



# Profiling of Protein Adducts of Estrogen Quinones in 5-Year Survivors of Breast Cancer Without Recurrence

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Che Lin, MD, PhD<sup>1,2</sup>, Ding-Ru Chen, MS<sup>1</sup>, Shou-Jen Kuo, MD<sup>1</sup>, Chi-Yen Feng, MD<sup>3</sup>, Dar-Ren Chen, MD<sup>1</sup> , Wei-Chung Hsieh, MD<sup>4</sup>, and Po-Hsiung Lin, PhD<sup>5</sup> 

## Abstract

**Aims:** The aim of this study was to simultaneously analyze estrogen quinone-derived adducts, including 17 $\beta$ -estradiol-2,3-quinone (E<sub>2</sub>-2,3-Q) and 17 $\beta$ -estradiol-3,4-quinone (E<sub>2</sub>-3,4-Q), in human albumin (Alb) and hemoglobin (Hb) derived from breast cancer patients with five-year postoperative treatment without recurrence in Taiwan and to evaluate the treatment-related effects on the production of these adducts.

**Settings and Design:** Cohort

**Methods and Material:** Blood samples derived from breast cancer 5-year survivors without recurrence were collected. Albumin and hemoglobin adducts of E<sub>2</sub>-3,4-Q and E<sub>2</sub>-2,3-Q were analyzed to evaluate the degree of disposition of estrogen to quinones and to compare these adduct levels with those in patients before treatment.

**Statistical Analysis:** All data are expressed as mean  $\pm$  standard deviation of three determinations. We used Student's *t*-test to examine subgroups. Data were transformed to the natural logarithm and tested for normal distribution for parametric analyses. Linear correlations were investigated between individual adduct levels by simple regression. Statistical analysis was performed using the SPSS Statistics 20.0.

**Results:** Result confirmed that logged levels of E<sub>2</sub>-2,3-Q-derived adducts correlated significantly with those of E<sub>2</sub>-3,4-Q-derived adducts (correlation coefficient  $r=.336-.624$ ). Mean levels of E<sub>2</sub>-2,3-Q-4-S-Alb and E<sub>2</sub>-3,4-Q-2-S-Alb in 5-year survivors were reduced by 60-70% when compared to those in the breast cancer patients with less than one year of diagnosis/preoperative treatment ( $P<.001$ ).

**Conclusions:** Our findings add support to the theme that hormonal therapy including aromatase inhibitors and Tamoxifen may dramatically reduce burden of estrogen quinones. We hypothesize that combination of treatment-related effects and environmental factors may modulate estrogen homeostasis and diminish the production of estrogen quinones in breast cancer patients.

## Keywords

biomarker, estrogen quinone, protein adducts, hormonal therapy

<sup>1</sup>Comprehensive Breast Cancer Center, Changhua Christian Hospital, Changhua, Taiwan

<sup>2</sup>Department of Optometry, Chung Hwa University of Medical Technology, Tainan, Taiwan

<sup>3</sup>Department of Surgery, Da-Chien Health Medical System, Taiwan

<sup>4</sup>Department of Laboratory Medicine, Da-Chien General Hospital, Miaoli, Taiwan

<sup>5</sup>Department of Environmental Engineering, National Chung Hsing University, Taichung, Taiwan

## Corresponding Author:

Po-Hsiung Lin, Department of Environmental Engineering, National Chung Hsing University, 145 Xingda Rd., South Dist., Taichung City 402, Taiwan.

Email: [pohsiunglin@yahoo.com](mailto:pohsiunglin@yahoo.com)



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## Introduction

Breast cancer is one of the common cancers found in Taiwanese women. The onset of breast cancer tends to occur at a younger age than in Western countries.<sup>1</sup> Development and application of biological markers to recognize high-risk individuals is needed. Conversion of 17 $\beta$ -estradiol (E<sub>2</sub>) to estrogen catechols, including 2-hydroxyestradiol (2-OH-E<sub>2</sub>) and 4-hydroxyestradiol (4-OH-E<sub>2</sub>), is mediated by CYP1A2/1A1 and CYP1B1.<sup>2-4</sup> Both estrogen catechols undergo oxidation to form quinones, including estrogen-2,3-quinone (E<sub>2</sub>-2,3-Q) and estrogen-3,4-quinone (E<sub>2</sub>-3,4-Q).<sup>5,6</sup> In combination with estrogen-derived mitogenesis, accumulation of estrogen quinones along with the subsequent generation of abasic sites and other types of DNA damage, contribute to estrogen-induced carcinogenesis.<sup>7-9</sup>

