. Higher serum EL was associated with a significantly reduced risk of death only for ER-negative tumors. | 102 |
| Retrospective cohort | Pre-and postmenopausal women with breast cancer who were operated on | Italy | Before surgery serum EL, measured by TR-FIA | Median (range) of serum EL (nmol/L): 20 (1- 145) | Women with EL levels ≥10 nmol/l had lower probability of all-cause and breast cancer-specific mortality. The protective effect was evident in postmenopausal women, in those with tumors ≥ 2 cm, in node-negative women, and in those who not receiving adjuvant chemotherapy. | 13 |

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| Prospective cohort | Postmenopausal women without previous cancer diagnosis | Denmark | Pre-diagnostic plasma EL, measured by TR-FIA | Median of plasma EL (nmol/L):<br>Alive: 29<br>Dead: 19 | Compared to deceased women, alive women had higher plasma EL at baseline, more often have low than high tumor grade, more often be diagnosed with an ER-positive tumor, were more often HRT users at baseline, had a smaller tumor, and were more likely to be free of positive lymph nodes at diagnosis.<br>Higher EL was associated with lower all-cause as well as breast cancer-specific mortality.<br>The association did not differ by ER status. | 103 |
| Prospective cohort | Postmenopausal women diagnosed with primary breast cancer | Germany | Post-diagnostic serum or plasma EL, measured by TR-FIA | Median of serum or plasma EL (nmol/L):<br>Deceased: 17.4<br>Still alive: 22.9 | Women with higher EL were diagnosed with tumors smaller in size, lower grade, ER-positive, elder, used tamoxifen, not smoking, and did not have chemotherapy.<br>There was an inverse association between serum EL and all-cause mortality, only in those with early but not advanced disease.<br>Hormone receptor status, smoking, fiber intake and HRT did not bring any significant difference. | 17 |
| Prospective cohort | Postmenopausal women with primary invasive breast cancer or in situ carcinoma | Germany | Post-diagnostic serum or plasma EL, measured by TR-FIA | Median (mean) of serum or plasma EL (nmol/L):<br>Still alive: 23.2 (37.2)<br>Deceased: 17.5 (26.4) | EL concentrations were inversely associated with decreased all-cause mortality, breast cancer specific mortality, and distant disease-free survival.<br>The effect was mediated by CRP. | 104 |
| Prospective cohort | Peri- or postmenopausal women with primary invasive or *in situ* carcinoma | Germany | Post-diagnostic serum or plasma phytoestrogens, measured by TR-FIA | Median of serum or plasma EL (ng/ml):<br>At baseline: 6.7<br>At follow up: 3.6 | EL levels were inversely associated with all-cause and breast cancer-specific mortality up to 4 years post-diagnosis blood draw. | 105 |

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| Prospective cohort | Postmenopausal women | Denmark | Pre-diagnostic and at diagnosis plasma EL, Measured by LC-MS/MS | Median of plasma EL (nmol/L):<br>Pre-diagnosis:<br>All women: 20<br>ACM: 16<br>BCSM: 17<br>Recurrence: 19<br>At-diagnosis:<br>All women: 19<br>ACM: 24<br>BCSM: 22<br>Recurrence: 16 | No association between high pre-diagnostic EL levels and breast cancer prognosis.<br>The association was significant for women who never used hormones or who smoked.<br>Significant inverse association with all-cause and breast cancer-specific mortality for those with 5 years between blood sample and diagnosis only. | 14 |
| Case-control | Women with breast cancer | Sweden | Plasma EL was measured by TR-FIA | Median of plasma EL (nmol/L):<br>Cases: 14.6<br>Controls: 16.3 | There was a tendency for interaction between polymorphism (rs2347867) in *ESR1* and EL concentrations.<br>EL levels and breast cancer risk were inversely associated among carriers of the minor allele AG and GG. | 106 |

FFQ, food frequency questionnaire, TR-FIA, time-resolved fluorescence immunoassay, LC-MS, Liquid chromatography-mass spectrometry. LC-ESI-MS/MS, liquid chromatography electrospray ionization tandem mass spectrometry. UHPLC-MS/MS, ultra-high-performance liquid chromatography-tandem mass spectrometry. BMI, body mass index, HER2, human epidermal growth factor receptor 2, ER, estrogen receptor, PR, progesterone receptor, EGF, epidermal growth factor. SHBG, sex hormone binding globulin. CRP, C-reactive protein. HR, hazard ratio, ND, not detected. VIP, Västerbotten Intervention Project, MONICA, Monitoring of Trends and Cardiovascular Disease study, MSP, The Mammary Screening Project. HRT, hormone replacement therapy.

* Phytoestrogen content was determined based on published laboratory data on the phytoestrogen contents of relevant food items Enterolignans food content values are based on *in vitro* production of EL and ED from certain foods according to published databases.

[#] Enterolignan production from different food items is determined based on *in vitro* fermentation with human fecal microbiota, which simulates colonic fermentation. The bioavailable enterolignans are measured and expressed in $\mu$g per 100 g of ingested foods.

## 2.9. *In vivo* and *in vitro* data on the association between EL and breast cancer

Based on findings from *in vivo* and *in vitro* model systems, authors put forward several mechanisms of EL action in the context of breast cancer. (Tables 2.3 and 2.4, respectively).

Animal models were used to investigate the relation between flaxseed (or its metabolite, EL) and breast cancer (Table 2.3). These studies evaluated the effect of flaxseed diets or EL administration on both human cancer cell xenografts, and chemically-induced tumors in rodent models. Flaxseed delays the development of breast tumors, and reduces tumor incidence as well as tumor burden[107]. It reduces cell proliferation and nuclear aberrations in the mammary glands in rats with established tumors[108,109]. 10% flaxseed diet diminishes the growth of MCF-7 tumor in athymic mice[110–112], and increases MCF-7 tumor regression[113]. Further, flaxseed enhances tamoxifen effect in athymic mice bearing MCF-7 xenograft.

*In vitro* studies proposed potential cellular and molecular targets of EL in breast cancer cells (Table 2.4), including downregulating cell proliferation-related genes, exhibiting an anti-metastatic activity, inhibiting cell growth, and suppressing the expression and activity of telomerase in breast cancer cells. Although the data showed that at high concentrations, EL suppresses proliferation, metastasis, and migration, at lower concentrations, EL exhibits estrogenic behavior, that it enhances the growth of ERα-positive cells, and stimulates the expression of estrogen target genes (Table 2.4).

While *in vitro* data reported the estrogenic behavior of EL, most *in vivo* findings did not support this observation. Generally, after EL administration in animal models, no uterotrophic effect or increased epithelial thickening is observed. However, Penttinen *et al.*[23] demonstrated that EL stimulates uterine stromal edema, and induces E2-responsive genes Cyclin D1, and Ki67.

## 2.10. Cytochrome P450 enzymes

Cytochrome P450 enzymes (CYPs) are membrane-bound heme-proteins responsible for detoxification, xenobiotic metabolism, and hormone biosynthesis. CYPs are expressed in many organs, playing a significant role in drug activation or deactivation, and carcinogenesis[114]. CYPs include 18 families, and 43 subfamilies[115]. CYP enzymes are transcriptionally activated in a receptor-dependent manner upon the exposure to exogenous or endogenous substrates.

**Table 2.3. *In vivo* studies on the association between lignans or EL and breast tumor-bearing animals.**

| Animal model | Intervention | Effect | Reference |
|---|---|---|---|
| Sprague-Dawley female rats | Flaxseed meals for 4 weeks followed by a single dose of DMBA for 24 h | Flaxseed reduces cell proliferation and nuclear aberrations in the mammary glands. | 109 |
| Female Sprague-Dawley rats with DMBA-established tumor | Flaxseed diet after 13 weeks of DMBA administration | Flaxseed regresses the growth and lowers the volume of the established tumors in the rats compared to control | 108 |
| Rats | N-methyl-N-nitrosourea (MNU) was injected. 2 days later, rats were fed flaxseed for 22 weeks | Flaxseed can delay MNU-established tumorigenesis with no effect on tumor multiplicity, size or incidence. | 107 |
| Ovariectomized athymic mice, injected with MCF-7, treated with E2 | 10% flaxseed or daily s.c. injections with enterolactone/ enterodiol (15 mg/kg body weight) for 3 weeks | Flaxseed and its lignans counteract the promoting effects of E2 on growth and angiogenesis in breast cancer via decreased secretion of the E2-induced VEGF. | 112 |
| Ovariectomized athymic mice with MCF-7 xenograft with or without E2 implant | 10% flaxseed diet with or without tamoxifen for 12-14 weeks | At low or high levels of E2, flaxseed inhibits the growth of human MCF-7 in nude mice.<br>It enhances the inhibitory effect of tamoxifen.<br>The mechanism involves decreased cell proliferation and increased apoptosis | 111 |
| Ovariectomized athymic mice with MCF-7 xenograft with E2 implant | Basal diet, 5 %, or 10% flaxseed diet with or without tamoxifen for 8 weeks | 5 and 10% flaxseed diets reduce tumor growth.<br>Combination of tamoxifen with flaxseed inhibits tumor growth better than tamoxifen alone or flaxseed alone.<br>Tamoxifen with flaxseed shows a higher expression of ERα especially compared to tam or flax alone. | 110 |

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| | | Flaxseed with or without tamoxifen reduces PgR expression compared with the control.<br><br>10% flaxseed diet reduces IGF-1 expression, with no significant difference in ERβ or cyclin D expression.<br><br>FS inhibits MCF-7 tumor growth in a dose-dependent manner and enhances the inhibitory effect of tamoxifen due to the modulation of ER and growth factor signal transduction pathways. | |
| Ovariectomized athymic mice with MCF-7 xenograft | Basal diet, 5 %, or 10% flaxseed diet with or without tamoxifen for 16 weeks | 5 and 10% flaxseed diet inhibits MCF-7 tumors in mice.<br><br>Flaxseed with tamoxifen reduces the expression of ERα, cyclin D1, HER2, and IGF-IR compared to tamoxifen alone. | 116 |
| Athymic mice with MDA-MB-453 xenograft | 10% flaxseed diet for 7 days before the xenograft, and 15 weeks post the xenograft | Flaxseed reduces primary tumor growth and lowers the lymph node metastatic incidence.<br><br>Flaxseed diet lowers the expression of Ki67, IGF-1, and EGFR. | 117 |
| Female Sprague Dawley rats treated with or without N-methyl-N-nitrosourea (MNU). | 5% flaxseed or 1.5 mg SDG/day for 4 weeks | Flaxseed or SDG can reduce plasma levels of IGF-I in rats treated with or without MNU.<br><br>Urinary lignan excretion is inversely related to plasma IGF-I concentrations. | 118 |
| Ovariectomized athymic mice with established MCF-7 tumors | Basal diet with daily injections of either enterolactone (10 mg/kg BW)<br><br>enterodiol(10 mg/kg BW), genistein (10 mg/kg BW), EL (3.33 mg/kg BW) + ED (3.33 mg/kg BW) + GEN (3.33 mg/kg BW), or vehicle (negative control) for 22 | Unlike GEN, EL and END (10 mg/kg BW), do not have growth-promoting effects.<br><br>Mammalian lignans increase tumor cell apoptosis. | 119 |

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| | weeks. | | |
| Ovariectomized athymic mice with MCF-7 xenograft<br><br>Low conc. of E2 | daily subcutaneous injections of EL (10 mg/kg BW), ED (10 mg/kg BW), GEN (10 mg/kg BW), EL (3.33 mg/kg BW) + ED (3.33 mg/kg BW) + GEN (3.33 mg/kg BW) (MIX), or vehicle for 22 weeks | EL alone does not affect uterus weight, but the combination with GEN results in a significantly larger uterus weight. | 120 |
| DMBA-induced female Sprague Dawley rats | 1 or 10 mg/kg EL administered daily (For 50 days) p.o. starting 9 weeks after the DMBA-induction. | EL at 10 mg/kg significantly reduces the total tumor volume during the 7-week period.<br><br>The inhibition of tumor growth is more pronounced in tumors that developed during the 7-week EL treatment period compared to tumors established before the start of the treatment.<br><br>EL increases the proportion of non-growing tumors and reduces the relative uterine weight.<br><br>EL is a weak inhibitor of aromatase in vivo. | 121 |
| Athymic mice with E2 implant and MCF-7 xenograft | Basal diet (BD)<br><br>BD + 100 mg/kg GEN, BD + 100 mg/kg EL, or their combination. | EL but not GEN inhibits E2-induced tumor growth and angiogenesis.<br><br>EL alone or in combination with GEN decreases the micro vessel areas.<br><br>EL but not GEN decreases extracellular cancer cell and stroma-derived VEGF and increases cancer cell-derived placenta growth factor.<br><br>EL and EL+GEN decrease E2-induced endothelial cell infiltration.<br><br>EL with GEN exhibits an inhibitory effect | 122 |

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| | | on the E2-stimulated VEGF secretion. | |
| Dimethylbenz[a]anthracene induced mammary cancer in rats.<br><br>Human MCF-7 breast cancer xenografts in athymic mice with E2 implant. | Control, Lariciresinol, p.o. daily 3 or 15 mg / kg body weight for 9 weeks.<br><br>BD or BD with Lariciresinol 20 or 100 mg/kg for 5 weeks. | Lariciresinol inhibits the growth of DMBA-induced tumors, especially in tumors that developed during the treatment period than those established already before the start of the treatments.<br><br>Lariciresinol (100 mg/kg) reduces MCF-7 tumor in mice.<br><br>Lariciresinol reduces micro vissel density in MCF-7 tumor-bearing mice.<br><br>Dietary lariciresinol increases the expression indices of ERβ and PR in the MCF-7 tumor tissue. | 123 |
| Ovarieoctomized athemic mice | Basal diet containing 100 mg/ kg EL or 100 mg/ kg genestein or 10% flaxseed. | EL and flaxseed reduce tumor growth and micro vessel density.<br><br>EL and flax like tamoxifen increase IL-1 receptor antagonist (IL-1Ra). | 124 |
| Female C57BL/6J mice bearing 3xERE-TATA-Luc transgene | Mice were injected ip with either 1 mg/kg 17β- estradiol dipropionate EP or 10 mg/kg EL for 12 or 24 h. | EL-induces significant luciferase expression only in the uterus and vagina with no edema, mitotic figures, or epithelial thickening.<br><br>EL significantly induces expression of Ki67 only on glandular epithelium<br><br>EL is a partial ER agonist with issue and possibly cell type-specific activity. | 23 |
| OVX athymic mice with tamoxifen implant | Basal diet alone or supplemented with SDG (1 g/kg in diet) or flaxseed oil (38.5 g/kg diet) or their combination for 8 weeks. | SDG and flaxseed oil regress palpable tumors in size compared to control group (tamoxifen alone).<br><br>Flaxseed oil and SDG combined with tamoxifen increase the apoptosis. | 125 |

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| | | SDG and flaxseed oil reduce PGR and cyclin D1 mRNA and ERα protein. | |
| Sprague Dawley rats with lignan-converting bacteria and DMBA-induced tumor. | Flaxseed-rich diet containing 0.34 g/kg SDG for 2 weeks before tumor induction. | Flaxseed does not affect cancer incidence.<br>Flax decreases tumor burden, size, and proliferation, and increases cell apoptosis.<br>No difference in the expression of genes encoding ERs, GPER, IGF-1 or EGFR. | 126 |
| Female athymic nude mice with MDA-MB-453 xenograft | Basal diet (BD), or BD supplemented with 10% flaxseed, or 0.2 g/kg SDG, or 36.53 g/kg flaxseed oil (FO), or SDG + FO for 6 weeks. | No estrogenic effect on the hormone-sensitive organs.<br>Tumor growth rate and cell proliferation decreased while the apoptosis increased in FS, FO, SDG+FO groups.<br>Metastasis reduced significantly in SDG+FO group. | 127 |

MNU: N-methyl-N-nitrosourea, DMBA, Dimethylbenz[a]anthracene, FO: flaxseed oil, FS, flaxseed, BD, basal diet, SDG, secoisolariciresinol diglucoside, GEN, Genistein, EGFR, epidermal growth factor receptor, IGF-1, insulin-like growth factor 1, VEGF, Vascular endothelial growth factor, BW, body weight, p.o. orally, s.c. subcutaneous, EL, enterolactone, ED, Enterodiol.

**Table 2.4. *In vitro* studies investigating the effect of EL in several human breast cancer cell lines.**

| Model system | EL concentration | Effect | Molecular target/ pathway affected | Reference |
|---|---|---|---|---|
| MCF-7, T47D | 10 μM | Estrogenic activity<br>Enhancing proliferation | ↑ PR | 128 |
| MCF-7 | 1 μM | Estrogenic effect | ↑ TFF1 | 28 |
| MCF-7 | 10 μM<br>for 72 h | Enhancement of cell proliferation | ↑ Erk1/2 and PI3K/Akt pathways<br>↑ CDK4, cyclin D1, cyclin E<br>↑ monocyte chemoattractant protein-1 (MCP-1) | 29 |
| MCF-7 | 0.5-50 μM<br>for 8-10 days | 0.5 and 10 μM EL: stimulation of cell growth.<br>>10 μM: growth inhibitory effect.<br>0.5 / 10 μM + 1 nM E2: reduced growth compared to each compound alone | Competition of EL and its sulfate with the estrogens for sulfokinases and sulfatases involved in estrogen metabolism.<br>Competing E2 for aromatase binding | 24 |
| MCF-7<br>MDA-MB-231 | 1 μM or 10 μM<br>for 48 h | Decrease in cell viability and survival | ↓ NF-κB activity<br>↓ *Csf2*, *Mmp9*, and *Tnf* | 129 |
| MDA-MB-231 | 25,50,75 μM at 24, 48 and 72 hrs | Anticancer, cytotoxic/ antiproliferative antimetastatic, antimigratory, and Anticlonogenic. | ↓ urokinase-type plasminogen activator (uPA) expression<br>↑ uPA inhibitor (PAI-1) | 130 |

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| | | | ↓ MMP-2 and MMP-9<br>↑ TIMP-1 and TIMP-2 | |
| T47D<br>MDA-MB-231 | (1, 10, 50, 100, 200 and 500 µM) for 24, 48 and 72 h. | Decreased cell viability<br>($IC_{50}$ at 72 h: 201, 112 µM for MDA-MB-231 and T47D, respectively)<br>Enhancing radio-sensitivity | ↓ DNA repair<br>↑ radiation-induced apoptosis<br>↑ chromosomal damages and aberrations | 131 |
| T47D-KBluc | 1-1000 µM<br>for 24 h | Estrogenic/antiestrogenic activity | ↑ pS2, PR at 10 µM | 132 |
| MCF-7 | 10 µM<br>for 3, 5 days | No significant effect on cell proliferation | ↑ ERα transcriptional activation | 63 |
| MCF-7 | 100 µM for 48 h | Inhibition cell viability<br>Reduced telomerase activity | ↓ human telomerase reverse transcriptase catalytic subunit hTERT protein | 27 |
| MDA-MB-231 | 25, 50, 100 µM | Anti-proliferative<br>Inhibiting migration and invasion | Accumulation of cells in the S phase<br>↓ Ki67, PCNA, FoxM1<br>↓ Cyclin E1, Cyclin A2, Cyclin B1, and Cyclin B2 genes<br>↓ phosphorylation of the FAK/paxillin pathway | 25 |

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| MDA-MB-231 | 25, 50, 75 µM | Anti-metastatic effect | ↓ N-cadherin and vimentin<br>↑ E-cadherin and occluding<br>↓ MAPK-p38), CD44<br>↓ ERK-1/2, NF-κB, and Snail | 26 |
| MDA-MB-231 | 25, 50 µM | Anti-metastatic activity | ↓ MMP2, MMP9 and MMP14<br>↓ cell adhesion, cell invasion and cell motility | 133 |
| MCF-7 | 1-50 µM | Inhibition of E2 synthesis | ↓ E1 production via aromatase pathway (10 µM EL)<br>↓ E2 production via 17_HSDβ type 1 (50 µM EL) | 134 |

PR: progesterone receptor, pS2: trefoil factor 1, Erk1: extracellular signal-regulated kinase 1, PI3K/Akt: Phosphatidylinositol 3-kinase/protein kinase B , CDK: Cyclin-dependent kinases, MMP: matrix metalloproteinase, TIMP: tissue inhibitors of metalloproteinases, PCNA: proliferating cell nuclear antigen,AMPK: AMP-activated protein kinase, FAK: focal adhesion kinase, hTERT: human telomerase reverse transcriptase, NF-κB: nuclear Factor-kappa B, MCP-1: monocyte chemoattractant protein-1, uPA: urokinase-type plasminogen activator, 17β-HSD: 17β-hydroxysteroid dehydrogenase, Csf2: colony-stimulating factor 2, Tnf: tumor necrosis factor, FoxM1: forkhead box protein M1.

