Abstract: Estrogen Receptor α (ERα) is reported to regulate the expression of many target genes by binding to specific estrogen response elements (EREs) in their promoters. c-myc is known to be over-expressed in most of the human carcinomas due to dysregulated transcription, translation, or protein stability. Estrogen (E) can induce the c-myc expression by binding to an upstream enhancer element in its promoter. This suggests that elevated estradiol (E2), a potent form of estrogen, levels could induce the expression of c-myc in breast cancer (BC). The expression of c-myc and estradiol were induced at Stage III and Stage IV of breast cancer. c-myc and estradiol expression was also associated with the established risk factors of breast cancer, such as BMI. Age at the time of the disease was also correlated with the relative expression of c-myc and estradiol (p < 0.0007 and p < 0.000001). The correlation coefficient (R = 0.462) shows a positive relationship between estradiol bound ER, ER, and c-myc. Docking energy \(-229 \text{ kJ/mol}\) suggests the binding affinity of estradiol bound ER binding to 500 bp upstream of proximal promotor of c-myc at three distinct positions. The data presented in this study proposed that the expression of c-myc and estradiol are directly correlated in breast cancer. The prognostic utility of an induced level of c-myc associated with the normal status of the c-myc gene and estradiol for patients with metastatic carcinoma should be explored further.

Keywords: c-myc; estrogen; estradiol; estrogen response elements (EREs)

1. Introduction

Estrogen is a steroid hormone that has critical roles in reproductive development, bone homeostasis, cardiovascular remodeling, and brain functions. However, estrogen also promotes mammary, ovarian, and endometrial tumorigenesis. Estrogen antagonists and drugs that reduce estrogen biosynthesis have become highly successful therapeutic agents for breast cancer. The three major forms of estrogen that have been reported are estrone (E1), the primary form of estrogen that the body makes after menopause, estradiol, (E2)
the most potent form of estrogen in the body during reproductive years, and estriol, (E3) a form that is active during pregnancy [1].

In premenopausal conditions, estrogen is produced in the ovaries. Post menopause, adipose tissues start producing estrogen; thus, high BMI, a risk factor for breast cancer, corresponds to higher estrogen levels, which subsequently could promote cancer [2]. ER and estradiol activity are prognostic and predictive factors used in the management of BC [3]. The c-myc gene serves as a “master regulator” of cellular metabolism and proliferation. It stimulates various metabolic adjustments that bring about malignant transformation. Estradiol, a circulating form of estrogen, induces c-myc by ER binding.

C-myc is a transcription factor that is constitutively and aberrantly expressed in over 70% of human cancers and belongs to the basic-helix-loop-helix-leucine zipper (bHLHZip) family present in the cell nucleus, where it acts to regulate cell growth, differentiation, metabolism, and death. The c-myc protein is a major transcription factor for pro-proliferative genes because it binds to enhancer box sequences (E-boxes) and recruits histone acetyltransferases (HATs). It is known to be over-expressed in most human carcinomas due to dysregulated transcription, translation, or protein stability [4]. c-myc preferably binds to sequence-specific target sites called E-box motifs, which are found within CpG islands [5]. ERs are known to regulate gene expression to mediate the actions of estrogen that has a regulatory role in the pathology of BC [6,7]. The effects of estrogen are largely mediated by estrogen receptor (ER) α and ERβ. The mechanisms underlying the aberrant expression of ER in breast cancer and other types of human tumors are complex [8]. Different isoforms of ER perform different roles in carcinogenesis. These forms interact with target genes by binding to specific estrogen response elements (EREs) in their promoters. On the other hand, ERs can indirectly interact with other domains of promoter bound-transcription factors such as activation protein 1 (AP1), nuclear factor κ-light-chain enhancer of activated B cells (NF-kB), or specificity protein (SP) 1 [8–10].

Estradiol, by activating the ER, makes a complex, binds to c-myc, and exerts its proliferative effect by regulating the levels of these proteins [11]. It is also known that estradiol stimulates c-myc exclusively at the transcriptional level by nuclear transcriptional activator mediation [12]. As estrogen rapidly induces the c-myc expression, it helps in the progression of the cell cycle. c-myc and ER have been used as significant soluble biomarkers in metastatic BC cells. c-myc is reported to be a vital target gene of ER, as estrogen stimulus upregulates the transcription of proto-oncogene c-myc in ER-positive BC, leading to cell proliferation [13]. Irregular estrogen signaling revamps normal metabolic pathways (including the metabolism of glucose, glutamine, and fatty acids) in cancerous cells, thus making the cell adapt to stressed conditions and stimulating cell proliferation. c-myc is involved in the regulation of glutamine metabolism through the interaction between HER2, estradiol, and ER in cells resistant to aromatase inhibitors [14]. The exact mechanism by which estradiol induces c-myc is not well-known [12].