Many new tools in the field of molecular profiling have been developed for breast cancer to accurately predict outcomes and to estimate the benefit of adjuvant treatment.<sup>10</sup> Tumor tissue markers such as Gene expression assays-oncotypeDX are used to predict outcomes.<sup>11</sup> Our previous investigation indicated that elevation of background levels of estrogen quinone-derived blood protein adducts and abasic sites in leukocytes are biomarkers of developing breast cancer.<sup>12-14</sup> Blood protein adducts have been used as surrogate markers for DNA adducts of several different classes of carcinogens.<sup>15-17</sup> Protein adducts, including serum albumin (Alb) and hemoglobin (Hb) adducts, seem to be reliable biomarkers of early prediction of disease. We used the Alb and Hb adducts of quinonoid metabolites of estrogen as biomarkers to reflect the cumulative burden of reactive estrogen quinones in breast cancer patients before treatment.<sup>18</sup> We concluded that body burden of E<sub>2</sub>-2,3-Q and E<sub>2</sub>-3,4-Q is an important indicator of breast cancer risk. In this study, we refine our analytical method and measure the concentration of estrogen quinones in blood proteins derived from Taiwanese breast cancer patients with greater than five years of post-operative treatment without recurrence (5-year survivors) and compare these estrogen quinone adducts with those of breast cancer patients with less than one year of diagnosis/preoperative treatment (BCP) and healthy controls.<sup>12-14</sup> We obtained evidence of significant reduction in the cumulative tissue doses of E<sub>2</sub>-2,3-Q and E<sub>2</sub>-3,4-Q adducts in breast cancer 5-year survivors when compared to those of BCP. We designate the products of reactions between estrogen quinones and blood proteins as E<sub>2</sub>-2,3-Q-1-S-Alb, E<sub>2</sub>-2,3-Q-4-S-Alb, and E<sub>2</sub>-3,4-Q-2-S-Alb for serum albumin, and E<sub>2</sub>-2,3-Q-1-S-Hb, E<sub>2</sub>-2,3-Q-4-S-Hb, and E<sub>2</sub>-3,4-Q-2-S-Hb for hemoglobin.

## Subjects and Methods

### Chemicals

E<sub>2</sub>-2, 4, 16, 16, 17-d<sub>5</sub> ([<sup>2</sup>H<sub>5</sub>]-E<sub>2</sub>) was from C/D/N Isotope (Canada H9R 1H1). Acetone, acetonitrile, ethyl acetate, and methyl alcohol were obtained from TEDIA (Charlotte,

NC288224, USA). E<sub>2</sub>, chloroform, N-Acetyl-L-Cysteine (NAC), L-Ascorbic acid (99%), trifluoroacetic acid anhydride (TFAA), methanesulfonic acid (MSA), human serum Alb, human Hb, and potassium nitrosodisulfonate were purchased from Sigma-Aldrich Inc. (St Louis, MO63178, USA).

### Synthesis of Adducts of Estrogen Quinones With N-Acetyl-L-Cysteine

The products of reactions between estrogen quinones and NAC are designated as E<sub>2</sub>-2,3-Q-1-S-NAC, E<sub>2</sub>-2,3-Q-4-S-NAC, and E<sub>2</sub>-3,4-Q-2-S-NAC, and those with Hb as E<sub>2</sub>-2,3-Q-1-S-Hb, E<sub>2</sub>-2,3-Q-4-S-Hb, and E<sub>2</sub>-3,4-Q-2-S-Hb. Authentic standards of E<sub>2</sub>-2,3-Q-1-S-NAC, E<sub>2</sub>-2,3-Q-4-S-NAC, and E<sub>2</sub>-3,4-Q-2-S-NAC were synthesized following the procedure as described previously by Chen et al (2011).<sup>18</sup>

### Synthesis of Adducts of Estrogen Quinones With Human Globin and Albumin

To 30 mg (.11 mmole) of 17 $\beta$ -estradiol, 10 mL of acetone and then 16 mL of 10% acetic acid in water (v/v) were added. After the addition of 50 mg of potassium nitrosodisulfonate, the mixture was shaken for 15 min at room temperature. A second portion of potassium nitrosodisulfonate (50 mg) was added and the mixture was shaken for another 15 min. The estrogen quinones were extracted from the solution three times with chloroform (2mLx3). Chloroform was removed under a gentle stream of N<sub>2</sub>. 20  $\mu$ L of acetonitrile was added to the residue and reactions were performed by adding estrogen quinones to a solution containing human globin and Alb (in 1X PBS; purchased from Sigma-Aldrich) for 30 min at 37°C. The reactions were terminated by adding 10 mM of ascorbic acid (final concentration) and by chilling in an ice bath. The modified protein was to serve as a positive control.<sup>18</sup>

