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# Perturbation of specific transcripts in peripheral blood mononuclear cells in breast cancer: A case-control pilot study

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## Abstract

**Introduction:** Breast cancer (BC) is the most common type of diagnosed cancer worldwide and the leading cause of cancer death in women and it is the second most frequent cancer-causing mortality for women worldwide. Peripheral blood-based biopsy for BC could be a promising tool for risk prediction and diagnosis. In this study, we aimed to evaluate the gene expression profile of PBMCs in Italian patients with BC.

**Methods:** In this case-control pilot study, we isolated PBMCs from 22 BC patients and 21 healthy controls and evaluated the expression of a panel of 52 target genes related to BC or circadian rhythm by a customized TaqMan Open Array Real-Time PCR panel.

**Results:** Among the differentially expressed genes, 22 remained unchanged. These unchanged genes are mainly involved in cellular processes, including the circadian clock, cellular responses to stress/stimuli, the immune system, signal transduction, and metabolism. We found a total of 30 significantly de-regulated genes. In particular, 8 genes, including PARP6, IGFR1, EZH2, VEGFA, NOTCH1, CD44, BCAR1, and CD24A, resulted significantly down-regulated in patients with BC compared to Controls, while 22 genes were significantly up-regulated in BCs patients compared to Controls. We found several already reported BC-associated genes up-regulated in PBMCs of our BC patients, but FOXO3, ARNTL, and ADAM17 emerged as the most strongly up-regulated. The enrichment pathways analysis highlight that de-regulated genes are mainly involved in the regulation of gene expression and transcription, signal transduction, and immune system response.

**Discussion:** The results of our pilot study demonstrated that the evaluation of PBMC gene signature could be a valuable tool for primary prevention and early diagnosis of BC in several high-risk settings, thus reducing the global mortality associated with this tumour.

**Take-home message:** Non-invasive screening programs, particularly those adopted in workplaces, may have a great impact on early diagnosis and good prognosis for BC. Our study provided proof of concept that the development of a screening test based on PBMC-derived gene expression biomarkers could be a viable route.

**Keywords:** Breast cancer; circadian rhythm; diagnosis; gene expression; PBMCs.

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## INTRODUCTION

Breast cancer (BC) is the second most common cause of death from cancer among women and the most common cancer in the world [1]. In addition, it is the most common cancer diagnosed in women, accounting for more than 1 in 10 new cancer diagnoses each year. The number of deaths in 2020, for both genders and all ages, was 6.9% of more or less 10 million cancer death worldwide, while the number of new cases in 2020 was 11.7% of more or less 20 million new cancer cases [2]. BC can also affect men (approximately 1%) even if it is much rarer than in women [3].

BC development is the result of a complex interplay between multiple risk factors. Among them, genetic mutations and family history account for 5–10% of BC cases, while the other cases are more commonly associated with lifestyle factors (e.g. obesity, sedentary habits, smoking, alcohol consumption), hormonal factors (e.g. early menarche, late menopause, and hormone replacement therapy), pregnancy-associated factors (e.g. fewer number of children and less breastfeeding), environmental factors, social psychological factors and ultimately exposure to adverse childhood experiences (e.g. child abuse and/or neglect, depression, and stressors) [4,5]. The most common cause of hereditary BC is germline pathogenic variants in BReast-CAnCER susceptibility gene 1 (BRCA1) or BReast-CAnCER susceptibility gene 2 (BRCA2) [6,7]. However, most women who develop BC have no family history of the disease, thus suggesting a crucial role of environmental factors in the development and progression of the disease [8]. Several lines of evidence highlighted that exposure to a wide variety of toxicants, most of them contained in everyday products and by-products, and also exposure to carcinogens during working life, was associated with BC development [8].

Occupational exposure to ionizing radiation is the most well-studied factor that can increase the risk of BC [9,10]. Moreover, mounting evidence suggests that night exposure to light and the production of stress-related mediators can contribute to BC risk, even though no direct association has been established between rotating shift work and the risk of disease [11]. In 2007, the International Agency for Cancer Research (IARC) Monograph working group listed shift work that involves circadian disruption as a class 2a or probable carcinogen [12]. Indeed, epidemiological studies, evaluating shift workers and people exposed to chronic jet lag, support the hypothesis of a major role in disrupting circadian rhythms in BC pathogenesis [13]. Another study highlights the correlation between male BC and exposure to organic solvents [14].