Regulation of the expression of CYP enzymes plays a principal role in influencing the drug pharmacokinetic response by altering its action, safety, or bioavailability. Expression of CYPs differs among individuals depending on several factors such as diet, drug, tobacco, and alcohol consumption[135].

## 2.11. CYP1A1 and AHR signaling

CYP1A1 is a phase I, heme-containing monooxygenase, involved in metabolizing wide range of drugs and endogenous substrates, including steroid hormones, vitamins, and fatty acids[136]. It is found mostly in the extrahepatic tissues. Environmental chemicals and pollutants are substrates for CYP1A1. CYP1A1 represents a downstream target of aryl hydrocarbon receptor (AHR) signaling (Figure 2.4). AHR is a ligand-activated transcription factor, found in the cytoplasm with a heat shock protein 90, a chaperon p23, and an immunophilin-related protein; XAP-2. Upon ligand binding, AHR undergoes conformational changes, and translocates from the cytosol to the nucleus, dissociating from other proteins, to dimerize with aryl hydrocarbon receptor nuclear translocator (ARNT). The ligand-bound AHR-ARNT complex then binds with the xenobiotic regulatory elements (XREs) located in the prompter region of AHR target genes such as CYP1A1, CYP1A2, and CYP1B1. The transcription of these genes is suppressed by the activity of aryl hydrocarbon receptor suppresser (AHRR), which binds ARNT, and inhibits the transcription of target genes[136].

## 2.12. CYP1A1 and cancer

CYP1A1 protein is found in the endoplasmic reticulum, and made up of 512 amino acids, and has a molecular weight of 58.1 kDa. CYP1A1 protein is a short-lived protein with a half-life of ~2.8 h[137]. Its expression is induced by activating AHR signaling pathway upon the exposure to polycyclic aromatic hydrocarbons, heterocyclic amines, and halogenated biphenyls[136]. Moreover, several pathways such as Wnt/$\beta$-catenin and estrogen receptor signaling pathways[137,138], were found to modulate CYP1A1 expression by interacting with AHR signaling. CYP1A1 is involved in procarcinogen activation, and is important in detoxification[136]. CYP1A1 was identified as one of the candidates which play a role in breast tumorigenesis. It metabolizes E2 into the oxidative product 2-hydroxyestradiol (2-OHE2)[139], which causes DNA damage, and development of breast cancer[140]. In addition, CYP1A1 polymorphisms affect the susceptibility of breast cancer[141]. Several CYP1A1 polymorphisms were studied concerning their association with breast cancer

risk[142]. They may alter the enzyme function and activity, leading to changes in the level of reactive metabolites which cause DNA damage[143]. Further, CYP1A1 expression increases in response to the exposure to environmental pro-carcinogenic compounds[137]. Also, it is elevated in malignant breast tumors compared to the adjacent ones[144]. As described by Rodriguez *et al.*[145], experimental depletion of CYP1A1 reduces cell proliferation, and enhances apoptosis. These data indicate that beside the role of CYP1A1 protein in xenobiotic metabolism, it regulates breast cancer cell proliferation.

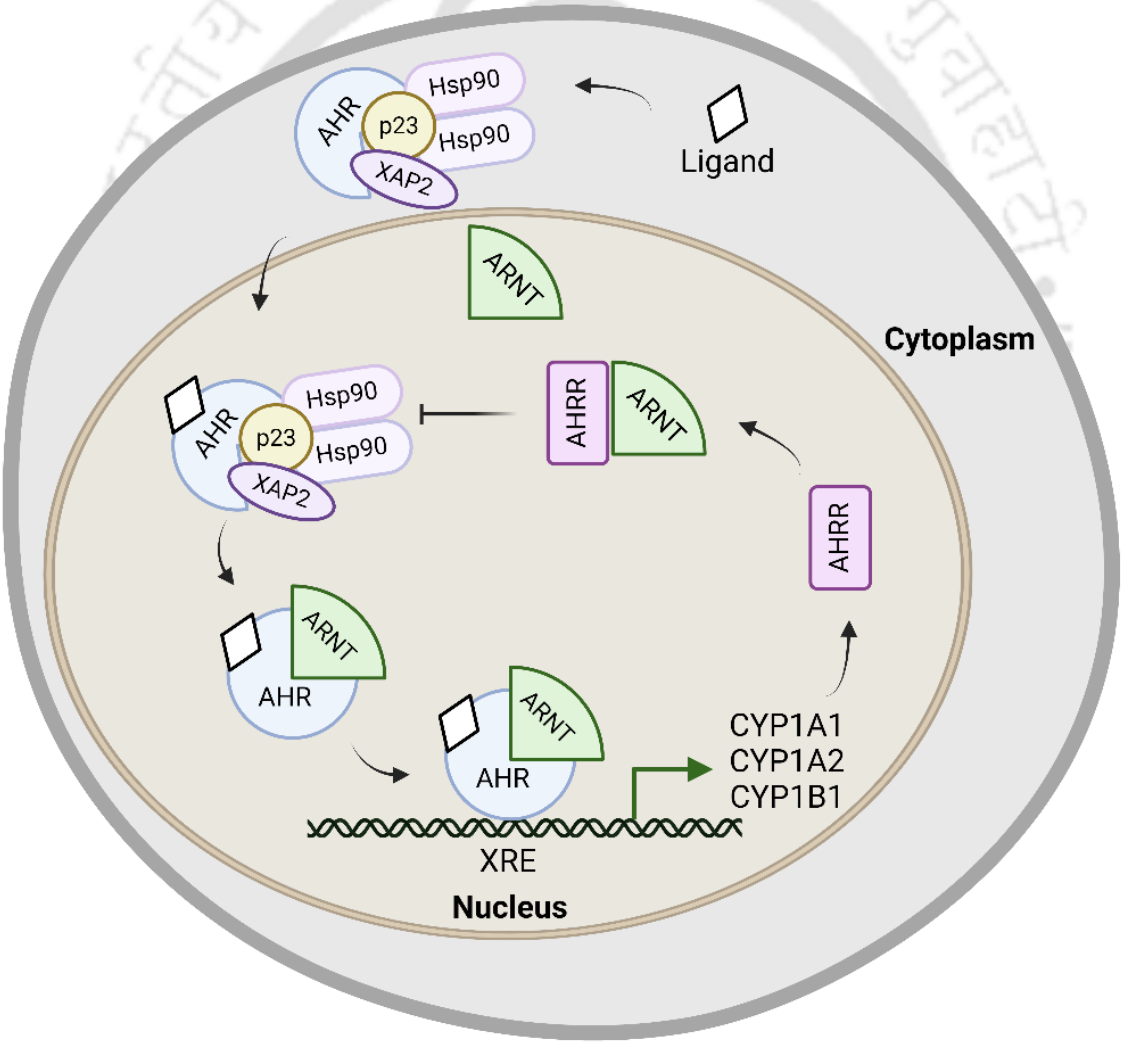


**Figure 2.4. AHR signaling**

## 2.13.ER-AHR crosstalk

ER and AHR are transcriptional factors. E2 exhibits its action by binding to ERα, whereas the action of dioxins such as 3-methylcholanthrene (3MC) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is mediated by AHR. E2 interferes with AHR signaling, down-regulating CYP1A1, the AHR downstream target gene. The mechanism was demonstrated by Marques *et al.*[138], Kharat *et al.*[146], and Beischlag *et al.*[147]. E2-liganded ERα inhibits AHR signaling by direct protein-protein interaction with AHR-ARNT complex, repressing the transcription of AHR downstream targets[147]. Additionally, Marques *et al.*[138] illustrated an involvement of potential epigenetic mechanism of CYP1A1 regulation by E2. They found that E2-bound ERα recruits Dnmt3b to the CYP1A1 locus, increasing the methylation in the promoter region. This, in turn, hinders AHR from binding to the regulatory sequence of CYP1A1, preventing its transcription (Figure 2.5). Alternately Ohtake *et al.*[148] put forth the mechanism of the modulation of ER signaling by AHR activation. The authors found that in the absence of E2, the ligand-activated AHR/ARNT complex can stimulate ER transactivation function, while in the presence of E2, it attenuates the E2-mediated activity of ER. The data revealed that the ligand-bound AHR/ARNT heterodimer recruits histone acetyltransferase containing p300 co-activator, and directly interacts with the unliganded ER. This causes binding of unliganded ER to ERE in the estrogen responsive gene promoters such as *TFF1* and *c-fos* leading to estrogenic response (Figure 2.5).

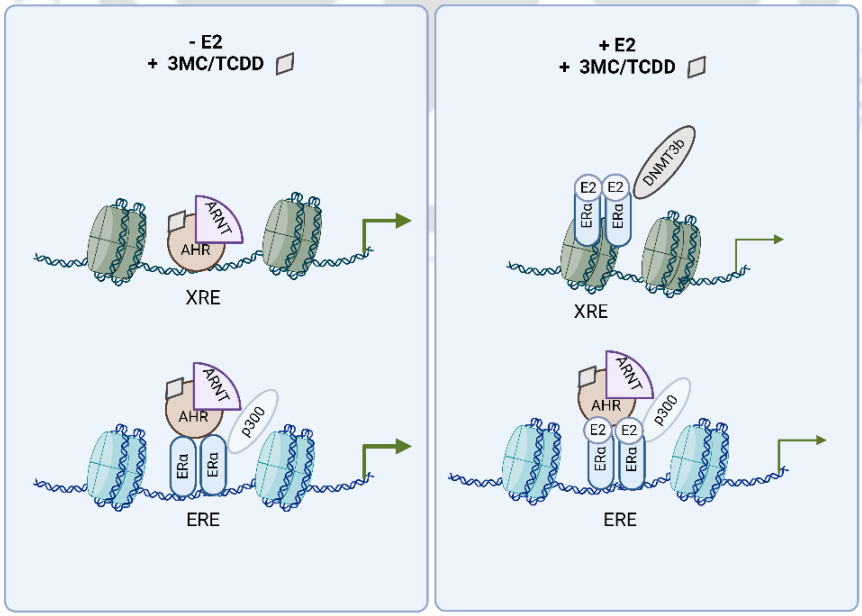


**Figure 2.5. The crosstalk between ERα and AHR signaling**

# Chapter 3

## Materials and methods

## 3.1. Plasticware, chemicals, and reagents

Details of the plasticware, chemicals, and reagents used in this research are provided in Appendix I.

## 3.2. Cell culture and treatments

### 3.2.1. Cell lines and cell culture

MCF-7, T47D, MDA-MB-231, and MDA-MB-453 breast cancer cell lines were procured from the National Centre for Cell Sciences, Pune, India. For routine expansion, the cells were grown in DMEM (for MCF-7) or RPMI-1640 (for T47D, MDA-MB-231, and MDA-MB-453) supplemented with 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin (M1 medium). For experiments involving treatment with E2, 4-hydroxy-tamoxifen (4OHT), or EL, phenol red-free DMEM or RPMI-1640, supplemented with 10% CS-FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin (M2 medium) was used. The cells were cultured under standard humidified conditions of 37 °C and 5% $CO_2$.

### 3.2.2. Sub-culturing and seeding

When the monolayer of the cells became 90% confluent, the cells were washed with DPBS, and trypsinized for 2-3 min at 37 °C. Thereafter, 1 ml of M1 was added to inhibit trypsin. 20 µl of cell suspension was mixed with 20 µl of 0.4 % trypan blue dye, and viable cells were counted using a hemocytometer. Seeding density was determined based on the experiment and duration of treatment.

### 3.2.3. Experimental protocols

- **Dose-response study**

  $2 \times 10^5$ cells were seeded in 35 mm dishes in M1. After 50% confluent, cells were washed with DPBS and fed with M2 for 24 h. Thereafter, the cells were treated with 0.1% DMSO (vehicle control), or indicated concentrations of E2 for 24 h, or EL for 72 h, respectively; with the treatment medium being replenished every 24 h.

- **Time-course study**

  $2 \times 10^5$ cells were seeded in 35 mm dishes in M1, and grown up to 50% confluency. The cells were washed with DPBS and fed with M2 for 24 h. The cells were then treated with 0.1%

DMSO (vehicle control), 10 nM E2, or 10 µM EL in M2 for the indicated period of time. Cells received fresh treatment medium every 24 h.

- **Effect of 4OHT on EL-mediated suppression of CYP1A1 mRNA**

Cells were grown up to the desired confluency as above. The cells were then maintained in M2 for 24 h. Thereafter, the cells were treated with 0.1% DMSO (vehicle control), 10 µM EL or 1 µM 4OHT, or both using M2 for 72 h. The treatment medium was changed every 24 h.

- **Effect of CH223191 treatment on EL-mediated modulation of CYP1A1**

The experiment was conducted as described above for studying the effect of 4OHT, except that 10 µM CH223191 was used.

- **Effect of fulvestrant treatment on EL-mediated modulation of CYP1A1**

MCF-7 cells were allowed to grow until 50% confluency. Then they were fed with M2 for 24 h, followed by incubation in M2 with or without 100 nM fulvestrant for another 24 h. After fulvestrant pre-treatment, cells were treated with 0.1% DMSO (vehicle control), or 10 µM EL in M2 for 72 h. Cells were fed with fresh M2 supplemented with vehicle or EL every 24 h.

- **Cell viability assays**

$7\times10^3$ cells were seeded in 35 mm dishes in M1 medium. After 48 h, the cells were fed M2 medium for 24 h, before commencing the experiments. For dose-response studies, cells were fed with M2 medium supplemented with varying concentrations of EL (0 to 40 µM) or 10 nM E2 for 0 h (baseline viable cell count) or 120 h (end-point viable cell count) in separate sets of culture dishes. Cells treated for 120 h were fed with fresh treatment medium every 24 h. For time-course studies, the cells were treated with vehicle (0.1% DMSO), 10 nM E2 or 10 µM EL in M2 medium for 0, 24, 48, 72, 96, and 120 h in separate sets of dishes. Upon completion of the experiments, the cells were washed with DPBS, trypsinized, and suspended in 1 mL medium. The cell suspensions were mixed with an equal volume of 0.4% trypan blue dye, and viable cells were counted using a hemocytometer.

## 3.3. Gene expression analysis

### 3.3.1. Primers

The primers used for PCR and qRT-PCR were designed manually as per the following parameters: length: 18-22 bp, CG content: 50-60%, melting temperature ($T_m$): 55-65 °C calculated using the formula 4 × (C+G) + 2 × (A+T) - 5. The primers were designed at two different exons separated by a large intron to avoid genomic DNA amplification. The details of the primers used in the present study are provided in Appendix II.

### 3.3.2. RNA isolation and cDNA synthesis

Total RNA was extracted using a reagent prepared in-house based on Chomczynski and Sacchi[149]. 2 µg of total RNA was reverse transcribed using High-Capacity cDNA Reverse Transcription kit, as per the manufacturer's instructions. The cDNA was diluted 10 times, and 2 µl (equivalent to 20 ng of total RNA) was used as template for PCR or qPCR.

### 3.3.3. RT-PCR

Total RNA was extracted, reverse transcribed, and subjected to PCR as described in the previous section. The reactions were carried out in a Veriti 96-Well Thermal Cycler (Applied Bio Systems, USA). After the completion of the PCR, the products were analyzed on 2% agarose gels. The images of ethidium bromide-stained bands were captured using the ChemiDoc™ XRS + System with Image Lab™ Software (Bio-Rad Laboratories, Hercules, CA, USA).

### 3.3.4. RT-qPCR

Following cDNA synthesis as described above, qPCR reactions were set up in PowerUp™ SYBR™ Green PCR master mix with gene-specific primers (Appendix II), and carried out in AriaMx Real-Time PCR System (Agilent, CA, US). Two technical replicate reactions were carried out for each biological replicate sample. The experiments were done with at least three biological replicates for every treatment group. Each biological replicate consisted of total RNA isolated from cells harvested from one dish. The qPCR data were analyzed using either the ($2^{-\Delta\Delta Ct}$) method for relative quantification[150], or by the ΔCt method. In this method as described previously[151]. In the latter, the Ct values obtained for gene of interest ($Ct^{test}$), and the internal control *RPL35a* ($Ct^{RPL35a}$) were processed. Their difference, ΔCt ($Ct^{test} - Ct^{RPL35a}$), served as a measure of the normalized expression of the test gene in each technical replicate. The average ΔCt of the technical replicates provided the normalized expression in each biological replicate. Higher ΔCt value reflected lower

gene expression and vice-versa. The ΔCt data obtained for each of the treatment groups were analyzed by one-way or two-way ANOVA, depending on the hypothesis and experimental design.

## 3.4. Total protein isolation and western blotting

Total protein was isolated from cells with Laemmli buffer[152] (quantified using the trichloroacetic acid (TCA) method[153]), or from the organic phase that was separated during the RNA extraction as described by Likhite *et al.*[154]. In this method, DNA was precipitated with absolute ethanol, and separated by centrifugation. Subsequently, the protein was precipitated by adding isopropanol to the supernatant, and incubation at -20 ºC for 30 min. The resultant protein pellets were washed with absolute ethanol containing 0.3 M guanidinium chloride. The protein was sonicated and dissolved in 1% SDS. Total protein obtained from each sample was quantified by Lowry's method[155]. 30 µg of total protein samples were resolved by 10% denaturing SDS-PAGE, and transferred onto nitrocellulose membrane. The blots were blocked with 1% (w/v) gelatin in 1X Tris-buffered saline containing 0.05% Tween 20 for 2 h at room temperature, then probed with anti-PCNA antibody, anti-CYP1A1 antibody, anti-ERα antibody, or anti-AHR antibody overnight at 4 ºC, or for 1h with anti-H3 antibody at room temperature. Blots were washed with 1X TBST (6 washes of 5 min each), then incubated with HRP-conjugated anti-rabbit, or anti-mouse secondary antibody for 1 h at room temperature. Blots were washed for 30 min (6 washes of 5 min each) with 1X TBST, and developed by Clarity Western ECL Substrate.

## 3.5. ChIP-seq analysis

ChIP-seq data corresponding to MCF-7 cells treated with E2 (ID: ERR022026), or vehicle (ID: ERR022025) were retrieved from Sequence Read Archive (SRA accession ID: ERP000380). The data were analyzed using GALAXY[156]. Read quality was assessed using FASTQC[157]. Thereafter, the quality scores were converted to Sanger quality type by FASTQ Groomer[158], followed by mapping the reads to reference human genome (hg19) using "Map with Bowtie for Illumina" tool[159]. Unmapped reads were filtered out by "Filter SAM or BAM, output SAM or BAM" tool[160]. Genomic regions with enriched sequencing reads were identified by MACS (Model-based analysis of ChIP-Seq) tool[161]. Resultant Wig files were converted to bigWig files using "Wig/BedGraph-to-bigWig" tool and the peaks representing ERα occupancy were visualized using UCSC genome browser[162].

## 3.6. Chromatin immunoprecipitation

Cells were fixed with 1 % (v/v) formaldehyde for 10 min, followed by addition of 125 mM glycine to stop the reaction. Subsequently, cells were washed with ice-cold DPBS, then lysed with lysis buffer (50 mM HEPES pH 7.5, 140 mM NaCl, 1 mM EDTA pH 8, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 1X protease inhibitor cocktail), and sonicated at an amplitude of 30% for 45 cycles; each cycle with a 10-sec pulse on, and a 25-sec pulse off. Lysates were clarified by centrifugation, and supernatants containing chromatin were collected. 80 µg of chromatin sonicated in ChIP lysis buffer was precleared by incubating with BSA- and herring sperm-coated Protein G plus-Agarose beads. 5% of the pre-cleared chromatin samples were kept aside as input, and the remaining were incubated with anti-ERα, anti-AHR, or normal rabbit IgG antibody for 4 h, at 4˚C. Immune complexes were pelleted by incubating with 20 µl of pre-coated Protein G plus-Agarose beads for 2 h, at 4˚C, followed by centrifugation. The pellets were washed extensively with a series of wash buffers[163]. Immunoprecipitated chromatin samples were eluted and reverse cross-linked as described earlier[163]. They were purified using Nucleospin Gel and PCR clean up kit from Machery-Nagel (Duren, Germany). ERα or AHR occupancy was assessed by PCR using two sets of primers targeting *TFF1* or *CYP1A1* promoter regions (Appendix II). The PCR reactions were carried out in Veriti 96 Well Thermal Cycler (Applied Bio Systems, USA). The products were resolved on 2% agarose gels. The images of ethidium bromide-stained bands were captured using ChemiDoc™ XRS + System with Image Lab™ Software (Bio-Rad Laboratories, Hercules, CA, USA).