### Synthesis of Isotopically Labeled Protein-Bound Internal Standards

Isotopically labeled protein bound internal standards were synthesized for the analysis of estrogen quinone-derived adducts according to the procedure previously described by Chen et al (2011).<sup>18</sup>

### Subjects

Inclusion criteria: Participants were female adults aged 20 years or over. The study size was arrived at a website calculation of Type I error rate .05, Power .7, ratio to control and different P0/P1 estimation. Exclusion criteria: those who were smoking or heavy drinkers as alcoholism victims or female cancers such as uterine endometrium cancers or ovary cancers. Healthy control participants, breast cancer patients with less than one year of diagnosis/preoperative treatment (BCP) and breast cancer patients with

greater than five years of postoperative treatment without recurrence (5-year survivors) were recruited in a medical center and hospitals in central Taiwan between April 2014 and September 2017 (n=47). The participants provided venous blood for protein adduct analyses and completed questionnaires regarding age, occupation, disease history, cigarette smoking, and alcohol consumption, etc. Eleven of Alb and fifteen of Hb samples cannot be recovered during protein extraction. Overall, 36 of Alb and 32 of Hb samples were analyzed for the background levels of estrogen quinone-derived adducts. Participants diagnosed with ER (+) and/or progesterone receptor (+) breast cancer and received hormonal therapy including aromatase inhibitor (AI) and Tamoxifen. Additionally, healthy controls and BCP were recruited between May 2009 and May 2012 as previously described.<sup>13</sup> None of the subjects had history of alcohol-drinking nor cigarette-smoking. The mean ages for healthy controls, BCP, and 5-year survivors were 41.7, 49.5, and 55.3, respectively. The protocols were approved by the Institutional Review Board Committee of Changhua Christian Hospital (CCH IRB No. 131216). Each participant was provided informed consents after receiving detailed explanation of this study. The informed consent was obtained from the patients before blood collection. All blood samples were maintained at  $-80^{\circ}\text{C}$  before protein isolation.

### Isolation of Human Serum Albumin

After bringing the serum to room temperature, Alb was isolated as follows. A saturated solution of  $(\text{NH}_4)_2\text{SO}_4$  was added dropwise to the plasma until the final concentration of ammonium sulfate was 2.5 M. The solution was mixed with a vortex mixer, and the immunoglobulins were removed by centrifuging for 30 min at 3000g. The protein was purified by dialysis against 4×4 L of 1 mM ascorbic acid at  $4^{\circ}\text{C}$  using Spectra-Por 2 dialysis tubing (MWCO 12000-14000). The dialyzed proteins were lyophilized, weighed, and stored at  $-80^{\circ}\text{C}$  prior to use.

### Isolation of Globin

After removal of red blood cell membranes by centrifuging at 30,000g at  $5^{\circ}\text{C}$  for 20 min, the supernatant containing hemoglobin was purified by exhaustive dialysis (Spectra-Pore 1, 6000-8000 MWCO) against 4×3.5 L of deionized water at  $4^{\circ}\text{C}$  for over 24 h. Globin was isolated by adding the Hb solution dropwise to 10 volumes of ice-cold acetone containing .1% HCl, washed twice with ice-cold acetone, lyophilized, and stored under  $-80^{\circ}\text{C}$ .

### Measurement of Adducts

All cysteinyl adducts arising from estrogen quinones were assayed by the procedure as described by Chen et al, 2011.<sup>18</sup> Briefly, to an 8-ml vial containing 10 mg of protein, isotopically labeled protein-bound internal standards for estrogen quinones were added. After bringing samples to complete dryness in a vacuum oven ( $70^{\circ}\text{C}$ ),

per-acetylation was achieved with the addition of 750  $\mu\text{L}$  of TFAA and the reaction was allowed to proceed at  $110^{\circ}\text{C}$  for 30 min. After cooling to room temperature, adducts were cleaved by adding 20  $\mu\text{L}$  of MSA and the mixture was heated at  $110^{\circ}\text{C}$  for an additional 30 min. The un-reacted anhydride was removed under a gentle stream of  $\text{N}_2$ . One and a half mL of hexane was added to the residue and the hexane layer was washed twice with 2 mL of .1 M Tris buffer (pH 7.4) and once with 1 mL of deionized water. After concentrating the samples to 50  $\mu\text{L}$ , a 2- $\mu\text{L}$  aliquot was analyzed by gas chromatograph (GC) and mass spectrometer (MS). All analyses were conducted using an Agilent 6890 series GC coupled to an Agilent 5973N MS. A HP-5MS fused silica capillary column (30 m, .25-mm i.d., .25- $\mu\text{m}$  film thickness) was used with He (99.999%) as the carrier gas at a flow rate of 1 mL/min. The MS transfer-line temperature was  $250^{\circ}\text{C}$  and the chemical ionization reagent gas (methane) pressure was  $2.3 \times 10^{-4}$  torr.