BC diagnosis depends on clinical examination and imaging techniques, including mammography, magnetic resonance imaging, and ultrasound. Still, confirmatory diagnosis requires histological examination of fine-needle aspiration cytology and core needle biopsy [15-17]. However, all these approaches are time-consuming and not applicable for the early detection of BC, which is crucial to ensure successful therapy and patient survival. Otherwise, several lines of evidence showed that peripheral blood-based biopsy for BC risk prediction and diagnosis could be a more accessible and less invasive tool [18].

In this research, we aimed to evaluate the possible perturbations of the expression of a panel of BC-related or circadian rhythm-associated genes in peripheral blood mononuclear cells (PBMCs) from patients with BC compared to healthy controls.

## **METHODS**

### ***Study design and procedure***

In this case-control pilot study, we enrolled 25 Italian females with BC and 25 without BC. The exclusion criteria for both groups were (a) a family history of BC; (b) a self-reported history of shift work; (c) previous exposition to ionizing radiation; (d) a history of other tumours; and (e) free for chronic diseases (i.e., type two diabetes or autoimmune diseases); (f) free of any therapy for at least 2 months. In addition, at the screening and sample collection, 3 patients with BC and 4 subjects without BC were excluded from the study for the following reasons: declined to participate (3), no sufficient sample to perform the analysis (2), and the physician's decision (2). Finally, 22 patients with BC and 21 healthy controls were included in the study.

### ***Study participants and sampling***

The BC patients were enrolled at the "Pietro Valdoni" Department of Surgery (La Sapienza University, Rome, Italy). In contrast, the healthy controls were enrolled at the Occupational Medicine Unit, Bambino Gesù Children's Hospital, IRCCS (Rome, Italy). PBMC were isolated by density-gradient centrifugation at  $600 \times g$  for 20 min at room temperature using Ficoll-Paque (Amersham Pharmacia Biotech, Amersham, UK). Next, the layer that contained PBMCs was removed, washed two times with phosphate-buffered saline, and stored at  $-80^{\circ}\text{C}$  until used for RNA extraction. Total RNA was isolated from the cells using the total RNA purification plus kit (Norgen Biotek Corp, Thorold, ON, Canada). Finally, genomic DNA was digested by using genomic DNA removal (Norgen Biotek Corp, Thorold, ON, Canada).

### ***Study instruments***

Two micrograms of total RNA were reverse transcribed using SuperScript™ VILO™ cDNA Synthesis Kit (Thermo Fisher Scientific-Invitrogen, Waltham, MA, USA) with random hexamer primers. All cDNA was used to analyze a customized panel of 52 genes previously associated with BC and circadian rhythms, and 4 housekeeping genes by a TaqMan Open Array Real-Time PCR platform (Thermo Fisher Scientific-Applied Biosystems) as already described and reported in Figure 1 [19]. Each sample was evaluated in quadruplicate. The relative quantity (RQ) of gene expression values was calculated using Thermo Fisher Cloud Resources. RQ of each gene in the different samples was normalized against their respective GAPDH, and the fold induction was calculated against the mRNA levels in the respective controls. RQ minimum and maximum values were calculated with a confidence level of 95%, using Benjamini-Hochberg's false discovery rate to adjust P values. The Maximum allowed Ct included in calculations is 40 and Cq confidence > 0.9.

Multivariate Student's t-tests were applied and values of  $P < 0.05$  were considered statistically significant.

**Data analysis**

Data were analyzed by unpaired Student's t-test using GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA). All values are presented as the mean  $\pm$  SD. Differences were considered significant at  $p \leq 0.05$ .

**Ethical aspects**

The study has received ethical approval from the Ethics Committee of the Bambino Gesù Children's Hospital (protocol code: 1670\_OPBG\_2018, approved on February 11, 2019), and all patients signed a written informed consent form.

