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Down regulation of estrogen receptors (ER*a* ; ER*β*) and atypical chemokine receptors (ACKR 2; ACKR3; ACKR4) to increase 17β-estradiol (E2) levels in MCF-7 in-vitro study

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ABSTRACT

Background: Oestrogen plays a vital role in breast development and is strongly related to breast cancer. This research article delves into this paradox. Inflammation is a cancer hallmark that involves chemokines that attract inflammatory immune cells and promote breast cancer spread. E2 as a potential estrogen can inhibit chemokine secretion, although the underlying mechanism remains unclear. Interestingly, atypical chemokine receptors (ACKRs), as anti-inflammatory G protein-independent transmembrane proteins, act as "scavengers," removing excessive chemokines, resulting in reduced inflammation, and most strikingly, these genes are essential for normal breast development. This finding suggested that ACKRs may act as tumour suppressors. This study investigated whether a higher E2 level can influence the expression of its own receptor type and ACKRs.

Methods: In this research, a relative gene expression study has been carried out on target genes estrogen receptors (ER*a*, ER*β*) and atypical chemokine receptors (ACKR2, ACKR3 & ACKR4) normalized with TOP1 endogenous control gene in MCF-7 breast cancer cells when treated with a higher E2 level including controls for calibration using RT-qPCR technique in designing the experimental assay.

Conclusion: These findings highlight the seemingly contradictory roles of E2. While it can fuel tumour growth, it might also have anti-inflammatory effects through cross-talk with expressed ACKR genes. A study with an extended time of E2 exposure on MCF-7 is further proposed to assess its effect at cellular level and an auxiliary analysis at protein level can strengthen the possibility of ERs-ACKRs interplay. Research & development in protein receptors field are valuable for evolving novel cancer therapies.

Trial Registration: Not applicable

Keywords: E2 – 17*β*-estradiol, ACKRs – atypical chemokine receptors ER*a* - estrogen receptor alpha, estrogen receptor beta, RT-qPCR – real-time quantitative polymerase chain reaction.

INTRODUCTION:

Breast cancer remains the second most common cancer worldwide, and it is rated as the most common cancer among women, with 2.3 million new cancer cases diagnosed in 2022, accounting for 11.5% of all new cancer cases [1].

Oestrogen plays a central role in breast cancer, and its influence is often mediated by estrogen receptors (ERs). Approximately two-thirds of breast cancers express ERα, while ERβ is expressed at lower levels in tumour tissue, which seems to have a suppressive effect on cancer progression. [2] 17β-Estradiol (E2) is the most abundant and dominant form of estrogen, and it binds efficiently to estrogen receptors (ERs), particularly ERα and ERβ leading to the formation of homo or heterodimers. The E2-activated ER dimer complex acts as a molecular switch, initiating and regulating various cell signaling pathways that control cell development and tissue growth in the human body. [3]

Inflammation, a cancer hallmark, involves a group of signaling molecules called chemokines. They bind to specific receptors on the cell surface, triggering a cascade of events leading to cellular movement, adhesion, and migration. This orchestrated dance of chemokines and their receptors influence various physiological and pathological processes, including tumorigenesis and metastasis. [4,5] The expression levels of several chemokines (e.g., CCL2, CCL3, CXCL8, and CXCL12) and their receptors (CCR2 and CXCR3) depend on ER activity. For example, CXCL12 expression in breast cancer cells is regulated by oestrogen, and blocking CXCL12 with antibodies can prevent oestrogen-induced proliferation. [6] However, the exact underlying mechanism remains unclear. In a human breast cancer cell line, MCF-7 cells can produce a large quantity of CCL2 (MCP-1) in response to interleukin-1a (IL-1a), while the addition of E2 inhibited CCL2 production in a dose-dependent manner. [7] To investigate the effect of tumour growth factor beta-1 (TGF-β1) on the breast cancer cell line MCF-7, the cells were treated with 0 (control), 1, 10, or 100 ng/mL rh-TGF-β1 for 48 h, after which total RNA was isolated for real-time PCR analysis of CXCR4 mRNA expression. TGF-β1 treatment led to a significant increase in CXCR4 mRNA expression in MCF-7 cells. [8]

These findings highlight the possibility of cross-talk between ERs and chemokine secreted by breast cancer cells in the tumour microenvironment (TME). Chemokine communicate with cells through two main types of receptors: classical G protein-coupled receptors, which are ligand-dependent, and atypical chemokine receptors (ACKRs). Unlike classical receptors, ACKRs do not directly trigger cellular responses but act as "scavengers," removing excessive chemokine and dampening inflammation through a process involving the beta(β)-arrestin protein. [9] Interestingly, some ACKRs, such as ACKR2 or D6/Decoy receptors, which are highly expressed in placental cells that produce oestrogen during pregnancy, seem to play a protective role. [10] Higher levels of ACKR2 in breast cancer patients are linked to better outcomes. [11] Similar to ACKR2, ACKR1 (DARC) acts as a "silent" receptor, sequestering chemokines that promote blood vessel growth in tumours, potentially limiting cancer progression. [12] Studies suggest that ACKR3 (CXCR7) might influence how breast cancers respond to hormonal therapies, particularly in tumours positive for ERα. [13] E2 can regulate cytokine production and induce macrophages and lymphocyte recruitment. In contrast, cytokines produced by immune cells can further stimulate E2 production, but the pathway involved in this interaction remains unclear. [14, 15] This research focused on the effect of E2 on ACKR gene modulation in hormone-dependent breast cancer.

The MCF-7 cell line is a valuable tool for breast cancer research. MCF-7 cells, which are derived from 'luminal A' breast cancer, are ER-positive, indicating that they express high levels of estrogen receptors. These cells are also poorly aggressive and non-invasive and have low metastatic potential. [16] Interestingly, several studies have shown the expression of atypical chemokine receptors (ACKRs) in MCF-7 cells. [11, 12, 13] ACKRs are a unique class of chemokine receptors that act as "scavengers," removing various chemokines and

potentially dampening inflammation. Building on prior research, this study focused on analyzing the changes in the mRNA expression levels of target genes of the ER α and ACKRs in MCF-7 cells subjected to high oestrogen (E2) exposure.

2. Methods

MCF-7 Cell Culture

MCF-7-HTB-22 cells were obtained from the ATCC-LGC standard protocol (UK), maintained in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM: F12) (without phenol red) supplemented with HEPES and sodium bicarbonate (NaHCO3) supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS, certified US origin) and 1% penicillin–streptomycin solution (GIBCO) and incubated at 37°C in a 5% CO2 incubator. For experimental layouts, complete culture media without FBS were used.

RT–qPCR assay development

a) RNA Optimization

MCF-7 cells were seeded in 6-well plates at cell densities 2.5 X 10^5, 5 x10^5, 7.5 X10^5, 1 X 10^6 & 2.5 X 10^6 cells/mL at time points 6, 12, 24 & 48 hours in culture for RNA optimization for RT–qPCR. This experiment included three biological replicates. Figure 1 shows the workflow for the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE). After incubation, the seeded cells were processed for total RNA isolation using RiboZol® reagent (AMRESCO, US), followed by NanoDrop ND-1000 spectrophotometer instrument for RNA quantification while RNA integrity assessment performed via gel electrophoresis (the appearance of two bands corresponding to 28S rRNA and 18S rRNA).

Further RNA samples were treated with DNase I to remove DNA as a potential contaminant in the assay.

