RESEARCH ARTICLE

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In Silico Analysis of the Role of Estrogen Signaling in the Expression of Metabolic Genes in Breast Cancer

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Abstract

Introduction: Estrogen exerts a multifaceted influence on breast cancer, particularly through its association with estrogen receptor (ER) and progesterone receptor (PR), which serve as pivotal prognostic and therapeutic markers. While the differential expression of metabolic genes and their prognostic relevance in breast cancer have been extensively studied, limited research has examined their regulation by estrogen signaling. This study adopts a novel approach by investigating the effect of estrogen signaling on the expression of a broad spectrum of metabolic genes in breast cancer. **Methodology:** Microarray data from breast cancer studies were retrieved from the *NCBI* Gene Expression Omnibus (GEO). Differential expression profiles of ER+PR+ versus ER−PR− samples across seven datasets were analyzed using GEO2R. The 250 most significantly overexpressed and underexpressed genes were identified, and genes with metabolic functions were filtered. Promoter and upstream sequences (up to 1000 bp) of the most common transcript variants were obtained from the UCSC Genome Browser (Hg38 cell line). Estrogen receptor elements (EREs) and CpG islands were subsequently identified. **Results:** Thirty-three unique metabolic genes were identified based on differential expression profiles. Out of these, 18 genes were identified as having EREs in their upstream regions- *CA12, CPA3, FBP1, STC2, NME5, DEGS2, ABAT, GAMT*, and *ARSG* were upregulated, whereas *B3GNT5, DPH2, PPARA, TNFRSF21, PHGDH, FOXL1, ME1, RNF145, and NUDT5* were downregulated. CpG islands closely corresponded to the EREs in *PPARA, PHGDH, ME1, RNF145, NUDT5, CA12, STC2, ABAT*, and *GAMT*. **Discussion:** The identification of *CA12*, consistent with previous findings on its role in oncogenesis and estrogen regulation, highlights the therapeutic potential of targeting this pathway. Furthermore, the additional genes identified expand our understanding of metabolic alterations in response to estrogen signaling in breast cancer, thereby offering new avenues for mechanistic exploration and the development of potential therapeutic targets.

Keywords: Estrogen- Breast Cancer- Metabolic Genes- Estrogen Receptor

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Introduction

Estrogen is the primary sex hormone in females, responsible for the development and regulation of the female reproductive system and secondary sexual characteristics. There are three major endogenous estrogens in females: Estrone (E1), Estradiol (E2), and Estriol (E3), with estradiol being the most potent. The aromatization of androgens forms estrogens in a complex process involving three hydroxylation steps, each requiring O_2 and NADPH. The aromatase enzyme complex is thought to include a P450 monooxygenase. Estradiol is formed if the substrate of this enzyme complex is testosterone, whereas estrone results from the aromatization of androstenedione (Figure 1) [1].

Being a steroid hormone, it readily diffuses across membranes and, once inside, binds to and activates the estrogen receptors (ERs), modulating genetic expression. Additionally, they bind to and activate membrane-bound ERs like ERα, ERβ, and GPR30. As stated previously, the actions of estrogen are mediated by ER, a dimeric nuclear protein that binds to DNA and modulates genetic expression. The estrogen-ER complex binds to specific sequences on the DNA called estrogen response elements

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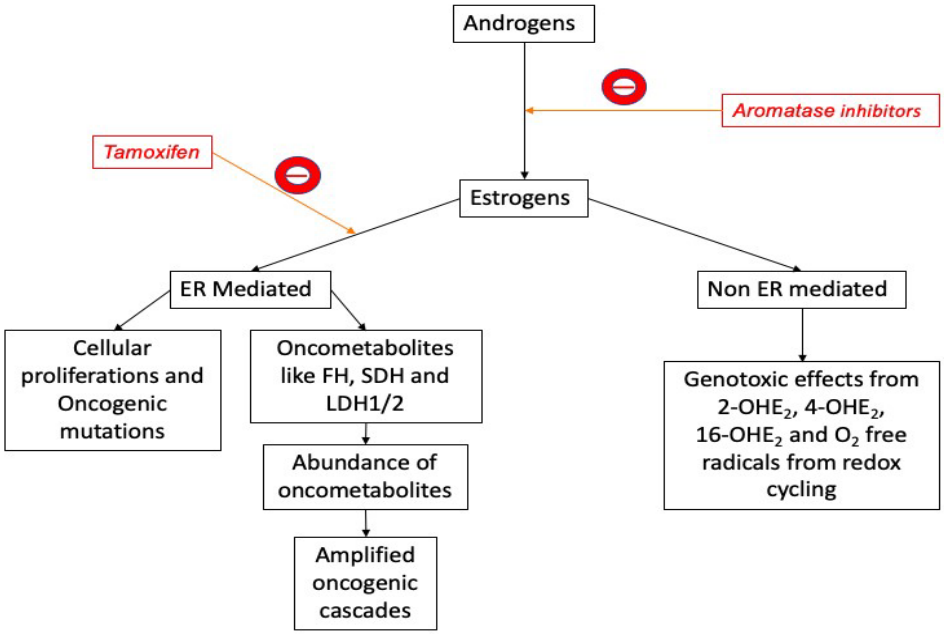

Figure 1. Effect of Estrogen in Breast Cancer Cell Lines

(EREs) to activate the transcription of specific target genes. Since estrogen enters all cells, its actions are dependent on the presence of the ER in the cell [2]. The ER is expressed in specific tissues, including the ovary, uterus, and breast.

The connection between breast cancer and estrogen has been recognized for more than 100 years since Beatson et al. demonstrated that bilateral oophorectomy resulted in the remission of breast cancer in premenopausal women [3]. Estradiol plays a vital role in the progression of breast cancer, and a majority of human breast cancers start as estrogen-dependent and express the ER. Its binding mediates the biological effects of estrogen to one of the structurally and functionally distinct ERs (ERα and Erβ) [4]. Endocrine therapy using Tamoxifen, a selective ER modulator [5], and aromatase inhibitors, which ablate peripheral estrogen synthesis, has been shown to improve disease-free survival substantially [6].