It has been reported that estrogen binds to an estrogen responsive promoter (ERP), and the interaction of c-myc and ERα thereby facilitates the formation of the ERα, c-myc, and TRRAP (transactivation/transformation domain associated protein) transcription activation complex, leading to chromatin remodeling and the enhanced transcription of c-myc target genes [8]. It is suggested that while c-myc is a renowned estrogen-target gene, it has binding sites for ER in distal intergenic regions located 67 kb upstream of the c-myc TSSP containing half-ERE and an AP-1 site along with protein complex of JunD and FOXB. (Figure 1) [15]. This provides insights for understanding the molecular mechanism underlying estrogen-induced c-myc expression and suggests that ER enhances and regulates the expression of c-myc gene in BC cells.
Figure 1. Binding of estradiol with estrogen receptor in collaboration of JunD and FOSB acts as enhancer 1.7 KB upstream of c-myc.

In this article, we discuss the co relation of c-myc, ER, and estradiol in BC and their correlation/inter-dependence in response to epidemiological factors.

2. Materials and Methods

2.1. Research Methodology

2.1.1. Study Design

Blood samples of total 142 female BC patients who alluded to the N.O.R.I. Cancer Hospital and P.I.M.S. Hospital Islamabad were contacted for this non-randomized research design (Table 1). Besides, 77 healthy age-matched female volunteers with no history of BC filled in as the control group. A total of 5 mL of the blood was collected for isolation of RNA in EDTA tubes and stored at \(-4^\circ C\) till research analysis. Informed consent was signed and approved by all individuals who participated in this study. All individuals under study were given questionnaires to fill out their clinic-pathological and lifestyle parameters (Table S2).

Table 1. Reference standard Table for BMI calculations.

| Reference BMI Range | BMI Range | Status    |
|---------------------|-----------|-----------|
| 0                   | 18.5      | Underweight |
| 18.5                | 24.9      | Normal weight |
| 24.9                | 29.9      | Overweight |
| 29.9                | 30        | Obese     |

2.1.2. Inclusion Criteria

Breast cancer patients were histologically confirmed for different stages of cancer (Stages I, II, III, IV). All epidemiological parameters of inclusion are enlisted in Table S1.
2.1.3. Exclusion Criteria

Patients who were positive for HIV and HCV or any other infection were excluded in this study. Patients diagnosed with multiple diseases and terminally ill patients were also excluded.

2.2. Expression Analysis

RNA Extraction/Quantitative RT-PCR

Total RNA was extracted using TRIzol® reagent (Invitrogen) according to manufacturer’s protocol. Gene-specific primers were designed using primer3 online tool and optimized before use (Table 2). Nucleotide blast was done using NCBI blast tool. In silico PCR was carried out using serial cloner and UCSC web browser. Product size was confirmed by confirmatory gel electrophoresis.

Table 2. Primer details of c-myc, ESR1, and β-globin.

| Primer Details | Product Size | Annealing Temperature |
|----------------|--------------|-----------------------|
| C-myc-F (forward) | 5'-TCGGATTCTCTGCTCCTCCTCTC-3′ | 157 bp | 57 °C |
| C-myc-R (reverse) | 5'-CTGGCCTCTTCTCCACAGAA-3′ | Product Size | Annealing Temperature |
| β-globin (forward) | 5'-GCTTCTGACAACTGTGTTCACTAGC-3′ | 115 bp | 59 °C |
| β-globin (reverse) | 5'-CACCAACTTCATCCACGTTCACC-3′ | Product Size | Annealing Temperature |
| ESR1 (forward) | 5'-GCTTACTGACCAACCTGGCAGA-3′ | 151 bp | 60 °C |
| ESR1 (reverse) | 5'-GGATCTCTAGCCAGACCAATTC-3′ | Product Size | Annealing Temperature |

mRNA quality and quantity were measured by the IMPLEN® P300 Nanophotometer P3 Implen GmbH, München, Germany, and cDNA synthesis was carried out using 500 ng of total RNA using Oligo (dT) primers and reverse transcriptase.

Gene expression analysis of c-myc and estradiol was carried out on Applied Biosystems™ 7500 real-time PCR, Thermo Fisher Scientific, Waltham, MA, USA using SYBR green master mix. RT-PCR conditions for c-myc were set as holding stage at 95 °C for 10 min, single cycling stage at 95 °C for 30 s and 57 °C for 1 min followed by melt curve cycle at 95 °C for 10 s with 10 °C difference for each step. To obtain Ct value for β-globin, same as mentioned above was used, except for Tm change, which is 59 °C in case of β-globin. Melt curve for c-myc generated by RT-PCR is shown in (Figure 2).

Relative gene expression is calculated using comparative Ct by Livak method. Ct is normalized to the housekeeping gene β-globin (Tm = 59 °C). Data was expressed in 2-fold standard means and RE means with ±SEM.

2.3. Enzyme-Linked Immunosorbent Assay

Estradiol in the blood samples of BC patients was extracted by using dichloromethane (Wako, Osaka, Japan). Estradiol is measured according to the manufacturer protocols (High Sensitivity Salivary 17β-estradiol Enzyme Immunoassay Kit, Salimetrics LLC, Carlsbad, CA, USA). MTP-300 microplate reader is used to measure absorbance in each well at 450 nm. R values were above 0.99 for the control dilution range of estradiol.