## 3.7. RNA-seq

### 3.7.1. Sample preparation

MCF-7 cells treated with vehicle or 10 µM EL for 72 h were lysed, and RNA was isolated using RNA extraction reagent prepared in- house as per Chomczynski and Sacchi[149]. Each biological replicate RNA sample was isolated from pooled lysates from three technical replicate dishes. The RNA samples (three biological replicates for each treatment group) were then subjected to mRNA enrichment, followed by library preparation, and sequencing on the NextSeq2K (Illumina) platform.

### 3.7.2. Library preparation and sequencing

mRNA enrichment was performed using KAPA mRNA capture kit (Roche, Switzerland). Libraries were prepared according to instructions accompanying the KAPA RNA HyperPrep kit (Roche, Switzerland). The double-stranded cDNAs were end-repaired, polyadenylated, and ligated with adapter sequences. Sequencing was performed using Nextseq2K Platform in 2 × 150 bases paired-end reads format.

### 3.7.3. Reads Quality Check and Trimming

The raw reads were in FASTQ format. The fastqc tool[157] was used to assess the read quality. Trimmomatic[164] was used to filter out adapters and low-quality reads from the FASTQ files. Trimmomatic was performed using the following parameters: ILLUMINACLIP:TrueSeq3-PE.fa:2:30:10:2:keepBothReads, LEADING:3, TRAILING:3, HEADCROP:10, and MINLEN:50. Prior to mapping, the filtered reads were re-assessed using the fastqc tool. After the quality trimming and assessment, the FASTQ files were used for mapping.

### 3.7.4. Read Mapping and Quantification

The trimmed reads were mapped using the Ensemble Homo sapiens GRCh38 genome as the reference genome (https://asia.ensembl.org/Homo_sapiens/Info/Index). Genome indexing and read mapping was carried out using the STAR aligner tool[165]. Samtools[166] was used to convert the mapped output files (sam files) into binary files (bam files). featureCounts tool[167] was utilized for quantification of mapped reads.

### 3.7.5. Normalization and differential gene expression analysis

The count data were analyzed by DESeq2 package in R to identify the differentially expressed genes. Wald statistic was applied with α equal to 0.05 followed by FDR correction. Subsequently, data were filtered as per log fold change (threshold = 0) and padj (adjusted p-values < 0.05), and the final gene list of significantly expressed genes was extracted.

## 3.8. Functional annotation

Gene ontology (GO) was done via clusterProfiler package in R[168], to identify the gene ontology terms over represented in the differentially regulated genes after RNA-seq analysis, according to three functional categories; biological processes (BP), cell components (CC), and molecular function (MF). Separate GO analysis for up-regulated genes and down-regulated genes was performed.

## 3.9. Gene Set Enrichment Analysis

GSEA software[169], with FDR correction of 25% was employed to find hallmark gene sets enriched in MCF-7 cells treated with 10 μM EL. The plot of normalized enrichment score (NES) was generated using ggplot2 package in R.

## 3.10. Statistical analysis

RT-qPCR data with multiple groups, such as those obtained from dose-response experiments were analyzed by one-way ANOVA. However, two-way ANOVA was used to examine the interaction between two variables (EL and 4OHT, EL and fulvestrant, or time and EL). Before applying one-way or two-way ANOVA, the homogeneity of variance was confirmed using the Levene test. TukeyHSD was used for multiple comparisons. All statistical analyses were performed at 5% level of significance ($p < 0.05$).

# Chapter 4

## The effect of EL on breast cancer cell proliferation

## 4.1. Introduction

Enterolactone is believed to be a key mediator of health-promoting effects of dietary lignan-rich diets, including breast cancer risk reduction[170]. However, the literature is divided on the association of dietary lignan intake, or serum EL concentration, with breast cancer risk. Independent epidemiological studies have produced conflicting results, either in favor of, or against any association (Table 2.2). The causal relationship between EL exposure and breast cancer risk is still in the realm of hypothesis, and bereft of mechanistic insights. Case-control studies on the relationship between serum EL and breast cancer risk, have yielded mixed results[15,171]. More recent systematic reviews, or meta-analyses have revealed limited evidence for the risk-reducing effect of dietary lignans, or serum EL, in post-menopausal, but not in pre-menopausal women[21,172,173]. It is therefore likely that the impact of EL on breast cancer risk may be governed by circulating hormones, or the status of the estrogen-ERα signaling axis in the breast epithelium[174,175].

EL, which is detectable in the serum of individuals on a routine diet[176], is significantly elevated in those maintaining vegetarian diets rich in dietary lignans, such as flaxseed and sesame[177]. However, the absolute level of serum EL is determined by gut microflora, along with other factors such as obesity, smoking, constipation, antibiotics, and intake of coffee, tea, and alcohol[40,178]. These factors lead to inter- and intra-individual variations in enterolactone levels in the serum or urine as reported in different populations (Table 2.2).

EL binds ERα *in vitro*[23]. It modulates ERα function, producing both estrogenic or antiestrogenic effects[63,132]. These effects were interpreted based on its ability to positively or negatively affect proliferation[24,129] over a wide range of concentration[28,132]. Estrogen responsive reporter constructs have revealed pro-estrogenic effects of EL[23,63]. Although the duality in the effect of EL on breast cancer cells is as intriguing as the perceived effects on breast cancer risk, it is likely that both are a result of its interaction with the estrogen-ERα signaling axis[29]. The true effect of EL on the breast epithelial cells may be concentration-dependent. If so, it follows, that large deviations in serum EL in women on diverse diet and lifestyle regimen[40] may obfuscate its true relationship with breast cancer risk.

The lack of consistency in the results of EL's effect on cell proliferation motivated us to independently assess the impact of EL on cell growth. In this chapter, the cell viability data

obtained after treating breast cancer cell lines with varying concentrations of EL (1 nM – 40 µM) are presented. Four breast cancer cell lines were used. MCF-7 and T47D served as ERα-positive models, while MDA-MB-231 and MDA-MB-453 served as ERα-negative models. This assessment enables better understanding of EL impact on cell proliferation in the presence or absence of ERα.

## 4.2. Results

### 4.2.1. Differential growth curves of MCF-7 and T47D cells cultured in M1 and M2

A study on the effect of EL on MCF-7 or T47D cell growth necessitated the choice of M2 as the treatment medium. M2, unlike M1, is devoid of phenol red, a known estrogenic agent[179], and contains CS-FBS, which is depleted of steroid hormones. Prior to the study of the effect of EL, we examined the growth of these cell lines in M1 or M2 with respect to time. A total of four combinations were tested, namely MCF-7 in M1, MCF-7 in M2, T47D in M1 and T47D in M2. Figure 4.1 shows the growth curves for each of the four combinations. We applied two-way ANOVA to test the effect of time, combination of medium and cell type, or their interaction. There were significant main effects of time ($p \approx 0$), and combination ($p \approx 0$). Notably, the interaction between time and combination was also significant ($p \approx 0$), suggesting that the effect of time depended on the combination of medium and cell-type. A post-hoc test revealed that MCF-7 cells cultured for 120 h in M1 yielded a significantly greater viable cell count compared to those cultured in M2 ($p = 0$; Figure 4.1, dark blue vs orange). In contrast, T47D cells outperformed MCF-7 cells in M1; the viable cell count of T47D cells after 120 h of growth being significantly greater than MCF-7 cells ($p = 0$, Figure 4.1, light blue vs dark blue). Furthermore, unlike MCF-7 cells, T47D cells appeared resilient in M2. This is reflected in the significantly higher viable cell count for T47D cells at the 120 h time-point, compared to MCF-7 cells ($p = 0$; Figure 4.1; red vs orange).

### 4.2.2. 10 µM EL enhances the growth of MCF-7 and T47D cells

#### 4.2.2.1. Dose-response

Dose-response experiments were performed to study the effect of EL on the growth of breast cancer cells in M2. ERα-positive (MCF-7, T47D), and ERα-negative (MDA-MB-231, MDA-MB-453) cells were treated with varying concentrations (0 to 40 µM) of EL, or 10 nM E2

in M2, for 0 (baseline viable cell count) or 120 h (end-point viable cell count). The results of the dose-response experiments are shown in Figure 4.2.

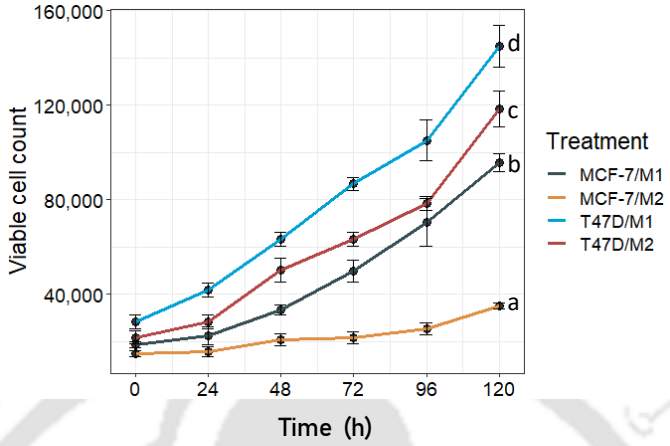


**Figure 4.1. Growth curves of MCF-7 and T47D cells cultured in M1 or M2 medium.** $7 \times 10^3$ cells were seeded in 35 mm dishes using M1. After 48 h, cells were further grown in M1 or M2 for 120 h, then counted as described in chapter 3. The legend on the right shows the combination of cell-type and the medium in cell-type/medium format. The data were analyzed by two-way ANOVA to test for the effect of time, combination, or their interaction on total viable cell count followed by the Tukey's HSD post-hoc test. Points on the graph represent mean total viable cell count (±SD, n = 3). a, b, c, and d, are letter codes showing statistical difference between combinations only for the 120 h data. The experiment was repeated two times with similar results.

The viable cell-count data were analyzed by two-way ANOVA to test the effects of time, and treatment, or their interaction. As expected, there was significant main effect of time ($p \approx 0$) for all the cell lines; the total viable cell count after 120 h being significantly greater than the baseline at all concentrations of EL (Figure 4.2, A-D). There was significant main effect of treatment in all the cell lines ($p = 0.036$ for MDA-MB-231, and $p \approx 0$ for others). Notably, the interaction between time and treatment was also significant in MCF-7, T47D, and MDA-MB-453 cells ($p \approx 0$), but not in MDA-MB-231 cells ($p = 0.08$). In all the cell lines, there was no significant difference in the baseline viability determined at 0 h across all treatments (Figure 4.2, A-D; green dots). A post-hoc analysis showed that total viable count at 120 h, with 10 nM E2 was significantly greater than control, or any concentration of EL in MCF-7 and T47D cells (Figure 4.2, A, B; $p << 0.001$). There was no effect of 10 nM E2 on MDA-MB-231 or MDA-MB-453 cells (Figure 4.2, C and D). Notably, EL had differential effect on viable cell count over 120 h of treatment, depending on the concentration and the cell type. In MCF-7 cells, there was a significant increase in viable cell count with 10 and 40 µM EL ($p \approx 0$, and $p < 0.0001$, respectively, Figure 4.2A). In T47D cells,

10 µM EL significantly increased the viable cell count ($p < 0.0001$), while 40 µM EL significantly decreased the viable cell count ($p < 0.05$) with respect control (Figure 4.2B). None of the concentrations of EL produced any significant increase or decrease in viable cell count in MDA-MB-231 cells (Figure 4.2C), although in some replicate experiments, we did observe marginal increase or decrease in cell viability (data not shown). In MDA-MB-453 cells, 40 µM EL caused a significant decrease in viable cell count ($p \approx 0$, Figure 4.2D).

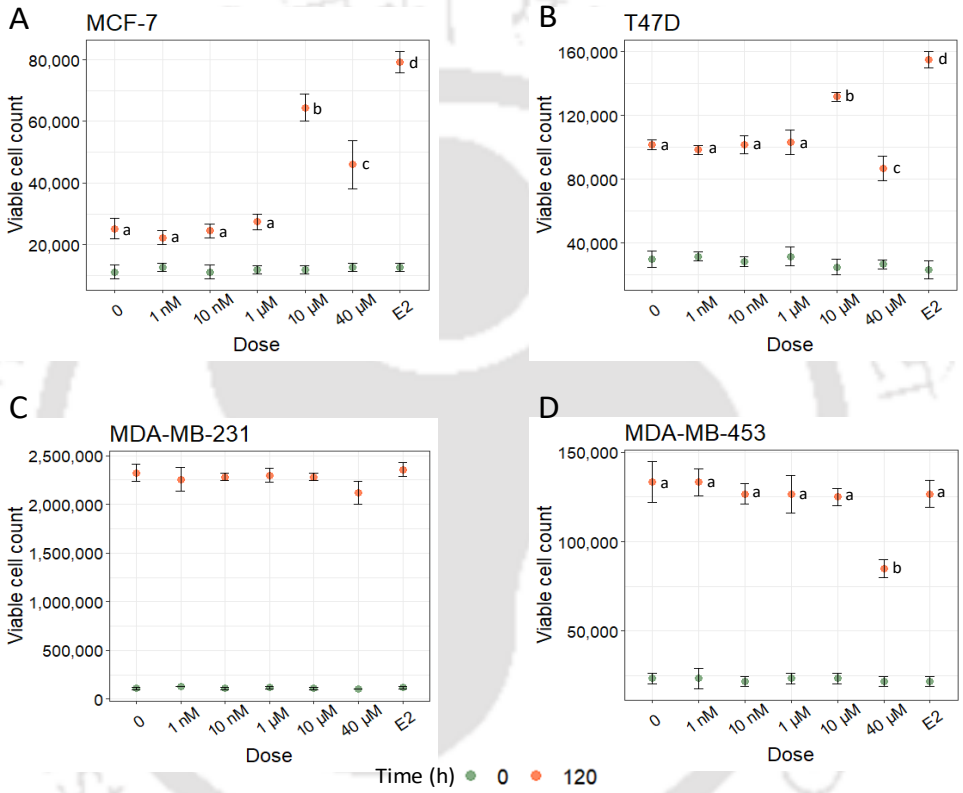


**Figure 4.2. Dose-response of breast cancer cell lines to EL.** $7 \times 10^3$ cells were seeded in 35 mm dishes using M1. After 48 h, the cells were fed with M2, and maintained for 24 h. Thereafter, the cells were fed with M2 containing indicated concentrations (0–40 µM) of EL, 10 nM E2, or 0.1% DMSO (vehicle control), for a period of 0 (baseline viable count) or 120 h. Thereafter, cells were counted as described in chapter 3. Each point represents mean viable cell count (±SD, n = 3). The data were analyzed by two-way ANOVA, to ascertain the main effects of time, treatments or concentration, or their interaction, followed by the Tukey's HSD post-hoc test. a, b, c, and d, are letter codes showing statistical difference between treatments only for the 120 h data. The experiment was repeated two times with similar results.

#### 4.2.2.2. Time-course

We performed time-course experiments to capture the growth curves of breast cancer cell lines treated with vehicle, E2 or 10 µM EL in M2. The data were analyzed by two-way ANOVA to examine the main effects of time, treatment or their interaction. Reflecting significant main

effect of time, the viable cell count increased significantly in all the cell lines ($p \approx 0$; Figure 4.3, A-D). The main effect of treatment was significant only in MCF-7 and T47D cells ($p \approx 0$; Figure 4.3, A, B). Interestingly, the interaction between time and treatment in the two ERα-positive cell lines was significant ($p \approx 0$, Figure 4.3, A, B), indicating difference in their growth curves in the presence of vehicle, E2 or EL. Differences in viable cell counts brought about by the treatments became magnified with time (Figure 4.3, A, B). Overall, at the 120 h time-point, EL produced a significantly greater number of viable counts in case of MCF-7 and T47D cells ($p < 0.001$), albeit to a lesser extent than E2 ($p \approx 0$). We examined the increase in viable cell counts obtained for both the cell lines after 120 h of vehicle or EL treatment. For MCF-7 cells the increase was 1.66 fold; the average viable cell count at 0, and 120 h being 9630 ± 1282 and 28148 ± 1283, respectively. In contrast, for T47D cells, the increase was 1.32 fold; the average viable count at 0, and 120 h being 26666 ± 2886 and 141666 ± 2886, respectively. Thus, the growth response of MCF-7 cells to 10 μM EL in M2 appeared to be robust in comparison to that of T47D cells.

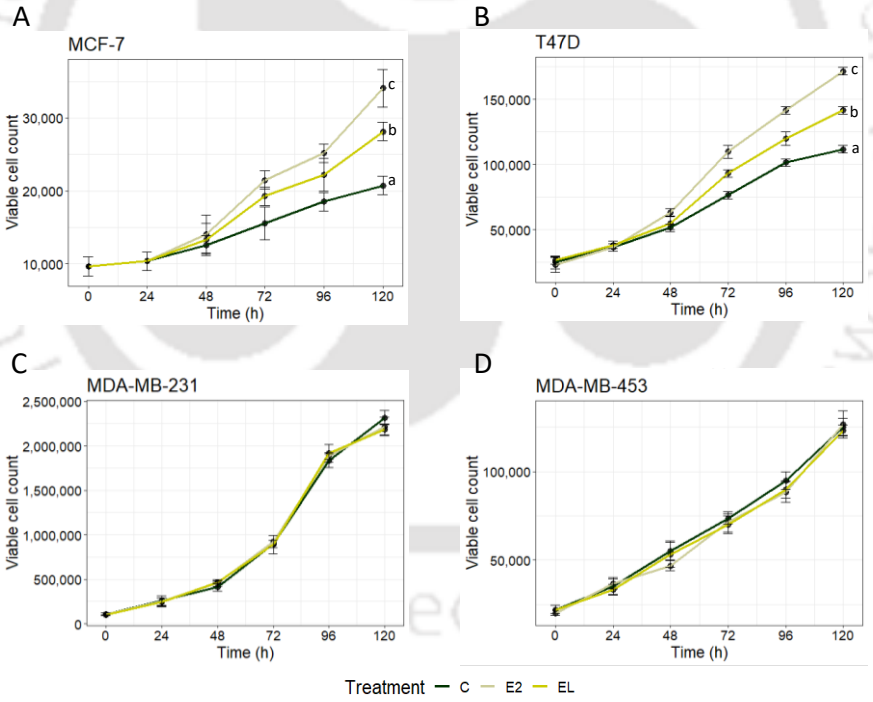


**Figure 4.3. Growth curves of breast cancer cell lines treated with EL.** $7 \times 10^3$ cells were seeded in 35 mm dishes using M1. After 48 h, the cells were fed with M2 for 24 h, then fed with M2 containing vehicle (0.1% DMSO), 10 μM EL, or 10 nM E2 for the indicated periods of time. At the indicated time-points, total viable cell count in every dish was determined by trypan blue dye exclusion method as described in chapter 3. Each point represents mean viable cell count (±SD, n = 3). The data were analyzed by two-way ANOVA, to ascertain the main effects of time, treatments or concentration, or their interaction, followed by Tukey's HSD post-hoc test. a, b, and c are letter codes showing statistical difference between treatments only for the 120 h data. The experiment was repeated two times with similar results.

### 4.2.3. EL-mediated restoration of cell growth in MCF-7 cells is associated with maintenance of PCNA protein expression

Data presented in Figure 4.1 showed compromised growth of MCF-7, and the resilience of T47D cells in M2. We wanted to compare the growth of both cell lines grown in M2 alone, or M2 supplemented with 10 nM E2 or 10 μM EL, with those grown in M1. The data were analyzed by two-way ANOVA. As expected, the growth curve of MCF-7 with M2 medium had a gentle slope indicating the compromised rate of cell proliferation (Figure 4.4A, dark green). The viable cell count at 120 h time-point for cells grown in M2 medium supplemented with E2 was comparable to that in M1 (Figure 4.4A, grey vs red). EL treatment in M2 significantly increased the viable cell count of MCF-7 cells ($p \approx 0$, Figure 4.4A, dark green vs yellow), albeit to a lesser extent compared to that achieved with M1 ($p = 0$; Figure 4.4A, red vs yellow), or M2 supplemented with E2 ($p \approx 0$; Figure 4.4A, grey vs yellow). Since the growth of T47D was already robust in M2 (Figure 4.4B, dark green), the impact of EL on its growth in M2 was not as robust as seen in MCF-7 cells ($p = 0.004$; Figure 4.4B, dark green vs yellow). Overall, viable cell counts for the 120 h time-point for T47D cells grown in M1, and M2 supplemented with EL or E2 were significantly different ($p = 0.004$ and $p= 0.001$; Figure 4.4B, grey vs yellow, and red vs yellow, respectively). The growth kinetic data correlated with the levels of PCNA protein expression in both the cell lines. Western blot analysis showed that after 72 h of culture in M2, PCNA protein level in MCF-7 cells falls (Figure 4.4C, lanes 5 and 7), but remains stable when cultured in M1 medium (Figure 4.4C, lanes 1 and 3). Furthermore, when cultured in M1 medium for 72 h, 10 μM EL had no effect on PCNA levels (Figure 4.4C, lanes 1 and 4). However, in the presence of M2 medium, 10 μM EL appeared to restore PCNA protein expression (Figure 4.4C, lanes 5 and 8). In contrast, irrespective of the medium (M1 or M2), EL did not have any impact on PCNA protein expression in T47D cells (Figure 4.4D).