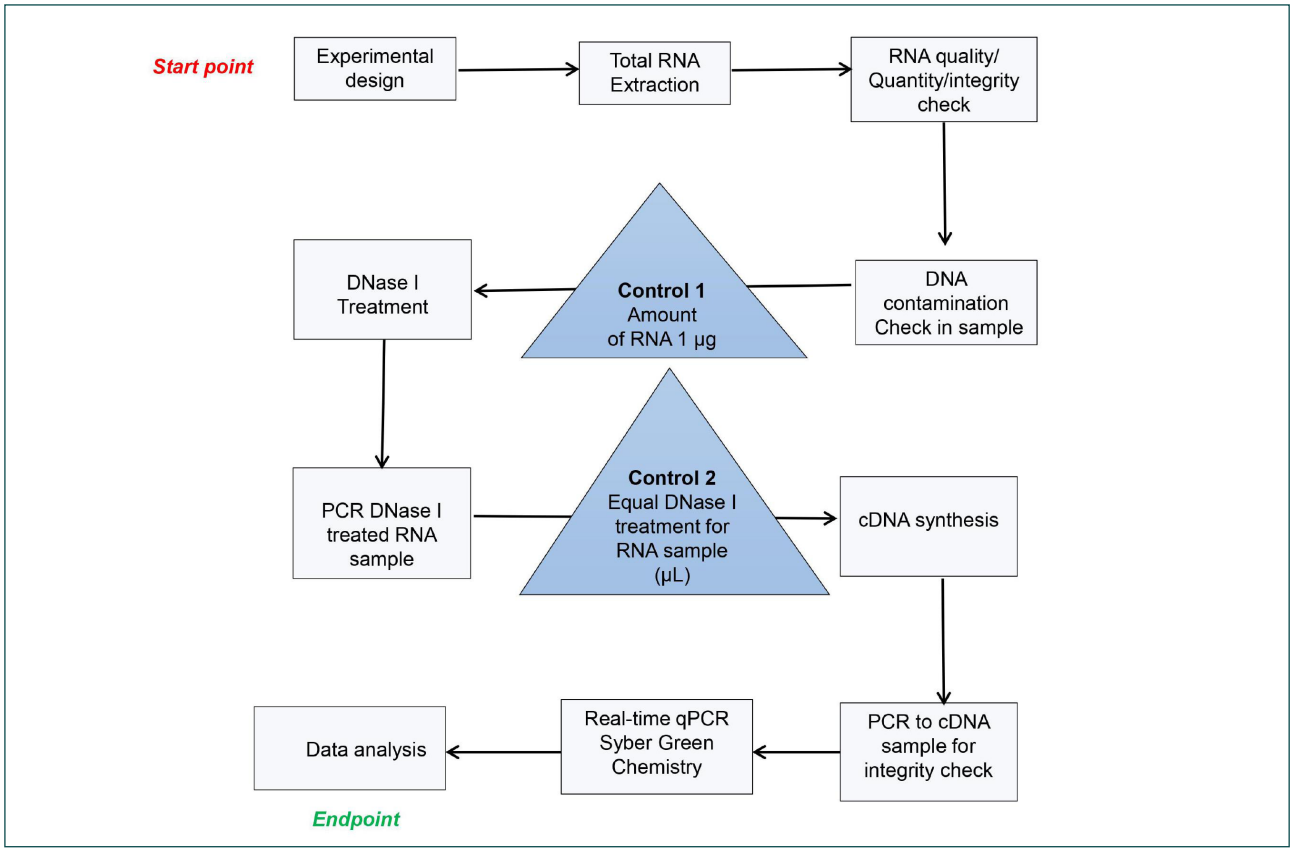

Figure 1: MIQE guideline for RT-qPCR experimental workflow and triangular shapes in blue are control steps that keeps a check on sample quality

DNase-cleaned total RNA samples were subjected to PCR and subsequently processed for cDNA synthesis using a High-Capacity cDNA Reverse Transcription Kit (with RNase inhibitor) of applied biosystems™. A no-reverse transcriptase control (NTC) was included for each experimental repeat. To check cDNA synthesis, samples with controls were checked for a single band product via PCR using the 18S rRNA primer.

b) Gradient PCR & New Primer Design

Primers for housekeeping genes (HKGs) and target genes (Table 1) were obtained from Invitrogen, UK. VWR® Red Taq DNA Polymerase Master Mix was used for PCR assay while Agilent Sure cycler 8800 instrument was used to set up the gradient PCR program with 35 cycles run including annealing (elongation) step of 30 seconds was set up at a range of temperature between 55-65°C i.e. 55°C, 57°C, 59°C, 61°C & 63 °C. Along with these parameters, cDNA samples from cell densities/time points were used with these primers to obtain the ideal annealing temperature required for qPCR assay design. The initial set of ERβ gene variant primers, i.e., ERβ1 and ERβ2/cx, from a published paper, Green AR et al., 2009, resulted in nonspecific binding/multiple bands (Supplementary figure 2). [17]

A new ERβ primer, ERβ_iso, was designed from the N-terminal region of the ESR2 (ERβ) gene. Using an in silico approach with 'Multialign', which is an online bioinformatics nucleotide alignment tool, (Supplementary figure 3)[55], the newly designed ERβ primers 'forward & reverse' were aligned to verify the specificity of the primers across known ERβ variants (details in Table 2).

c) Selection of the endogenous control

The 18S rRNA, HPRT1, TBP, and TOP1 HKGs were subjected to RNA optimization for qPCR analysis to evaluate the stability ranking based on the lower standard deviation (SD) relative to the mean Cq value. Furthermore, the comparative ΔCq approach was used for the initial two lowest-ranking SD mean Cq values, i.e., TOP1 vs. 18S rRNA with E2 treatment samples (section 2.3). The stability ranking based on the lower standard deviation (SD) relative to the meanΔCq value was evaluated for both HKGs as endogenous controls for target gene normalization. The StepOne system was used for data acquisition.

E2 Treatment and Alamar Blue Assay for MCF-7 Cell Viability

Using working solutions of 1 mM E2, various concentrations of E2 (1 μM, 100 nM, and 1 nM) were freshly prepared in culture media supplemented with less than ≤1% DMSO. A cell viability optimization assay was performed by seeding MCF-7 cells at various cell densities in 96-well plates (n=5). The plate was incubated overnight at 37°C with 5% CO_2, after which the complete media was replaced with fresh media containing 20 μL of AB dye per well. Plate readings (at 570 nm and 595 nm absorbance) were collected at multiple time points: 3 h, 6 h, 12 h, 24 h, 30 h, 36 h, 48 h, and 72 h. The data were used to plot a graph for different cell densities showing the % Alamar Blue reduction (ABR) at different time points. For the cell viability assay, a cell density from assay optimization was used in replicates (n=5). After incubation, the media was then replaced with complete media (without FBS) supplemented with media containing E2 at concentrations of 1 μM, 100 nM, or 1 nM along with the respective vehicle controls. After E2 treatment for 6 h and 18 h, 20 μL AB was added to the plate, and the absorbance at 570 nm and 595 nm was measured using a Bio-Trick ELISA plate reader.

qPCR analysis of target genes

An initial RNA optimization design with a 6-well plate was used for preparing E2-treated samples at two time points, 6H and 18H. The experiment was conducted for three biological replicates: Run 1, Run 2, and Run 3. For target gene expression analysis, samples treated with 1 nM, 100 nM, and 1 μM E2, including the respective vehicle control (≤ 0.1% DMSO v/v) and calibrator control (no treatment), were included at two time points. The samples were subjected to RNA optimization, and qPCR was performed using a qScript™ One-Step SYBR® Green

Table 1. Details of primers used for the study.

Gene	Forward Primers	Reverse Primers	Nucleotide Sequence No.	Product size (bp)	Reference
18S rRNA	5'-CAA CTT TCG ATG GTA GTC G-3'	5'-CTT TCC TTG GAT-GTG GTA-3'	K03432	110	[17]
HPRT1	5'- TGACACTGGCAAAACAATGCA -3'	5'- GGTCCTTTTCACCAGCAAGCT -3'	NM_000194	94	[49]
TOP1	5'-GAC GAA TCA TGC CCG AGG AT-3'	5'-CAG TGT CCG CTG-TTT CTC CT-3'	NM_003286.2	381	Design in-house
TBP	5'- TTCGGAGAGTTCTGGGATTGTA -3'	5'- TGGACTGTTCTTCACTCTTGGC -3'	NM_001172085.1 NM_003194.4	227	[50]
ERα	5'-TGG GCT TAC TGA CCA ACC TG -3'	5'-CCT GAT CAT GGA GGG TCA AA-3'	NM_000125	99	[17]
ERβ_iso	5'-TCA CAT CTG TAT GCG GAA CC-3'	5'-TACTTGGACCTGGTCATTG TC-3'	Refer Table.2	140	Design in-house
ACKR2	5'- CCT GCT CCT TGC TAC CAT AGT ATG G -3'	5'-CAC CAA GAC ACA ACC AAT ACG GGA G-3'	NM_001296.4	232	[11]
ACKR3	5'- AAA AGC GTC CTG CTC TAC AC-3'	5'- AGA CTG GGA TGG TGA GGA CAA -3'	NM_020311.2	199	Design in-house
ACKR4	5'-TGG ATG GCT GCC ATC TTG-3'	5'-CTT CAT GAG TGT CCT TGC TGT G-3'	NM_0.16557.3 NM_178445.2	203	Design in-house

q-PCR Kit. The mean fold change in the expression of three biological replicates of each target gene normalized to that of TOP1, which was used as an endogenous control, was calculated via the ΔΔCq comparative method given by Livak KJ & Schmittgen TD, 2001. [18]

3. Results

RNA optimization for RT–qPCR

MCF-7 cells at 2.5 x 10^5 cells/mL for 48 hours of incubation had a total RNA yield ≥ 1 μg and the required RNA integrity for downstream analysis.