A1	1	2	3	4	5	6	7	8
a	Hs99999901_s1 18S	Hs99999903_m1 ACTB	Hs01561483_m1 ABCC1	Hs00988721_m1 ABCC4	Hs01041915_m1 ADAM17	Hs01007998_m1 ALDH2	Hs00154147_m1 ARNTL	Hs01582072_m1 AURKA
b	Hs01547079_m1 BCAR1	Hs00375126_m1 BCAS3	Hs00215240_m1 BCAS4	Hs00153353_m1 BIRC5	Hs01556193_m1 BRCA1	Hs00609073_m1 BRCA2	Hs04405695_m1 CD24	Hs01075864_m1 CD44
c	Hs00231857_m1 CLOCK	Hs00172734_m1 CRY1	Hs00901993_m1 CRY2	Hs00266431_m1 CSNK1E	Hs01076090_m1 EGFR	Hs01082166_m1 EMSY	Hs01001580_m1 ERBB2	Hs01046816_m1 ESR1
d	Hs00544830_m1 EZH2	Hs00417598_m1 IGFR1	Hs00818121_m1 FOXO3	Hs99999905_m1 GAPDH	Hs00231032_m1 HDAC2	Hs00153153_m1 HIF1A	Hs02800695_m1 HPRT1	Hs00174029_m1 KIT
e	Hs01091188_m1 KLHL1	Hs00364282_m1 KRAS	Hs00907398_m1 MCM4	Hs00234508_m1 MTOR	Hs00232074_m1 MYCN	Hs01062014_m1 NOTCH1	Hs00231212_m1 NPAS2	Hs00220252_m1 PARP6
f	Hs01579625_m1 PDE4D	Hs00242988_m1 PER1	Hs01007553_m1 PER2	Hs00213466_m1 PER3	Hs00907957_m1 PIK3CA	Hs02621230_s1 PTEN	Hs01056457_m1 PTK2	Hs04189704_m1 PTPRC
g			Hs00962580_m1 SATB1	Hs01086966_m1 TIMELESS	Hs01032137_m1 TOP2A	Hs01034249_m1 TP53		
h			Hs00361186_m1 TWIST1	Hs00900055_m1 VEGFA	Hs00983720_m1 WHSC1	Hs00256555_m1 WHSC1L1		

**Figure 1.** Gene expression panel analyzed by using Open Array real-time PCR. Arrangement of the array panel and TaqMan assays used for each gene spot repeated in quadruplicate.

**RESULTS**

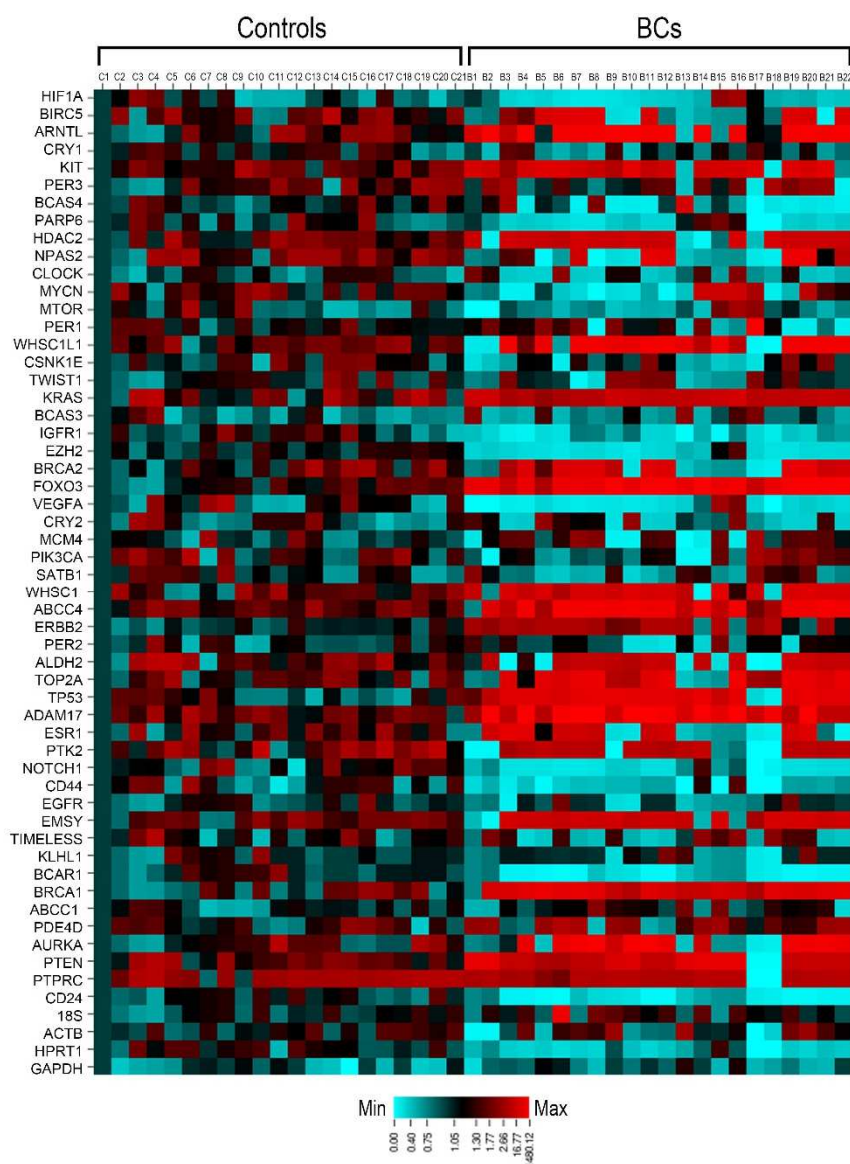
**Gene expression analysis in PBMCs of BCs and Controls**

Here, we evaluated the gene expression in PBMCs isolated from 21 control subjects (mean age 50.76 $\pm$ 9.98 years) and 22 patients with a diagnosis of BCs of which characteristics are reported in Table 1. Among the 22 histopathologically confirmed cases of BC, 16 patients (73%) had a diagnosis of ductal cancer while the remaining 6 (27%) had a lobular cancer type.