Metabolomics has emerged as an important area of study in the case of cancers, including breast cancer. Oncogenes like *MYC* and *TP53* have been found to affect the metabolism in breast cancers [7]. Differences in metabolism have been discovered between ER+ and ER- breast cancers, such as differences in the metabolism of glutamine and alanine [8]. Besides, there is evidence for a primary effect of estrogen on mitochondrial function. ERβ interacts with mitochondrial import proteins or cytosolic chaperone proteins and is imported into the mitochondrial membrane. It can then affect the transcription of mitochondrial genes by interaction with estrogen response elements (EREs) or other transcription factors (TFs) [9]. Glutamine metabolism is interconnected with several other metabolic alterations in breast cancer, and the observed differences in its regulation between ER-positive and ER-negative subtypes further highlight distinct oncometabolic profiles between these two forms of the disease. Although these differences in metabolite profile do not correspond to molecular subtypes in studies [10, 11], differences in the number of different oncometabolites have been shown to relate to clinical progression and outcomes such as an increase in glutamine and lactate in the cell leads to increased aggression of the breast cancer [7]. Also, it has been found that serine synthesis plays an important role in tumor growth in particularly ER-negative breast cancer. Phosphoglycerate dehydrogenase enzyme gene is amplified in ER-negative breast cancer but not in ER+ breast cancer, which in turn facilitates the diversion of carbon from glycolytic pathway into serine and glycine synthesis and increases tumor cell proliferation, and increases the synthesis of α-ketoglutarate [12–14]. Serine activates pyruvate kinase M2 (*PKM2*), and its level affects TP53 induced metabolic remodeling [15, 16]. Although the exact role of *PKM2* in oncogenesis is not clearly defined, it is differentially expressed in neoplasms and has a role in oncogenic processes like *HIF1α* activation [17–20].

In fact, it has long been known that ERRα controls cellular energy homeostasis by affecting mitochondrial metabolism, glycolysis, gluconeogenesis, lipid metabolism, etc. [21], and ERRα has been shown to have a significant effect on cellular metabolism such that ERRα antagonists have been considered as potential therapeutics in breast cancer [22]. ERRα lies in the promoter region of enzymes with roles in pyruvate metabolism, tricarboxylic acid (TCA) cycle, and carbohydrate metabolism [23, 24].

Altogether, estrogen signaling controls and integrates various metabolic pathways like lipid metabolism, fatty acid oxidation, TCA cycle, oxidative phosphorylation (OXPHOS), and mitochondrial functions. Studies have shown that estrogen signaling in breast cancer alters its metabolism to adapt to reduced perfusion, decreased local pH, and nutrient deprivation [25]. Increased dependence

on glutamine metabolism has also been demonstrated in breast cancer models that are estrogen-independent and antiestrogen-resistant compared to parental cell lines [26]. All these points relate to the role of differential estrogen signaling in the regulation of oncometabolite, which the study discusses.

Materials and Methods

Collection of Breast Cancer Microarray Datasets, Preprocessing and Identification of Consistently Altered Metabolic Genes

Gene Expression Omnibus (GEO) of the National Center for Biotechnology Information (NCBI) was used to fetch the mRNA expression profiles. The inclusion criteria were that the datasets must be of human samples, and each study had at least 2 ER+ PR+ and ER- PR- samples. Any studies devoid of case reports, review articles, abstracts, non-human subjects, and cell-line-based experimental study designs were excluded. To avoid potential analytical bias, the NCBI GEO2R tool [27] was utilized to directly identify genes showing differential expression between ER+PR+ samples (as test) and ER-PR- samples (as control). Subsequently, genes showing significant differential expression, with a p-value < 0.05 and $|\log_2$ (fold change)|>0 were selected as differentially expressed genes (DEGs).

Selection of Progression Markers

The 250 most significant DEGs were then identified from each study, which included both the overexpressed and underexpressed genes. These genes were then pooled together, and the 250 most significantly overexpressed and underexpressed genes were filtered out. The investigators then manually annotated all these genes for their function and role in metabolism in the cancer cells based on the existing literature. The unique genes that clearly had metabolic functions were identified. This way, downregulated genes and upregulated genes were identified for further study of the effect of estrogen.

Prediction of Estrogen Receptor Elements

UCSC Genome Browser [28] was used to get the promoter/upstream sequences upto 1000bp in the most common transcript variants in these genes in the Hg38 cell line. These upstream sequences were then used to identify EREs using the EREFinder algorithm developed by Anderson et al. [29]. Binding affinity (Kd) cutoffs were employed to detect EREs. Genes found to harbor EREs within their upstream regions, within a 1000bp range, were further examined to identify CpG islands in those regions [30] (Figure 2).

Protein-protein interaction network (PPIN) and enrichment analyses

We input all the ERE up and downregulated metabolism-related DEGs individually into the STRING v12.0 database for establishing a PPIN corresponding to the lowest confidence (i.e., interaction score>0.150). Thereafter, the ERE DEGs participating in PPIN were subjected to pathway enrichment analysis. We used the enrichr web-based tool and fetched the top 10 significant (p-value<0.05) Pathways corresponding to the Reactome library.

Bioinformatics validation using cBioPortal and UALCAN

Functionally enriched up and downregulated ERE DEGs were used as input for mutational analysis via