Characterization and new primer design

For characterization, primers for the target gene and HKGs were used for gradient PCR. A single band with appropriate product size and band \strength to various annealing temperatures was captured in the gel image for each primer set to the respective target gene and HKGs (Figure 2).

The new primer named ERβ_iso led to a single band of the expected size of 140 bp, which was compared to that of the initial ERβ primer sets with multiple bands on the gPCR gel (Figure 3).

Additionally, the PCR efficiency (E) for the newly designed ERβ primer set was calculated using the slope (m) from the linear equation of the standard curve generated with the respective primer set dilution (Table 3).

Table 2*. Listed known mRNA (variants) and protein (isoforms) for human ESR2 or ERβ

Nucleotide (mRNA)	Nucleotide Accession Number	Coding sequence (CDS)	Protein	Protein Accession Number	Total Amino Acid	Tissue Distribution ESR2 gene	Reference
Transcript variant a	NM_001437.2 AF051427.1	1592 bp 2011 bp	ERβ isoform 1	NP_001428.1 AAC05985.1	530 a.a	High expression: Testis and ovary Medium expression: Spleen and uterus Low expression: Adipocytes,Thymus,mammarry gland, placenta, brain	[51]
Transcript variant b ERb2 Transcript variant k Transcript variant l ERb2/cx	NM_001040275.1 AF051428.1 NM_001291712.1 NM_001291723.1 AB006589.1	1487 bp	ERβ isoform 2	NP_001035365.1 AAC05751.1 NP_001278641.1 NP_001278652.1 BAA31966.1	495 a.a	High expression: Testis, ovary & Thymus Medium expression: Spleen and uterus Low expression: Adipocytes, mammary gland, placenta, brain	[51], [52]
ERβ isoform 3 mRNA	AF060555.1	1541 bp	ERβ isoform 3	AAC15234.1	513 a.a	Testis and ovary	[53]
Transcript variant d	NM_001214902.1 DQ838582.1	1445 bp	ERβ isoform 4	NP_001201831.1 ABH09189.1	481 a.a	High expression: Testis, Low expression: Spleen, thymus, ovary, mammary gland and uterus	[53], [54]
Transcript variant f	NM_001271876.1 DQ777076.1	1424 bp	ERβ isoform 5	NP_001258805.1 ABG88022.1	474 a.a	Ovary cDNA	[54]
ERβ isoform 5 mRNA	DQ838583.1	1419 bp	ERβ isoform 5	ABH09190.1	472 a.a	Breast cancer cell lines	[53]
Transcript variant g	NM_001271877.1	1518 bp	ERβ isoform 6	NP_001258806.1	439 a.a	Testis	[52]

*NCBI database source

Table 3. Details of PCR efficiency to new primer set ERβ _iso

Gene	Forward and Reverse primer (5'-3')	Product (bp)	R^2	E (%)
ERβ _iso	FWD 5'-TCA CAT CTG TAT GCG GAA CC-3' REV 5'-TACTTGGACCTGGTCATTG TC-3'	140	0.99	90 %

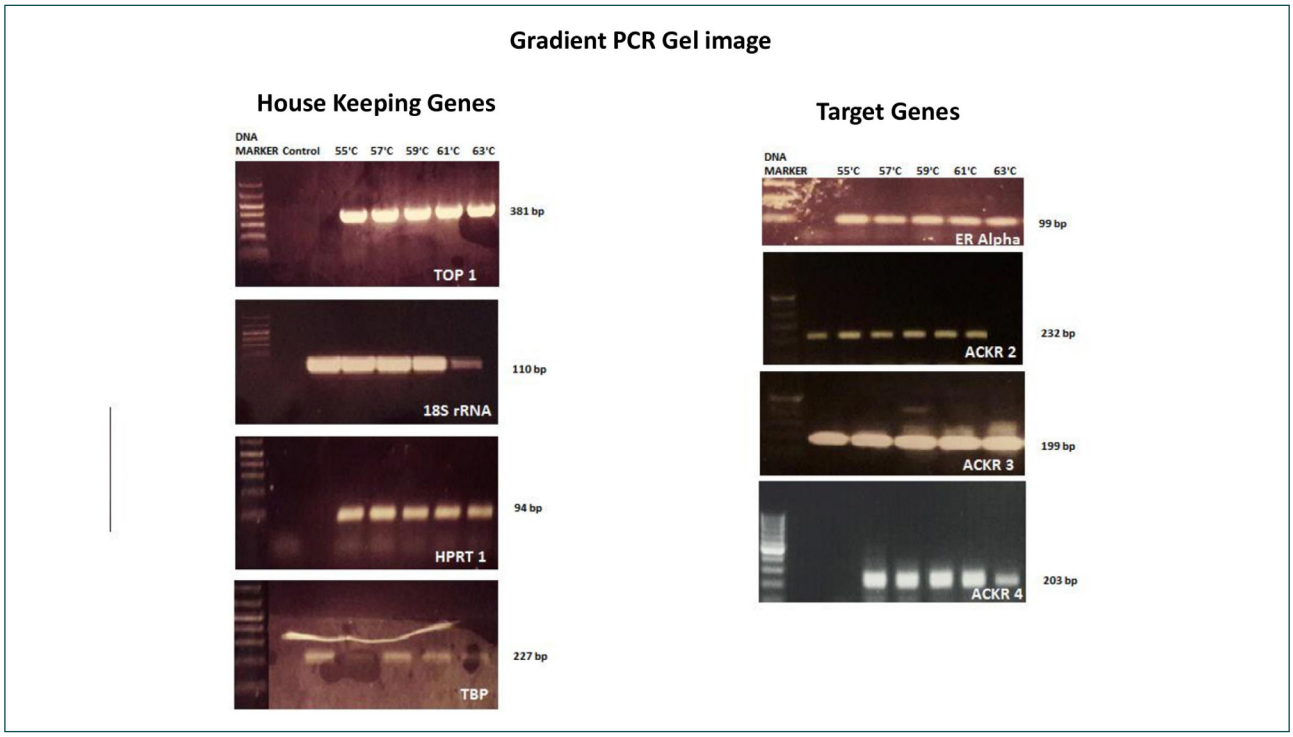

Figure 2: Gradient PCR gel image for 18S rRNA, TOP1, HPRT1, and TBP as HKGs and ERα, ACKR2, ACKR3, ACKR4 as target gene