**Table 1.** Characteristics of patients with BC.

	Patients/tumor characteristics (N=22)
Age (mean, years)	61.33
Lobular	6
Ductal	16

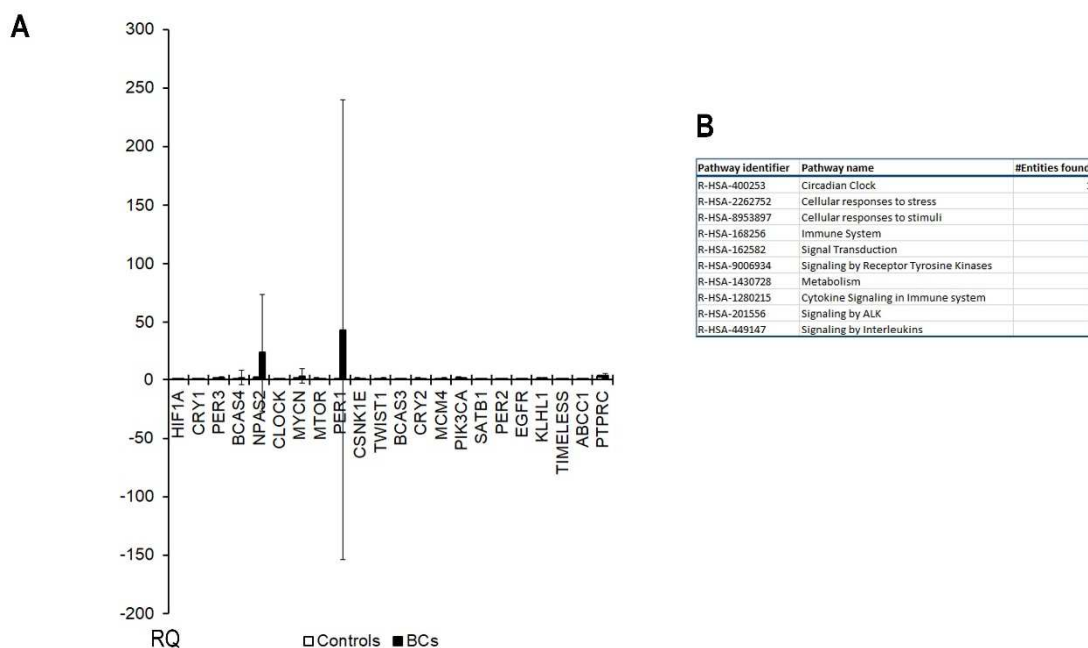
To perform gene expression analysis, we customized a panel containing probes for 52 target genes, of which ten are involved in the regulation of circadian rhythm (i.e., ARNTL, CLOCK, CRY1, CRY2, MCM4, NPAS2, PER1, PER2, PER3, TIMELESS). In contrast, the other ones are BC-associated genes. As shown by the heatmap of the mean RQ expression for each gene, we observed differential levels of gene expression among the 52 transcripts in the patients belonging to the two groups, BCs and Controls (Figure 2).



**Figure 2.** Gene expression profiling in patients with BCs vs healthy controls.

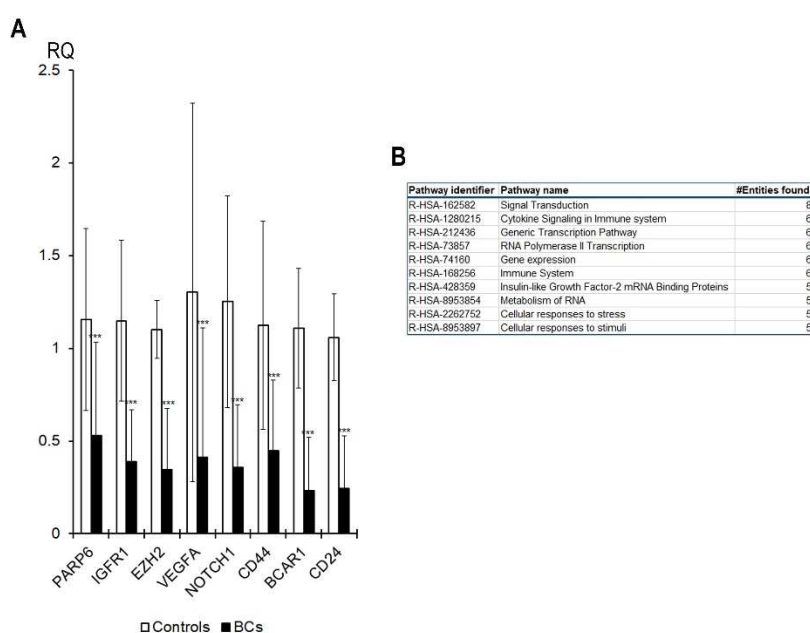
RQ for each gene analyzed by using Open Array real-time PCR reported as a heatmap. Single rows represent gene expression of target genes in a single patient (column). RQ for gene expression was normalized on the expression of the GAPDH gene and used as a reference sample for the control C1.