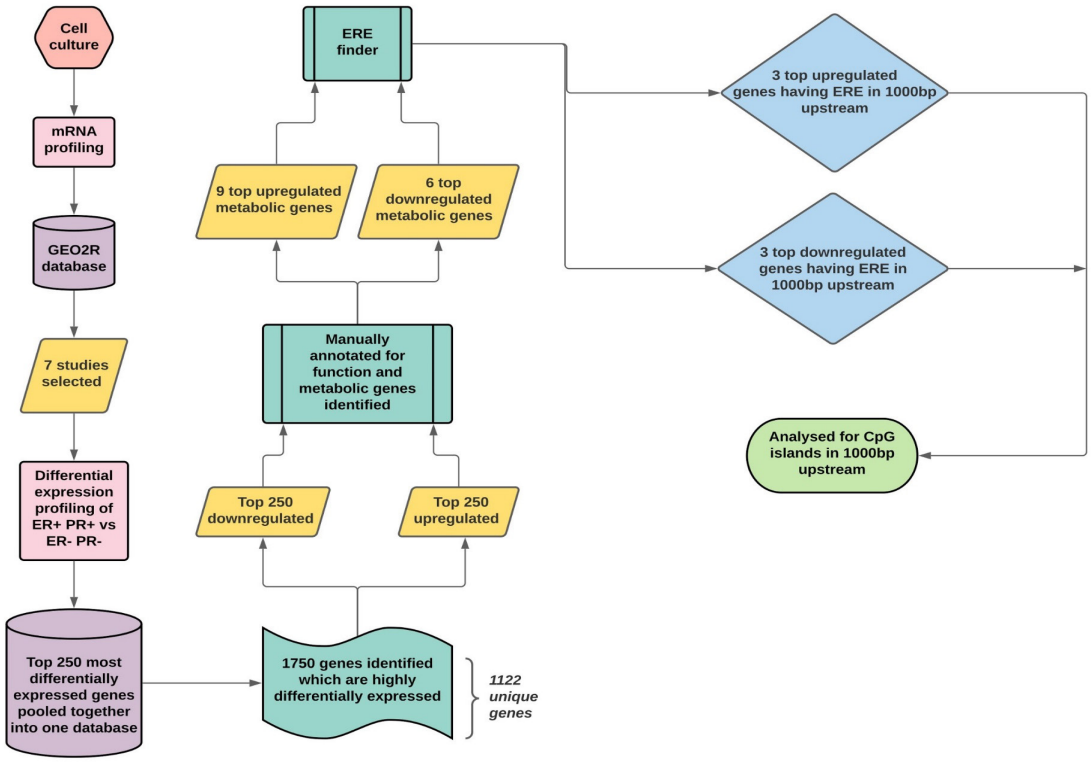

Figure 2. Methodology of the Study. ERE, Estrogen Receptor Element

cBioPortal web-based tool. UALCAN online tool (https://ualcan.path.uab.edu/) [31] was accessed thereafter to examine the promoter methylation level of most altered ERE DEGs on the basis of individual cancer stages, major subclasses, nodal metastasis, and *TP53* mutation status.

Results

Metabolic genes are consistently altered in human ER+ Breast Cancer

7 relevant studies (Table 1) were identified as per the

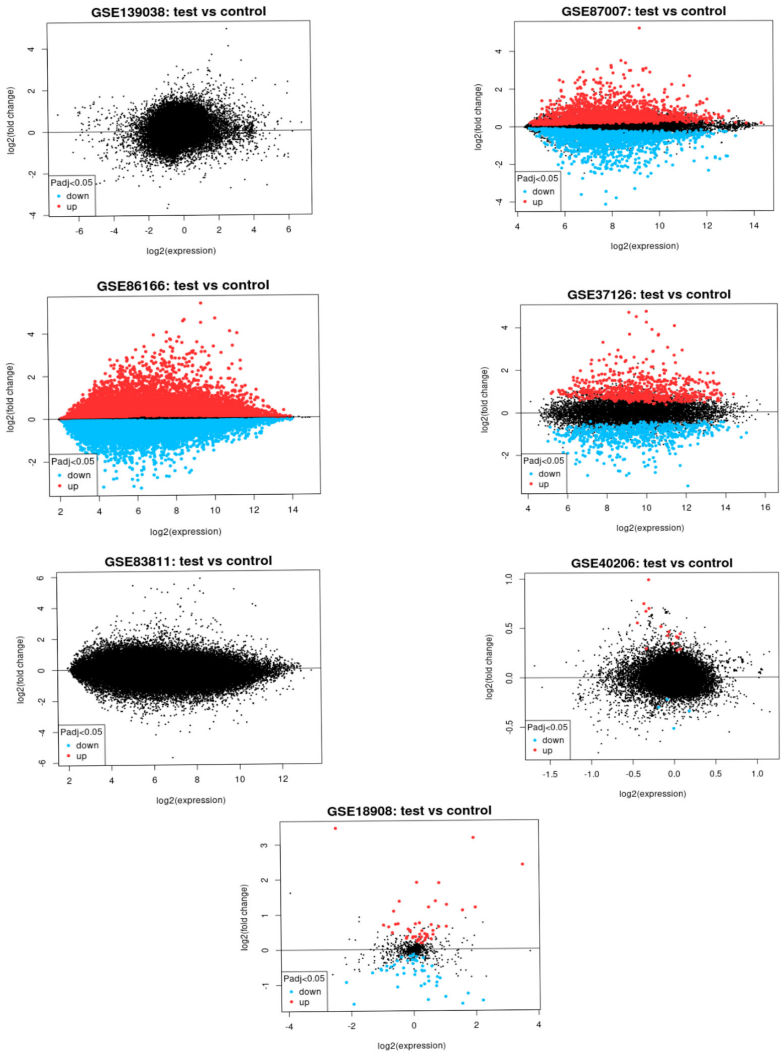

Figure 3. Mean Difference Plots of the 7 Selected Studies with x-axis Having $\log_2$ (expression) and y-axis having $\log_2$ (fold change) values.

Table 1. 7 Selected Studies Meeting Inclusion and Exclusion Criteria

S.no.	GSE No.	Title	Investigator
1	GSE83811	Expression Data from ALDH1+ breast cancer stem cells	Tiezzi et al. [72]
2	GSE37126	Portrait of early-onset breast cancer in Brazilian patients: germline mutation screening of *BRCA1, BRCA2* and *TP53* genes and tumor expression profiling	Carraro et al. [73]
3	GSE139038	Gene expression profiling in paired normal, apparently normal and breast tumour tissues	Rajkumar et al. [74)
4	GSE87007	CDK4 phosphorylation status and a linked gene expression profile predict sensitivity to Palbociclib [tumors]	Raspé et al. [75]
5	GSE40206	Differential expression of genes and protein networks in the primary breast tumor that proceed to distant metastasis	Kondaiah et al. [76]
6	GSE86166	Evaluation of Invasive Breast Cancer Using a 12-Chemokine Gene Expression Score: Correlation With Clinical Outcomes	Soliman et al. [77]
7	GSE18908	Breast tumor subtypes correlate with prognosis	Minana B [78]

above mentioned inclusion and exclusion criteria from NCBI-GEO. From these 7 studies, 1750 DEGs entries were pooled together. Figures 3 and 4 illustrate the mean difference and volcano plots of the seven studies. After excluding the entries that had adjusted p-value>0.05, 879 entries remained. After manually annotating the 250 most upregulated and downregulated metabolic genes, 33 unique genes were identified for further studying, out of which 20 were downregulated, and 13 were upregulated. After studying the upstream regions of these genes for EREs, 18 genes were identified as having EREs in their upstream regions-*CA12, CPA3, FBP1, STC2, NME5, DEGS2, ABAT, GAMT, ARSG* were upregulated, and *B3GNT5, DPH2, PPARA, TNFRSF21, PHGDH, FOXL1, ME1, RNF145, NUDT5* were downregulated. CpG islands closely corresponded to the EREs in *PPARA, PHGDH, ME1, RNF145, NUDT5, CA12, STC2, ABAT* and *GAMT*. The results are tabulated in Table 2 and Table 3.