2.4. DNA Molecular Docking&Protein-Ligand Docking

There are few studies where DNA intercalator are being used for therapeutic potential. Molecular docking was carried out to explain the structural affinity between 17β-estradiol and c-myc.

Preparation of Ligand and Receptor Molecule

Molecular properties of the ligand are extracted by using Molinspiration tool®. SDF file is converted to pdbqt format before use.
Receptor molecule for DNA docking was 500 bp genetic fragment upstream of c-myc proximal promotor. Firstly, we searched for the promotor binding sites of c-myc (NG_007161.2 Homo sapiens MYC proto-oncogene, on chromosome 8, 14,518 nucleotides) using primer 2.0 tool, which is freely available on http://www.cbs.dtu.dk/services/Promoter/ (accessed on 25 June 2021) [16]. It gave us 11 marginal predicted sites docking score < 0.6 and three highly likely predicted sites at positions 700, 5900, and 7300 with predicted scores of 1.186, 1.246, and 1.019, respectively (jobid = 60CE40B1000036637FE816BA). Results were confirmed by another tool fprom.pl on Linux-based server; it delivered an enhancer at position 5262 with +9.0 docking score [17]. As reported earlier by [15], half ERE binding sites were available ≈67 kb away from promotor regions, so we utilized 500 bp sequence around 2nd promotor region and 5262 bp enhancer position for DNA and ligand molecular docking to align these with the structure of ER bound estradiol (2j7x pdb format) on HDOCK server.

![Melt Curve](image)

**Figure 2.** c-myc melt curve generated by 7500 ABI system for confirmation of c-myc product amplification.

Receptor molecule for protein docking is prepared by removing water and adding hydrogen to the pdb structure. Further we did add kollman charges to our protein and grid it and saved in pdbqt format for docking studies.

2.5. Statistical Analysis

Microsoft Excel is used to arrange data in spreadsheets, and csv format is used for R import and GraphPad prism import. Excel add-in “DATA ANALYSIS” was used to create bar graphs, poly-regression analysis, one-way ANOVA, and Welch’s t test. Livak method of normalizing Ct values was used to calculate expression analysis [18]. Box and whisker graphs were made using software GraphPad prism 7® version 7.04 by GraphPad software.
3. Results

3.1. BMI Association with c-myc and Estradiol

BMI and menopausal status (data not mentioned here) show a strong affiliation with the elevated expression of estradiol and c-myc in breast cancer patients. BMI is calculated using the standard BMI Reference Range as given by the US Department of Health and Human Services (Figure 3). Out of 142 patients, only 126 could answer about their weight and height. The patients who knew only about their height and not weight and vice versa were excluded. BMI index was calculated on the basis of the World Health Organization’s (WHO) recommendations. We observed that patients had significantly higher BMI, with 30% obese and 27% overweight patients. Collectively, this amounts to 57% of total patients. Correlation studies suggested that c-myc and estradiol are affected by BMI by the correlation coefficients of 0.46 and 0.50, respectively. These results are significant at $p < 0.05$, $df = 95\%$, $n = 126$ (chi square = $1.6 \times 10^{-10}$).

![Figure 3. Correlation studies between c-myc, estradiol, and BMI status of breast cancer patients Using Psych package in R software. (A): Positive correlation ($R = 0.46$) exists in c-myc and BMI status. (B): Positive correlation ($R = 0.50$) between estradiol and BMI status (image depicting $R^2$ value is then converted to R correlation coefficient. * Correlation is significant at 0.01 level (two tailed).](image)

3.2. Age Related c-myc Regulation in Breast Cancer Lymphocytes

The relative expression of c-myc in patients was normalized against healthy control. Patients were divided in four distinct age groups (average age at onset of the disease = ±46.6 years): age group 21–35 (Figure 4A), age group 36–45 (Figure 4B), age group 46–50 (Figure 4C), and age group > 60 (Figure 4D). We observed that overall, the mean RE of c-myc is relatively high in patients as compared to healthy control, as shown in Figure 4E ($p$-value < 0.0001). Research analysis provided a clearer view of elevated c-myc RE in an age-related fashion. We observed that after certain age, i.e., age > 60, the c-myc-related expression is less elevated as compared to age groups < 60 years, which could be due to the decreased level of estrogen. We calculated the relative risk from binomial pairwise comparison after performing the Pearson chi-square goodness of fit test, and results showed that the relative risk for age group 36–45 and age group 46–60 was >1, while the relative risk for age groups 21–35 and >60 was <0.143. This is due to the age-specific expression pattern of estrogen. Estradiol level were also significantly elevated in patients with $p$ value < 0.001 as compared to age matched healthy control (Figure 5).
The relative expression of c-myc in patients was normalized against healthy control. Overall expression of estradiol among breast cancer patients (Average RE = 16.31, SD = 7.0, SEM = 1.32) and healthy control (Average RE = 10.99, SD = 1.76, Sqrt of sample size = 5, SEM = 0.07, df = 1, p-value < 0.0001). Research analysis provided a clearer view of elevated c-myc RE in an age-related fashion. We observed that after certain age, i.e., age > 60, the c-myc-related expression pattern of estrogen. Estradiol level was also significantly elevated in patients with p-value < 0.001 as compared to age matched healthy control (Figure 5).