Interestingly, among the differentially expressed genes, 22 genes remained unchanged (Figure 3A). Furthermore, the pathways enrichment analysis by Reactome (<https://reactome.org/>) showed that these unchanged genes are mainly involved in cellular processes, including a circadian clock, cellular responses to stress/stimuli, the immune system, signal transduction, and metabolism (Figure 3B).



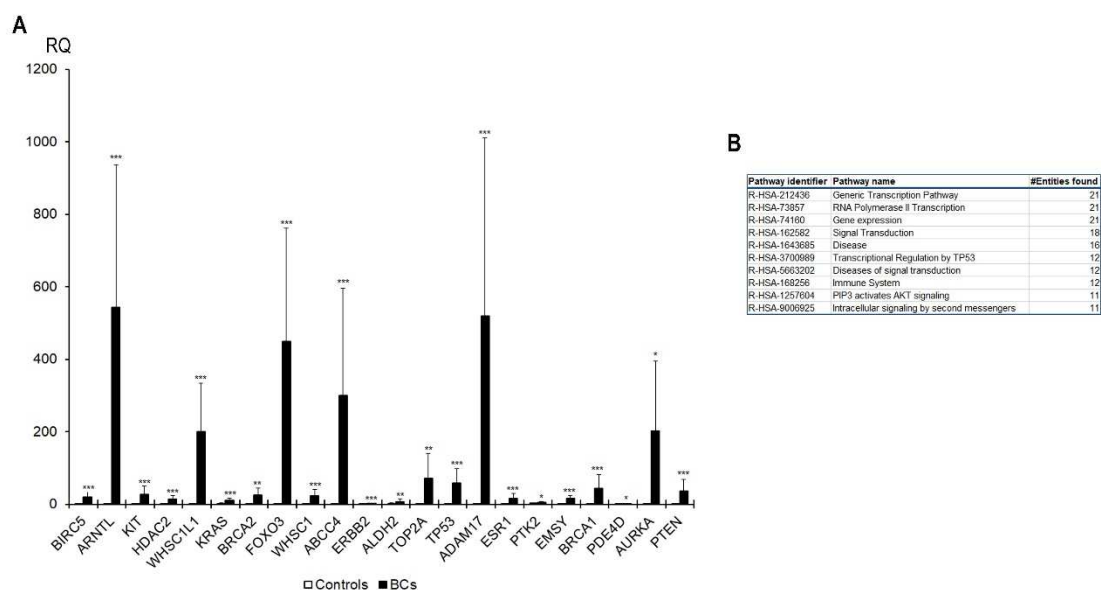
**Figure 3. Analysis of unchanged genes in PBMCs from patients with BC and healthy individuals. A) mRNA quantification of non-significantly de-regulated genes in PBMCs of BC patients and Controls. B) Classification of top ten significant enrichment pathways from Reactome of unchanged genes.**

Otherwise, we found 30 significantly de-regulated genes in BC patients compared to Controls. As reported in Figure 4A, 8 genes, including PARP6, IGFR1, EZH2, VEGFA, NOTCH1, CD44, BCAR1, and CD24A, resulted in significantly down-regulated in patients with BC compared to Controls. The pathways enrichment analysis by Reactome highlighted that these genes are involved mainly in the regulation of signal transduction, gene expression and transcription, immune system regulation, and cell response to different factors (Figure 4B).



**Figure 4.** Analysis of down-regulated genes in PBMCs from healthy individuals and patients with BC. **A)** mRNA quantification of down-regulated genes in PBMCs of Controls and BC patients. **B)** Classification of top ten significant enrichment pathways from Reactome of unchanged genes. \*\*\*p<0.001.