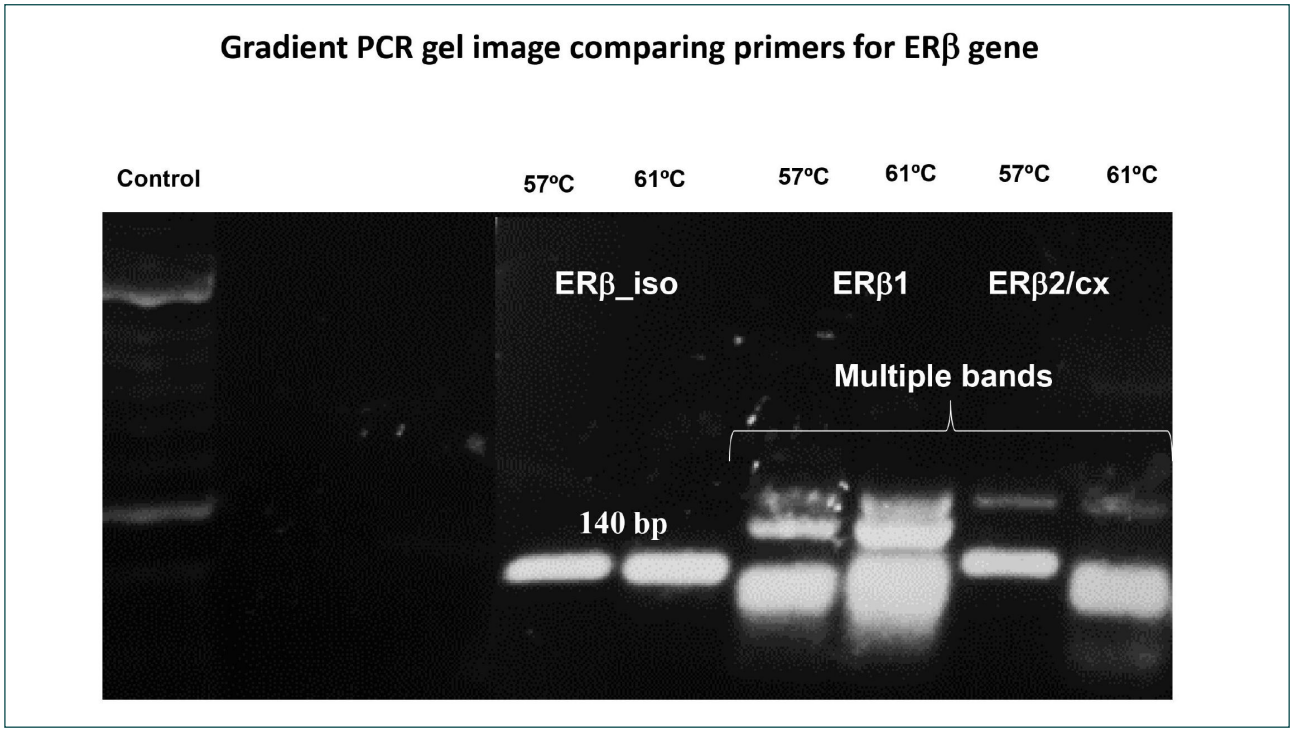

Figure 3: Gradient PCR gel image comparing primers to ERβ gene

Normalization gene selection

The SDs of the mean Cq values for HKGs with RNA optimized cDNA samples from three biological replicates were analysed using qPCR. 18S rRNA displayed early cycle detection, necessitating 100-fold dilution of the samples. Nevertheless, 18S rRNA had the lowest standard deviation (SD) from the average Cq value across the other HKGs (https://doi.org/10.5281/zenodo.11127511, Annexure I A.);[55] however, its use, which requires dilution of the sample step, might introduce variability. Therefore, the comparative ΔCq approach was used for comparing two of the lowest SD ranking mean Cq HKGs, i.e., E2 treatment samples (section 2.3) were used for each target gene to calculate the SD mean ΔCq for TOP1 vs. 18S rRNA as an endogenous control. Comparative ΔCq approach analysis revealed that TOP1 was a better endogenous control than 18SrRNA, with a low SD to the mean ΔCq (https://doi.org/10.5281/zenodo.11127511, Annexure I B.).[55]

E2 treatment Cell viability assay

Cell viability optimization assay using different cell densities and time points for MCF-7 cells. As a result, a seeding density of 7.8×10^4 cells/mL was considered the optimal cell density range, with a 70% reduction in the alamar blue concentration after 24 hours of incubation (Figure 4).

The data obtained through a cell viability assay with E2 treatment revealed no significant difference across samples (Figure 5).

Additionally, DMSO vehicle controls < 1% v/v to preparing of E2 solution did not induce any apparent cytotoxic effects on MCF-7 cells in culture media.

Relative gene expression

The E2 treatment test samples were subjected to qPCR with the following target genes for normalization: ERα, ERβ and ACKR2, ACKR3, ACKR4 and TOP1. Using the comparative ΔΔCq method, the mean fold changes for individual target genes were calculated (https://doi.org/10.5281/zenodo.11127511, Annexure II),[55] and the data were plotted in figures 6 (a) to 6(e).

Statistical analysis was performed with the nonparametric Mann–Whitney U test using the Minitab tool for measuring the significance of the data (Supplementary Table 1 & 2).[55]

4. Discussion

MCF-7 as an in-vitro study model

MCF-7 cells being well-established characteristics as hormone-responsive breast cancer cells provide an excellent model system for investigating the molecular biology of oestrogen action in in-vitro breast cancer research. The MCF-7 cell line is capable of rapid genetic alterations & therefore a useful model for understanding the genetic development of breast cancers. Different amounts of ER expression have been seen in MCF-7 sub-lines by treating MCF-7 wild type with anti-oestrogen medications, exposing them to E2 for either a short or long period, and adjusting the composition of the media. They have also been widely utilized to investigate the short- and long-term effects of estrogen on gene expression. [19,20] When treated with E2, long-term estrogen deprivation (LTED) MCF-7 cells have exhibited apoptosis, whereas long-term estrogen exposure (LTEE) MCF-7 cells have experienced significant global gene expression alterations. [21]. In the process of creating long-term estrogen-deprived MCF-7 cells, estrogen was removed from the culture by using charcoal-stripped serum in the media. This resulted in estrogen hypersensitivity, altered gene expression, and increased activation of the ERα (classical/genomic pathway) or AKT, ERK1/2-MAPK, and PI3K/AKT/mTOR (mammalian target for rampamycin) growth factor/non-genomic pathways.[22]

E2 binds to distinct estrogen receptors (ERs), ERα & ERβ, forming functional heterodimers that are co-expressed in tissue and cell lines while E2-ERβ inhibits the expression of genes regulated by ERα. [23] It is critical to identify the molecular mechanism controlling the expression of both ERs in order to comprehend the cellular and biological processes of estrogen-mediated gene regulation in both normal and diseased breast tissue. Expression of ERβ has been detected in human tumor biopsy samples and several human breast epithelial cell lines using