Protein Protein Interaction Network (PPIN) and enrichment analyses

Unweighted and undirected PPINs as shown in Figure 5A-B comprised 6 nodes and 8 linking edges for upregulated ERE metabolism-related DEGs whereas 5 nodes and 5 linking edges for downregulated ERE metabolism-related DEGs. Undirected chord plots as shown in Figure 5C-D shows the association of top 10 significant pathways for up and downregulated ERE metabolism-related DEGs participating in PPINs. The most significant pathways in the case of upregulated PPIN are creatine metabolism (p-value=2.99×10^{-3}) and other metabolism while downregulated PPIN-participating ERE DEGs were and PPARA activates gene expression (p-value=3.30×10^{-4}).

Bioinformatics validation using cBioPortal and UALCAN

Mutational analysis was performed for functionally enriched ERE DEGs using cBioPortal using the breast invasive carcinoma (TCGA, firehose legacy) dataset. All these DEGs (i.e., *sSTC2, FBP1, ABAT, CA12, GAMT, PPARA, TNFRSF21, PHGDH*) were altered in 14% patient samples (i.e., 160/1108). A total of 2%, 0.73%, 5%, 1%, 1%, 2%, 2%, 3% mutation frequencies were reported for *STC2, FBP1, ABAT, CA12, GAMT, PPARA, TNFRSF21, PHGDH*. As evidenced, *ABAT* reported the highest mutation frequency out of all ERE DEGs. Based

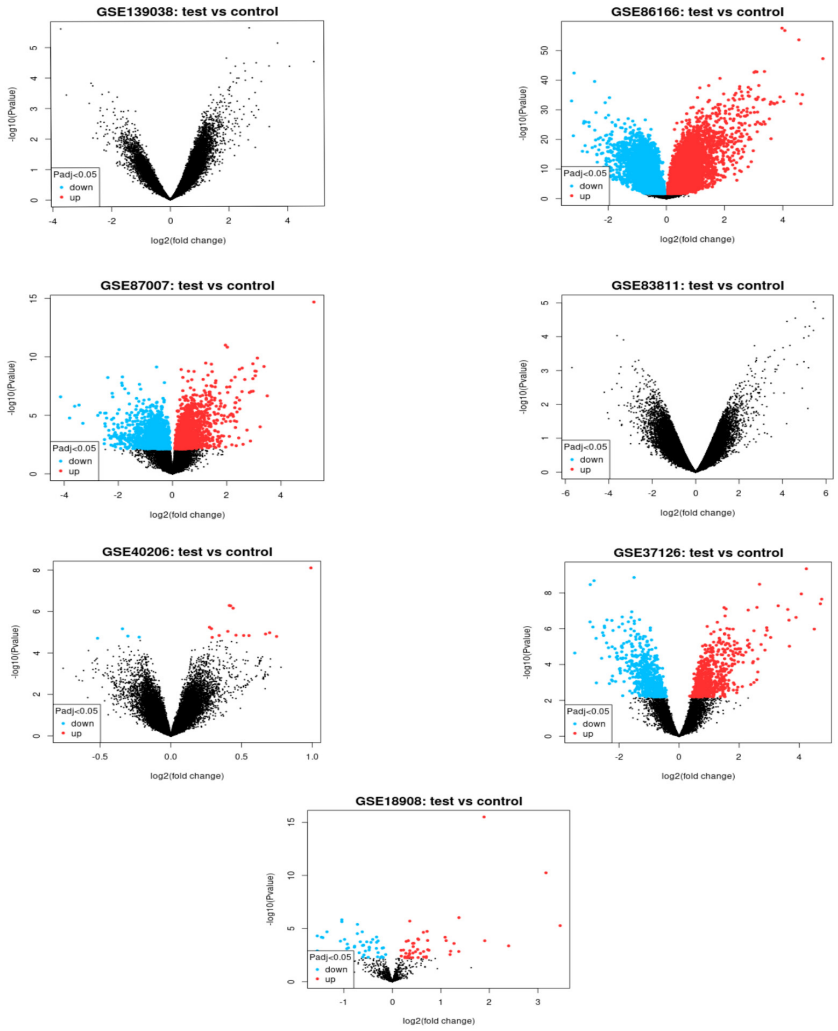

Figure 4. Volcano Plots of the 7 Selected Studies with x-axis having $\log_2$ (fold change) and y-axis having $-\log_{10}$ (p−value).

Table 2. ERE Status and CpG Island Status of Downregulated Metabolism-associated Genes.

S.No.	Gene Symbol	Locus	Function	ERE	CpG Island upto 1000bp upstream*	Remarks
1	*B3GNT5*	Chr3 (q27.1)	Codes for a member of beta-1,3-N-acetylglucosaminyltransferase family. Involved in Glycolipid metabolism	ERE present between -691 and -686	Start site: -228 End site: -8 Length- 221 GC content- 68%	-
2	*DPH2*	Chr1 (p34.1)	Involved in dipthamide biosynthesis.	ERE present between -566 and -556	Start site: -424 End site: -25 Length- 400 GC content- 55%	-
3	*PPARA*	Chr22 (q13.31)	Codes PPAR-alpha, a nuclear transcription factor which is a key regulator of lipid metabolism.	ERE present between -501 and -491	Start site- -1000 End site- -2 Length- 999 GC content- 81%	ERE is present in a CpG island
4	*TNFRSF21*	Chr6 (p12.3)	Codes a member of the tumor necrosis factor receptor superfamily, which regulates lipid metabolism through PPAR-alpha	ERE present between -506 and -496	Start site: -387 End site: -49 Length- 339 GC content- 51%	-
5	*PHGDH*	Chr1 (p12)	Codes the enzyme which is involved in the early steps of L-serine synthesis; Involved in amino acid metabolism	ERE present between -866 and -856	Start site: -867 End site: -23 Length- 845 GC content- 68%	ERE is present in a CpG island
6	*FOXL1*	Chr16 (q24.1)	Codes a member of the forkhead/winged helix-box (FOX) family of transcription factors, and plays a critical role in many cellular processes including metabolism regulation.	ERE present between -131 and -121	Not present	-
7	*ME1*	Chr6 (q14.2)	Codes a cytosolic NADP-dependent enzyme that generates NADPH for fatty acid biosynthesis	ERE present between -566 and -556	Start site: -798 End site: -10 Length- 789 GC content- 64%	ERE is present in a CpG island
8	*RNF145*	Chr5 (q33.3)	Involved in cholesterol biosynthesis through its effect on SREBPF2	ERE present between -356 and -346	Start site: -415 End site: -26 Length- 390 GC content- 61%	ERE is present in a CpG island
9	*NUDT5*	Chr10 (p14)	Codes an enzyme which catalyzes the hydrolysis of modified nucleoside diphosphates; involved in purine metabolism	ERE present between -46 and -36	Start site: -916 End site- -6 Length- 911 GC content- 52%	ERE is present in a CpG island