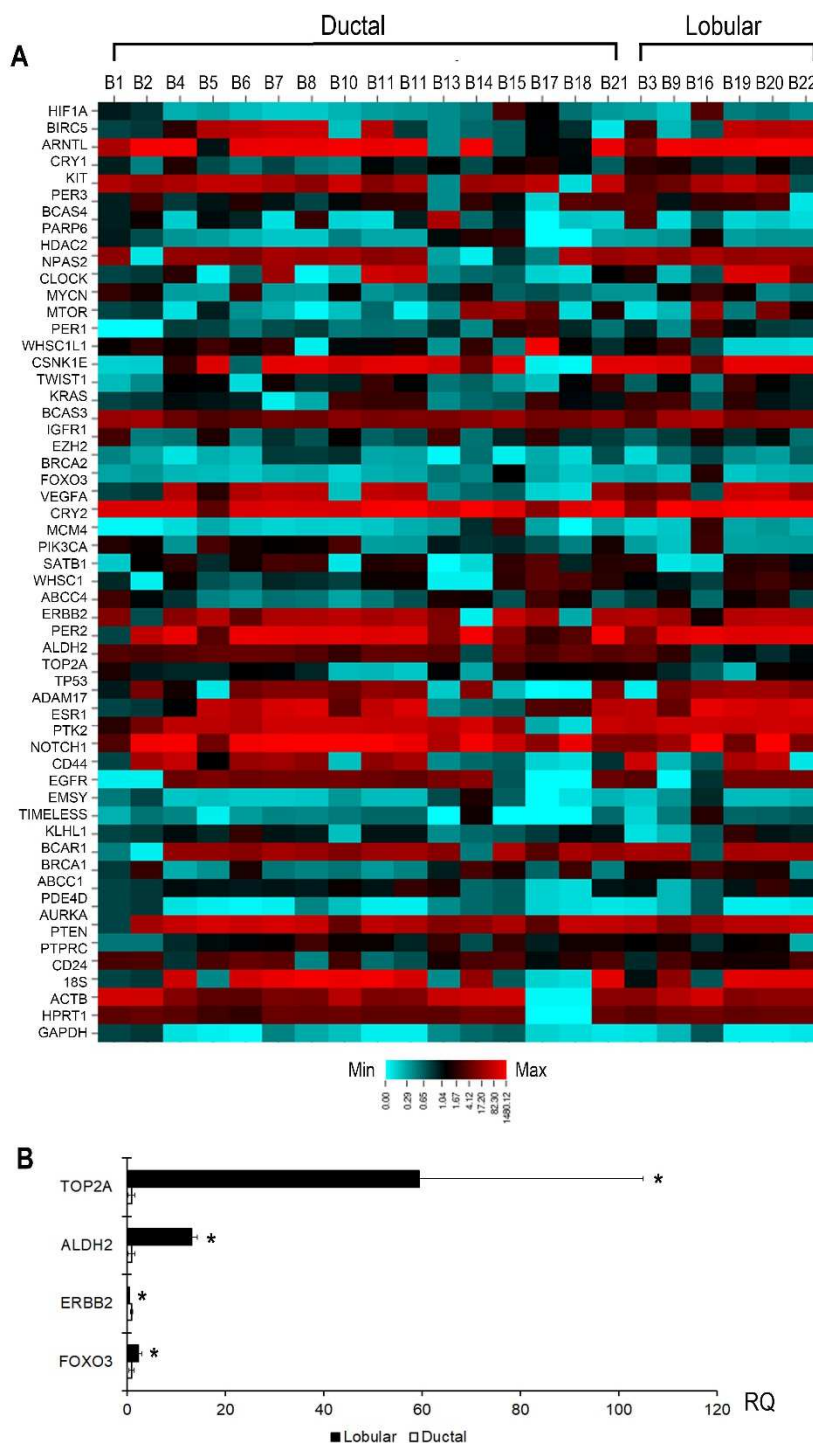
We also found that 22 genes were significantly up-regulated in BCs patients compared to Controls (Figure 5A). The pathways enrichment analysis by Reactome highlighted that these genes are mainly involved in the regulation of gene expression and transcription, while the other ones are involved in the regulation of intracellular signal transduction and immune system response (Figure 5B).



**Figure 5.** Gene expression analysis in PBMCs from healthy individuals and patients with BC. **A)** mRNA quantification of up-regulated genes in PBMCs of BC patients and Controls. **B)** Classification of top ten significant enrichment pathways from Reactome of unchanged genes. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001.