For characterization of adducts in the assay, our GC-MS was set to scan from m/Z 50 to m/Z 750 in electron impact and negative ion chemical ionization (NICI) modes. The GC oven temperature was held at  $75^{\circ}\text{C}$  for 2 min and increased at  $6^{\circ}\text{C}/\text{min}$  to  $145^{\circ}\text{C}$ , where it was held for 10 min. Late-eluting compounds were removed by increasing the oven temperature at  $50^{\circ}\text{C}/\text{min}$  to  $260^{\circ}\text{C}$ , where it was held for 5 min. The ion source temperature was set at  $150^{\circ}\text{C}$  and the injection-port temperature was  $250^{\circ}\text{C}$  in all cases.

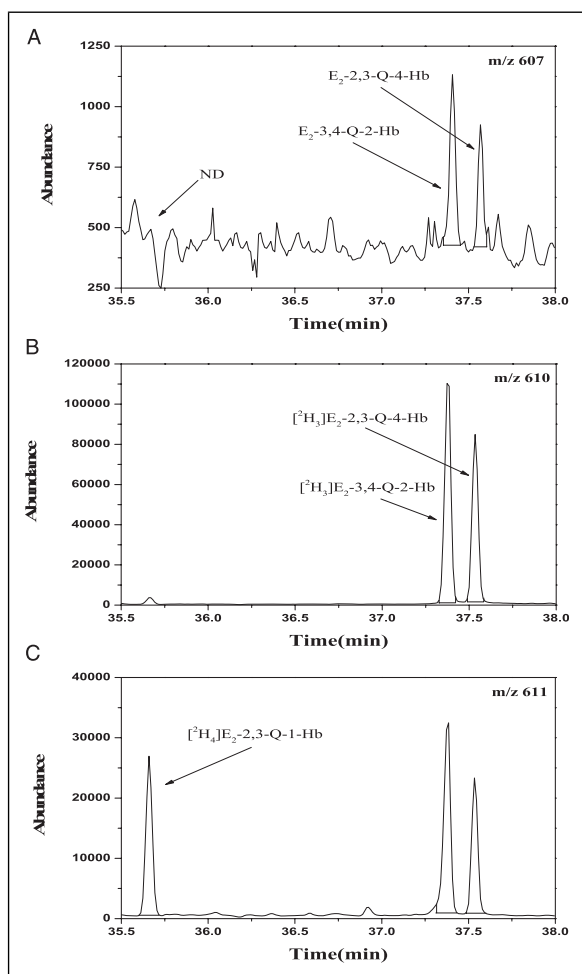
For quantitative analysis of adducts, all conditions were similar to those described above for GC-NICI-MS except that the mass spectrometer was set to selected ion monitoring (SIM) mode. Fragment ions of the TFA-derivatives of estrogen quinone-derived adducts were monitored in NICI mode as follows:  $\text{E}_2$ -2,3-Q-1-S-TFA,  $\text{E}_2$ -2,3-Q-4-S-TFA, and  $\text{E}_2$ -3,4-Q-2-S-TFA (m/Z 607); [ $^2\text{H}_3$ ]  $\text{E}_2$ -2,3-Q-4-S-TFA and  $\text{E}_2$ -3,4-Q-2-S-TFA (m/Z 610); [ $^2\text{H}_4$ ]  $\text{E}_2$ -2,3-Q-1-S-TFA (m/Z 611). For  $\text{E}_2$ -2,3-Q-1-S-TFA,  $\text{E}_2$ -2,3-Q-4-S-TFA, and  $\text{E}_2$ -2,3-Q-2-S-TFA, the quantitation was based on calibration against [ $^2\text{H}_3$ ]  $\text{E}_2$ -2,3-Q-4-S-TFA, [ $^2\text{H}_3$ ]  $\text{E}_2$ -3,4-Q-2-S-TFA, and [ $^2\text{H}_4$ ]  $\text{E}_2$ -2,3-Q-1-S-TFA, respectively. Standard curves were prepared over a range of .1–500 pmole by deriving authentic standards equivalent to those of samples.

### Precision and Limit of Detection of the Assay

Based on a signal-to-noise ratio of 3, the limit of detection of the assay corresponds to 10 pmole/g for all adducts, assuming 10 mg of proteins were used for the assay. The precision, as indicated by estimated coefficients of variation, was estimated to be less than 15% (n=5).