* o/e ratio=0.60, Minimal length=200, any CpG island which has GC content less than 50% is filtered out

Table 3. ERE status and CpG Island Status of Upregulated Metabolism-Associated Genes

S.no.	Gene symbol	Locus	Function	ERE	CpG island upto 1000bp upstream*	Remarks
1	*CA12*	Chr15 (q22.2)	Codes an enzyme responsible for catalyzing the reversible hydration of carbon dioxide to form bicarbonate (HCO3-) and hydrogen (H+) ions	2 EREs present between -809 and -796 and -326 and -301	Start site: -765 End site: -25 Length- 741 GC content- 56%	2nd ERE is present in a CpG island
2	*CPA3*	Chr3 (q24)	Codes a member of the carboxypeptidase A family of zinc metalloproteases; involved in protein metabolism	ERE present between -581 and -571	Not present	-
3	*FBP1*	Chr9 (q22.32)	Codes a a gluconeogenesis regulatory enzyme; involved in Glucose metabolism	ERE present between -446 and -436	Not present	-
4	*STC2*	Chr5 (q35.2)	Codes a secreted, homodimeric glycoprotein; has a role in cell metabolism regulation	ERE present between -746 and -736	2 CpG Islands present Start site: -997 End site: -605 Length- 393 GC content- 55% and Start site- 541 End site- 997 Length- 457 GC content- 61%	ERE is present in the 1st CpG island
5	*NME5*	Chr5 (q31.2)	Codes a protein involved in purine and pyrimidine metabolism	ERE present between -611 and -601	Not present	-
6	*DEGS2*	Chr14 (q32.2)	Codes a bifunctional enzyme that is involved in the biosynthesis of phytosphingolipids; involved in sphingolipid metabolism	ERE present between -761 and -751	Start site: -574 End site: -11 Length- 564 GC content- 71%	-
7	*ABAT*	Chr16 (p13.2)	Codes 4-aminobutyrate aminotransferase (ABAT); responsible for catabolism of gamma-aminobutyric acid	ERE present between -611 and -601	Start site: -742 End site: -15 Length- 728 GC content- 64%	ERE is present in a CpG island
8	*GAMT*	Chr19 (p13.3)	Codes a methyltransferase; involved in Urea cycle and amino acid metabolism	ERE present between -446 and -436	Start site: -1000 End site: -27 Length- 974 GC content- 93%	ERE is present in a CpG island
9	*ARSG*	Chr17 (q24.2)	Codes a sulfatase involved in sphingolipid and protein metabolism	ERE present between -761 and -751	Start site: -295 End site: -14 Length- 282 GC content- 52%	-

* o/e ratio=0.60, Minimal length=200, any CpG island which has GC content less than 50% is filtered out

on cancer-type summary analysis, alteration frequencies (overall) of *STC2, FBP1, ABAT, CA12, GAMT, PPARA, TNFRSF21, PHGDH* were illustrated by barplots shown in Figure 6A-H. Types of mutations and their percentage frequencies are summarized in Table 4. Promoter methylation levels of *abat* correlated with individual cancer stages, major subclasses, nodal metastasis, and *tp53* mutation status across breast invasive carcinoma (TCGA, firehose legacy) dataset were illustrated by box-and-whisker plots in Figure 7A-D.

Table 4. Mutation Types and Their Frequencies for All Functionally Enriched ERE DEGs.

Gene symbol	Amplification (%)	Deep Deletion (%)	Missense Mutation (%)
STC2	1.29	0.18	0.18
FBP1	0.28	0.28	0.18
ABAT	4.59	-	0.55
CA12	0.64	0.09	0.09
GAMT	-	1.1	0.09
PPARA	0.55	0.64	0.37
TNFRSF21	1.65	0.37	0.18
PHGDH	2.48	0.09	-

Discussion

Estrogens and ERs play pivotal roles in regulating metabolism not only in normal physiological conditions but also in disease states. Even in invertebrates, which lack sexual reproduction, ER-like proteins exist and regulate metabolic processes, underscoring the significance of ER signaling in non-reproductive functions, including metabolism. Studies on metabolic changes in postmenopausal women following estrogen loss suggest that estrogen signaling plays a crucial role in regulating energy metabolism. On the other hand, cancer cells, which proliferate uncontrollably, employ various strategies to maintain their proliferation rate even under conditions of limited nutrient availability. This adaptive capacity of cancer cells to adjust their metabolic