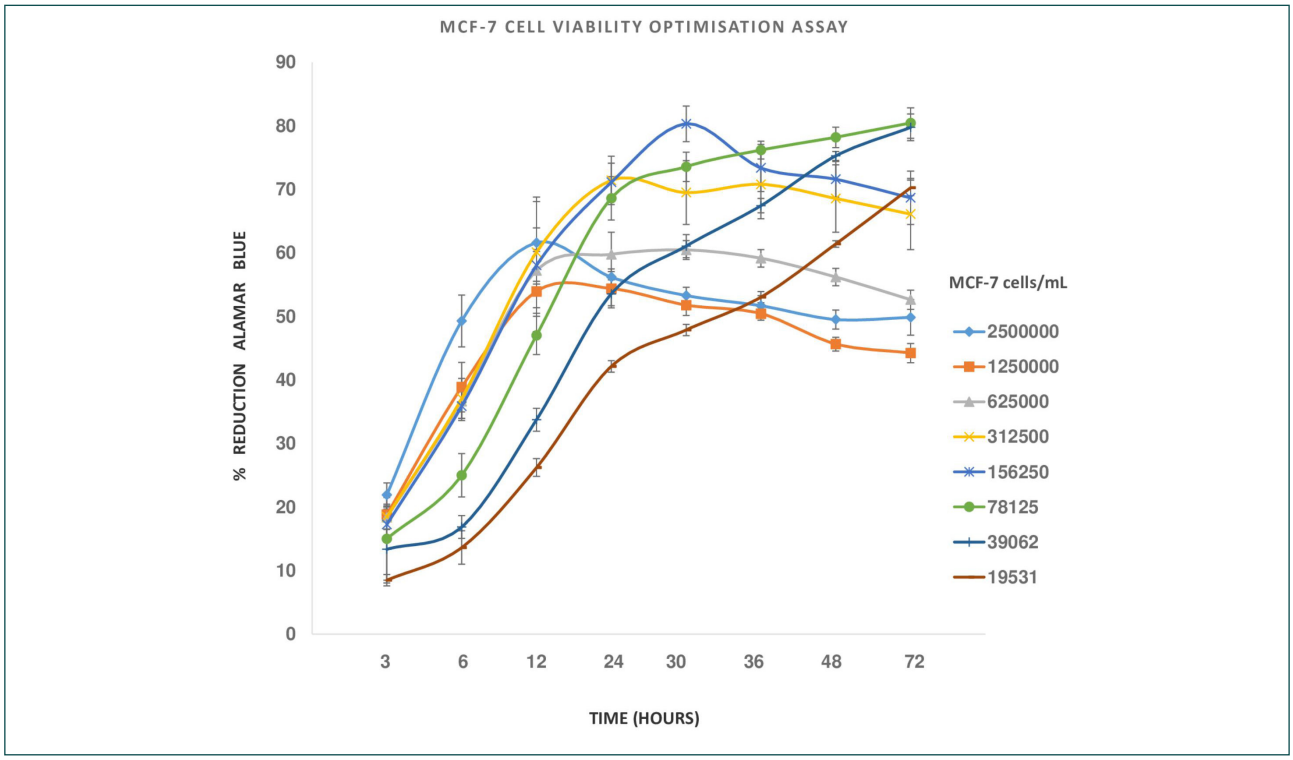

Figure 4: Graph is showing colour line for various cell densities of MCF-7, % reduction of AB on the y-axis and time points on the x-axis (plate readout made at absorbance 570 nm and 595nm)

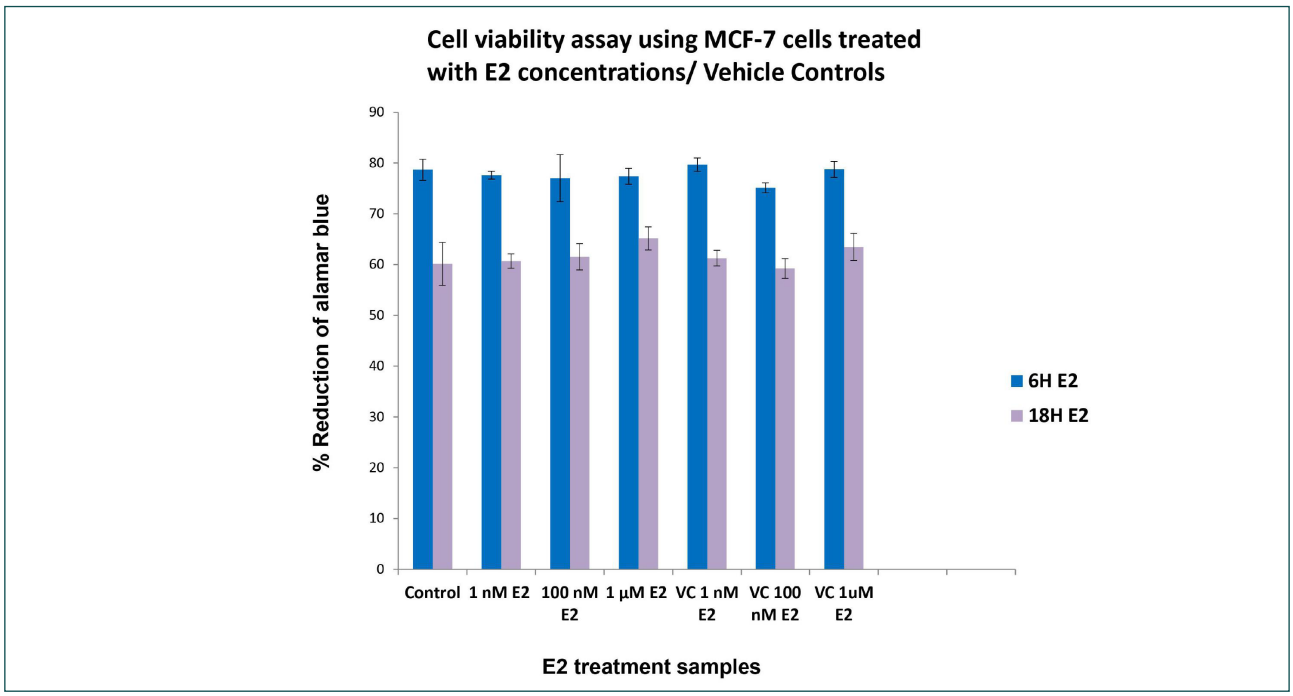

Figure 5. Bar chart represents cell viability for MCF-7 cells treated with E2 concentrations along with vehicle control showing % ABR two time point of 6H and 18 H respectively

RT-PCR. The co-expression of ERβ and ERα mRNA in breast tumor and cell lines support possible role of ERβ in human breast cancer progression. [24] In a study on human breast tissue samples using RT-PCR prognostic factors including tumor grade and node status were investigated for co-expression of ERa & ERb isoforms. Tumor samples with co-expression of ERα & ERβ were node positive and tended to be a higher grade. [25]
Previous studies have demonstrated the suitability of DMEM: F12 phenol red-free media supplemented with 10% serum for maintaining and culturing MCF-7 cells for in vitro assays, including 3D models. [26] In this in-vitro study, serum-free media DMEM: F12 (no phenol red) without FBS is used to investigate the effect of higher E2 concentration on gene expression modulation of the ERs & inflammatory mediator receptors in MCF-7. Higher E2 exposure at 18H time point in MCF-7 cultured in serum-free media has shown down-regulation of ERα & ERβ relative gene mRNA level expression when compared to control & vehicle control samples.

Learning from the design of the experiment (DoE)

Real-time quantitative PCR (RT–qPCR) is widely considered the "gold standard" technique for mRNA quantification. However, its effectiveness relies heavily on standardized protocols. Although it is the most common method for mRNA quantification, concerns exist regarding its consistency across studies. To address this issue, the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines, which provide a checklist of 85 parameters to ensure assay design quality and control, ultimately enhance the reliability of results and facilitate the evaluation of RT–qPCR data. [27,28] These guidelines were followed throughout the design of the experiment (DoE). By adhering to the MIQE guidelines, we aimed to ensure the robustness and reproducibility of our findings. For the StepOne RT–qPCR assay design, the primer set desirably amplify a single product. The primers were checked with gPCR before RT–qPCR, which led to the identification of multiple band/nonspecific amplification with the initial primer set of the ERβ variant gene, which was obtained from a published paper. Hence, it is essential to verify the primers prior to performing RT–qPCR assays as per experimental design. Additionally, bioinformatics online tools, in this case 'Multialign', assisted in-silico confirmation of the newly designed set of ERβ primers that goes common for known ERβ (mRNA) variants. HKG selection is a crucial step in optimizing the qPCR assay; 18S rRNA, HPRT1, and TBP were used for normalization, while TOP1, an HKG, was used in this study for relative gene expression analysis. TOP1 was considered the most stable endogenous control gene for normalization to 18S rRNA based on comparative ΔCq approach analysis. The data as anneAnnexurehas been provided in the thesis repository link. (https://doi.org/10.5281/zenodo.11127511).[55] The target gene mRNA levels were confirmed via PCR, while the TOP1 gene was chosen as an endogenous control for normalization of gene expression via RT–qPCR. The E2 treatment samples for three biological replicates were run in duplicate for each target gene individually, and using the comparative ΔΔCq method.[18] the mean fold change or $2^{-\Delta\Delta Cq}$ for the target genes was calculated (https://doi.org/10.5281/zenodo.11127511, Annexure II).[55]
Alamar blue cell viability assay has been assessed several times on different types of cells for cytotoxicity reliability i.e. immortalized and cancer cell line [29,30] such as human lymphocytes[31], primary neuronal cell culture[32], and fibroblast [33] In a study using fluorescent Hoechst reagent, an increase in cell proliferation of MCF-7 was measured with 1 nM E2 when compared to no treatment control but assay was attained in 5% DC-FBS with IMEM media.[34] In this study, Alamar blue cell viability assay using higher E2 concentrations and vehicle controls (≥ 0.1% DMSO v/v) at two-time points, 6 h and 18 h, did not lead to any significant changes across the E2 treatment groups (Figure 5); however, a decrease in the %ABR was observed across the two time points (Figure 5), possibly because of the higher seeding density. Therefore, a lower seeding density of ≥1000 cells per well of a 96-well plate is further advisable for a similar experimental setup.