### Statistical Analysis

All data are expressed as mean  $\pm$  standard deviation (SD) of three determinations. We used Student's *t*-test to examine subgroups. Some Data were transformed to the natural logarithm and tested for normal distribution for parametric analyses. Linear correlations were investigated between individual



**Figure 1.** GC-NICI-MS chromatogram obtained in selected ion monitoring mode following the analysis of Hb derived from a 5-year survivor. (A) Ions  $m/z$  607 corresponds to TFA derivatives of  $E_2$ -2,3-Q and  $E_2$ -3,4-Q. (B) The corresponding deuterated internal standard ions were  $m/z$  610 for  $[^2H_3]E_2$ -2,3-Q-4-S-TFA and  $[^2H_3]E_2$ -3,4-Q-2-S-TFA and (C)  $m/z$  611 for  $[^2H_4]E_2$ -2,3-Q-1-S-TFA.

adduct levels by simple regression. Two-tailed statistical significance was indicated by a  $P$  value less than .05. Statistical analysis was performed using the SPSS Statistics 20.0.

## Results

### Measurements of Estrogen Quinone-Derived Adducts in Human Serum Alb Derived From Breast Cancer 5-year Survivors

To examine the background levels of estrogen quinone-derived adducts in serum Alb derived from breast cancer 5-year survivors, we refined our previous protocol to allow measurements of all estrogen quinone-derived adducts in serum Alb. Figure 1 depicts the typical GC-NICI-MS chromatogram obtained in SIM mode after derivatization of 10 mg of Alb derived from a breast cancer 5-year survivors. This figure clearly demonstrates the presence of cysteinyl adducts of estrogen quinone on Alb after adducts cleavage. Levels of  $E_2$ -2,3-Q-1-S-Alb,  $E_2$ -2,3-Q-4-S-Alb, and  $E_2$ -3,4-Q-2-S-Alb were summarized in Table 1. Adducts of  $E_2$ -2,3-Q-4-S-Alb and  $E_2$ -3,4-Q-2-S-Alb were detected in serum Alb of the study population. Levels of  $E_2$ -2,3-Q-4-S-Alb and  $E_2$ -3,4-Q-2-S-Alb were estimated to be between 43-933 and 10-177 pmole/g in these subjects, respectively.

### Measurements of Estrogen Quinone-Derived Adducts in Human Hb Derived From Breast Cancer 5-year Survivors

Adducts of  $E_2$ -2,3-Q-4-S-Hb and  $E_2$ -3,4-Q-2-S-Hb were detected in Hb of the participants. Levels of  $E_2$ -2,3-Q-1-S-Hb,  $E_2$ -2,3-Q-4-S-Hb, and  $E_2$ -3,4-Q-2-S-Hb were summarized in Table 2. We observed that the concentrations of  $E_2$ -2,3-Q-4-S-Alb and  $E_2$ -3,4-Q-2-S-Alb were estimated to be between 143-345 and 195-706 pmole/g in these subjects, respectively.

**Table 1.** Measurements of the background levels of estrogen quinone-derived Alb adducts in healthy controls, BCP\* and 5-year survivors<sup>†</sup>.

Adducts	Healthy Controls (n=75)	BCP (n=152)	5-Year Survivors (n=36)
$E_2$ -2,3-Q-1-S-Alb (pmole/g-Alb)			
Mean (SD <sup>‡</sup> )	ND <sup>§</sup>	ND <sup>§</sup>	ND <sup>§</sup>
Median	ND <sup>§</sup>	ND <sup>§</sup>	ND <sup>§</sup>
Range	ND <sup>§</sup>	ND <sup>§</sup>	ND <sup>§</sup>
$E_2$ -2,3-Q-4-S-Alb (pmole/g-Alb)			
Mean (SD <sup>‡</sup> )	295 (116)	403 (252)	193 (192)
Median	282	330	112
Range	66.6~785	61.7~1330	43~933
$E_2$ -3,4-Q-2-S-Alb (pmole/g-Alb)			
Mean (SD <sup>‡</sup> )	138 (57.9)	688 (333)	51 (36)
Median	119	664	41
Range	66.6~378	188~1590	10~177
Ratio of mean levels of $E_2$ -3,4-Q-2-S-Alb to $E_2$ -2,3-Q-4-S-Alb	.468	1.71	.264

BCP\*: breast cancer patients with less than one year of diagnosis/preoperative treatment; 5-year survivors<sup>†</sup>: breast cancer patients with five year postoperative treatment without recurrence; SD<sup>‡</sup>: standard deviation; ND<sup>§</sup>: not detected. The limit of detection is defined as 10 pmole/g when 10 mg of proteins are assayed.