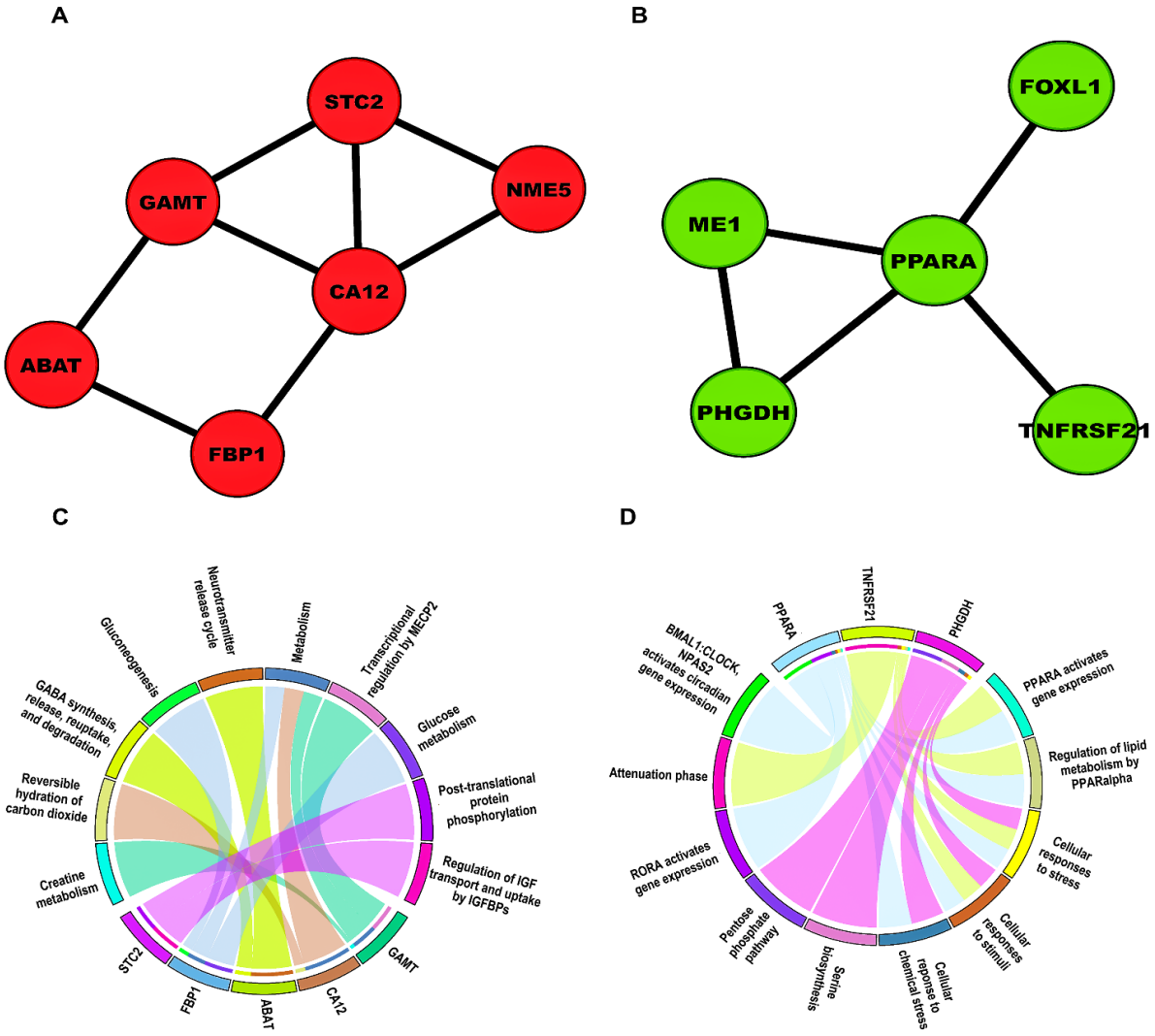

Figure 5. Undirected and Unweighted PPINs in the Case of (A) upregulated ERE DEGs and (B) downregulated ERE DEGs. The red and green colored nodes signify the expression status of DEGs in case of ER+PR+ w.r.t. ER-PR-, respectively. Chord plots showing the association of top 10 significant pathways with PPIN participating DEGs in case of (A) upregulated ERE DEGs and (B) downregulated ERE DEGs.

processes in response to changing nutrient conditions is termed metabolic plasticity. Identifying consistently altered metabolic targets is crucial for advancing basic, translational, and clinical studies on cancer metabolism. In this study, we have unveiled, for the first time, metabolic genes consistently dysregulated in ER+ breast cancer. These genes, when contextualized within their associated biochemical pathways, reveal the suppression of well-established breast cancer metabolic functions, such as lipid metabolism, and the up-regulation of energy-yielding processes like glycolysis, as consistent features of breast cancer.

In breast cancer cells, there is an upregulation of key glucose transporters and glycolytic enzymes to support the synthesis of building blocks required for cell proliferation, leading to a metabolic shift towards glycolysis, commonly known as the 'Warburg effect' [32]. Hypoxia-dependent upregulation of ER signaling stimulates the expression of glucose transporters (GLUT-1, GLUT-2, and GLUT-5) [32]. Elevated glucose levels in the media activate AKT signaling and suppress the TCA cycle [33]. E2 treatment upregulates the c-Myc–hnRNP axis and the expression and activity of glycolytic enzymes, such as PFKFB3, resulting in increased levels of FBP1 and glucose uptake [34, 35]. Additionally, in breast cancer cells, E2 regulates the balance between glycolysis and OXPHOS by upregulating pyruvate dehydrogenase (PDH) in the absence of glucose [36]. E2 also promotes addiction to the pentose phosphate pathway by upregulating glucose-6-phosphate dehydrogenase (G6PD) enzyme [36]. In addition, *FBP1* overexpression was associated with reduced HIF-1α protein expression and mRNA levels of *PDK1, LDHA, GLUT1,* and *VEGF* under hypoxic conditions [37]. It has been recently demonstrated that HIF-1α is a transcriptional target of ERα, and high HIF-1α expression is linked to tamoxifen resistance in ERα+ tumors [38]. Conversely, the hypoxia response reduces ERα expression and cell proliferation [39]. This well-established crosstalk between ERα and hypoxia response pathways opens up new therapeutic avenues for treating hypoxic ER+ breast cancers.