Figure 4. (A–E) Comparison of mean Relative Expression (RE = 2^∆ΔCt) of c-myc in breast cancer patients and healthy controls (RE calculated using Livak method from two-fold Ct values). Error bars indicate standard deviation. Asterisks indicate a significant difference determined using Welch’s t-test (* p > 0.05). (A) RE of age group 21 to 35 years patients n = 13, SEM = 4.1, (Average 2^∆ΔCt = 10.32) and healthy control (Average 2^∆ΔCt = 0.99). n = 23, SD = 0.1, Sqrt of sample size = 4.8, SEM = 0.01, df = 1, p-value = 0.0027. (B) 2^∆ΔCt of age group 36 to 45 years, patients (Average 2^∆ΔCt = 5.81) and healthy control (Average 2^∆ΔCt = 1.95). n = 39, SD = 3.6, Sqrt of sample size = 6.2, SEM = 0.15, df = 1, p-value = 0.01. (C) 2^∆ΔCt of age group 46 to 60 years patients (Average RE = 7.2) and healthy control (Average RE = 0.8). n = 41, SD = 0.48, Sqrt of sample size = 3.46, SEM = 0.01, df = 1, p-value < 0.0001. (D) RE of age group > 60 years. patients (Average RE = 2.49) and healthy control (Average RE = 0.8). n = 25, SD = 1.76, Sqrt of sample size = 5, SEM = 0.07, df = 1, p-value = 0.0134. (E) Overall RE of patients (Average RE = 8.4 n = 116, SD = 14.9, SEM = 0.25) and healthy control (Average RE = 0.99, n = 76, SD = 2.17) df = 1, p-value < 0.0001 (** = p-value 0.001, *** = p-value 0.0001).
3.3. Expressional Level of ESR1 in Leucocytes of Breast Cancer Patients

We study the expression of ESR1 in leucocytes of breast cancer patients (142) and 77 healthy control and normalized it with housekeeping gene by Livak $2^{\Delta\Delta Ct}$ method. Primer sequence used and annealing temperatures are mentioned in Table 2. The mean RE ($2^{\Delta\Delta Ct}$) in patient is higher than in control group. Welch’s $t$-test shows significance of results at ($p$-value < 0.00623) for 142 patients and 77 control samples. We did divide the ESR1 data into different stages of the disease, and on analysis of variance (anova) bases, we find F value (2.8) with significance of Pr(>F) = 0.0237 (Figure 6). Lastly, we compare the RE of both cmyc and ESR1 to find correlation between them and find strong spearman correlation coefficient R = 0.88 (Figure 6).

3.4. Crosstalk between c-myc, ER and Estrogen in Breast Cancer Leucocytes

The protein expression of estradiol from the peripheral blood of the patients gave insight into the correlation of genetic parameters under study with disease progression. It was found that c-myc and estradiol were induced in ER+ve blood samples as compared to ER−ve leucocytes (Figure 7).

3.5. Correlation of c-myc and Estradiol in Breast Cancer Lymphocytes

A correlational study was conducted on twofold Ct values of c-myc and estradiol. The results presented in (Figure 8b) showed a positive correlation between the two with a correlation coefficient of 0.469.
Asterisks represent significance at 0.05 level.

We study the expression of ESR1 in leucocytes of breast cancer patients (142) and 77 healthy control and normalized it with housekeeping gene by Livak \( \Delta\Delta^{Ct} \) method. (A) The mean RE (\( 2^{\Delta\Delta^{Ct}} \)) in patient is higher than in control group. Welch’s \( t \)-test showed the significance of results at (\( p \)-value < 0.00623) for 142 patients and 77 control samples. (B) analysis of variance (anova) \( F \)-value (2.8) with significance of \( Pr(>F) = 0.0237 \). (C) c-myc and ESR1 showed strong spearman correlation coefficient \( R = 0.88 \). Asterisks represent significance at 0.05 level.

We confirm our results on RStudio software and found similar results (\( R = 0.50 \)) with the Psych package (Figure 8B). There was a positive correlation and interdependence between the high expression of estradiol and c-my in breast cancer patients, which validated our studies.
3.5. Correlation of c-myc and Estradiol in Breast Cancer Lymphocytes

For the next step to check our predicted results in relation to stages of the disease, we concluded that the levels of c-myc and estradiol are induced in the metastatic phases of the disease. Stage III involves the nodal status, and Stage IV, where metastases occur in other parts of the body, showed elevated expression of biomarkers (Figure 9).

3.6. Up-Regulation of c-myc & Estradiol at Metastatic Stages

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3.7. Multilinear Regression and Correlation Analysis between Estradiol, c-myc, ER, and Stages of Breast Cancer Patients

Multilinear regression and correlation analysis were performed between estradiol, c-myc, ER, and stages of breast cancer patients. Results in Figure 10 showed that there is a negative correlation between stage and ER status, \((R = -0.54)\) whereas a positive correlation between c-myc and estradiol was observed \((R = 0.50)\). Similarly, a strong positive correlation was observed between ER and estradiol \((R = 0.76)\). Estradiol and c-myc showed a positive correlation with disease stage with \((R = 0.60)\) and \((R = 0.32)\), respectively (Figure 10).