**Table 2.** Measurements of the background levels of estrogen quinone-derived Hb adducts in healthy controls, BCP\* and 5-year survivors<sup>†</sup>.

Adducts	Healthy Controls (n=147)	BCP (n=143)	5-Year Survivors (n=32)
E <sub>2</sub> -2,3-Q-1-S-Hb (pmole/g-Hb)			
Mean (SD <sup>‡</sup> )	ND <sup>§</sup>	ND <sup>§</sup>	ND <sup>§</sup>
Median	ND <sup>§</sup>	ND <sup>§</sup>	ND <sup>§</sup>
Range	ND <sup>§</sup>	ND <sup>§</sup>	ND <sup>§</sup>
E <sub>2</sub> -2,3-Q-4-S-Hb (pmole/g-Hb)			
Mean (SD <sup>‡</sup> )	83 (39)	478 (204)	217 (41)
Median	72	436	211
Range	36~293	146~1473	143~345
E <sub>2</sub> -3,4-Q-2-S-Hb (pmole/g-Hb)			
Mean (SD <sup>‡</sup> )	151 (72)	956 (321)	368 (125)
Median	139	913	341
Range	69~453	153~2392	195~706
Ratio of mean levels of E <sub>2</sub> -3,4-Q-2-S-Alb to E <sub>2</sub> -2,3-Q-4-S-Alb	1.82	2.00	1.70

BCP<sup>\*</sup>: breast cancer patients with less than one year of diagnosis/preoperative treatment; 5-year survivors<sup>†</sup>: breast cancer patients with five year postoperative treatment without recurrence; SD<sup>‡</sup>: standard deviation; ND<sup>§</sup>: not detected. The limit of detection is defined as 10 pmole/g when 10 mg of proteins are assayed.

### Correlation of E<sub>2</sub>-2,3-Q Derived Adducts With Those of E<sub>2</sub>-3,4-Q

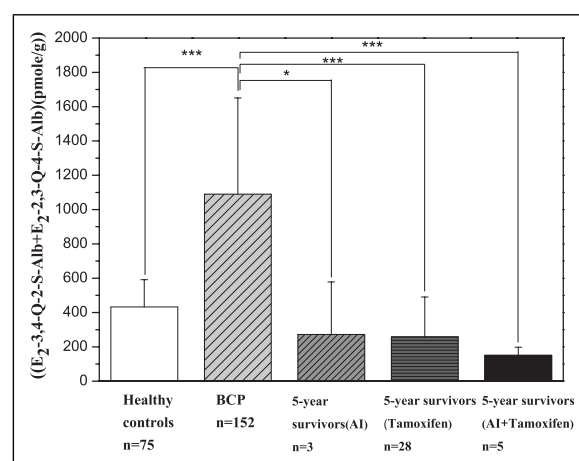
To examine the relationship between E<sub>2</sub>-2,3-Q and E<sub>2</sub>-3,4-Q adducts, we performed simple regression analysis after natural logarithmic transformation of these adducts. Results showed that logged levels of E<sub>2</sub>-2,3-Q adducts correlated with logged levels of E<sub>2</sub>-3,4-Q adducts with correlation coefficients at .624 and .336 for Alb and Hb, respectively ( $P < .001$ ).

### Discussion

We acknowledge the limitations of our current study. The relatively small size of the healthy controls, BCP and 5-year survivors hindered observation of both biomarker efficacy as the exploratory endpoint of the different patient group comparison and the breast cancer long term recurrence risk estimation as an individual patient concern. The short duration of the follow-up period before the 20-year analysis prevented us from assessing the biomarker efficacy. The racial diversity of study participants is restricted to ethnicities in Taiwan.

Another limitation of our study is about Student's *t*-test that is unreliable when variances differ between underlying populations. The *t* distribution tends to the normal distribution for large sample sizes and our non-normality of the samples could not be ignored.

