Relationship Between Plasma Estradiol Levels and Estrogen-Responsive Gene Expression in Estrogen Receptor–Positive Breast Cancer in Postmenopausal Women

Anita K. Dunbier, Helen Anderson, Zara Ghazoui, Elizabeth J. Folkerd, Roger A’Hern, Robert J. Crowder, Jeremy Hoog, Ian E. Smith, Peter Osin, Ashutosh Nerurkar, Joel S. Parker, Charles M. Perou, Matthew J. Ellis, and Mitch Dowsett

Abstract

Purpose
To determine whether plasma estradiol (E2) levels are related to gene expression in estrogen receptor (ER)–positive breast cancers in postmenopausal women.

Materials and Methods
Genome-wide RNA profiles were obtained from pretreatment core-cut tumor biopsies from 104 postmenopausal patients with primary ER-positive breast cancer treated with neoadjuvant anastrozole. Pretreatment plasma E2 levels were determined by highly sensitive radioimmunoassay. Genes were identified for which expression was correlated with pretreatment plasma E2 levels. Validation was performed in an independent set of 73 ER-positive breast cancers.

Results
The expression of many known estrogen-responsive genes and gene sets was highly significantly associated with plasma E2 levels (eg, TFF1/pS2, GREB1, PDZK1 and PGR; \( P < .005 \)). Plasma E2 explained 27% of the average expression of these four average estrogen-responsive genes (ie, AvERG; \( r = 0.51; P < .0001 \)), and a standardized mean of plasma E2 levels and ER transcript levels explained 37\% \( (r, 0.61) \). These observations were validated in an independent set of 73 ER-positive tumors. Exploratory analysis suggested that addition of the nuclear coregulators in a multivariable analysis with ER and E2 levels might additionally improve the relationship with the AvERG. Plasma E2 and the standardized mean of E2 and ER were both significantly correlated with 2-week Ki67, a surrogate marker of clinical outcome \( (r = -0.179; P = .05; \text{ and } r = -0.389; P = .0005, \text{ respectively}) \).

Conclusion
Plasma E2 levels are significantly associated with gene expression of ER-positive breast cancers and should be considered in future genomic studies of ER-positive breast cancer. The AvERG is a new experimental tool for the study of putative estrogenic stimuli of breast cancer.

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Introduction

Approximately 80\% of invasive breast cancers present in developed countries as estrogen receptor (ER)–positive and are usually treated with endocrine therapy in the form of antiestrogens or estrogen suppressants—aromatase inhibitors (AIs) in postmenopausal women. ER-positive tumors, although highly heterogeneous, consist largely of tumors described as luminal1 and are now recognized as being genetically distinct from ER-negative tumors. There is considerable clinical interest in identifying molecular factors that reflect the variable dependence of ER-positive tumors on estrogenic stimulation and, by extension, the likelihood of these tumors benefiting from endocrine therapy2; however, few genomic studies have focused specifically on the ER-positive subgroup.

Although expression of progesterone receptor (PgR), which is strongly influenced by estrogens in vitro3 and in vivo,4,5 has been evaluated in this manner, it is clear that, as a single factor, PgR does not fully reflect estrogen dependence: many PgR-negative tumors respond to tamoxifen or AIs.4,6 Trefoil factor 1 (TFF1), originally known as pS2,7 is used often as a marker in laboratory studies8 but infrequently for clinical studies. Other studies of ER-positive breast cancer cells in vitro have identified additional genes for which expression is dependent on estrogenic stimulation,9 but most have not been translated to clinical utility.
Plasma levels of estradiol (E2) vary markedly between postmenopausal women and are significantly correlated with risk of development of breast cancer. Exogenous estrogens increase risk, particularly when administered with progestins. Plasma levels of endogenous E2 are, however, not known to influence breast cancer biology significantly; conversely, it is widely considered that intratumoral synthesis of estrogens provides the predominant estrogenic influence.

The primary objective of this study was to determine whether plasma E2 levels correlated with gene expression in ER-positive breast cancers and, if so, whether associated genes were known to be estrogen regulated. Secondary objectives were to determine the relative contribution of plasma E2 and estrogen-regulated genes and to assess the relationship of these genes with clinical and biologic measures of clinical associations with estrogen-regulated genes and to assess the relationship of these genes with clinical and biologic measures of clinical significance.