### 4.2.4. 4OHT blocks EL-mediated increase in viable cell count in MCF-7 and T47D cells

We studied the effect of 4OHT on EL-induced growth of MCF-7 and T47D cells. Cells were treated with vehicle or 10 μM EL, alone or in combination with 1 μM 4OHT for 0 (baseline) or 120 h. The data were analyzed by two-way ANOVA to test the main effects of time, treatment, or their interaction. There were significant main effects of time, treatment, and interaction in both

the cell lines. The baseline viability of MCF-7 or T47D cells in the treatment groups was not significantly different (red dots, Figure 4.5, A, B).

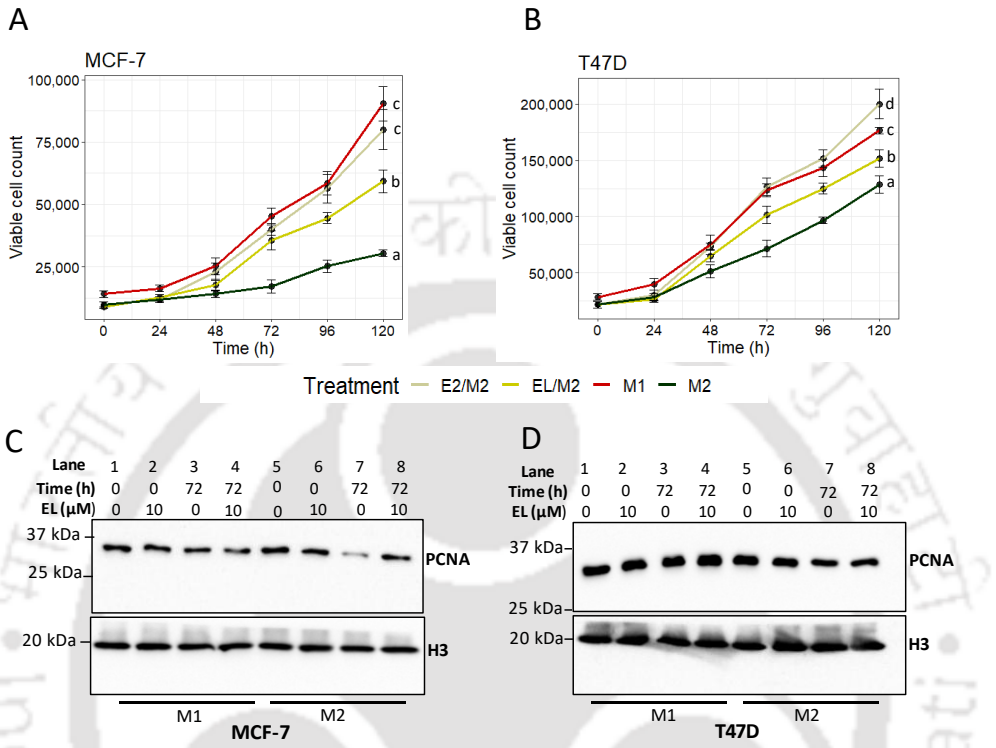


**Figure 4.4. Induction of MCF-7 cell proliferation by 10 μM EL in M2 is associated with restoration of PCNA expression.** A, B. Growth curves of MCF-7 (A) and T47D (B) cells. $7 \times 10^3$ cells were seeded in 35 mm dishes using M1. After 48 h, the cells were fed with M2, and maintained for 24 h. Thereafter the cells were grown in M2 containing vehicle (0.1% DMSO), 10 μM EL, or 10 nM E2 for indicated periods of time. The growth curves were compared to that of cells maintained in M1 medium. At the indicated time-points, total viable cell count in every dish was determined by trypan blue dye exclusion method as described in chapter 3. Each point represents mean viable cell count (±SD, n = 3). The data were analyzed by two-way ANOVA, to ascertain the main effects of time, treatment, or their interaction, followed by TukeyHSD post-hoc test. a, b, c, and d, are letter codes showing statistical difference between treatments, only for the 120 h data. C, D. Western blot analysis of PCNA protein expression. MCF-7 (C) or T47D (D) cells were treated with vehicle (0.1% DMSO), or 10 μM EL in M1 or M2 for 0 or 72 h. Total protein was extracted, and 30 μg of each protein sample was subjected to western blot analysis using PCNA-specific antibody, as described in chapter 3. H3 served as an internal control.

The significant effect of time is reflected in the significant increase in viable cell count in all treatment groups (Figure 4.5, A, B). The post-hoc analysis revealed that after 120 h of treatment, the viable cell count was significantly greater with 10 μM EL compared to control (Figure 4.5, A, B; C vs EL). While 1 μM 4OHT alone had a significant negative effect on the viable cell count

(Figure 4.5, A, B; C vs 4OHT), it significantly reduced the viable cell count brought about by EL (Figure 4.5, A, B; EL vs EL+ 4OHT).

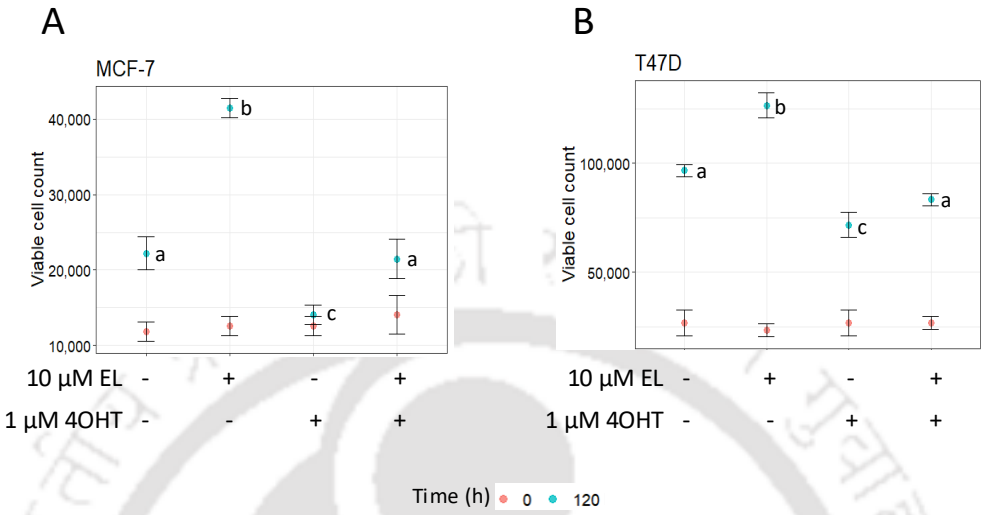


**Figure 4.5. Effect of 4OHT on 10 µM EL-mediated increase in cell viability.** A, B. 7 × $10^3$ MCF-7 (A) or T47D (B) cells were seeded in 35 mm dishes using M1. After 48 h, the cells were fed with M2, and maintained for 24 h. The cells were then fed with 10 µM EL, 1 µM 4OHT, or both for 0 (baseline viability) or 120 h. Control cells were treated with vehicle (0.1% DMSO). The data were analyzed by two-way ANOVA to test the effects of time, treatment or their interaction. TukeyHSD was applied for the post-hoc test for ascertaining significant difference between groups. a, b, and c are letter codes showing statistical difference between pairs of treatments only for the 120 h data.

## 4.3. Discussion

In the light of the epidemiological data on the possible link between enterolactone exposure and breast cancer risk[21], investigations into the pro- or anti-estrogenic effects of EL[10,29] cannot be overlooked. Pro-estrogenic molecules are expected to induce proliferation, and modulate the expression of estrogen target genes in ERα-positive and estrogen responsive cells. EL qualifies as a pro-estrogenic molecule on both counts. Studies on ERα-positive breast cancer cells have repeatedly demonstrated proliferative effect of EL only up to 10 µM, but not higher concentrations[24,28,128]. The present study confirms this result via elaborate dose-response (Figure 4. 2) and time-course experiments (Figure 4.3).

Zhu *et al.*[29] have demonstrated that EL-mediated proliferation of MCF-7 cells is associated with activation of cyclin-CDK complexes[29], which is typical of estrogen-mediated induction of cell proliferation[180]. Our analysis of PCNA expression, a marker of cell proliferation, provides an

independent perspective on EL action. The time-dependent fall in PCNA expression in MCF-7 cells cultured in M2 (Figure 4.4C), a medium depleted of growth supporting hormones, is restored by 10 μM EL. This supports the growth-promoting effect of 10 μM EL in MCF-7 cells, although the effect is significantly less comparable to 10 nM E2. Notably, T47D cells, which inherently proliferate faster than MCF-7 cells (Figure 4.1), were resilient to M2 medium and did not show any fall in PCNA levels (Figure 4.4D). This could be due to autocrine proliferative mechanisms that continue to support the growth of T47D cells, despite hormone depletion. As a result, the growth response of MCF-7 cells to 10 μM EL is more robust compared to that of T47D cells (Figure 4.2, A, B and 4.3, A, B).

Physical binding of EL to ERα[23] raises the possibility that its actions are mediated, at least in part, via the classical estrogen receptor in ERα-positive breast cancer cells. It leads to the hypothesis that 10 μM EL-mediated induction of MCF-7 or T47D proliferation could be mediated via ERα. Our results show that 10 μM EL does not enhance the proliferation of the ERα-negative MDA-MB-231 or MDA-MB-453 cells (Figure 4.2 and 4.3), which strengthens the hypothesis. Zhu *et al.*[29] showed that ICI 182,780 (fulvestrant), which selectively degrades ERα, inhibits the proliferation of MCF-7 cells induced by 10 μM EL. Our observation that 4OHT, a selective estrogen receptor modulator, prevents enhanced proliferation of MCF-7 by 10 μM EL, complements their result. Taken together these are compelling evidences in favor of a significant role for ERα in EL-induced cell proliferation at 10 μM concentration.

The contrasting growth responses of ERα-positive and ERα-negative cell lines to EL is evident. While the observed effects of 4OHT on growth in ERα-positive cell lines, provide compelling evidences to suggest ERα-mediated effects of EL, this study does not specifically address the role of ERβ. Penttinen *et al.*[23] showed that EL can bind both ERα, or ERβ, albeit with lesser affinity compared to E2. Furthermore, reporter assays showed that EL can transactivate both ERα, and ERβ[63]. Thus, ERβ-mediated effects of EL cannot be ruled out. It is also known that while the proliferative effect of E2 is mediated via ERα, ERβ counters this effect, leading to repression of cell proliferation [181]. Furthermore, though structurally similar, ERα, and ERβ are different proteins with different amino acid compositions[182]. The change in the conformation of these receptors upon ligand binding are subtly different leading to differential recruitment of coactivators or

corepressors[183], which in turn results in differential effects on gene expression, and contrasting cell biological effects. MCF-7, or T47D cells express both the receptors. However, the expression of ERα is higher[184], which possibly explains the induced viable cell counts with 10 μM concentration of EL. In contrast, MDA-MB-453 cells lack ERα, but express ERβ, which may be the underlying reason behind reduced cell viability with 40 μM EL. Hence, it is likely that the net effect of EL on breast cancer cells depends on the relative expression levels of the two classical estrogen receptors and the concentration of EL.

# Chapter 5

## Genomic correlates associated with EL-mediated proliferation in MCF-7

## 5.1. Introduction

Lignans are found in nearly all plants, and are frequently consumed by several populations[185,186]. The potential of lignan-rich regimes against breast cancer was discussed in the literature, relating intake of lignans or their circulating metabolite, EL, with breast cancer risk (Table 2.2). However, the studies yielded conflicting findings either in favor or against any association. *In vitro* data demonstrated that EL binds ERα, and displays estrogenic properties[23]. As shown in the previous chapter, and as per Zhu *et al.*[29] and Welshon *et al.*[128], 10 µM EL increases the growth of ERα-positive breast cancer cells, while at higher concentrations, it does not[24]. However, the mechanisms of EL that affect cellular pathways and modify cell growth are not well understood.

Despite the availability of the next generation sequencing technology, the genome-wide transcriptomic effect of EL in relation to breast cancer has remained unaddressed. Previously, Zhu *et al.*[29] performed a microarray expression profiling of MCF-7 breast cancer cells treated with 10 µM EL for 72 h. However, the analysis was limited to 150 estrogen-responsive genes. In addition, Damdimopoulou *et al.*[187] conducted a microarray analysis using uteri of gonadectomized mice treated with 10 mg/kg EL or 50 µg/kg E2 for 24 h. The analyses revealed a high correlation between EL and E2 gene expression profiles[29], and the ability of EL to regulate fraction of the estrogen target genes[187].

The objective of the present chapter was to generate next generation sequencing-based transcriptomic (RNA-seq) data that captures the transcriptomic alterations associated with EL-induced MCF-7 cell proliferation. This chapter presents RNA-seq data obtained from MCF-7 breast cancer cells treated with 10 µM EL for a period of 72 h. For EL, an estrogen-mimicking nutrient that binds to estrogen receptors, transcriptomic analysis provides insights into the genes differentially regulated in response to EL exposure. This helps in clarifying the molecular mechanisms and biological functions of EL in the context of nutrient-gene interactions, while also revealing the signaling changes triggered by EL-ER binding. Additionally, given the possible link between EL and breast cancer risk, RNA-seq is useful in assessing the effects of EL on breast tumors.

## 5.2. Results

### 5.2.1. RNA sequencing

MCF-7 cells were treated with 0.1% DMSO (vehicle control) or 10 µM EL for 72 h as per the protocol described in section 3.7.1. Upon completion of the experiment, total RNA was isolated. The integrity of RNA was first checked using agarose gel electrophoresis. Total RNA images are shown in figure 5.1. RNA concentration and RNA integrity number (RIN) for each sample determined using Tapestation are presented in Table 5.1.

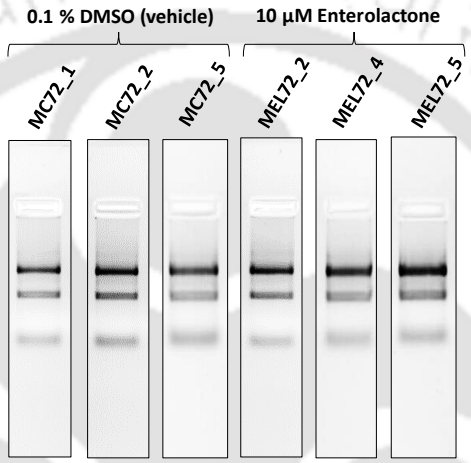


**Figure 5.1.** Images of total RNA before library preparation and sequencing from MCF-7 cells treated with 0.1% DMSO (MC72_1, MC72_2, and MC72_5), or 10 µM enterolactone (MEL72_2, MEL72_4, and MEL72_5) for 72 h.

**Table 5.1. Concentrations and RIN values of total RNA samples.**

| Treatment | Sample | Concentration (ng/µl) | RIN value | rRNA Ratio |
|---|---|---|---|---|
| Vehicle (0.1% DMSO) | MC72_1 | 513.1 | 9.9 | 2.00 |
| | MC72_2 | 427.2 | 9.9 | 1.93 |
| | MC72_5 | 303 | 9.9 | 1.79 |
| 10 µM Enterolactone | MEL72_2 | 705.6 | 10 | 1.95 |
| | MEL72_4 | 611 | 10 | 1.90 |
| | MEL72_5 | 533.4 | 10 | 1.91 |

mRNA enrichment and library preparation were performed as mentioned in section 3.7.2. The raw reads were in FASTQ format, and were submitted to GEO with accession number GSE216876. Read quality check and trimming were done as described in section 3.7.3. After the quality trimming and assessment, the FASTQ files were used for mapping. The number of read pairs passed after quality trimming and assessment is shown in Table 5.2. Read mapping and quantification were performed as discussed in section 3.7.4.

**Table 5.2. Summary of the sequencing read data.**

| Treatment | Sample | File Names (paired-end) | Total Reads | |
|---|---|---|---|---|
| | | | Before trimming | After trimming |
| Vehicle (0.1% DMSO) | MC72_1 | MC72_1_S32_R1 | 45796213 | 32233033 |
| | | MC72_1_S32_R2 | 45796213 | 32233033 |
| | MC72_2 | MC72_2_S33_R1 | 28475957 | 18205563 |
| | | MC72_2_S33_R2 | 28475957 | 18205563 |
| | MC72_5 | MC72_5_S34_R1 | 47187139 | 26069695 |
| | | MC72_5_S34_R2 | 47187139 | 26069695 |
| 10 µM Enterolactone | MEL72_2 | MEL72_2_S36_R1 | 65882250 | 38038490 |
| | | MEL72_2_S36_R2 | 65882250 | 38038490 |
| | MEL72_4 | MEL72_4_S37_R1 | 30204826 | 19561867 |
| | | MEL72_4_S37_R2 | 30204826 | 19561867 |
| | MEL72_5 | MEL72_5_S38_R2 | 32138604 | 21621616 |
| | | MEL72_5_S38_R2 | 32138604 | 21621616 |

Quality assessment was done using unsupervised clustering analysis. This included performing hierarchical clustering and generating the correlation heatmap, followed by principal component analysis (PCA). Figure 5.2A represents the correlation heatmap of 0.1% DMSO-treated versus 10 µM EL-treated samples. PCA plot (Figure 5.2B) indicates that treatment and control groups are separated along PC1 (principal component 1) and the replicates within individual groups are separated along PC2 (principal component 2) indicating that EL-treated samples are different from control samples.

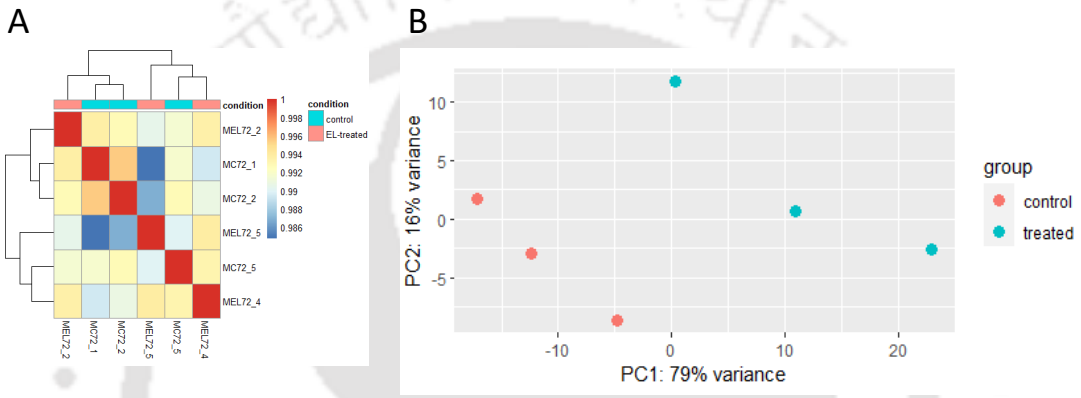


**Figure 5.2. RNA-seq data visualization.** A. Correlation heatmap for 0.1% DMSO-treated samples versus 10 µM EL-treated samples with their respective biological replicates. B. Principle component analysis (PCA) plot. Red dots represent control samples, and blue dots represent treated samples. Samples with similar gene expression profile are clustered together. A and B plots were generated by DESeq2 package in R.

### 5.2.2. Impact of EL on MCF-7 transcriptome

The read-count data, which were obtained after adaptor trimming and filtering, followed by read alignment, were analyzed to determine differentially expressed genes using DESeq2 package in R. As shown in the volcano plot presented in figure 5.3A, 1141 genes were modulated by 10 µM EL in MCF-7 cells, based on a threshold value of 0 for $\log_2$FC, and 0.05 for Wald statistic and FDR. These comprised 727 upregulated and 414 downregulated genes as illustrated in the heatmap in figure 5.3B. The top 25 upregulated, and 25 downregulated genes are presented in Table 5.3 and Table 5.4, respectively.

### 5.2.3. Gene Ontology analysis

On the derived set of significantly modulated genes, GO analysis was performed.