Krishnamurthy et al (2012) reported that tamoxifen inhibits estrogen-induced DNA damage and mammary tumorigenesis in the aromatase transgenic mouse model through up-regulation of quinone reductase. Tamoxifen-treated mice showed a significant increase quinone reductase level over the corresponding controls.<sup>19</sup> A novel mechanism of tamoxifen prevention against breast cancer has been proposed where



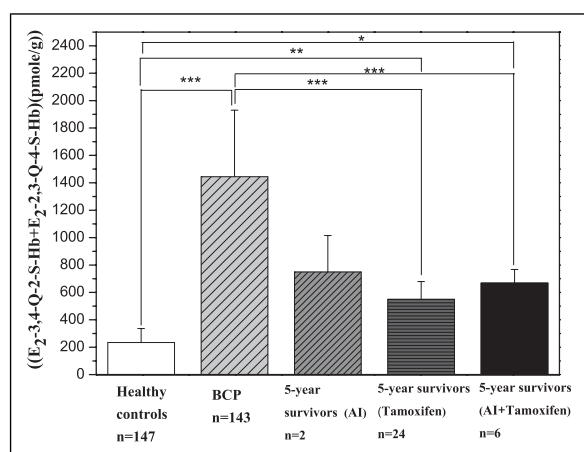
**Figure 2.** Levels of E<sub>2</sub>-2,3-Q-4-S-Alb and E<sub>2</sub>-3,4-Q-2-S-Alb in healthy controls, breast cancer patients with less than one year of diagnosis/preoperative treatment (BCP), and breast cancer patients with five-year postoperative treatment without recurrence (5-year survivors). Aromatase inhibitor (AI); Symbols (\*\*\*) are statistically significantly different from control ( $P < .001$ ).

upregulation of NQO1 induced by tamoxifen may inhibit estrogen-induced DNA damage and tumorigenesis in human MCF-10A cells and in transgenic mice.<sup>19,20</sup> Additionally, various environmental factors and genetic events may influence the production of estrogen quinones in humans as well.<sup>21-23</sup> Cytochrome P450 1A1 and 1B1 mediate metabolic activation of estrogen to generate estrogen catechols and quinones, which are the critical metabolites implicated in the initiation and progression of breast cancer.<sup>24</sup> Several critical enzymes may modulate the formation of estrogen quinone-DNA adducts and oxidative DNA

lesions. Genetic predisposition of polymorphisms in these protective enzymes may affect their activities.<sup>25</sup> O-methylation of estrogen catechols mediated by catechol-o-methyl transferase plays a critical role in blocking the oxidation of the estrogen catechols. Other protective enzymes, such as glutathione-S-transferase and NAD(P)H quinone oxidoreductase 1 (NQO1), have key roles in the disposition of estrogen

quinones through conjugation with glutathione and quinone reduction pathway, respectively.<sup>26</sup> However, environmental factors, including diet, life style, and occupation, may also modulate gene expression of these enzymes. These influences may reduce or increase levels of formation of reactive metabolites of estrogen. Several studies have demonstrated that the protective agents in the diet modulate the expression of enzymes responsible for estrogen disposition.<sup>27,28</sup>

To evaluate the cumulative body burden of estrogen quinones in breast cancer 5-year survivors, levels of estrogen quinone-derived adducts in serum Alb and Hb were measured and compared to those observed in healthy controls and BCP.<sup>13,14</sup> Results from our analyses indicated that Alb adducts of estrogen quinones were detected in the breast cancer 5-year survivors, with overall adduct levels ( $E_2$ -2,3-Q-4-S-Alb plus  $E_2$ -3,4-Q-2-S-Alb) being dramatically reduced (~70%) and comparable to those observed in healthy controls **Figure 2**. Similar observation was detected in Hb adducts of estrogen quinones where overall adduct levels ( $E_2$ -2,3-Q-4-S-Hb plus  $E_2$ -3,4-Q-2-S-Hb) were decreased by ~60% **Figure 3**. This is the first report of the treatment-related effects, including surgery and hormonal therapy, on the reduction of estrogen quinones in breast cancer survivors **Figure 2** and **Figure 3**. Additionally, we noticed that ratio of  $E_2$ -3,4-Q-2-S-Alb to  $E_2$ -2,3-Q-4-S-Alb in breast cancer 5-year survivors was estimated to be .264, whereas .468 and 1.71 was observed for healthy controls and BCP, respectively **Table 1**. As distinguished by age, similar degree of reduction in estrogen quinone-derived



**Figure 3.** Levels of  $E_2$ -2,3-Q-4-S-Hb and  $E_2$ -3,4-Q-2-S-Hb in healthy controls, breast cancer patients with less than one year of diagnosis/preoperative treatment (BCP), and breast cancer patients with five-year postoperative treatment without recurrence (5-year survivors). Symbols (\*\*\*) are statistically significantly different from control ( $P < .001$ ).

**Table 3.** Levels of estrogen quinone-derived Alb adducts in healthy controls, BCP\* and 5-year survivors† as distinguished by age.