Transcriptome analysis by Porras et al. identified ca12

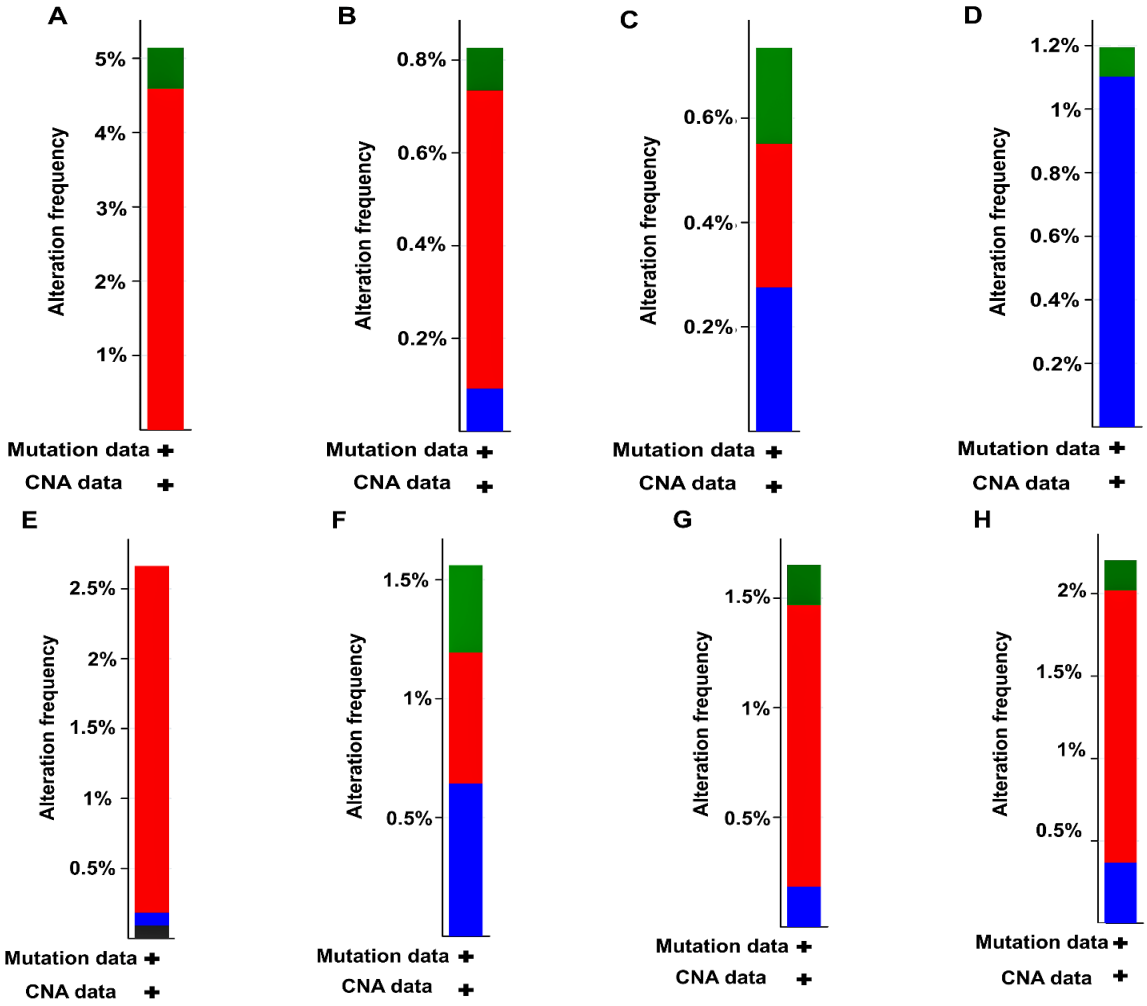

Figure 6. Barplots Showing Alteration Frequencies of (A)ABAT, (B) CA12, (C) FBP1, (D) GAMT, (E) PHGDH, (F) PPARA, (G) STC2, (H)TNFRSF21 across TCGA breast invasive carcinoma dataset.

as an estrogen target gene closely correlated with ESR1 and regulated by ERα and GATA3 [40]. Immunostaining in 118 tumors showed strong positive correlations of *CA12* with ERα and GATA3, with comparable expression in ER+PR- and ER+PR+ tumors, indicating active estrogenic signaling in some ER+PR- cancers. However, *CA12* is downregulated in TNBC and basal-type aggressive tumors, as shown by Vargara et al. and Gobo database analysis [41]. LINC02568 also regulates *CA12*, maintaining pH homeostasis and suggesting *CA12* as a potential biomarker, particularly due to its membrane localization [42]. Barnett et al. demonstrated that ER transcriptionally regulates *CA12* via a hormone-responsive enhancer, highlighting its co-expression with ER in breast tumors [43].

Carboxypeptidases, including CPA3 [44, 45] and the splice variant CPE-ΔN [46], are linked to various cancers. CPA3 may serve as a biomarker for ER+ breast cancer, supported by its association with other cancers [44, 45, 47]. *ABAT,* and *STC2*, identified by Bouras et al. [8, 48], and NME5 show significant associations with ER+ breast cancer [49], with potential roles in endocrine resistance. The *ESR1* Y537S mutation, linked to endocrine therapy resistance, correlates with genes like *DEGS2, FMN1*, and *IRS1* [50]. GAMT, associated with creatine metabolism, also shows promise as an ER+ biomarker, alongside underexplored estrogen-regulated enzymes like ARSA and ARSB [51, 52].

B3GNT5, correlated with *COX2* expression, promotes cell proliferation and metastasis via glycosphingolipid biosynthesis [53, 54] and may serve as a target for AR antagonists in TNBC [55]. Overexpression of *PPARα,* stimulated by arachidonic acid, drives proliferation in ER+ breast cancers, particularly MCF7 cells [56]. PHGDH has been linked to poor outcomes and chemotherapy sensitization [57], while NUDT5 and TNFRSF21 influence estrogen signaling and therapy response [58].

Tumor suppressor FOXL1 inhibits proliferation and metastasis, showing therapeutic potential in ER+ cancers [59]. Similarly, metabolic genes like *ME1*, implicated in glycolysis and obesity-linked estrogen metabolism [60], and diphthamide synthesis enzymes like DPH2, connected to NF-κB activation, warrant further study [61, 62]. RNF145, an ER-anchored E3 ligase involved in sterol regulation, may also link metabolism to ER+ breast cancer [63]. While its implications in cancer and estrogen contexts remain unexplored, its involvement in sterol regulation suggests a compelling avenue for investigating the link between RNF145 and ER+ breast cancer.