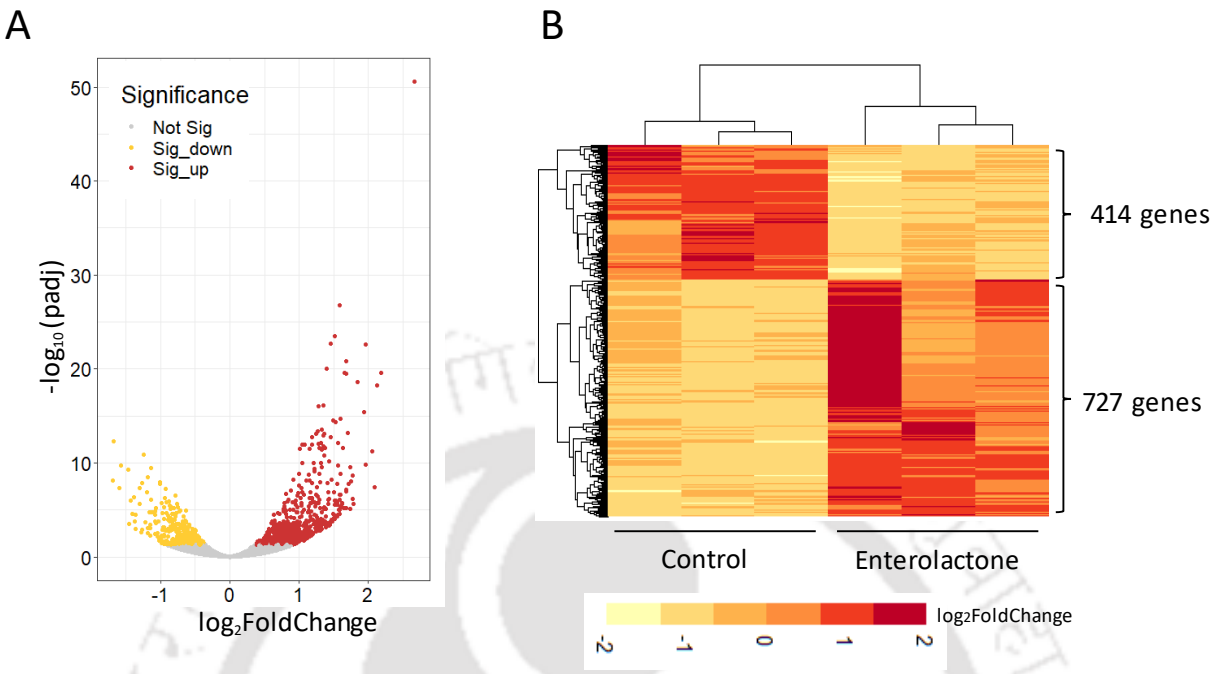


**Figure 5.3. Summary of RNA-seq data.** A. Volcano plot. Genes significantly regulated by 10 µM EL are represented as colored dots according to their –log10Padj (<0.05) and $log_2$FC (threshold = 0). Red dots (n = 727) represent the upregulated genes, while the yellow dots (n = 414) represent the downregulated genes. B. Expression heatmap of genes significantly regulated by EL. The upregulated genes are represented in shades of red, downregulated are represented in shades of yellow.

Under the category of cellular compartment, over-represented terms associated with EL-induced genes included *chromosome*, *chromosomal region*, *centromeric region*, and *condensed chromosome* (Figure 5.4A, left panel, green bars). In contrast, those associated with EL-repressed genes included *collagen-containing extracellular matrix*, *extracellular matrix*, *external encapsulating structure*, *extracellular region*, and *cell periphery* (Figure 5.4A, right panel, green bars).

Over-represented terms under the broad category of biological processes associated with EL-induced genes included *chromosome segregation*, *cell division*, *cell cycle process*, *mitotic cell cycle process*, and *cell cycle* (Figure 5.4A, left panel, yellow bars). In contrast, those associated with EL-repressed genes included *regulation of signaling*, *regulation of cell communication*, *regulation of signal transduction*, *blood vessel morphogenesis*, *and regulation of multicellular organismal process* (Figure 5.4A, right panel, yellow bars).

**Table *5*.3. List of top 25 upregulated genes upon 10 µM EL treatment for 72 h in MCF-7 cells.**

| Ensembl ID | Symbol | $log_2FC$ | padj | Description |
|---|---|---|---|---|
| ENSG00000160182 | *TFF1* | 2.67 | 0.0000 | Trefoil factor 1 |
| ENSG00000147255 | *IGSF1* | 2.19 | 0.0000 | Immunoglobulin superfamily member 1 |
| ENSG00000131747 | *TOP2A* | 2.12 | 0.0000 | Topoisomerase (DNA) II alpha |
| ENSG00000254337 | | 2.09 | 0.0000 | (Novel Transcript) is an RNA Gene, and is affiliated with the lncRNA class. |
| ENSG00000137807 | *KIF23* | 2.05 | 0.0000 | Kinesin family member 23 |
| ENSG00000068489 | *PRR11* | 1.96 | 0.0000 | Proline rich 11 |
| ENSG00000196208 | *GREB1* | 1.96 | 0.0000 | Growth regulation by estrogen in breast cancer 1 |
| ENSG00000156802 | *ATAD2* | 1.93 | 0.0000 | Atpase family, AAA domain containing 2 |
| ENSG00000151892 | *GFRA1* | 1.84 | 0.0000 | GDNF family receptor alpha 1 |
| ENSG00000109805 | *NCAPG* | 1.78 | 0.0000 | Non-SMC condensin I complex subunit G |
| ENSG00000088325 | *TPX2* | 1.78 | 0.0000 | TPX2, microtubule nucleation factor |
| ENSG00000065328 | *MCM10* | 1.77 | 0.0000 | Minichromosome maintenance 10 replication initiation factor |
| ENSG00000112984 | *KIF20A* | 1.74 | 0.0000 | Kinesin family member 20A |
| ENSG00000109321 | *AREG* | 1.74 | 0.0000 | Amphiregulin |
| ENSG00000118193 | *KIF14* | 1.73 | 0.0000 | Kinesin family member 14 |
| ENSG00000198826 | *ARHGAP11A* | 1.71 | 0.0000 | Rho gtpase activating protein 11A |
| ENSG00000129173 | *E2F8* | 1.70 | 0.0000 | E2F transcription factor 8 |

| | | | | |
|---|---|---|---|---|
| ENSG00000090889 | *KIF4A* | 1.68 | 0.0000 | Kinesin family member 4A |
| ENSG00000178999 | *AURKB* | 1.68 | 0.0000 | Aurora kinase B |
| ENSG00000144554 | *FANCD2* | 1.68 | 0.0000 | Fanconi anemia complementation group D2 |
| ENSG00000161888 | *SPC24* | 1.68 | 0.0000 | SPC24, NDC80 kinetochore complex component |
| ENSG00000011426 | *ANLN* | 1.67 | 0.0000 | Anillin actin binding protein |
| ENSG00000135476 | *ESPL1* | 1.66 | 0.0000 | Extra spindle pole bodies like 1, separase |
| ENSG00000051341 | *POLQ* | 1.64 | 0.0000 | DNA polymerase theta |
| ENSG00000154839 | *SKA1* | 1.63 | 0.0000 | Spindle and kinetochore associated complex subunit 1 |

**Table 5.4. List of top 25 downregulated genes upon 10 µM EL treatment for 72 h in MCF-7 cells.**

| Ensembl ID | Symbol | $log_2FC$ | padj | Description |
|---|---|---|---|---|
| ENSG00000233052 | | -1.69 | 0.000 | (Novel Transcript) is an RNA Gene, and is affiliated with the lncRNA class |
| ENSG00000108551 | *RASD1* | -1.68 | 0.000 | Ras related dexamethasone induced 1 |
| ENSG00000087085 | *ACHE* | -1.59 | 0.000 | Acetylcholinesterase |
| ENSG00000136928 | *GABBR2* | -1.57 | 0.000 | Gamma-aminobutyric acid type B receptor subunit 2 |
| ENSG00000189058 | *APOD* | -1.46 | 0.000 | Apolipoprotein D |
| ENSG00000278484 | | -1.45 | 0.000 | (Novel Transcript) is an RNA Gene, and is affiliated with the lncRNA class |
| ENSG00000118523 | *CCN2* | -1.43 | 0.000 | Cellular communication network factor 2 |
| ENSG00000116701 | *NCF2* | -1.41 | 0.000 | Neutrophil cytosolic factor 2 |
| ENSG00000125851 | *PCSK2* | -1.39 | 0.000 | Proprotein convertase subtilisin/kexin type 2 |
| ENSG00000085741 | *WNT11* | -1.37 | 0.000 | Wnt family member 11 |
| ENSG00000162496 | *DHRS3* | -1.36 | 0.000 | Dehydrogenase/reductase 3 |
| ENSG00000171476 | *HOPX* | -1.36 | 0.001 | HOP homeobox |
| ENSG00000146674 | *IGFBP3* | -1.31 | 0.000 | Insulin like growth factor binding protein 3 |
| ENSG00000187122 | *SLIT1* | -1.30 | 0.000 | Slit guidance ligand 1 |
| ENSG00000174640 | *SLCO2A1* | -1.27 | 0.002 | Solute carrier organic anion transporter family member 2A1 |
| ENSG00000260239 | *LINC02533* | -1.27 | 0.001 | Long intergenic non-protein coding RNA 2533 |
| ENSG00000053108 | *FSTL4* | -1.26 | 0.000 | Follistatin like 4 |
| ENSG00000146859 | *TMEM140* | -1.24 | 0.000 | Transmembrane protein 140 |

| | | | | |
|---|---|---|---|---|
| ENSG00000274928 | *KRT89P* | -1.22 | 0.000 | Keratin 89 pseudogene |
| ENSG00000100234 | *TIMP3* | -1.20 | 0.000 | TIMP metallopeptidase inhibitor 3 |
| ENSG00000179776 | *CDH5* | -1.19 | 0.003 | Cadherin 5 |
| ENSG00000100373 | *UPK3A* | -1.18 | 0.001 | Uroplakin 3A |
| ENSG00000184254 | *ALDH1A3* | -1.18 | 0.000 | Aldehyde dehydrogenase 1 family member A3 |
| ENSG00000065325 | *GLP2R* | -1.16 | 0.000 | Glucagon like peptide 2 receptor |
| ENSG00000237187 | *NR2F1-AS1* | -1.15 | 0.006 | NR2F1 antisense RNA 1 |

### 5.2.4. Gene Set Enrichment Analysis (GSEA)

GSEA was applied to identify hallmark gene sets which were enriched in EL-regulated genes. The significantly enriched gene-sets, based on FDR < 25%, are indicated by green bars in Figure 5.4B. The enrichment of G2/M checkpoint genes is noteworthy, which correlates with the ability of 10 µM EL to induce growth of MCF-7 cells. The enrichment plot for the G2/M checkpoint gene-set is shown in Figure 5.4C. There were 115 leading edge genes within this gene-set. Out of these, the modulation of *CDC20*, *CENPF*, *TOP2A*, *STIL*, and *GINS2* was validated by RT-qPCR (Figure 5.4E). The EL-regulated genes were also enriched in the estrogen-response-late, estrogen-response-early, and DNA repair gene sets. The enrichment plot for the estrogen-response-late gene set is shown in Figure 5.4D. There were 69 leading edge genes in this set. Out of these, the modulation of *TFF1*, *TFF3*, *CA2*, *CDC20*, *CXCL12*, *GINS2*, *IGSF1*, *KIF20A*, *MYB*, *PCP4*, *PRLR*, *STIL*, *TOP2A*, and *XBP1* was validated by RT-qPCR (Figure 5.4E). The effect of 10 nM E2 on the expression of the EL-modulated genes was also examined. In general, the induction brought about by 10 µM EL was lower than that produced by 10 nM E2 (Figure 5.4E).

### 5.2.5. 10 µM EL enhances the expression of TFF-1 mRNA in MCF-7 cells

The mechanism of estrogen-mediated induction of *TFF1* mRNA is well known[188]. It involves ERα, and its binding to the estrogen response element in the *TFF-1* locus. As expected, E2 induced *TFF1* mRNA expression. 4OHT blocked this estrogen-mediated induction of *TFF-1* mRNA (Figure 5.5A). 10 µM EL also increased the *TFF1* mRNA levels in MCF-7 (Figure 5.5A) cells, although the fold-increase was significantly lesser compared to that brought about by 10 nM E2 (Figure 5.5A). 4OHT blocked the EL-mediated induction of *TFF1* mRNA (Figure 5.5A). Furthermore, EL-mediated induction of *TFF-1* RNA was associated with increased binding of ERα to the estrogen response element within the *TFF-1* locus (Figure 5.5B).

## 5.3. Discussion

This chapter addresses the transcriptomic changes associated with EL-mediated proliferation. The functional diversity in the genes that are modulated by EL is apparent from the results of GO, and GSEA. Induced proliferation entails rapid cell cycle progression and higher frequency of cell division.

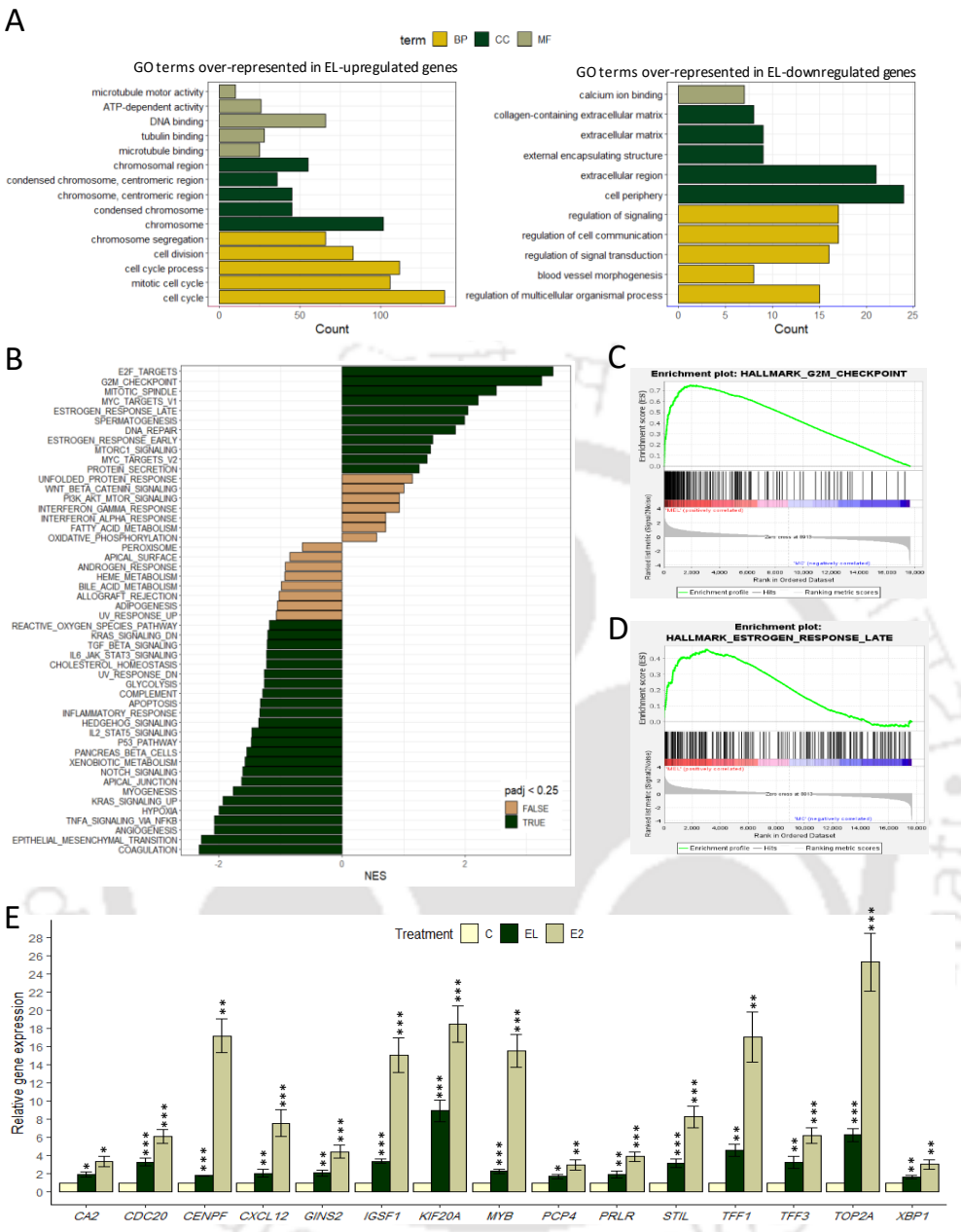


**Figure 5.4. Ontology and enrichment analysis of EL-modulated genes in MCF-7 cells.** A. Top enriched GO terms associated with upregulated (left panel) and downregulated (right panel) under broad categories, namely biological processes (BP), cellular components (CC), and molecular functions (MF). clusterProfiler package in R was used for the analysis with 5% FDR cut-off. B. Gene set enrichment analysis. The analysis was performed using GSEA software. Green bars represent the hallmark gene-sets enriched by EL, based on 25% FDR cut-off. C, D. Enrichment plots of G2/M checkpoint and estrogen-response-late gene sets. E. Validation of selected leading-edge genes from the G2/M checkpoint and estrogen-response-late gene-sets. Total RNA from cells treated for 72 h, with vehicle (0.1% DMSO), 10 μM EL, or 10 nM E2, were subjected to RT-qPCR analysis. RPL35a served as internal control. The expression in EL- or E2-treated samples was expressed relative to those in vehicle controls using the ΔΔCt method (Livak and Schmittgen, 2001). Bars represent mean relative gene expression (±SD). For each gene, the fold induction by EL, or E2 was analyzed by t-test. *p < 0.05, ** p < 0.01, ***p < 0.001.

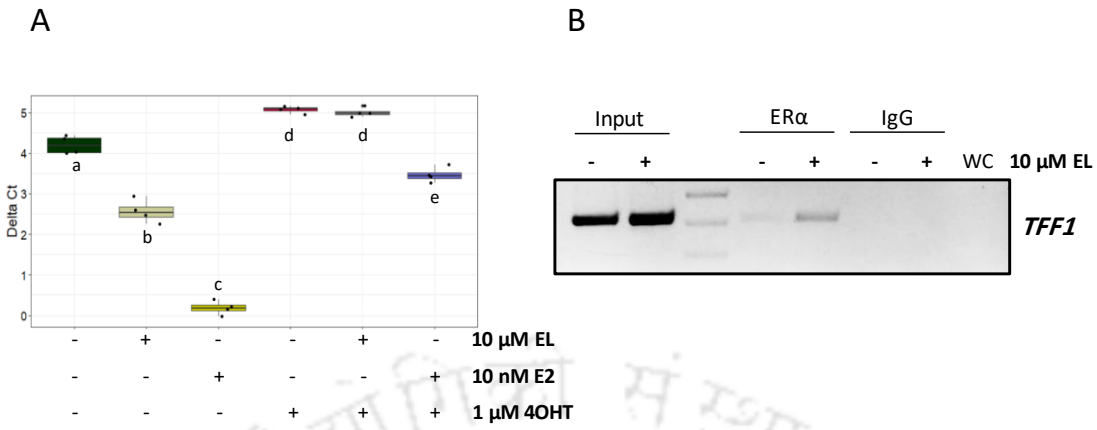


**Figure 5.5. ERα mediates EL effect.** A. Total RNA was isolated from MCF-7 cells treated with vehicle, 10 µM EL, 1 µM 4OHT, or both for 72 h, and subjected to RT-qPCR analysis. For each sample the average Ct value for TFF1 mRNA ($Ct^{TFF1}$), and RPL35a ($Ct^{RPL35a}$) were determined. The difference $Ct^{TFF1}$ - $Ct^{RPL35a}$, which is referred to as ΔCt, was considered as a measure of the normalized expression; RPL35a serving as an internal control. Thus, higher the ΔCt value, lower the expression in a given sample. The data were analyzed by ANOVA followed by TukeyHSD. The boxplot shows the mean relative expression (±SD, n = 4). a, b, c, d, and e represent the statistical difference between pairs of treatment. B. Sonicated chromatin samples prepared from vehicle- or 10 µM EL-treated cells were immunoprecipitated with non-specific IgG or ERα-specific antibody. Immunoprecipitated DNA samples were subjected to PCR using primer pairs designed to specifically amplify the region encompassing an estrogen response element.

In the face of induced proliferation, cells channelize energy for DNA synthesis, chromosomal duplication and segregation, and negotiation of cell cycle checkpoints. Cellular processes that entail communication with the environment are not a priority. Over-representation of terms, such as those associated with chromosomes and cell cycle (Figure 5.4A, left panel) in the EL-induced genes, and over-representation of terms associated with membrane, and signaling (Figure 5.4A, right panel) in the EL-downregulated genes essentially reflect this notion.