Adducts (pmol/g) mean (SD) <sup>‡</sup>		Healthy Controls (n=138)	Breast Cancer Patients (n=143)	5-Year Survivors (n=36)
$E_2$ -2,3-Q-4-Alb	<50	194 (112) n=102	396.7 (242.) n=73	94.8 (27.8) <sup>§  </sup> n=10
$E_2$ -2,3-Q-4-Alb	>50	192 (106) n=36	402 (275) n=70	299 (402) n=26
$E_2$ -3,4-Q-2-Alb	<50	61.7 (41.0) n=102	725 (321) n=73	38.3 (15.6) <sup>  </sup> n=10
$E_2$ -3,4-Q-2-Alb	>50	62.7 (36.7) n=36	626 (348) n=70	61.5 (45.4) <sup>  </sup> n=26

Note: BCP\*: breast cancer patients with less than one year of diagnosis/preoperative treatment; 5-year survivors†: breast cancer patients with five-year postoperative treatment without recurrence; SD<sup>‡</sup>: standard deviation. Symbols (§) represent values of 5-year survivors are statistically significantly different from healthy controls ( $P < .05$ ). Symbols (||) represent values of 5-year survivors are statistically significantly different from breast cancer patients ( $P < .05$ ).

**Table 4.** Levels of estrogen quinone-derived Hb adducts in healthy controls, BCP\* and 5-year survivors as distinguished by age.

Adducts (pmol/g) mean (SD) <sup>‡</sup>		Healthy Controls (n=147)	Breast Cancer Patients (n=143)	5-Year Survivors (n=32)
$E_2$ -2,3-Q-4-Hb	<50	82.8 (35.8) n=114	488.3 (212.4) n=73	209.0 (23.1) <sup>§  </sup> n=10
$E_2$ -2,3-Q-4-Hb	>50	85.0 (50.2) n=33	476.9 (211.5) n=70	220.0 (47.1) <sup>§  </sup> n=22
$E_2$ -3,4-Q-2-Hb	<50	150.8 (63.0) n=114	985.5 (334.7) n=73	301.4 (76.3) <sup>§  </sup> n=10
$E_2$ -3,4-Q-2-Hb	>50	150.4 (101.1) n=33	936.6 (321.3) n=70	397.5 (133.1) <sup>§  </sup> n=22

Note: BCP\*: breast cancer patients with less than one year of diagnosis/preoperative treatment; 5-year survivors†: breast cancer patients with five-year postoperative treatment without recurrence; SD<sup>‡</sup>: standard deviation. Symbols (§) represent values of 5-year survivors are statistically significantly different from healthy controls ( $P < .05$ ). Symbols (||) represent values of 5-year survivors are statistically significantly different from breast cancer patients ( $P < .05$ ).

adducts in 5-year survivors was observed except for levels of E<sub>2</sub>-2,3-Q-4-S-Alb in survivors with age greater than 50 (299 and 402 pmol/g for survivals and BCP, respectively, P>.05). Table 3 and Table 4. This evidence strongly suggests a treatment-related effect on the disposition of estrogen to quinones. Involvement of changes in diet and life style may also contribute to the reduction of estrogen quinones in these breast cancer 5-year survivors.

Overall, our findings add further support to the theme that surgery and hormonal therapy, including AI and Tamoxifen, in cancer patients may dramatically reduce burden of estrogen quinones, DNA damage, and tumorigenesis.<sup>19,20</sup> We concluded that treatment-mediated modulation of estrogen disposition and the subsequent decline of burden of estrogen quinones may play roles in the prevention of breast cancer recurrence.

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### Authors contributions

CL and PHL conceived and designed the study. Ding-Ru Chen, CYF, CL, WCH, and Dar-Ren Chen collected blood samples. Ding-Ru Chen, SYH, PHL, and CL performed instrumental and statistical analyses and interpretation of data. PHL wrote the first draft of the manuscript, which was revised with contributions from WCH, CL, SJK, and Dar-Ren Chen. All authors read and approved the final manuscript. PHL supervised the overall study progress. All authors read and approved the final manuscript.

### Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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### Ethics Statement

This study was approved by the Institutional Review Board Committee of Changhua Christian Hospital (CCH IRB No. 131216).<sup>29</sup> The informed consent was obtained from the patients before blood collection.

### Ethics Approval and Consent to Participate

The study protocol was reviewed by the Human Ethics Committee of the Changhua Christian Hospital, Taiwan (No. 131216). Each subject provided informed consent after receiving a detailed explanation of the study.

### Availability of Data and Material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### ORCID iDs

Dar-Ren Chen  <https://orcid.org/0000-0002-0897-4374>

Po-Hsiung Lin  <https://orcid.org/0000-0003-1905-2778>

### Supplemental Material

Supplemental material for this article is available online.

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