![Figure 10](image.png)

*Figure 10. Multilinear regression and correlation analysis between estradiol, c-myc, ER, and stages of breast cancer. A negative correlation exists between stage and ER status. \((R = -0.54)\) A positive correlation exists among c-myc and estradiol \((R = 0.50)\), and a positive correlation exists among ER and estradiol \((R = 0.76)\). Estradiol is also positively correlated to stage of the disease \((R = 0.60)\), while c-myc showed a weak positive correlation with stage \((R = 0.32)\). This figure produced \(R^2\) value, which was then converted to \(R\) correlation coefficient. * Correlation is significant at 0.05 level (two tailed) ** Correlation is significant at 0.01 level (two tailed).*

3.8. Molecular Docking

The system generated 10 models for docking affinity, with model 1 having the lowest energy. However, docking model 10 showed flexible binding and a greater number of H-bonds involved. Keeping in mind that the lowest energy bonds are best to describe the molecular docking, one of the best fit models, 2, with the lowest binding energy and lowest RMSD values \((1.72\text{Å})\) is presented in (Figure 11).

The bonding affinity between c-myc and estradiol is predicted using HDOCK and CB-DOCK tools \([20,21]\). HDOCK is a free available online server used to perform protein-DNA docking. It is based on the hybrid algorithm of template-based modeling.

While CB-DOCK is used to check direct binding of estradiol to the c-myc protein crystal structure, CB-DOCK is a blind docking server available for free online. It is based on the cavity detection-guided easy approach bioinformatic tool.

3.8.1. Genetic c-myc and Receptor Bound Estradiol

The results confirm the reported molecular docking of both estradiol and c-myc with binding energies \(< -215\text{ kcal/mol}\), LG scores of 5.606, and a MaxSub score \(< 0.8\), as shown in Figure 12A–C. An aligned length of 229 of Chain A of the estradiol bonded ER with \(R = 0.931\) was observed. The identification with sequence id was 95.8\% \((\text{jobid} = 60ce3ba192bf6)\) (Figure 10). With this score, the strong binding could be observed between selected region of c-myc and receptor bound estradiol (Figure 12).
Figure 10. Multilinear regression and correlation analysis between estradiol, c-myc, ER, and stages of breast cancer. A negative correlation exists between stage and ER status. (R = −0.54) A positive correlation exists among c-myc and estradiol (R = 0.50), and a positive correlation exists among ER and estradiol (R = 0.76). Estradiol is also positively correlated to stage of the disease (R = 0.60), while c-myc showed a weak positive correlation with stage (R = 0.32). This figure produced R² value, which was then converted to R correlation coefficient. * Correlation is significant at 0.05 level (two-tailed) ** Correlation is significant at 0.01 level (two-tailed).

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Figure 11. (A) Bioactivity score of 17β-estradiol obtained from molinspiration® by providing smile structure. (B) 3D structure. (C) 2D structure of 17β-estradiol as predicted by molinspiration web server.

Figure 12. (A) Overall result of all models generated by HDOCK server. (B) Binding of ER bound estradiol with c-myc gene 67 kb upstream of the promoter region at lowest energy score of −229 kcal/mol (Model 2 at HDOCK) binding with LG score 5.606 and MaxSub < 0.8. (C) estradiol interacts with c-myc protein results available for job 60 ce3ba192b6.

3.8.2. Root Mean Square Deviation (RMSD)

RMSD is commonly used quantifiable parameter of the similarity amongst two superimposed atomic aligns. We redo the docking only with catalytic domain and checked for the appropriate method to improve the DNA crystal structure. RMDS calculated by the system was <2.0 Å of the ligand, aligned the same docking pose, and double-checked the coverage accuracy (Table 3).
### Table 3. Binding energies and RMSD of Model 2 and 10.

| Model No. | Ligand       | Receptor                          | RMSD  | Binding Energy |
|-----------|--------------|-----------------------------------|-------|----------------|
| Model 1   | 17β-estradiol| C-myc (Crystalized B-DNA) 500 bp around ERE site | <2.0 Å | −227.9 kJ/mol  |
| Model 10  | As above     | As above                          | 1.59 Å | −217 kJ/mol    |

#### 3.8.3. Prospective TF Targets of ESR1 Using Chip-Seq Atlas

For the identification of possible target bindings of the antigen ESR1, we used online available tools (chIP-seq atlas) [22–27]. We generated targets specifically for ESR1 for specifically blood and breast cell types. In the chIP-seq data, we did not find the binding of ESR1 directly to the c-myc on any available distance of ±1 kb, ±5 kb, and ±10 kb regions, which is not inconsistent with our study. Previous studies showed the binding of a complex (ESR1, ESR2, JUND, FOS) at ERE sites of c-myc (Figure 1) [4,28,29]. Similarly, on the other hand, we did find an experimentally determined gene neighborhood, gene fusion cooccurrence, and coregulation among c-myc and ESR1 using a string database version 11.5 https://string-db.org/ accessed on 15 April 2022 [23]. Figure 13 depicts said relationship between two experimentally studied factors. A better approach needs to be made for chIP-seq analysis to seek in-depth analysis of genetic complex binding on ERE sites.