Overall, the observed mixture of both up-regulated

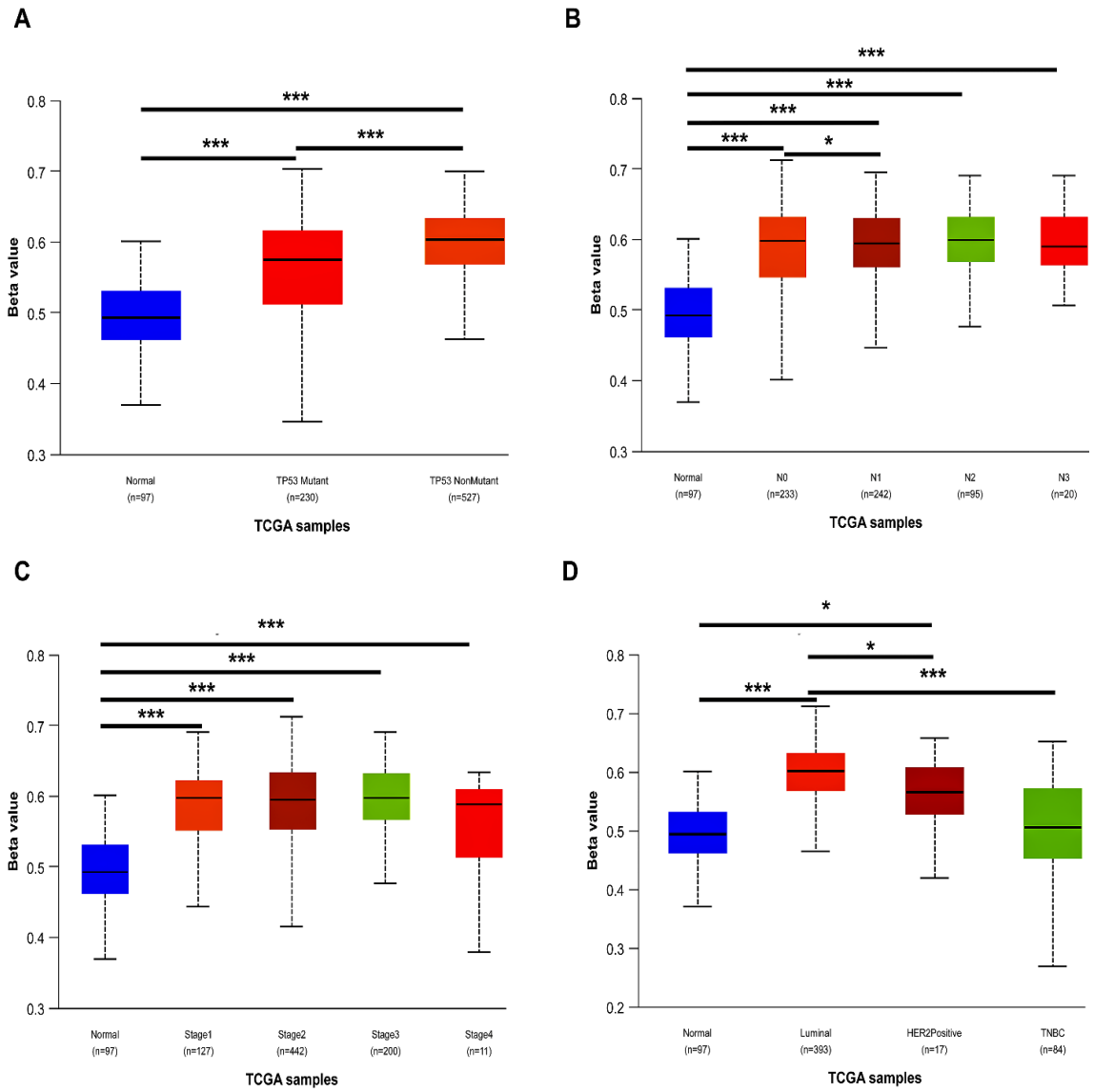

Figure 7. Box-and-whisker plots displaying promoter methylation distribution of ABAT w.r.t. (A) TP53 mutation status, (B) nodal metastasis status, (C) individual cancer stages, (D) major subclasses across TCGA breast invasive carcinoma dataset. *p-value <0.05, **p-value<0.01, ***p-value<0.001.

and down-regulated genes in most metabolic pathways underscores the complexity of metabolic dysregulation in ER+ breast cancer. Isoforms of metabolic genes consistently expressed in opposite directions may represent clinically significant alterations in breast cancer.

While this study presents a valuable first step in linking estrogen signaling to specific metabolic genes in breast cancer, suggesting a potential direct transcriptional regulatory mechanism via EREs in key metabolic genes, it also opens up several avenues for further exploration. The analysis is based solely on microarray-derived transcriptomic data and in silico predictions, without functional assays to confirm ER binding at the identified promoter regions. Additionally, the study does not assess protein-level expression or enzymatic activity, which can differ from mRNA levels due to post-transcriptional modifications; hence, complementary proteomic and metabolomic analyses would be important to establish the biological significance of these findings. While CpG islands were identified near EREs, their methylation status remains unexplored, leaving room for future epigenetic investigations. Moreover, the heterogeneity among ER+ subtypes was not specifically addressed, which could further refine the interpretation of expression patterns. The potential influence of other receptors, including HER2 and the androgen receptor, which also modulate cancer metabolism, was not within the scope of this study but requires consideration in future work. Additionally, while data from multiple GEO studies enriches the analysis, it may also introduce batch effects and clinical variability, highlighting the need for harmonization and meta-analytic strategies. In this context, like other disease and cancer models, incorporation of bioinformatics and the use of advanced 3D models of breast cancer and its subtypes, particularly those derived from stem cells or cancer stem cells [18, 19, 20, 64–71], is also encouraged, as they can provide valuable insights into the interplay between metabolism, hormonal regulation,

and tumor heterogeneity. Lastly, expanding from gene-level observations to pathway-level analyses could provide a more integrated understanding of the metabolic reprogramming governed by estrogen signaling. Taken together, these directions underscore the importance of building upon this foundational work with experimental validation and systems biology approaches to fully elucidate the therapeutic potential of estrogen-regulated metabolic pathways in breast cancer.

In conclusion, these 18 genes that were identified have functions in metabolism and are significantly differentially expressed in ER+PR+ breast cancer cell lines as compared to ER-PR- cell lines. Also, these genes have upstream EREs and hence are regulated by estrogen signaling. Since the CpG island does correspond to the EREs we can say that DNA methylation has a role in the epigenetic control of the regulation of these genes by estrogen.

Of these genes, CA12 has very high-affinity ERE in its upstream region, and there are studies pointing to its role in oncogenesis and its regulation by estrogen [43]. This shows that our methodology was able to identify genes involved in estrogen signaling, and the other genes also potentially are involved in metabolic changes in response to estrogen signaling.

Delineating the mechanism of action of these metabolic genes and their regulation by upstream modulators like estrogen will open up novel pathways of oncogenesis and help in identifying potential therapeutic targets. Further work is needed to clearly quantify the effect of estrogen on the expression of these metabolic genes and their oncometabolite, which can potentially affect cancer progression, disease survival, and metastasis.

In conclusion, our hypothesis based on the current in-silico analysis is that these 18 genes that were identified can be targets for estrogen stimulation that can affect the concentration of different oncometabolites in the tumor cells and their variable expression profiles may hence be predictors of clinical outcomes or targets for modulation via novel therapeutics.

Author Contribution Statement

AM, S, PS, and OSS drafted the manuscript with assistance from IG, JT, TS, and AR. SK, PS, RD, and OSS performed bioinformatic analysis. PR assisted in clinical correlation. SK and RD oversaw the entire work.

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Conflict of Interest

The authors declare no potential conflict of interest

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