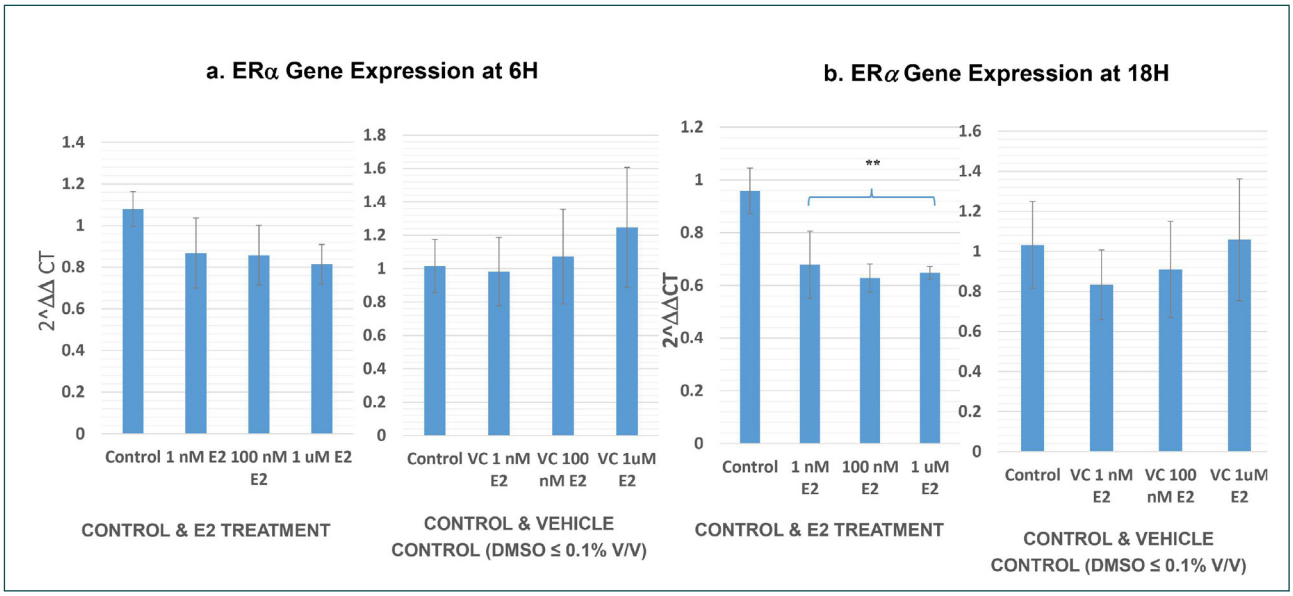

Figure 6. (a). Above graph is representation of 2-ΔΔCq or mean fold value for ERα target gene normalised with TOP1 gene using three biological repeats of E2 treatment experiment runs a. 6H & b. 18H time point. Data analysis achieved using non-parametric statistical tool Mann-Whitney's U-test (https://doi.org/10.5281/zenodo.11127511, Supplementary table 1) significance in data was explored. For significant difference p value symbol * p<0.05, **p< 0.005, *** p<0.0005 value is defined

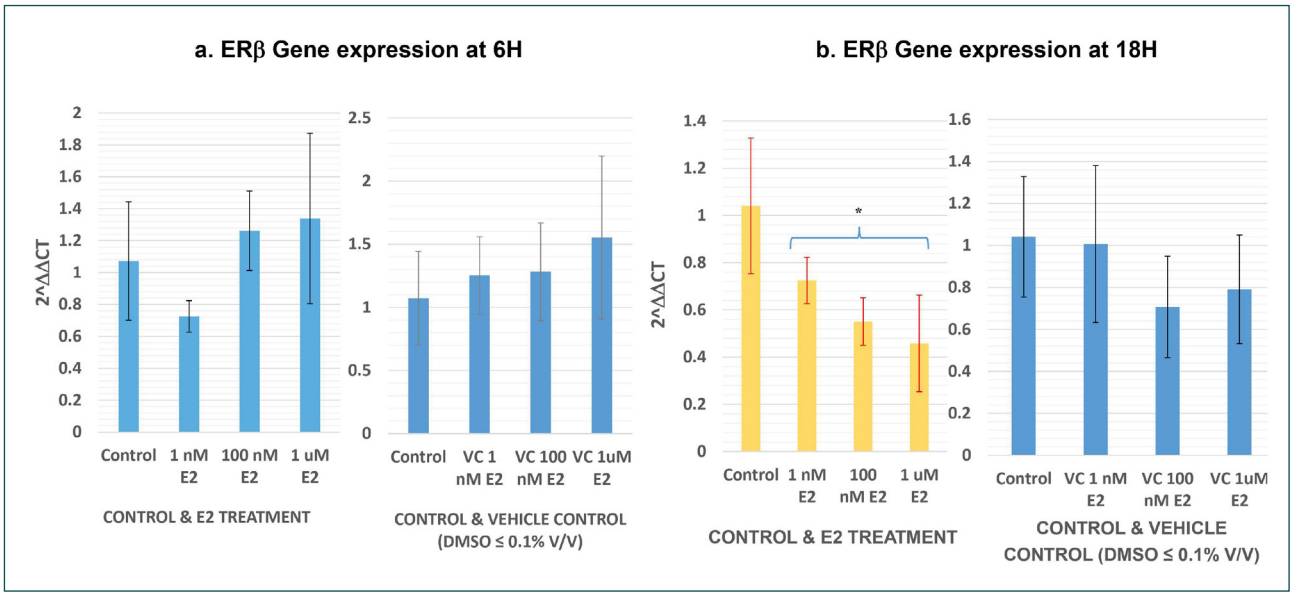

Figure 6. (b). Above graph is representation of 2-ΔΔCq or mean fold value for ERβ target gene normalised with TOP1 gene using three biological repeats of E2 treatment experiment runs a. 6H & b. 18H time point. Data analysis achieved using non-parametric statistical tool Mann-Whitney's U-test (https://doi.org/10.5281/zenodo.11127511, Supplementary table 1) significance in data was explored. For significant difference p value symbol * p<0.05, **p< 0.005, *** p<0.0005 value is defined