![Figure 13. Interaction of myc, ESR1, and ESR2. Red color is for fusion of genes, green for gene neighborhood, blue for gene cooccurrence, and pink is for experimentally determined interactions.](image)

### 4. Discussion

#### 4.1. Positive Association of c-myc and Estradiol Exists in Peripheral Bc Blood

In the present study, we investigated the role of c-myc, estradiol, and ER in breast cancer patients. We found that c-myc and estradiol are induced among all age groups as compared to the healthy control (Figure 4). We also observed that the age groups more exposed to estrogen production showed induced expressions of both estrogen and c-myc in leukocytes (Figures 4 and 5). BMI is an indicator of the induced expression of estradiol, which in turn induces the c-myc expression in patients [24]. The luteal phase induces estradiol before menopause, and women get more exposed to the risk of breast cancer. Other reasons for the induced expression of c-myc are the binding of more estradiol to estrogen receptors and increased transcription [15].
4.2. c-mycIs an Estrogen-Dependent Gene in Breast Cancer

We observed that higher estrogen levels were correlated with the expression of c-myc (Figures 4 and 5) Similarly, in ER+ve patients, both estradiol and c-myc expression are induced (Figure 7). The multilinear correlational studies in Figure 10 suggest the interdependence of c-myc and estradiol, which needs to be investigated more in further studies.

In BC cells, the c-myc oncogene is thought to be a conventional estrogen-induced gene [25]. Other significant estrogen-regulated genes in breast cancer (e.g., TFF1/pS2) have had their molecular regulation explored, but c-myc is known for its complex mode of regulation [26]. For many years, the exact method was unknown, until a putative estrogen-responsive site, ERE, was discovered in the basal promoter region of the c-myc gene in a previous project [26].

Utilizing a cellular model with inducible AP-1 predominant negative expression, they discovered that c-myc is an estrogen-induced and AP-1-dependent gene. These findings added to our knowledge of the estrogen, i.e., steroid hormone signaling pathway in breast carcinomas, allowing us to dig into the biomolecular processes of estrogen elevation of the c-myc gene expression. Many ER-binding sites are found in distal intergenic regions instead of promoter regions, according to genome-wide analyses (Figure 12). We discovered an estradiol-bound ER cooperating factor that may modify the function of c-myc in a systematic way as distal enhancer.

4.3. Estradiol Affinity as Ligand to the DNA Structure

Previous studies suggest that estrogen induces c-myc gene expression via an upstream enhancer activated by the estrogen receptor (ER) [4] Estradiol is already established woman hormonal medicine used to treatment menopausal symptoms. Estradiol binding to DNA as a ligand molecule bring the idea of molecular docking [26] Many reports suggest that HRTs in longer run develops breast cancer due to continual deposition of estradiol in the body. We suggest an infancy stage method by expressional studies of estradiol and c-myc level in breast cancer patient via RT-PCR along with molecular docking the idea of targeting therapies at molecular level [9,22]. A future inhibitory study of direct targeting of ER bound estradiol at molecular level can help in reduction of estradiol in the body which in return reduces the cause of breast cancer in woman on HRT for long.

4.4. Anticancer Drug Screening Using Docking

Docking results in Figure 12 give support and affirmation to RT-PCR studies of c-myc and estradiol in breast cancer patients [26]. Our finding, together with those of other similar studies as performed by [26–28], suggest that various drugs based on estrogen used for pharmacological treatment mimic naturally existing estradiol. Investigating the DNA-based molecular docking processes of 17β estradiol and ER bound 17β estradiol would help in understanding the therapeutic efficacy of various inhibitory drugs in the case of breast cancer and enhancers in case of HRT treatments. As a result, it will offer new insight into anticancer drug screening.

ScRNA-Seq Data and Molecular Docking

Estrogen receptor ESR1 and its allies belong to the nuclear receptor family. Reactome Pathways shows the sharing of transcription regulation pathway with c-myc by binding to a half ERE site with the help of AP1, SP1, and junD. Jin Li et al. analyzed a single-cell RNA seq of a nuclear receptor family and its behavior as a ligand molecular in triple negative breast cancer [29].

5. Conclusions

Taken together, our data suggest that c-myc and estradiol are induced in an age-related fashion in breast cancer patients. Strong correlations exist, which suggests that both estradiol and c-myc are cooperative and dependent on each other with the help of an estrogen receptor’s presence.
Our work suggests that estradiol binds to somewhere around 5262 bp (selected region after defining a promotor for docking) away from the promotor along with complex protein structure and induces the expression of c-myc in the leukocytes of breast cancer patients. By identifying the structural and functional affinity of estradiol, the model in this study elucidates that estradiol behaves like an enhancer ligand for c-myc and exhibits many key structural points to be considered in future therapeutic studies for breast cancer.