The enrichment of the hallmark G2/M checkpoint genes on the one hand, captures the systemic shift towards proliferative state under the influence of 10 µM EL. On the other hand, enrichment of estrogen-response-late and estrogen-response-early genes reflects the involvement of the ERα signaling axis. It is worth noting that CDC20, STIL, TOP2A, and GINS2 were among the leading-edge genes in both sets. CDC20, a cell cycle modulator, controls the chromosomal segregation during mitosis[189]. It is over-expressed in breast tumors compared to normal tissues[190], and known as an oncoprotein[191]. GINS2 is a subunit of one of the core components of eukaryotic replicative helicase, whose role is involved in DNA replication by opening the double-stranded DNA at the replication fork[192]. GINS2's expression is higher in breast cancer tissues compared to

normal ones[193], and it was identified as a metastasis promoting-protein in breast cancer patients[194]. Further, TOP2A, is a nuclear enzyme involved in DNA replication and transcription via controlling DNA topology[195]. High TOP2A in breast cancer patients is thought to negatively affect cancer prognosis[196]. TOP2A overexpression is also associated with increased tumor grade and Ki67 index[197]. Besides, centriolar assembly protein (STIL), is a cytosolic protein, which promotes centriole duplication in proliferating cells[198]. It is upregulated in many tumors, including breast tumors[199]. The induction of the expression of the aforementioned genes, which are also induced by E2, suggests estrogen-like effect of EL at 10 μM concentration. It also aligns with the intertwining of genomic and proliferative effects of estrogen. Both viability assays, and gene expression studies have repeatedly shown that the effect produced by EL is significantly lower than that produced by E2. There is a substantial difference in their affinities to the ERα[65], which possibly underlies the quantitative difference in their effects. However, being different chemical entities, they may induce conformational changes in ERα, differently, though subtly, just like in case of selective estrogen receptor modulators. That may lead to differential effects on gene expression. This begs for an independent interrogation into the extent of overlap between genes regulated by EL and E2, both qualitatively and quantitatively.

EL induces ERα transcriptional activity in a dose-dependent manner[63]. It induces *TFF1*[28], and progesterone receptor expression in MCF-7 cells, and induces prolactin synthesis in pituitary cells[128]. *In vivo*, it produces uterine stromal edema, one of the signs of estrogenic activity[23]. Notably, these effects are mediated by EL at a concentration of 10 μM. Furthermore, antiestrogen inhibits EL-mediated induction of progesterone receptor in MCF-7 cells[128]. Here we show that 10 μM EL significantly induces the expression of *TFF1* in MCF-7 cells via a mechanism that involves ERα binding to the estrogen response element within the *TFF1* locus (Figure 5.5). Through a microarray-based expression profiling of a limited set of estrogen-responsive genes, Zhu *et al.*[29] showed a significant correlation between the effects of E2 and EL on gene expression. Taken together, these are compelling evidences in favor of a significant role for ERα in EL-induced cell proliferation and alteration of gene expression at 10 μM concentration.

Collectively, the present chapter provides a genomic perspective on the estrogenic effect of EL in MCF-7 cells. It does not address the opposite effect of EL on breast cancer cell proliferation at higher concentrations, which is also important in understanding the epidemiological difference

in the effects of EL in pre- and post-menopausal women. The observed increase in viability and estrogen-like effect on gene expression in MCF-7 cells counters the beneficial effects of EL against breast cancer as interpreted from epidemiological studies and *in vitro* data that show anti-proliferative and anti-metastatic properties of EL. It is likely that the true effect of EL on the mammary gland is a function of its concentration, and the existence of a functional estrogen-ERα signaling axis.

# Chapter 6

## EL regulation of CYP1A1 expression

## 6.1. Introduction

Given the pro- or antiestrogenic activity of EL, it is likely that EL exposure that results from the intake of a plant lignan-rich diet may have a positive or negative impact on mammary gland tissue homeostasis. Epidemiological data from diverse study populations have been analyzed to examine the relationship with EL exposure and breast cancer (Table 2.2). These studies have yielded conflicting results. While some studies have found an inverse association between plant lignan intake or EL exposure, and breast cancer risk or the associated mortality[12,13,84], others have found no such association[14,200,201]. Zeleniuch-Jacquotte *et al.* even found that EL exposure is associated with increased risk of breast cancer in pre-menopausal women[15]. However, all meta-analyses conducted so far have consistently found the beneficial effects of plant lignans or serum EL in post-menopausal women[16–21]. But, the mechanistic basis has remained unknown.

EL binds ERα[23], and displays both estrogenic and antiestrogenic activities in various experimental models. Notably these estrogen-like activities have been demonstrated at low (up to 10 μM) concentrations of EL. Higher concentrations of EL (> 10 μM) are growth-inhibitory in MCF-7 cells[24,128], and negatively impact DNA synthesis[202]. There is no evidence to state that the apparent anti-estrogenic and growth inhibitory effects of high concentrations of EL are mediated via ER. However, Mousavi and Aldercreutz showed that while low concentration of EL alone stimulated the growth of MCF-7 cells, it inhibited E2-induced growth[24]; a reflection of its partial agonistic activity[65]. EL also interferes with estrogen metabolism via inhibition of aromatase (CYP19) and 17 β-hydroxysteroid dehydrogenase (17β-HSD)[203]. It increases the synthesis of sex hormone binding globulin (SHBG)[204], which sequesters E2 in the serum; thereby reducing its bioavailability.

CYP1A1 is a phase I xenobiotic-metabolizing enzyme. It belongs to cytochrome P450 family, and is involved in metabolizing wide range of drugs and endogenous substrates. It is found mostly in the extrahepatic tissues. Otherwise suppressed, it is induced by environmental substrates via the aryl hydrocarbon receptor (AHR)[136]. CYP1A1 is implicated in breast tumorigenesis. It metabolizes 17-β estradiol into the oxidative product 2-hydroxyestradiol (2-OHE2)[139], which causes DNA damage, and development of breast cancer. Also, reports indicated that the polymorphism of *CYP1A1* gene may determine individual's susceptibility to breast cancer[141,205]. CYP1A1 is expressed in 90% of breast tumours[206], and positively correlated with the tumor

grade[144]. In the absence of the xenobiotic exposure, CYP1A1 expression but not its enzymatic activity is implicated in the growth and survival pathway of breast cancer cells[145], indicating the cell cycle-promoting role of CYP1A1.

This chapter discusses the effect of EL on CYP1A1 regulation, and its implications in breast cancer. The data presented in this chapter allow a reconciliation of the beneficial effect in the face of estrogen-like activity of EL, and provide important insights into how EL may afford a protective effect against breast cancer in post-menopausal women.

## 6.2. Results

### 6.2.1. 10 µM EL down-regulates CYP1A1 in MCF-7 cells

In the previous chapter we analyzed RNA-seq data generated from control-, and EL-treated MCF-7 cells to examine the transcriptomic alterations induced by 10 µM EL in ERα-positive breast cancer cells. In line with its known estrogenic property, 10 µM EL not only induced the viability of MCF-7 cells, but also induced a transcriptomic response similar to that induced by estrogen. GSEA showed that EL-modulated genes were positively enriched in G2/M checkpoint, and late estrogen response gene-sets. GSEA also revealed a negative enrichment of the hallmark xenobiotic metabolism genes, which sets the background for the present study. The enrichment plot for this gene-set is shown in figure 6.1A. As illustrated in figure 6.1B, the leading-edge genes of this gene-set contained few cytochrome P450 family members, including CYP1A1, which appeared down-modulated by EL. The distribution of normalized counts in three biological replicates each of control-, and EL-treated cells showed down-modulation of CYP1A1 mRNA by 10 µM EL (Figure 6.1C). This result was independently confirmed by RT-qPCR (Figure 6.1D).

### 6.2.2. EL, like E2, modulates CYP1A1

E2 is known to downmodulate *CYP1A1* mRNA[207] in MCF-7 cells. Our dose-response, and time-course experiments with E2, were in agreement (Figure 6.2A, B; top panels). At all concentrations tested, E2 significantly decreased *CYP1A1* mRNA expression (Figure 6.2A, top panel). In a time-dependent manner, control cells showed progressive increase in *CYP1A1* mRNA, while 10 nM E2-treated cells showed progressive decrease (Figure 6.2B, top panel).

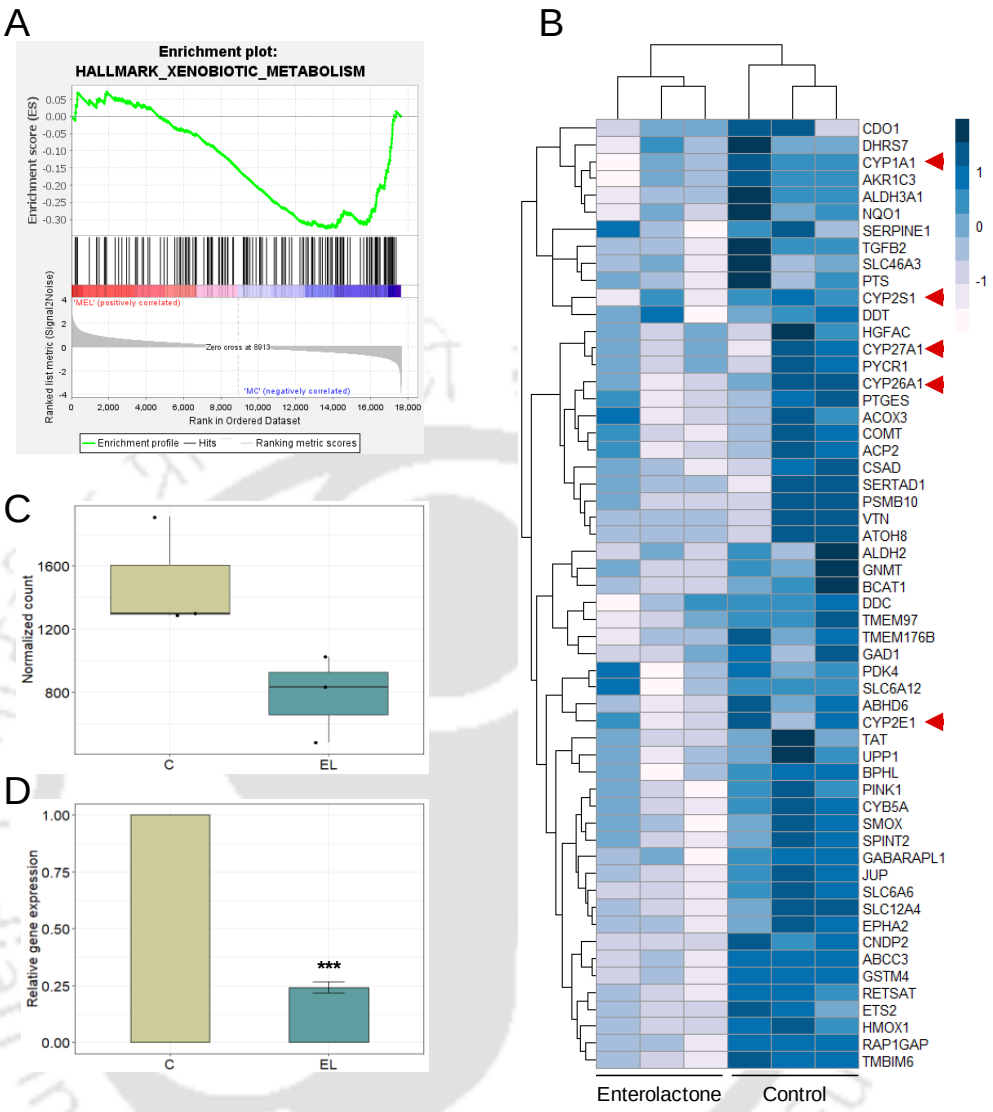


**Figure 6.1. EL modulates xenobiotic response genes in MCF-7 breast cancer cells.** In a previous chapter, genes modulated by 10 µM EL in MCF-7 cells were examined for enriched gene-sets using the GSEA software. **A**. Enrichment plot for hallmark xenobiotic metabolism gene-set. **B**. Heatmap showing the pattern of expression of the leading-edge genes within the xenobiotic metabolism gene-set in control and EL-treated cells (n = 3 for each). The color bar represents levels of expression. Cytochrome P450 family members are marked by red arrowheads. **C**. A box plot showing the distribution of normalized counts corresponding to CYP1A1 in three samples each of control and 10 µM EL-treated cells. The mean normalized counts in control- and EL-treated groups are significantly different as inferred from the Wald's t-test, which was as a part of the DESeq2 analysis (padj = 0.01). **D**. RT-qPCR validation. Total RNA samples extracted from control or 10 µM EL-treated cells were subjected to RT-qPCR analysis of CYP1A1 mRNA expression as described in Materials and Methods. The data were analyzed by the relative quantitation $2^{-\Delta\Delta Ct}$ method using RPL35a as the internal control. The expression in control cells was set to 1, and that in EL-treated cells were expressed relative to control. Bars represent mean relative CYP1A1 mRNA expression ± SD (n = 3). The data were analyzed by a one-tailed one-sample t-test to examine whether the mean relative expression in EL-treated samples is significantly less than one. ***$p < 0.001$. The x-axis labels C, and EL in panels B, C, and D denote control and 10 µM EL-treated cells, respectively.

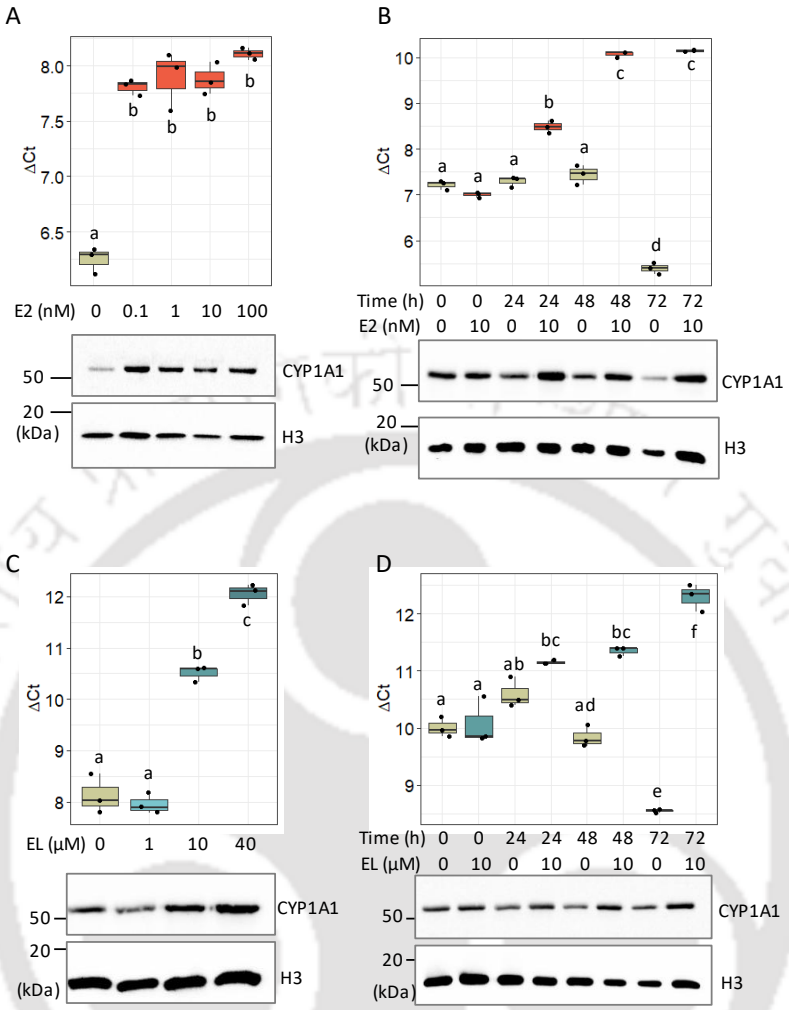


**Figure 6.2. EL, like E2, modulates CYP1A1 mRNA and protein in MCF-7 cells. A, C.** Dose-response study. MCF-7 cells were treated with indicated concentrations of E2 (A) or EL (C) in M2 medium for 24, or 72 h, respectively. Control cells were treated with 0.1% DMSO. **B, D.** Time-course study. MCF-7 cells were treated with 0.1% DMSO (vehicle), 10 μM EL or 10 nM E2 in M2 medium for indicated periods of time. Upon completion of the experiments, total RNA and protein were isolated and subjected to RT-qPCR (top panels), and western blotting analysis (bottom panels) to analyze the expression of CYP1A1 mRNA and protein, respectively. The RT-qPCR data were analyzed by ΔCt method. Here, ΔCt is a measure of CYP1A1 mRNA expression relative to RPL35a, which served as an internal control. Higher the ΔCt value, lower the expression in a given sample. The boxplots show the distribution of ΔCt (n = 3 for each experimental group). The RT-qPCR data in A and C were analyzed by one-way ANOVA. The RT-qPCR data in B and D were analyzed by two-way ANOVA to ascertain the main effects of treatment, or time, or their interaction. While the main effects of EL or E2 ($p \approx 0$), and time ($p \approx 0$) were significant, their interaction was also significant ($p \approx 0$). Note that in the absence of E2 or EL stimulation (B, D), the CYP1A1 mRNA expression significantly increases with time as reflected by decrease in the ΔCt values. On the other hand, in the presence of E2 or EL stimulation, there is progressive decrease in CYP1A1 mRNA expression. TukeyHSD was used for multiple comparison. In all the box plots, the statistical difference between pairs of treatment groups are illustrated by lower-case letters. For western blotting, total protein was isolated from the phenolic phase that is separated during total RNA extraction as described in Materials and Methods. The blots were probed with specific antibodies against the indicated proteins. Histone-H3 served as an internal control. The chemiluminescence images shown here correspond to one out of the three biological replicates.

Given that the literature informs little on the effect of E2 treatment on CYP1A1 protein, dose-response and time-course experiments with E2 were conducted to analyse CYP1A1 protein expression by western blotting. In contrast, all tested concentrations of E2 (0.1 to 100 nM) increased CYP1A1 protein (Figure 6.2A, bottom panel). Results from time-course experiments conducted up to 72 h, showed that while CYP1A1 protein level progressively diminished in control cells, it increased with 10 nM E2 treatment. These results demonstrated the contrasting dynamics of *CYP1A1* mRNA and protein expression post E2 stimulation.

EL binds ERα[23], and is well-known to generate estrogenic responses[28,29,128]. Thus, down-modulation of *CYP1A1* mRNA by 10 µM EL (Figure 6.1) appeared to be yet another manifestation of its estrogenic property. The results shown in figure 6.2A, B served as a reference against which the effect of EL could be assessed. Dose-response and time-course experiments revealed that EL also down-modulated the expression of *CYP1A1* mRNA (Figure 6.2C, D; top panels), but increased CYP1A1 protein (Figure 6.2C, D; bottom panels).

### 6.2.3. EL-mediated regulation of CYP1A1 mRNA is ERα-dependent

4OHT, a selective estrogen receptor modulator significantly blocked EL-mediated decrease in *CYP1A1* mRNA (Figure 6.3), indicating the involvement of ERα. To confirm this, we tested the effect of fulvestrant, a selective estrogen receptor degrader. We first confirmed the depletion of ERα protein by fulvestrant treatment, which also led to increased *CYP1A1* mRNA in a time-dependent manner (Figure 6.4). This indicated that under basal conditions, ERα exerts a suppressing effect on *CYP1A1* mRNA. Figure 6.5 shows that EL-induced loss of *CYP1A1* mRNA (top panel) and accumulation of CYP1A1 protein (bottom panel) were blocked by fulvestrant. Overall, EL appeared to mimic estrogen action at the *CYP1A1* locus; except that EL increased ERα protein (Figure 1C, bottom panel), while E2 is known to decrease it [208].

These data motivated us to examine the *CYP1A1* locus for the presence of potential ERα binding sites. Analysis of ChIP-seq data (GSE25710) revealed two sites of estrogen-induced ERα occupancy in the *CYP1A1* locus (site 1 and site 2, Figure 6.6A). The JASPAR tool also predicted a potential estrogen response element (ERE) in site 1 (Figure 6.6A). ChIP experiments employing

primers targeting site 1 (amplicon I, Figure 6.6A), confirmed that EL-mediated downmodulation was associated with enhanced ERα binding (Figure 6.6B, left panel).