**Gene expression analysis in BCs comparing ductal and lobular carcinoma pattern**

For a better interpretation of the results obtained in BC patients, the gene expression data was divided into two subgroups: patients diagnosed with invasive ductal carcinoma and those diagnosed with lobular carcinoma. As shown in Figure 6A, the heatmap representation of RQ of gene transcripts showed a different pattern of expression for several genes. However, the statistical analysis among the two groups revealed that only 4 genes are significantly de-regulated. In particular, the TOP2A, ALDH2, and FOXO3 genes were significantly up-regulated in lobular respect to the ductal type of BC. At the same time, only ERBB2 was down-regulated in lobular respect to the ductal type of BC (Figure 6B).



**Figure 3.** Gene expression analysis in PBMCs comparing ductal and lobular BCs. **A)** RQ for each gene and patient reported as a heatmap. Single rows represent gene expression of target genes in a single patient (column). RQ for gene expression was normalized on the expression of the GAPDH gene and used as a reference sample for the control C1. Arrangement of the array panel and TaqMan probes used for each gene spot repeated in quadruplicate. **B)** RQ quantification of significantly downregulated genes in PBMCs of lobular BCs compared to ductal BCs. \*  $p < 0.05$ .



## DISCUSSION

BC is the most common type of diagnosed cancer worldwide and the leading cause of cancer death in women [20]. A recent study published by a group of IARC researchers estimated that in 2020 BC accounted for 1 in 8 cancer diagnoses worldwide, with approximately 2.3 million new cases and about 685 000 deaths globally [21]. Based on these numbers, the authors, in making future forecasts on the progress of the disease, estimated that in 2040 new cases per year will increase by approximately 40%, while deaths will increase by about 50%. The study also confirmed that women aged 50 and over exhibited the highest incidence of all new cases (70%), and 81% of all deaths fell into this age group. Moreover, it has been highlighted that ethnicity and geographical differences may affect the rate of both incidence and mortality.

A lot of evidence suggests that shift work contributes to BC risk. The potential primary culprit for this association is the lack of melatonin, a cancer-protective agent whose production is severely diminished in people exposed to light at night [22]. In addition, the production of stress-related mediators also alters regular sleep-wake cycles [23,24]. An additional proven occupational risk factor for BC is radiation. Indeed, a recent Italian study investigated a broad range of aspects of radiation management among a national cohort of cardiologists suggesting the need for specific training programs to reduce radiation exposure [25]. In addition, there is a considerable literature evidence addressing the issue where it is established that ionizing radiation exposure may lead to BC development [26-28].

All these lines of evidence emphasize as primary prevention, and early diagnosis are essential tools to reduce global mortality. In this scenario, screening programs, particularly those adopted in workplaces, may greatly impact early diagnosis and good prognosis.

Previous studies have supported the value of identifying specific gene signatures by transcriptome analysis on peripheral blood cells to assess early diagnosis in different tumors, including BC [29-31]. Indeed, Hensler et al. [29] analyzed the gene expression profiling of 147 PBMC samples from BC patients using an expression panel of BC-associated genes, revealing a differentially expressed pattern in PBMCs according to tumour grade and the presence of metastasis. Moreover, it has been demonstrated that differences in PBMC gene expression allowed us to discriminate between patients with BC and Controls, but also to distinguish among different BCs subtypes by identifying a specific immune-related gene signature [30]. Accordingly, a recent interesting study reinforced the immunosuppressive role of PBMCs in BC patients, thus highlighting that gene signature in PBMCs of patients may reflect their inability to trigger an immune response against tumours [31].

In the present work, we compared the PBMCs transcriptome of BC patients with healthy control subjects, to identify a specific non-invasive gene expression pattern that could be associated with the diagnosis of BC in female subjects with no familiarity and whose working conditions are devoid of risk factors such as shift workers and radiation exposure. We identified several genes that were down-regulated in BCs compared to control subjects. In particular, we found reduced expression levels of PARP6, a new member of the PARP family. Still, poorly investigated in BC but found down-regulated in other types of cancers, where it suppresses cancer proliferation and metastasis [32]. As expected, the tumour suppressor gene BRCA1 was down-regulated [33]. We also observed a CD44/C24 down-regulation that, according to a previous study, is a pattern frequently found in BC [34]. Furthermore, Hensler et al. found that besides these two markers also, VEGFA was down-regulated in PBMCs from patients with BC, data confirmed in our patients [29]. Also, the EZH2 gene was down-regulated in our BC group, even though it was found overexpressed in different malignant tumours, including BC [35]. However, the biological functions of EZH2 in different kinds of tumour cells are still to be determined, in fact, in myeloid malignancies has been suggested that this gene may exert a dual function acting as a tumour suppressor gene or as an oncogene [36]. Finally, regarding NOTCH1 down-regulation found in our BC group, even if this factor plays a pro-tumorigenic role, there is growing evidence suggesting that it may also act as a potent tumor suppressor in both solid tumours and haematological malignancies [37]. In the same way, IGF1R down-regulation could be mainly related to its role in determining cell lineage in BC [38].