A study to identify the measures to reduce the expression of estradiol and c-myc and control their interdependence could possibly reduce the chances of metastasis and disease prognosis. To establish the strong dependency between the trio, further investigation is necessary.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app12146853/s1, Table S1: Clinico-pathological Parameters; Table S2: Genetic Expression Parameters and calculations.

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Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| BC | Breast cancer |
| c-myc | Homo sapiens MYC proto-oncogene |
| ER/ERα/Erβ | Estrogen Receptor/estrogen receptor alpha/estrogen receptor beta |
| HER2/neu | human epidermal growth factor receptor 2 |
| E-boxes | Enhancer boxes |
| HATs | histone acetyltransferases |
| CpG islands | CpG islands are short stretches of DNA with an unusually high content and a higher frequency of CpG dinucleotides. |
| GC | Estrogen response elements |
| EREs | ACTIVATOR PROTEIN 1 |
| AP-1 | specific protein |
| CDKs | Cyclin-dependent kinases |
| NRs | nuclear transcriptional activators |
| p21 | CDK-interacting protein 1 |
| ERP | exported repetitive protein |
| TRRAP | Transformation/Transcription Domain Associated Protein |
| AF-1/2 | Activation function 1/2 |
| N.O.R.I | Nuclear Medicine Oncology and Radiotherapy Institute |
| EDTA | Ethylenediamine tetraacetic acid |
| mRNA | messenger ribonucleic acid |
| HIV | human immunodeficiency virus |
| HCV | hepatitis C virus |
RT-PCR Reverse transcription polymerase chain reaction
Ct cycle threshold
SEM standard error of mean
SD standard deviation
DNA deoxyribonucleic acid
bp base pair
ANOVA Analysis of Variance
WHO world health organization
BMI body mass index
N number of samples
df degree of freedom
RE relative expression
ME Mean expression
LG score Levitt-Gerstein score
Max Sub score maximum subarray score
kcal/mol kilo calorie per mole