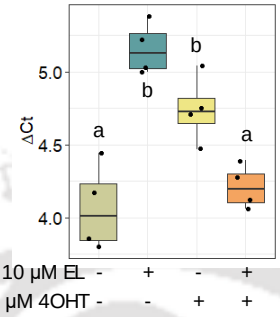


**Figure 6.3. 4OHT blocks EL-suppression of CYP1A1 mRNA.** Total RNA was isolated from MCF-7 cells treated with vehicle, 10 μM EL, 1 μM 4OHT, or both for 72 h in M2, and subjected to RT-qPCR analysis. The data were analyzed by ΔCt method. The box plot shows the distribution of ΔCt values (n = 4). The data were analyzed by two-way ANOVA to ascertain the main effects of EL, or 4OHT, or their interaction. While there was significant main effect of EL ($p = 0.02$), but not 4OHT ($p = 0.2$), their interaction was significant ($p \approx 0$). a and b denote the statistical difference between pairs of treatment groups, which was determined by TukeyHSD.

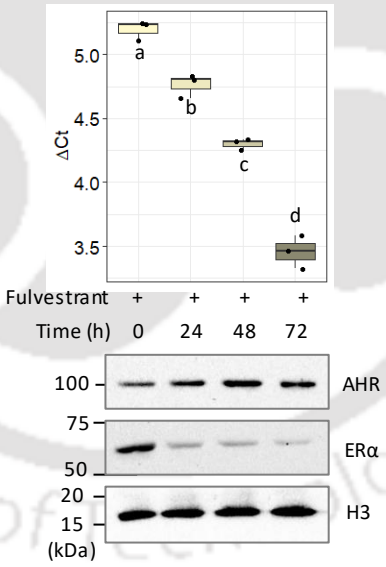


**Figure 6.4. Fulvestrant modulates CYP1A1 expression in MCF-7 cells.** Cells were treated with 100 nM fulvestrant in M2 medium for the indicated periods of time. Top panel. RT-qPCR. Total RNA was isolated and subjected to RT-qPCR. The data were analyzed by ΔCt method. The boxplot shows the distribution of ΔCt values in each of the treatment groups (n = 3 for each time-point). The data were analyzed by one-way ANOVA followed by TukeyHSD for pair-wise comparison of group means. Letters a, b, c, and d represent statistical difference between pairs of treatment groups. Bottom panel. Western blotting. Total protein isolated from the phenolic phase that is separated during total RNA extraction, was used for western blotting using antibodies specific to ERα. The blots were then stripped and probed sequentially with AHR, and Histone-H3 (internal control). Shown are the results obtained from one out of the three biological replicates. Note the increase in AHR protein concomitant to decrease in ERα.

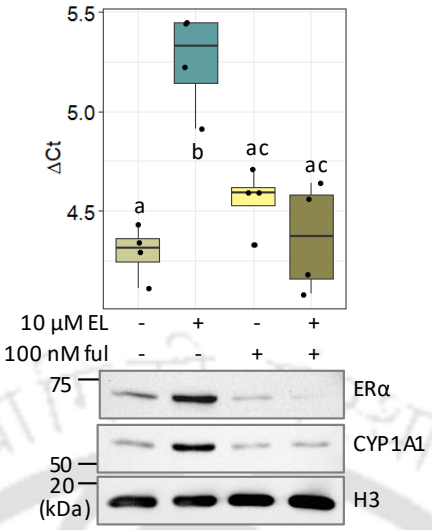


**Figure 6.5. Fulvestrant affects EL-mediated regulation of CYP1A1 in MCF-7 cells.** Top panel shows RT-qPCR data. Cells were pre-treated with vehicle, or 100 nM fulvestrant (ful) in M2 medium for 24 h, followed by treatment with vehicle, or 10 µM EL treatment for 72 h. Total RNA was isolated and subjected to RT-qPCR. The data were analyzed by ΔCt method. The boxplot shows the distribution of ΔCt values in each of the treatment groups (n = 4 biological replicates). The data were analyzed by two-way ANOVA followed by TukeyHSD for pair-wise comparison of group means. Letters a, b, and c represent statistical difference between pairs of treatment groups. Bottom panel shows western blotting. Total protein isolated from the phenolic phase that is separated during total RNA extraction, was used for western blotting using antibodies specific to ERα, CYP1A1, and Histone-H3 (internal control). The blots were stripped and probed sequentially with CYP1A1 and Histone-H3 antibodies. Shown are results obtained from one out of the four biological replicates.

### 6.2.4. EL-bound ER antagonizes AHR to suppress CYP1A1 mRNA

Compelling experimental data show that the interplay of opposing estrogenic and xenobiotic signals determines the net *CYP1A1* mRNA expression, wherein, estrogen-activated ERα directly interferes with xenobiotic-induced, and AHR-dependent transcriptional activation of *CYP1A1*[138]. Given that our experiments were conducted without xenobiotic exposure, both EL, and E2 seem to impact the basal *CYP1A1* expression. A precipitous fall in *CYP1A1* mRNA levels by CH223191, an inhibitor of AHR nuclear translocation (Figure 6.7, top panel), showed that basal *CYP1A1* mRNA expression is AHR-dependent. CYP1A1 protein, in contrast was increased (Figure 6.7 bottom panel). EL-treatment, over and above CH223191, did not change the mRNA or protein expression any further (Figure 6.7). We hypothesized that EL-mediated ERα activation interferes with AHR-mediated transactivation at the *CYP1A1* locus. To test the hypothesis, we performed ChIP experiments employing AHR-specific antibody, and examined the representation of site 1 in the immunoprecipitated chromatin samples.

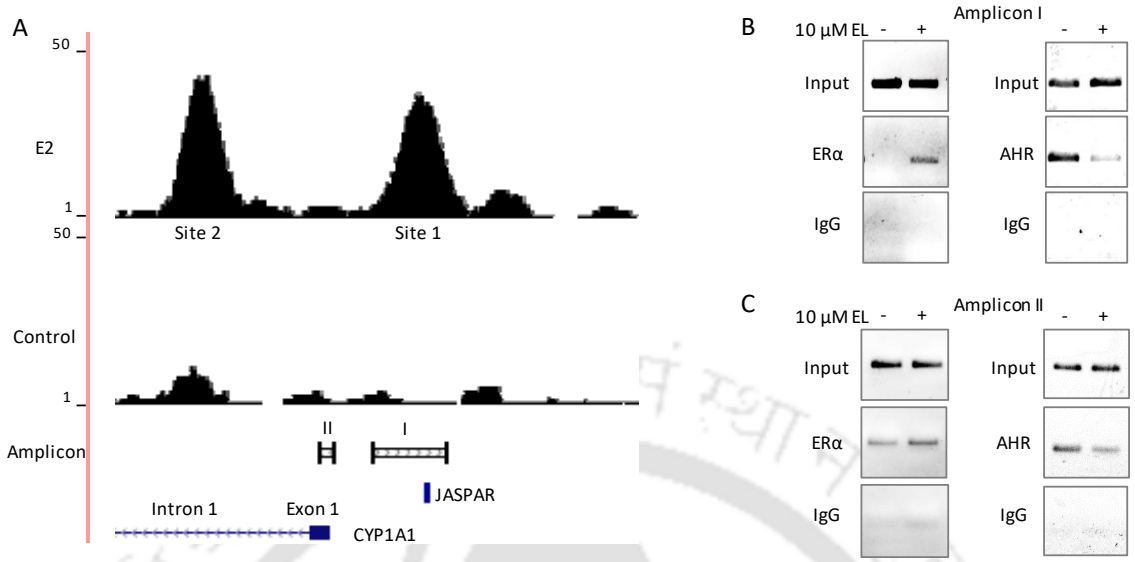


**Figure 6.6. EL enhances ERα occupancy within CYP1A1 locus in MCF-7 cells. A**. A snapshot of the CYP1A1 locus. Site 1, and Site 2 are ERα-enriched regions deduced from the ChIP-seq data (GSE25710). ERE predicted by JASPAR is indicated in blue. The amplicon track shows the regions amplified by two pairs of ChIP primers (labeled as I and II). The primer pair for amplicon II was from Marques *et al.* [138] **B, C**. Results of ChIP experiments with primer pairs generating amplicon I (**B**), or amplicon II (**C**), respectively. Sonicated chromatin samples prepared from vehicle- or 10 µM EL-treated cells were immunoprecipitated with non-specific IgG, ERα- or AHR-specific antibody. Immunoprecipitated DNA samples were subjected to PCR. Shown are results obtained from one out of the three biological replicates.

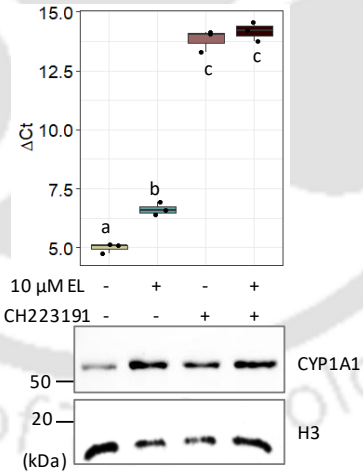


**Figure 6.7. Effect of CH223191 on EL's modulation of CYP1A1 in MCF-7 cells.** Top panel. Total RNA was isolated from MCF-7 cells treated with vehicle, 10 µM EL, 10 µM CH223191, or both for 72 h in M2, and subjected to RT-qPCR analysis. The data were analyzed by ΔCt method. The box plot shows the distribution of ΔCt values (n = 3 for each treatment group). The data were analyzed by two-way ANOVA to ascertain the main effects of EL, or CH223191, or their interaction. While there was significant main effect of EL ($p = 0.01$), but not CH223191 ($p \approx 0$), their interaction was significant ($p = 0.01$). a, b, and c denote the statistical difference between pairs of treatment groups, which was determined by TukeyHSD. Bottom panel. Western blotting. Total protein isolated from the phenolic phase that is separated during total RNA extraction, was used for western blotting. The blots were probed with specific antibodies against CYP1A1 and Histone-H3. Histone-H3 served as an internal control.

Concomitant to the EL-induced ERα occupancy (Figure 6.6B, left panel), there was a decrease in AHR occupancy, at site 1 (Figure 6.6B, right panel). In parallel, we examined the representation of another site demonstrated by Marques *et al.*[138] to be the seat of ERα-AHR interplay. As shown in Figure 6.6C, EL treatment increased ERα occupancy (left panel), while decreasing AHR occupancy at this site (right panel).

### 6.2.5. EL restores CYP1A1 expression in MCF-7 cells grown in M2

CYP1A1 protein is expressed in 90% of breast tumours[206], and positively associated with cell proliferation[145]. In the previous chapter, we showed that MCF-7 cell growth is compromised in M2 medium compared to those grown in M1 medium (Figure 4.4A). We have observed that MCF-7 cells grown for 72 h in M2 showed less CYP1A1 protein expression than those grown in M1 (Figure 6.8). Moreover, 10 µM EL, which restored growth and PCNA expression in cells grown in M2 (Figure 4.4A, C) also restored CYP1A1 protein (Figure 6.8). It may be recalled that, E2, which induces the proliferation of ERα-positive breast cancer cells, also increased CYP1A1 protein, although, like EL, it downmodulated the mRNA (Figure 6.2A, B).

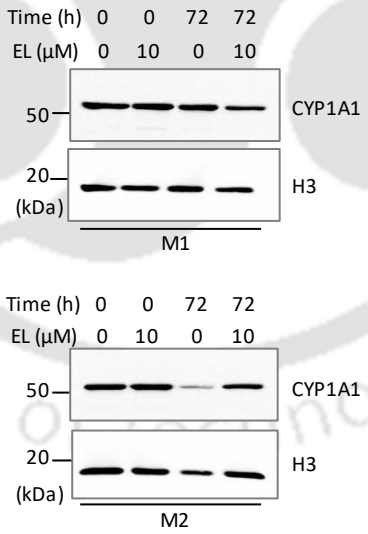


**Figure 6.8. EL restores CYP1A1 protein expression in MCF-7 cells grown in M2.** MCF-7 cells were allowed to grow until 50% confluent. Then the cells were fed M1 or M2 medium for 24 h, followed by treatment with 0.1% DMSO (vehicle), or 10 µM EL in M1 or M2 for 0 or 72 h. Total protein was isolated from the phenolic phase, which is separated during total RNA extraction as described in Materials and Methods. The blots were probed with specific antibodies against the indicated proteins. Histone-H3 served as an internal control. The chemiluminescence data shown are one of the three biological replicates.

### 6.2.6. EL antagonizes E2 to reduce CYP1A1 protein

As mentioned above, E2 or EL induced CYP1A1 protein. Also, as found in Chapter 4, EL or E2 induced cell proliferation in ERα-positive breast cancer cells. Given that EL exerts partial agonist/antagonistic activity, we checked whether EL-E2 co-treatment impacts CYP1A1 expression. MCF-7 cells were treated with increasing concentrations of EL with or without 10 nM E2 in M2 for 72 h. EL, like E2, induced the expression of the classical estrogen-regulated *TFF1* mRNA (Figure 6.9, top panel). Similarly, while E2 or EL independently decreased or increased the levels of *CYP1A1* mRNA and protein respectively, EL significantly attenuated the E2 effect (Figure 6.9, middle and bottom panels).

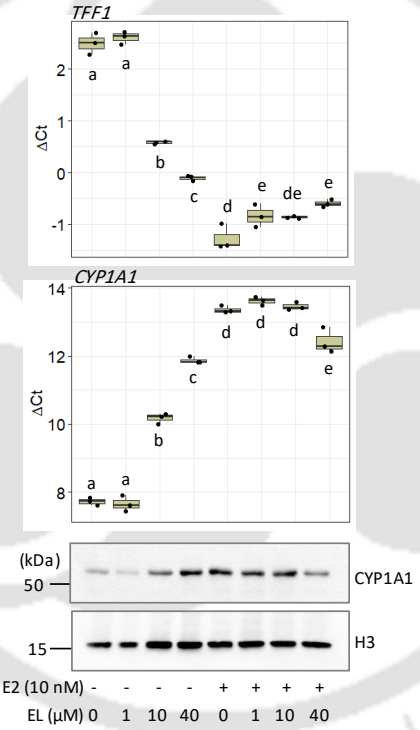


**Figure 6.9. EL attenuates E2-mediated effect on CYP1A1 expression.** MCF-7 cells were treated with vehicle, or the indicated concentrations of EL, alone or in combination with 10 nM E2 for 72 h in M2. Top panel. Total RNA was isolated, and subjected to RT-qPCR analysis to check the expression of TFF1 (top panel) or CYP1A1 mRNA (middle panel). The data were analyzed by ΔCt method. The box plots show the distribution of ΔCt values (n = 3 for each treatment group). The data were analyzed by two-way ANOVA to test the main effects of E2, EL, or their interaction. There were significant main effects of E2 ($p \approx 0$), and EL ($p \approx 0$). The interaction between E2 and EL was also significant ($p \approx 0$). TukeyHSD was used for pair-wise comparison of group means. The lower-case letters represent the statistical difference between pairs of treatment. Total protein was isolated from the phenolic phase that is separated during total RNA extraction as described in Materials and Methods, and used for western blotting (bottom panel). The blots were probed with specific antibodies against the indicated proteins. Histone-H3 served as an internal control.

## 6.3. Discussion

The results conclusively show that EL suppresses *CYP1A1* mRNA in an ERα-dependent manner via interference with AHR binding and transcriptional activation of the *CYP1A1* promoter. In contrast, like E2, EL increases the level of CYP1A1 protein. While EL alone produces EL-like effects of CYP1A1 expression, it antagonizes the effects of E2, which is consistent with the partial agonistic property of EL, as demonstrated earlier[65]. EL-mediated regulation of *CYP1A1* is a not only a manifestation of E2-like effects of EL, but also bears interesting clinical implications in the field of breast cancer prevention and treatment.

CYP1A1 is a mediator of the xenobiotic response. It metabolizes pro-carcinogens into carcinogens[136]. However, it is one among a large repertoire of metabolic enzymes. Besides *CYP1A1*, other cytochrome P450 family members, such as *CYP2S1*, *CYP27A1*, *CYP26A1*, and *CYP2E1*, are also a part of the leading-edge genes within the negatively enriched xenobiotic response gene-set (Figure 6.1B). Since these enzymes acting on endogenous and exogenous substrates can potentially lead to the generation of reactive intermediates[209], down-modulation of these genes possibly underlies EL's beneficial effects in breast cancer. But, the induction of CYP1A1 protein by EL obscures the relevance of CYP1A1 in EL's protective effect. Firstly, increased CYP1A1 enzymatic activity on xenobiotics can lead to enhanced conversion of xenobiotics into carcinogens. Secondly, CYP1A1 converts E2 to 2-hydroxyestradiol (2-OHE2), which is more reactive, and may promote carcinogenesis by facilitating the generation of reactive oxygen species. On the other hand, domination of CYP1A1 activity over CYP1B1, which converts E2 to 4-hydroxyestradiol (4-OHE2), is beneficial from the cancer perspective; given the contrasting anti-proliferative and genotoxic nature of 2-OHE2 and 4-OHE2, respectively [139]. Thus, any explanation for the beneficial effects of EL against breast cancer, only on the basis of differential modulation of *CYP1A1* mRNA or protein in cultured cells observed with a high concentration of 10 µM EL, may not be straightforward. However, non-enzymatic activity of CYP1A1 associated to cell survival and proliferation is discussed hereunder, which, in the light of a preliminary observation from this study, may be the basis of the protection imparted by EL in post-menopausal women.

CYP1A1 is expressed in majority of breast tumors[144,206]. Experimental depletion of CYP1A1 expression reduces proliferation and increases apoptosis via AMPK activation, and de-

phosphorylation of AKT, ERK1/2 and p70S6K[145]. Carnosol, a pharmacological agent reduces CYP1A1 protein levels, also impairs proliferation[145]. The proliferative potential of MCF-7 cells in phenol red-free, and charcoal-stripped serum-containing medium (M2) is significantly lower than those grown in routine medium (M1) (Figure 4.4A). We have observed that MCF-7 cells grown for 72 h in M2 showed less CYP1A1 protein expression than those grown in M1 (Figure 6.8). Moreover, 10 µM EL, which restored growth and PCNA expression in cells grown in M2 (Figure 4.4C) also restored CYP1A1 protein (Figure 6.8). E2, which induces the proliferation of ERα-positive breast cancer cells, also increased CYP1A1 protein, although, like EL, it down-modulated the mRNA (Figure 6.2A, B). These are clear indications of the positive correlation between CYP1A1 protein expression and proliferative state. The correlation of CYP1A1 protein expression with estrogenic input is also borne out by the observation that breast tumors in pre-menopausal women have higher expression of CYP1A1 protein than those in post-menopausal women[144]. Rodriguez and Potter[145] showed that CYP1A1 protein knockdown, but not inhibition of its enzyme activity, impairs cell proliferation. Thus, it appears that cell proliferation and xenobiotic metabolism are mutually exclusive functional attributes of CYP1A1. Given that E2 or EL induces CYP1A1 protein, the question as to how EL may exert a protective effect is a question that begs attention. EL is known to exert partial agonist/antagonistic activity[65]. In agreement with this notion, we found that although EL, like E2, induced the expression of the classical estrogen-regulated *TFF1* mRNA, EL attenuated the induction brought about by E2 (Figure 6.9, top panel). Similarly, while E2 or EL independently decreased or increased the levels of *CYP1A1* mRNA and protein, respectively, EL significantly attenuated the E2 effect (Figure 6.9, middle and bottom panels). This parallels the observation by Mousavi and Adlercreutz[24] that both induce proliferation, but EL can attenuate E2-mediated proliferation. This property of EL may be a significant contributor to its beneficial effects. We propose that in the face of diminishing levels of estrogen in post-menopausal women, EL concentrations achieved through lignan-rich diet may be better able to antagonize estrogen to keep levels of CYP1A1 protein low, thereby reducing the chances of breast tumors.

# Chapter 7

## Conclusions and future prospects

## 7.1. Conclusions

The data presented in this thesis bring to the fore a mechanistic insight about EL action in the context of breast cancer management. The results may be of interest in the fields of cancer biology, cancer prevention, and nutrigenomics. Our findings align with the previous observations about EL estrogenicity in breast cancer cells. In MCF-7 cells, 10 μM EL enhanced the viability, and significantly increased the expression of *TFF1* mRNA, an estrogen-induced transcript. The binding of ERα to the estrogen response element within the *TFF1* locus further demonstrated the pro-estrogenic effect of 10 μM EL.

EL is a biologically active compound. It has a nutrigenomic effect attributed to its ability to modulate several molecular targets. Our transcriptomic analysis provides the genetic understanding of EL action in breast cancer cells. Analysis of RNA-seq data obtained from vehicle (0.1% DMSO)- or 10 μM EL-treated MCF-7 cells revealed modulation of expression of diverse sets of functionally related genes, which reflected cell cycle progression. The manner in which 10 μM EL regulated the hallmark G2/M checkpoint, and estrogen-response-late genes correlated with proliferation-inducing, and estrogen-like effects of EL on MCF-7 cells.