Noteworthy, we found several up-regulated genes in PBMCs of our BC patients, most of which are already known to be BC-associated genes, including BRCA1, BRCA2, BIRC5, ERBB2, ESR1, KRAS, KIT, WHSC1L1, WHSC1 ABCC4, ALDH2, TOP2A, TP53, PTK2, EMSY, PDE4D, AURKA, and PTEN, that are crucial for both prognosis and response to therapy [39,40]. However, 3 genes, ADAM17, ARNTL, and FOXO3, emerged as strongly up-regulated in BC patients compared to control subjects. The up-regulation of ADAM17 and FOXO3 genes in PBMCs is in agreement with previous studies demonstrating that the tissue expression of their encoded proteins is significantly high in patients with shorter overall survival [41,42]. Regarding instead the ARNTL gene, which encodes for a well-known important player in the control of circadian rhythms called BMAIL1, we found that it is the only circadian gene up-regulated in PBMCs from BC patients compared to healthy controls, even if our patients have never experienced shift work. This result could be in line with a non-canonical function of BMAIL1 recently reported in BC [43].

In addition, in our analysis, we found only 4 genes differentially expressed in BC (i.e., FOXO3, ALDH2, TOP2A, and ERBB2) compared the pattern of ductal or lobular localization of the tumour. The transcription factor FOXO3 was previously described as a relevant protein in the progression of BC, where its low tissue levels were correlated with poor overall survival [44]. Accordingly, we found a lower gene expression of FOXO3 in PBMCs from ductal BC than in lobular BC. Over-expression of the ALDH2 gene, encoding for an aldehyde dehydrogenase, was often reported as a cancer stem cell marker, and it was negatively correlated with the relapse-free survival of patients with BC. Our data demonstrated an up-regulation of this gene in PBMCs from patients with lobular BC [45].

Interestingly, ERBB2, involved in BC progression and metastasis, was up-regulated in PBMCs from patients with ductal BC, as already reported [46]. Moreover, the TOP2A gene, encoding for topoisomerase II alpha enzyme and located near the ERBB2 locus, was up-regulated in PBMCs from the lobular type of BCs. This data contrasts with a previous study reporting that high TOP2A and ERBB2 expression and tumour size remain the only independent parameters for predicting poor survival, thus suggesting a more aggressive tumour phenotype [47].

Despite our findings adding new information on the possible application of specific gene expression signatures in PBMCs as a viable tool for diagnosing BC, some other questions remain open due to the limitations of this study. Indeed, BC displays tremendous heterogeneity among different patients due at least partly to varying molecular alterations and divergent cells of origin. Therefore the small size of the study population and the lack of information about staging, tumour size, and metastasis limit the strength of the present study. Moreover, another limitation of the present study is the failure to consider the environmental and historical factors of the subjects examined.

Nonetheless, our data open a scenario on the importance of evaluating these gene expression profiles on a population of shift and radio-exposed workers potentially subject to high-risk BC.

## CONCLUSION

In conclusion, once validated in large population studies, the transcriptional targets identified in this pilot study may help define a panel of potentially useful molecular descriptors for early non-invasive diagnosis of BC. Furthermore, these potential biomarkers could be adaptable as a screening program, particularly in workplaces, thus having a significant impact on early diagnosis and good prognosis [48,49].

**Author Contributions:** Conceptualization: AA, SZ. Methodology: NP, MRB, EC. Formal analysis: AA, NP, VC. Investigation: GA, RB, TG, AC, GD. Data curation: AA. Writing—original draft preparation: NP, VC. Writing—review, and editing: MRB, EC, GA, TG, AC, GN, LS, GD, AA, and SZ. Supervision: AA, GD, SZ. Funding acquisition: AA. All authors have read and agreed to the published version of the manuscript.

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**Institutional Review Board Statement:** The study was conducted under the Declaration of Helsinki and approved by the Ethics Committee of Bambino Gesù Children's Hospital (protocol code: 1670\_OPBG\_2018, approved on February 11, 2019).

**Informed Consent Statement:** Informed consent was obtained from all subjects involved in the study.

**Conflicts of Interest:** The authors declare no conflict of interest.

**Data Availability Statement:** Some or all data and models that support the findings of this study are available from the corresponding author upon reasonable request.

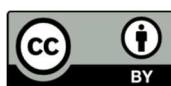
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