References
1. Hua, H.; Zhang, H.; Kong, Q.; Jiang, Y. Mechanisms for estrogen receptor expression in human cancer. *Exp. Hematol. Oncol.* 2018, 7, 24. [CrossRef] [PubMed]
2. Key, T.J.; Appleby, P.N.; Reeves, G.K.; Roddam, A.W.; Helzlsouer, K.J.; Alberg, A.J.; Strickler, H.D. Endogenous Hormones and Breast Cancer Collaborative Group. Circulating sex hormones and breast cancer risk factors in postmenopausal women: Reanalysis of 13 studies. *Br. J. Cancer* 2011, 105, 709–722.
3. Faheem, M.; Mahmood, H.; Khurram, M.; Qasim, U.; Irfán, J. Estrogen receptor, progesterone receptor, and Her 2 Neu positivity and its association with tumour characteristics and menopausal status in a breast cancer cohort from northern Pakistan. *Eancermedicalscience* 2012, 6, 283. [PubMed]
4. McEwan, M.V.; Eccles, M.R.; Horsfield, J.A. Cohesin is required for activation of MYC by estradiol. *PLoS ONE* 2012, 7, e49160. [CrossRef]
5. Zeller, K.I.; Zhao, X.; Lee, C.W.; Chiu, K.P.; Yao, F.; Yustein, J.T.; Ooi, H.S.; Orlov, Y.L.; Shahab, A.; Yong, H.C.; et al. Global mapping of c-myc binding sites and target gene networks in human B cells. *Proc. Natl. Acad. Sci. USA* 2006, 103, 17834–17839. [CrossRef] [PubMed]
6. Carroll, J.S.; Liu, X.S.; Brodsky, A.S.; Li, W.; Meyer, C.A.; Szary, A.J.; Eeckhoute, J.; Shao, W.; Hestermann, E.V.; Geistlinger, T.R.; et al. Chromosome-Wide Mapping of Estrogen Receptor Binding Reveals Long-Range Regulation Requiring the Forkhead Protein FoxA1. *Cell* 2005, 122, 33–43. [CrossRef] [PubMed]
7. Prall, O.W.J.; Rogan, E.M.; Musgrove, E.A.; Watts, C.K.W.; Sutherland, R.L. C-myc or Cyclin D1 Mimics Estrogen Effects on Cyclin E-Cdk2 Activation and Cell Cycle Reentry. *Mol. Cell. Biol.* 1998, 18, 4499–4508. [CrossRef]
8. Cheng, A.S.L.; Jin, V.X.; Fan, M.; Smith, L.T.; Liyanarachchi, S.; Yan, P.S.; Leu, Y.-W.; Chan, M.W.Y.; Plass, C.; Nephew, K.P.; et al. Combinatorial Analysis of Transcription Factor Partners Reveals Recruitment of c-myc to Estrogen Receptor-α Responsive Promoters. *Mol. Cell* 2006, 21, 393–404. [CrossRef]
9. Clusan, L.; Le Goff, P.; Flouriot, G.; Pakdel, F. A Closer Look at Estrogen Receptor Mutations in Breast Cancer and Their Implications for Estrogen and Antiestrogen Responses. *Int. J. Mol. Sci.* 2021, 22, 756. [CrossRef]
10. Viedma-Rodriguez, R.; Baiza-Gutman, L.; Salamanca-Gómez, F.; Diaz-Zaragoza, M.; Martínez-Hernández, G.; Espanza-Garrido, R.R.; Velázquez-Flores, M.A.; Arenas-Aranda, D. Mechanisms associated with resistance to tamoxifen in estrogen receptor-positive breast cancer (Review). *Onco. Rep.* 2014, 32, 3–15. [CrossRef]
11. Mukherjee, S.; Conrad, S.E. c-myc Suppresses p21WAF1/CIP1 Expression during Estrogen Signaling and Antiestrogen Resistance in Human Breast Cancer Cells. *J. Biol. Chem.* 2005, 280, 17617–17625. [CrossRef] [PubMed]
12. Dubik, D.; Shiu, R.P. Transcriptional regulation of c-myc oncogene expression by estrogen in hormone-responsive human breast cancer cells. *J. Biol. Chem.* 1998, 263, 12705–12708. [CrossRef]
13. Oloomi, M.; Moazzezy, N.; Bouzari, S. Comparing blood versus tissue-based biomarkers expression in breast cancer patients. *Heliyon* 2020, 6, e03728. [CrossRef] [PubMed]
14. Kulkoyluoglu-Cotul, E.; Arca, A.; Madak-Erdogan, Z. Crosstalk between Estrogen Signaling and Breast Cancer Metabolism. *Trends Endocrinol. Metab.* 2019, 30, 25–38. [CrossRef]
15. Wang, C.; Mayer, J.A.; Mazumdar, A.; Fertuck, K.; Kim, H.; Brown, M.; Brown, P.H. Estrogen Induces c-myc Gene Expression via an Upstream Enhancer Activated by the Estrogen Receptor and the AP-1 Transcription Factor. *Mol. Endocrinol.* 2011, 25, 1527–1538. [CrossRef]
16. Knudsen, S. Promoter 2.0: For the recognition of PolII promoter sequences. *Bioinform. Oxf. Engl.* 1999, 15, 356–361. [CrossRef]
17. Solovyev, V.V.; Shahmuradov, I.A.; Salamov, A.A. Identification of Promoter Regions and Regulatory Sites. *In Computational Biology of Transcription Factor Binding; Humana Press: Totowa, NJ, USA, 2010; pp. 57–83.*
18. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2$-\Delta\Delta$CT method. *Methods* 2001, 25, 402–408. [CrossRef]
19. RStudio Team. RStudio: Integrated Development for R. RStudio, PBC, Boston, MA, USA. Available online: http://www.rstudio.com/ (accessed on 30 March 2020).
20. Yan, Y.; Tao, H.; He, J.; Huang, S.Y. The HDOCK server for integrated protein–protein docking. *Nat. Protoc.* 2020, 15, 1829–1852. [CrossRef]
21. Liu, Y.; Grimm, M.; Dai, W.T.; Hou, M.C.; Xiao, Z.X.; Cao, Y. CB-Dock: A web server for cavity detection-guided protein–ligand blind docking. *Acta Pharmacol. Sin.* 2020, 41, 138–144. [CrossRef]
22. Data, R.E.; Williams, S.B.; Roberts, D.D.; Gralnick, H.R. Platelets adhere to sulfatides by von Willebrand factor dependent and independent mechanisms. *Thromb. Haemost.* 1991, 5, 581–587. [CrossRef]
23. Szklarczyk, D.; Gable, A.L.; Nastou, K.C.; Lyon, D.; Kirsch, R.; Pyysalo, S.; Doncheva, N.T.; Legey, M.; Fang, T.; Bork, P.; et al. The STRING database in 2021: Customizable protein–protein networks, and 581 functional characterization of user-uploaded gene/measurement sets. *Nucleic Acids Res.* 2021, 49, 10800. [CrossRef] [PubMed]
24. Benz, C.C. Impact of aging on the biology of breast cancer. *Crit. Rev. Oncol. Hematol.* 2008, 66, 65–74. [CrossRef] [PubMed]
25. Shang, Y.; Brown, M. Molecular determinants for the tissue specificity of SERMs. *Science* 2002, 295, 2465–2468. [CrossRef] [PubMed]
26. Wierstra, I.; Alves, J. The c-myc promoter: Still MysterY and challenge. *Adv. Cancer Res.* 2008, 99, 113–333. [PubMed]
27. Gilad, Y.; Senderowitz, H. Docking studies on DNA intercalators. *J. Chem. Inf. Model.* 2014, 54, 96–107. [CrossRef]
28. Aldakheel, F.M.; Alduraywish, S.A.; Mateen, A.; Alqahtani, M.S.; Syed, R. Molecular and docking studies of tetramethoxy hydroxyflavone compound from Artemisia absinthium against carcinogens found in cigarette smoke. *Open Chem.* 2021, 19, 1148–1154. [CrossRef]
29. Jin, L.; Li, Y. Structural and functional insights into nuclear receptor signaling. *Adv. Drug Deliv. Rev.* 2010, 62, 1218–1226. [CrossRef]