EL acts via ERα to antagonize AHR-mediated transactivation that results in downmodulation of *CYP1A1* mRNA. It possibly imparts beneficial effects by inhibiting the oncogenic effects of genotoxic agents produced by xenobiotic response to pro-carcinogens, such as polycyclic or heterocyclic aromatic hydrocarbons. EL, like E2, induces CYP1A1 protein. However, consistent with the property of partial agonism/antagonism, it blocks E2-mediated increase of CYP1A1 protein, thereby negatively influencing the proliferation of mammary epithelial cells.

## 7.2. Future prospects

The effects of EL presented in this thesis were observed at higher concentrations of EL, which present a major caveat. Hence, the question remains as to how high the concentration of EL in the mammary tissue could be achieved through consumption of lignan-rich diet so as to be able to negate E2 action in mammary epithelial cells. However, the results open the scope for further investigations to explore whether increased intake of lignan-rich diet or supplementation of EL will confer protection against breast cancer in post-menopausal women.

Breast cancer surpassed lung cancer as the leading cause of cancer incidence among women globally. In 2022, there were 2,296,840 incidents (23.8% of all cancer cases), and 666,103 deaths (15.4% of cancer deaths) among women worldwide. The number of new cases is estimated to increase by more than 106% in 2045. In India, breast cancer is the most diagnosed cancer among women[210]. The number of new cases in 2022 was 192,020 (26.6% of total cancer incidents), while the number of deaths was 98,337 (10.7% of total cancer deaths). However, India is one of the regions that have the lowest incidence rates of breast cancer[211]. India has a large percentage of vegetarians who consume legumes, vegetables, and roots[212]. In a study by Kunisue *et al.*, the concentration of phytoestrogens was determined in urine samples of several populations[213]. Among the analyzed samples, the highest levels of EL were found in urine samples from India. Epidemiologically, several research groups addressed the relationship between lignan intake or EL concentration in the plasma and breast cancer in different cohorts (Table 2.2). However, this relationship is poorly understood in the Indian population, which begs more attention.

Observational studies highlighted the beneficial effect of EL against breast cancer among postmenopausal women. However, as discussed in the chapter 6, EL might have a positive effect among premenopausal women as well. This is reflected in few epidemiological studies that report a decrease in breast cancer risk among women with higher lignan intake, or higher EL concentrations in the plasma. The possible reason of EL's inability to manifest its actual effect in pre-menopausal women is the high levels of circulating E2, that EL levels achieved by diet alone are not sufficient to antagonize E2 to decrease CYP1A1 protein expression. Further, McCann[78] showed that breast cancer risk-reducing effect of EL can be modified by the genotype of the steroidogenic enzyme, *CYP17*. CYP1A1 is another steroid-metabolizing enzyme. *CYP1A1* polymorphisms play a role in breast cancer susceptibility. Various cohorts of women may have different *CYP1A1* polymorphisms, which alter the enzyme expression and activity, thus affect the metabolism and modify risk association. Epidemiological studies addressed the link between EL and breast cancer in the context of menopausal status or estrogen receptor status, but not in the context of the genetic variation of *CYP1A1*. Hence, breast cancer risk linked with EL levels in relation to *CYP1A1* polymorphisms warrants more investigations.

This knowledge might enable the governmental agencies to decide about promoting lignan-rich foods such as flaxseed and sesame seeds as lifestyle dietary intervention, to include them in the diet for better prevention against breast cancer.

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# *Appendix*

**Appendix I. List of chemicals and reagents with their sources.**

| Item | Cat. No. | Company | Source |
|---|---|---|---|
| DMEM with phenol red | AT-007 | HiMedia | Mumbai, India |
| RPMI-1640 with phenol red | AT-028 | HiMedia | Mumbai, India |
| DMEM without phenol red | AT-190 | HiMedia | Mumbai, India |
| RPMI-1640 without phenol red | AT-120 | HiMedia | Mumbai, India |
| FBS | RM10432 | HiMedia | Mumbai, India |
| CS-FBS | RM10416 | HiMedia | Mumbai, India |
| Trypsin-EDTA | TCL-034 | HiMedia | Mumbai, India |
| Antibiotic solution 100 X liquid (10,000 U penicillin and 10 mg streptomycin per ml in citrate buffer) | A018 | HiMedia | Mumbai, India |
| DPBS | TS-1006 | HiMedia | Mumbai, India |
| Enterolactone | 45199 | Sigma-Aldrich | MO, USA |
| 17β-estradiol | E8875 | Sigma-Aldrich | MO, USA |
| 4-hydroxy-tamoxifen | H7904 | Sigma-Aldrich | MO, USA |
| Fulvesrant | 14409 | Sigma-Aldrich | MO, USA |
| CH223191 | C8124 | Sigma-Aldrich | MO, USA |
| Trypan blue dye | T8154 | Sigma-Aldrich | MO, USA |
| High-Capacity cDNA Reverse transcription kit | 4368814 | Applied Biosystems | USA |
| PowerUp ™ SYBR™ Green Master Mix for qPCR | A25743 | Thermo Scientific | PA, USA |
| Protein G plus-Agarose beads | IP04-1.5ML | Merck Millipore | Burlington, USA |
| Nitrocellulose membrane | SF110B | HiMedia | Mumbai, India |

| | | | |
|---|---|---|---|
| ERα antibody (ChIP) | 8644 | Cell Signaling Technology | MA, USA |
| ERα antibody (Western blot) | 8002 | Santa Cruz Biotechnology | USA |
| AHR antibody | 83200S | Cell Signaling Technology | MA, USA |
| CYP1A1 antibody | PA1-340 | Thermo Scientific | PA, USA |
| Anti-PCNA antibody | 13110 | Cell Signaling Technology | MA, USA |
| Anti-H3 antibody | BB-AB0055 | BioBharti LifeSciences | Kolkata, India |
| Goat anti-rabbit HRP-tagged secondary antibody | 7074S | Cell Signaling Technology | MA, USA |
| Horse anti-mouse HRP-tagged secondary antibody | 7076S | Cell Signaling Technology | MA, USA |
| Clarity Western ECL Substrate | 1705060 | BIO RAD | California, USA |
| Cell culture plasticware | | Eppendorf | Hamburg, Germany |
| | | Thermo Fisher Scientific | PA, USA |
| All other reagents, salts, and buffers | | Merck | Darmstadt, Germany |
| | | Sisco Research Laboratories | Mumbai, India |
| | | Sigma-Aldrich | MO, USA |

**Appendix II. List of primers**

| Gene | Application | Primer sequence (5′→3′) | Amplicon (base pair) | Annealing temperature (°C) |
|---|---|---|---|---|
| *RPL35a* | RT-qPCR | Forward- CGGCCTCCAAGCTCTCTAAG<br>Reverse- CAGGTCCAGGGGCTTGTACT | 131 | 60 |
| *CYP1A1* | RT-qPCR | Forward- ACCTTTGAGAAGGGCCACATCCG<br>Reverse- TGACTGTGTCAAACCCAGCTCCAAAG | 154 | 60 |
| *CYP1A1* (Amplicon I) | ChIP | Forward- AGTCCCAATTCCAAGGCGTC<br>Reverse- CCTTCGCCATCCATTCCGAT | 406 | 60 |
| *CYP1A1* (Amplicon II) | ChIP | Forward- CGTACAAGCCCGCCTATAAA<br>Reverse- CTGGGATCACAAGGATCAGG | 92 | 60 |
| *CDC20* | RT-qPCR | Forward- AGTGCCGTGGATGCCCATTC<br>Reverse- GGCCATGGTTGGGTACTTCC | 123 | 60 |
| *TFF3* | RT-qPCR | Forward- CCCTGGAGTGCCTTGGTGTT<br>Reverse- AGCAATCACAGCCGGGCAAG | 122 | 60 |
| *XBP-1* | RT-qPCR | Forward- GGCTCGAATGAGTGAGCTGG<br>Reverse- CAGCAACCAGGGCATCCATC | 158 | 60 |
| *STIL* | RT-qPCR | Forward-CATGCACACACCCAAGACTGAG<br>Reverse- CAGGGCATCAGAGACTGTGC | 147 | 60 |
| *KIF20A* | RT-qPCR | Forward- GCCAAGCCACACACAGGTTC<br>Reverse- TAGATGAGCCAGTTCTGCCC | 127 | 60 |
| *GINS2* | RT-qPCR | Forward- GCCCAGCCCTTACTACATGG | 168 | 60 |

| | | | | |
|---|---|---|---|---|
| | | Reverse- GCATGTGCCTCCTGCTGTCT | | |
| *CA2* | RT-qPCR | Forward- GCCAAGTATGACCCTTCCCT<br>Reverse- AAGTGCCATCCAGGGGTCCT | 151 | 60 |
| *PCP4* | RT-qPCR | Forward- AGTGAGCGACAAGGTGCTGG<br>Reverse- GCACGTTCTGTCTCTGGTGC | 122 | 60 |
| *PRLR* | RT-qPCR | Forward- CCTGGGACAGATGGAGGACT<br>Reverse- CCAAAGTGGCAGGAGTTGGG | 119 | 60 |
| *MYB* | RT-qPCR | Forward- GCCTGGACGAACTGATAATGC<br>Reverse- CGGAGCCTGAGCAAAACCCA | 136 | 60 |
| *IGSF1* | RT-qPCR | Forward- GACACCTGAGGATGAAGGGG<br>Reverse- CCCAGGATGGGCTGTCAAAG | 139 | 60 |
| *CXCL12* | RT-qPCR | Forward- TGAACGCCAAGGTCGTGGTC<br>Reverse- GTTGGCTCTGGCAACATGGC | 128 | 60 |
| *CENPF* | RT-qPCR | Forward- GGCTGCACAGAAGTTAGCG<br>Reverse-GGAGGATGGTGCCTGAATCTAC | 140 | 60 |
| *TOP2A* | RT-qPCR | Forward- GTGGCAAGGATTCTGCTAGTCC<br>Reverse- ACCATTCAGGCTCAACACGCTG | 135 | 60 |
| *TFF1* | RT-qPCR | Forward- GGGTCCCCTGGTGCTTCTAT<br>Reverse- AGCCGAGCTCTGGGACTAA | 140 | 60 |
| *TFF1* | ChIP | Forward- CATTG CCTCCTCTCTGCTCC<br>Reverse- ACTGTTGTCACGGCCAA GCC | 423 | 60 |

# List of abbreviations

| | |
|---|---|
| ANOVA | Analysis of variance |
| µg | Microgram |
| µL | Microliter |
| µM | Micromolar |
| 17β-HSD | 17 β-hydroxysteroid dehydrogenase |
| 2-OHE2 | 2-hydroxyestradiol |
| 3MC | 3-methylcholanthrene |
| 4-OHE2 | 4-hydroxyestradiol |
| 4OHT | 4-hydroxy-tamoxifen |
| AF-1 | Transactivation function 1 |
| AF-2 | Transactivation function 2 |
| AHR | Aryl hydrocarbon receptor |
| AHRR | Aryl hydrocarbon receptor suppresser |
| AKT | Protein kinase B |
| ARNT | Aryl hydrocarbon receptor nuclear translocator |
| BD | Basal diet |
| BP | Biological processes |
| bp | Base pair |
| BSA | Bovine serum albumin |
| BW | Body weight |
| CA2 | Carbonic anhydrase 2 |
| CC | Cellular components |
| CDC20 | Cell division cycle 20 |
| CDK | Cyclin-dependent kinase |
| cDNA | Complementary deoxyribonucleic acid |

| | |
|---|---|
| CENPF | Centromere protein F |
| ChIP | Chromatin immunoprecipitation |
| COMT | Catechol-O-methyltransferase |
| CS-FBS | Charcoal-stripped fetal bovine serum |
| CXCL12 | C-X-C Motif Chemokine Ligand 12 |
| CYP1A1 | Cytochrome P450 Family 1 Subfamily A Member 1 |
| CYP1B1 | Cytochrome P450 Family 1 Subfamily B Member 1 |
| CYP26A1 | Cytochrome P450 family 26 subfamily A member 1 |
| CYP27A1 | Cytochrome P450 family 27 subfamily A member 1 |
| CYP2E1 | Cytochrome P450 family 2 subfamily E member 1 |
| CYP2S1 | Cytochrome P450 family 2 subfamily S member 1 |
| DMEM | Dulbecco's Modified Eagle Medium |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| DPBS | Dulbecco's phosphate beffered saline |
| E1 | Estrone |
| E2 | 17β-estradiol |
| E3 | Estriol |
| ECL | Enhanced chemiluminescence |
| ED | Enterodiol |
| EDTA | Ethylenediaminetetraacetic acid |
| EGF | Epidermal growth factor |
| EGFR | Epidermal growth factor receptor |
| EL | Enterolactone |
| EREs | Estrogen-responsive elements |
| ERK | Extracellular-signal-regulated kinase |
| ERα | Estrogen receptors alpha |

| | |
|---|---|
| ERβ | Estrogen receptors beta |
| FBS | Fetal bovine serum |
| FDR | False discovery rate |
| FO | Flaxseed oil |
| ful | Fulvestrant |
| GEN | Genistein |
| GEO | Gene expression omnibus |
| GINS2 | GINS Complex Subunit 2 |
| GO | Gene ontology |
| GPER1 | G protein-coupled estrogen receptor 1 |
| GSEA | Gene Set Enrichment Analysis |
| h | Hours |
| HER2 | Human epidermal growth factor receptor 2 |
| HRP | Horseradish peroxidase |
| HSD | Honestly significant difference |
| kDa | Kilodalton |
| LBD | Ligand binding domain |
| M1 | Culture medium with phenol red and fetal bovine serum |
| M2 | Experimental medium with charcoal-stripped fetal bovine serum, and phenol-red free |
| MAPK | Mitogen-activated protein kinase |
| MAT | Matairesinol |
| MF | Molecular functions |
| min | Minute |
| mL | Milliliter |
| MMP | Matrix metalloprotease |
| mRNA | Messenger ribonucleic acid |

| | |
|---|---|
| MYB | Proto-Oncogene transcription factor |
| NES | Normalized enrichment score |
| NF-κb | Nuclear factor kappa B |
| nM | Nanomolar |
| OVX | Ovariectomy |
| p70S6K | p70 ribosomal S6 kinase |
| PCNA | Proliferating cell nuclear antigen |
| PCP4 | Purkinje Cell Protein 4 |
| PCR | Polymerase chain reaction |
| PR | Progesterone receptor |
| PRLR | Prolactin receptor |
| RIN | RNA integrity number |
| RNA | Ribonucleic acid |
| RNA-seq | RNA sequencing |
| RPL35a | Ribosomal protein L35a |
| RPMI | Roswell Park Memorial Institute Medium |
| RT-qPCR | Reverse transcription quantitative PCR |
| SDG | Secoisolariciresinol diglucoside |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| SECO | Secoisolariciresinol |
| SHBG | Sex hormone binding globulin |
| STIL | Centriolar assembly protein |
| TBST | Tris-buffered saline-Tween 20 |
| TCA | Trichloroacetic acid |
| TCDD | 2,3,7,8-tetrachlorodibenzo-p-dioxin |
| TFF1/pS2 | Trefoil factor 1 |
| TFF3 | Trefoil factor 3 |

| | |
|---|---|
| TIMP | Tissue inhibitors of metalloproteinase |
| TOP2A | DNA Topoisomerase II Alpha |
| uPA | Urokinase-type plasminogen activator |
| VEGF | Vascular endothelial growth factor |
| XBP1 | X-box binding protein 1 |
| XREs | Xenobiotic regulatory elements |

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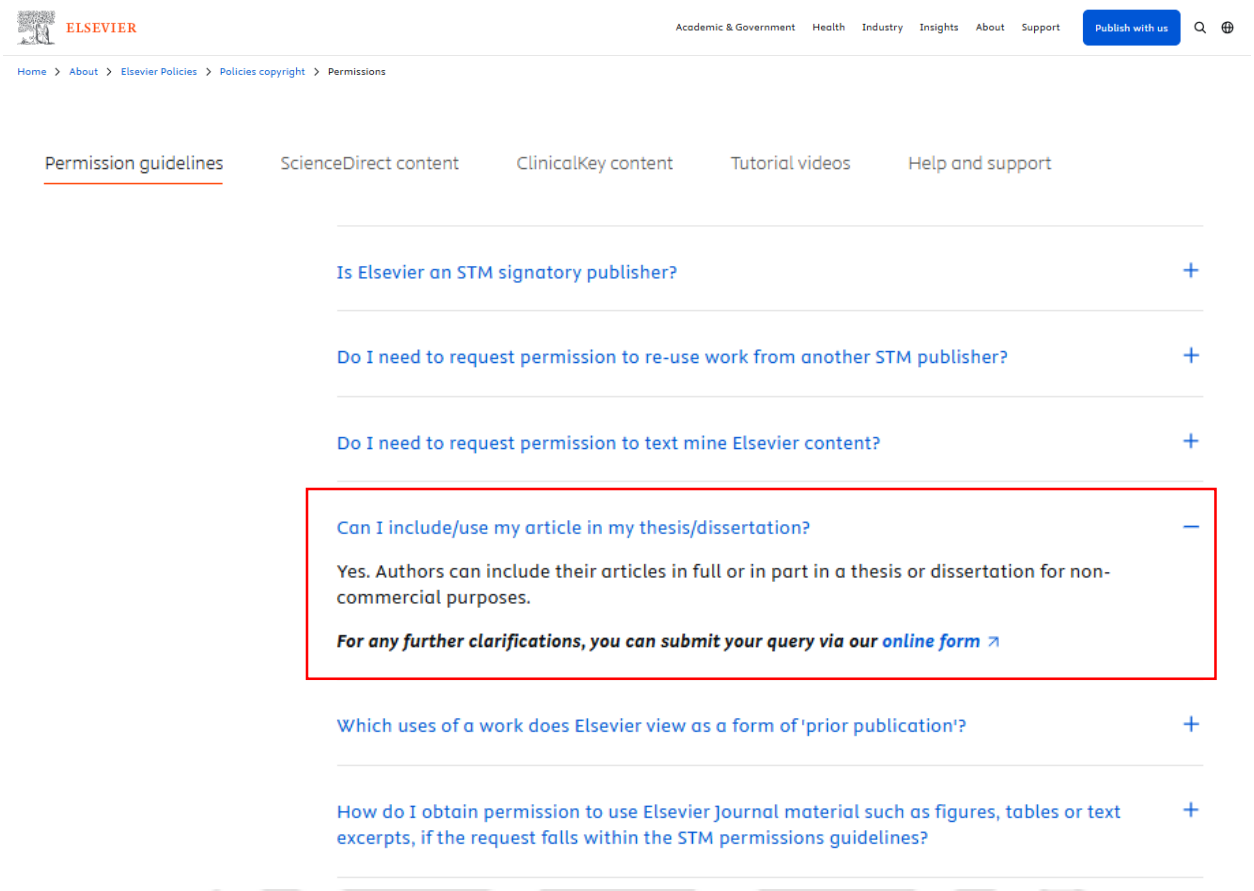

# List of publications and presentations

## Publications from thesis work:

1. J. Hatwik, N. Sonia, A. M. Limaye, The CYP1A1 connection between enterolactone and breast cancer risk (in bioRxiv). https://doi.org/10.1101/2024.06.06.597836.

2. Hatwik J, Patil HN, Limaye AM. Proliferative response of ERα-positive breast cancer cells to 10 μM enterolactone, and the associated alteration in the transcriptomic landscape. Gene. 2023 Sep 25;881:147640. doi: 10.1016/j.gene.2023.147640. Epub 2023 Jul 13. PMID: 37453722.

3. Hatwik J, Pal U, Limaye AM. Transcriptomic data of MCF-7 breast cancer cells treated with 10 μM enterolactone. Data Brief. 2023 Mar 28;48:109098. doi: 10.1016/j.dib.2023.109098. PMID: 37077651; PMCID: PMC10106490.

## Poster presentations:

1. Juana M. Hatwik, Hrishikesh N. Patil, Anil M. Limaye. Proliferative response of ERα-positive breast cancer cells to 10 μM enterolactone, and the associated alteration in the transcriptomic landscape. Presented in "Research conclave" held in Guwahati, India (2023).

2. Juana M. Hatwik, Hrishikesh N. Patil, Anil M. Limaye. Proliferative response of ERα-positive breast cancer cells to 10 μM enterolactone, and the associated alteration in the transcriptomic landscape. Presented in the Indian Association for Cancer Research (IACR), held in Pune, India (2024).