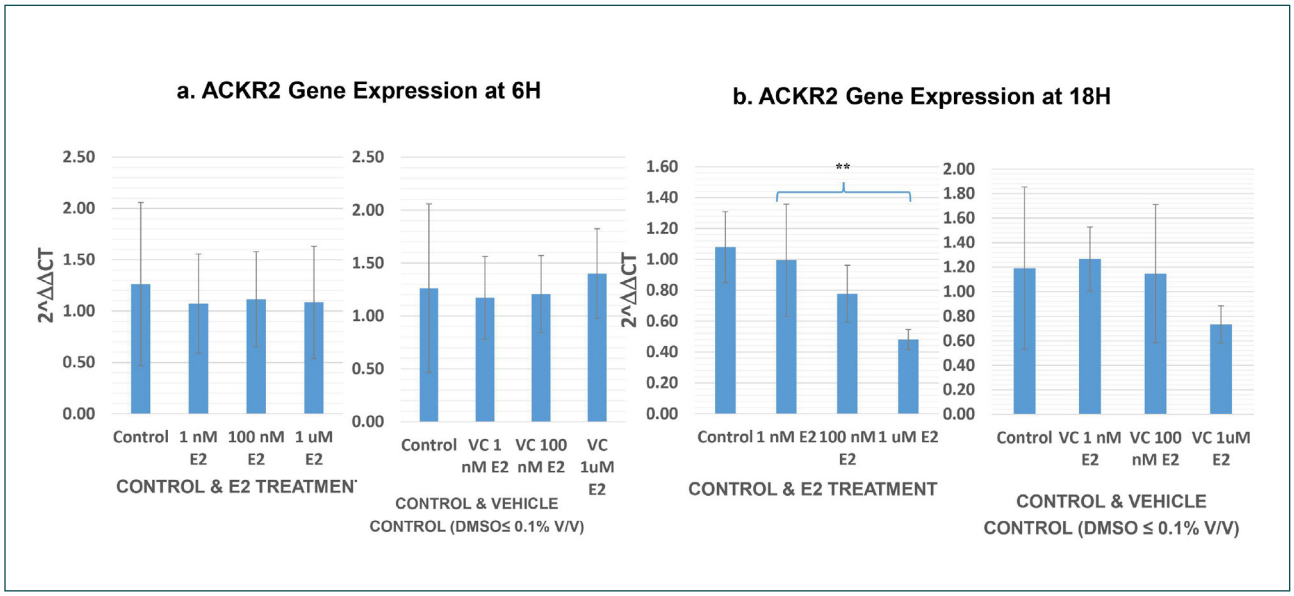

Figure 6. (c). Above graph is representation of 2-ΔΔCq or mean fold value for ACKR2 target gene normalised with TOP1 gene using three biological repeats of E2 treatment experiment runs a. 6H & b. 18H time point. Data analysis achieved using non-parametric statistical tool Mann-Whitney's U-test (https://doi.org/10.5281/zenodo.11127511, Supplementary table 2) significance in data was explored. For significant difference p value symbol * p<0.05, **p< 0.005, *** p<0.0005 value is defined.

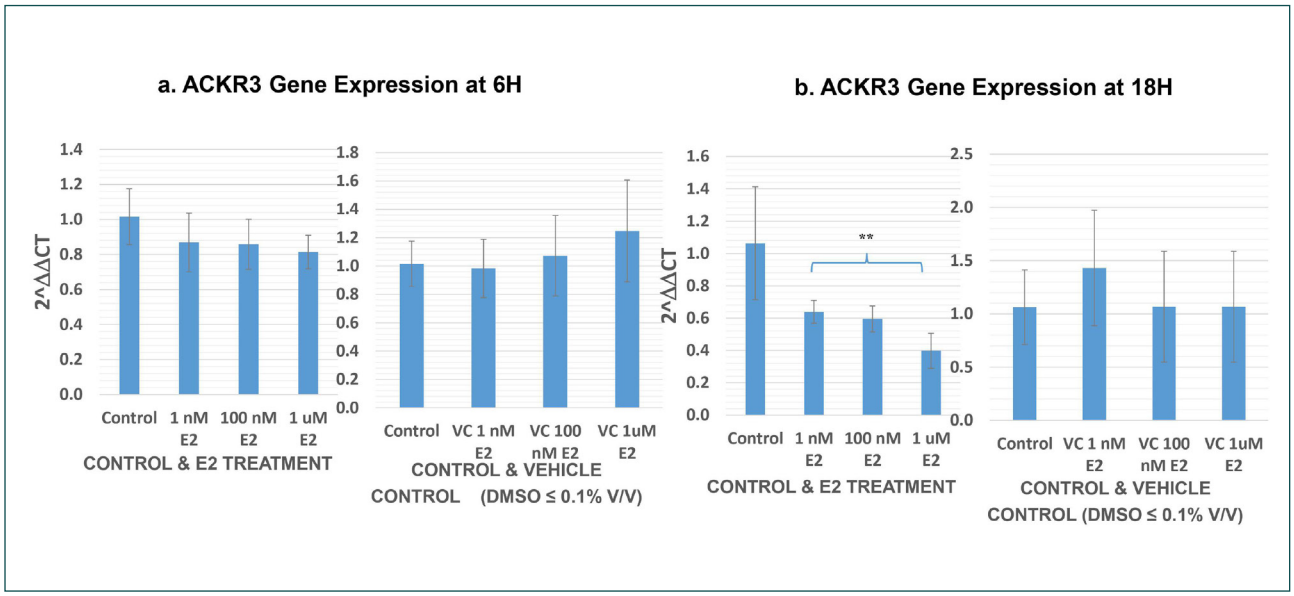

Figure 6. (d). Above graph is representation of 2-ΔΔCq or mean fold value for ACKR3 target gene normalised with TOP1 gene using three biological repeats of E2 treatment experiment runs a. 6H & b. 18H time point. Data analysis achieved using non-parametric statistical tool Mann-Whitney's U-test ((https://doi.org/10.5281/zenodo.11127511, Supplementary table 2)) significance in data was explored. For significant difference p value symbol * p<0.05, **p< 0.005, *** p<0.0005 value is defined.

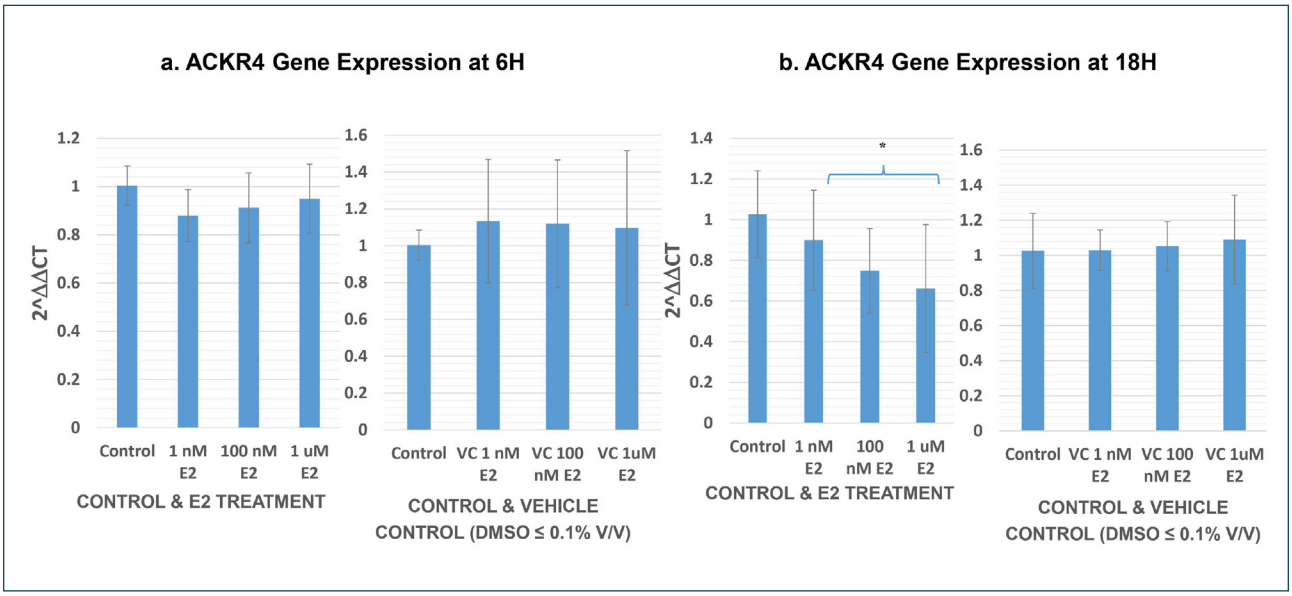

Figure 6. (e). Above graph is representation of 2-ΔΔCq or mean fold value for ACKR4 target gene normalised with TOP1 gene using three biological repeats of E2 treatment experiment runs a. 6H & b. 18H time point. Data analysis achieved using non-parametric statistical tool Mann-Whitney's U-test test ((https://doi.org/10.5281/zenodo.11127511, Supplementary table 2.) significance in data was explored. For significant difference p value symbol * $p<0.05$, ** $p< 0.005$, *** $p<0.0005$ value is defined.

Estrogen receptor signaling mechanisms: Combining genomic and non- genomic effects on target genes

When MCF-7 cells express only ERα, estrogen promotes cell proliferation and leads to the formation of tumors. In a study, the transfection of the ERβ gene into MCF-7 cells inhibited the proliferation of the cells & mRNA expression in vitro and stopped the development of tumors in a mouse xenograft model when exposed to E2. These findings showed that the effects of ERα and ERβ on tumor formation and cell proliferation in MCF-7 are opposing. [35] In this study, ERα, ERβ gene co-expression were significantly down regulated at 1 μM E2 concentration level when compared to those of the control or vehicle controls (≤ 1% DMSO v/v) at the 18H time point [refer to figure 6(a) to figure 6(b)]. No significant change in gene expression was observed at 6 h for the target genes [refer to figure 6 (a) to figure 6 (b)] A study has demonstrated that the signaling pathway needed for E2-ERβ to trigger a cellular response is the ERE estrogen-response-elements dependent pathway. G-protein coupled receptor (GPCR), is one of the many genes that the E2-ERβ complex has activated. However, the nature of the regulatory element that is essential for the E2 responsiveness of the discovered genes, including GPCR, is unknown.[36] E2 level is known to modulate the inflammatory mediator as chemokine expression. MCF-7 cells produce a large quantity of CCL2 (MCP-1) in response to interleukin-1a (IL-1a) while addition of E2 to MCF-7 cells inhibited CCL2 production in a dose-dependent manner. [37] Chemokines can trigger signalling via conventional GPCRs or through atypical chemokine receptors (ACKRs). In this study for the first time effect of E2 in gene modulation of ACKRs been established. Currently, four atypical chemokine receptors have been are described (ACKR1, ACKR2, ACKR3 and ACKR4). ACKRs are expressed in various cells and tissues, including breast cancer cells. These receptors' main function is related to the internalization and degradation of chemokines, as well as to the inflammation control.

During this study mRNA expression of the ACKR 1 gene was not evident using MCF-7 cells in gradient PCR experimental method.

ACKR2 is expressed in breast cancer cells having a protective role but it is not clear if normal epithelial cells express this receptor.[38] Atypical chemokine receptor 2

(ACKR2) or decoy receptor 6 (D6) is known to attenuate lymph node metastasis and negatively correlate with clinical tumour stage in breast cancer but signalling pathway involved is still not clear. [11]

ACKR4 also known as CCRC1 or CCX-CKR is a scavenger receptor for homeostatic chemokines CCL19, CCL21, CCL25 and weakly to CXCL13. [39] Overexpression of ACKR4 in breast cancer metastatic cell line MDA-MB-231 has shown inhibition of cell proliferation in vitro but progressive to tumour growth in an in-vivo model. Low levels of ACKR4 expression are correlated with poor prognosis in breast patients. [40]

ACKR3 expression in breast cancer tumour cells is shown important for proliferation and survival. [41] ACKR3 promotes cancer cell growth by inducing Erk1/2 phosphorylation and inhibiting apoptosis in breast cancer cell line in MCF-7 cell line. [42]

In this study, ACKR2, ACKR3, and ACKR4 genes were expressed at mRNA level & were significantly down regulated with a higher E2 concentration level when compared to those of the control or vehicle controls (≤ 1% DMSO v/v) at the 18H time point [refer to figure 6(c) to figure 6(e)]. No significant change in gene expression was observed at 6 h for the target genes [refer to figure 6 (c) to figure 6 (e)].

Limitation of the study

There are accumulating evidences that membrane and cytoplasmic ER subtypes can facilitate non-genomic or rapid signaling with the effect of E2. GPCR can get activated by ERs after E2 exposure and can control gene expression through the activation of transcription factors e.g. β-arristen. [43] Plasma membrane associated ER when bind to E2 result in activation of ERK1/2-MAPK signal transduction which are involving GPCR activation. [44] ACKR2 expressed in MCF-7, are known to act like sponge and recirculate on cell membrane after ligand degradation in lysosomal vesicle through plasma membrane internalisation β-arrestin dependent signalling. [45]

ACKR3 or CXCR7 expressed in MCF-7, generally function as a signalling receptor by phosphorylation of MAPKs or serine/threonine Akt pathway through β-arrestin activation.[46]

ACKR4 expressed in MCF-7 also internalises chemokine by recruiting β-arrestin and after degradation these receptors become desensitised. [47] It is not confirmed yet if it does not activate any signal transduction pathway. [48]

E2-ERβ is known to regulate various genes including the GPCR while β-arristen has emerged as a key signal transducer that activates the ACKRs via a rapid signaling pathway. Therefore considering β-arristen gene's expression would have reinforced the notion that E2 modulates the ACKRs gene expression via E2-ERs linked non-genomic/ rapid signalling pathway.

5. Conclusion

Exposure to a high concentration of oestrogen (E2) significantly altered the gene expression in breast cancer cells. After 18 hours, MCF-7 cells, a type of hormone-responsive breast cancer cell, showed changes in messenger RNA (mRNA) levels for several key genes. These genes included those for estrogen receptors (ERα and ERβ) and atypical chemokine receptors known as ACKRs (ACKR2, ACKR3, and ACKR4). Interestingly, higher E2 levels led to a decrease (downregulation) in the activity of these genes. This suggests that E2 might play a role in regulating these genes in breast cancer. Further studies are warranted to investigate transcriptional factors involved in the E2-mediated downregulation of ERs and ACKRs opening the possibility of potential cross-talk between genomic/ non-genomic (rapid) signaling pathways. This study in a 3D/xenograft model with protein level analysis is vital for establishing a possible link between E2-ERs & ACKRs cross-talk and such studies are crucial for developing new cancer therapies.

Abbreviation

AB – Alamar blue dye

ABR- Alamar blue reduction

ACKRs – atypical chemokine receptors

ACKR1 or DARC - Atypical chemokine receptor 1 or

Duffy Antigen receptor for chemokine
ACKR2 or D6 - Atypical chemokine receptor 2 or Decoy Receptor 6
ACKR3 or CXCR7 - Atypical chemokine receptor 3
ACKR4 - Atypical chemokine receptor 4 (CCRL1/CCX-CKR/CCBP2)
bp – base pair
CCL2 or MCP-1 - Chemokine (C-C motif) ligand 2 or Monocyte Chemoattractant protein-1
CCL3 or MIP-1a - Chemokine (C-C motif) ligand 3 or Macrophage Inflammatory protein 1 alpha
CXCL8 - Chemokine (C-X-C motif) ligand 8
CXCL12 or SDF-1 - Chemokine (C-X-C motif) ligand 12 or Stromal derived factor 1
CCR2 - C-C chemokine receptor type 2
CXCR3 - C-X-C motif chemokine receptor type 3
CXCR4 - C-X-C motif Chemokine receptor type 4
Cq - Threshold cycle value
ΔCq - delta threshold cycle value
ΔΔCq – Delta delta threshold cycle
DMEM: F12 - Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
DMSO – Dimethyl Sulfoxide
DoE - Design of the experiment
2D – two- dimentional
3D – three-dimensional
E2 – 17β-estradiol
ERs – estrogen receptors
ERα - estrogen receptor alpha
ERβ – estrogen receptor beta
gPCR – gradient polymerase chain reaction
HKGs – Housekeeping genes
HPRT1 - Hypoxanthine Phosphoribosyl transferase 1
IL-1α - Interleukin 1 alpha/ hematopoietin 1
MIQE - Minimum Information for Publication of Quantitative Real-Time PCR Experiments
RT-qPCR – real-time quantitative polymerase chain reaction
m-RNA – messenger ribonucleic acid
rRNA – ribosiomal ribonucleic acid
RNA - Ribonucleic acid
18S rRNA - 18 subunit ribosomal ribonucleic acid
SD – Standard deviation
TGF-b1 - Transforming growth factor beta 1
TME - Tumour microenvironment
TBP - TATA-binding protein gene
TOP1 - Topoisomerase 1 gene
mM – Milli- Molar
μM – micro-molar
nM – nano- molar
μL – micro-liter
$2^{-\Delta\Delta Cq}$ - Fold change

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Ethics approval and consent to participate
Not Applicable

Availability of data and materials
All data generated or analysed during this study are included in this published article [supplementary / Annexure files]. The thesis is available under citation "Talashi S. Gene expression analysis of oestrogen receptors and atypical chemokine receptors in response to 17-β estradiol in MCF-7. Zenodo; 2024. https://doi.org/10.5281/zenodo.11127511".

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Conflict of interest
There are no conflicts of interest to declare.

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