**Materials and Methods**

**Patient Samples**

Core-cut tumor biopsies (14-gauge) were obtained from 112 postmenopausal women with stage I to III B, ER-positive, early breast cancer before and after 2 weeks of anastrozole treatment in a neoadjuvant trial. Patients who received gefitinib during the first 2 weeks were excluded. Tissue was later stored in RNA (Ambion, Austin, TX) at −20°C. Two 4-μm sections from the core were stained with hematoxylin and eosin to confirm the presence of tumor and the histopathology. Total RNA was extracted by using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA): samples with RNA integrity values less than 5 were excluded from additional analysis. ER status and Ki67 values by immunohistochemistry were already available.

The validation set was derived from baseline frozen biopsy samples obtained from 73 patients receiving neoadjuvant letrozole therapy in a phase II clinical trial. 

**Gene Expression Analysis and Data Preprocessing**

RNA amplification, labeling, and hybridization on HumanWG-6 v2 Expression BeadChips (Illumina, San Diego, CA) were performed according to the manufacturer’s instructions at a single Illumina BeadStation facility. Tumor RNA of sufficient quality and quantity was available to generate expression data from 104 pretreatment biopsies. Data was extracted by using BeadStudio (Illumina) software and was normalized with variance-stabilizing transformation (VST) and Robust Spline Normalization method in the Lumi package.

Probes that were not detected in any samples (detection $P > 0.1$) were discarded from additional analysis. The gene expression approach (Agilent Technologies) and gene expression data sets for the validation study are described in Crowder et al.

**Data Analysis**

**Primary analysis.** Multiple correlation analysis was performed in BRB-Array Tools (http://linus.nci.nih.gov/BRB-ArrayTools.html). A statistical significance level for each gene for testing the hypothesis that the Spearman correlation between gene expression and plasma estradiol was zero was calculated, and $P$ values then were used in a multivariate permutation test, from which false discovery rates were computed. Gene set enrichment analysis (GSEA) was carried out on the ranked lists of correlated genes by using the GSEA preranked function of the software across the curated gene sets of the Broad Institute Molecular Signatures Database (MSigDB). Pathway analysis by using Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA) was conducted on the list of genes correlated with $P < .005$. Other statistical analyses were performed in SPSS for Windows (SPSS, Chicago, IL), S-Plus (TIBCO Software, Palo Alto, CA), and Graphpad Prism (Graphpad Software, La Jolla, CA).

Intrinsic subtypes were assigned with a 50-gene subtype predictor. Data from the exclusively ER-positive tumors in this study were normalized before subtyping analysis with data from an additional 64 tumors, including 23 ER-negative cancers, analyzed concurrently on Illumina arrays.

Secondary and exploratory analyses. Multivariable analysis was performed in a forward stepwise fashion, and the most significant additional variable (satisfying $P < .05$) was added at each stage. Occurrences with missing values for any of the variables in the model were excluded from analysis (Appendix Table A1). Statistical significant results were assessed when data availability allowed in the validation set.

<p>| Table 1. Genes Positively Correlated With Plasma Estradiol Concentration |
|-----------------------------|---------------------------------|------------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Rank</th>
<th>Gene Symbol</th>
<th>Genbank Accession No.</th>
<th>Spearman Correlation Coefficient</th>
<th>Parametric P</th>
<th>Known Estrogenic Function</th>
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<td>1</td>
<td>TFF3</td>
<td>NM_003225</td>
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<td>7.00E-07</td>
<td>Estradiol-responsive gene in vitro and in vivo$^{10,22,23}$</td>
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<td>2</td>
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<td>13</td>
<td>SERPINA3</td>
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<td>15</td>
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<td>.0029109</td>
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<td>16</td>
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<td>17</td>
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</tbody>
</table>

NOTE. Ranked according to Spearman correlation. The 10 most strongly correlated genes are shown together with genes known to be associated with estrogen signaling with $P < .005$. © 2010 by American Society of Clinical Oncology
Blood Samples and Hormone Measurement

Baseline plasma E2 levels before anastrozole or letrozole treatment were determined by a highly sensitive radioimmunoassay after ether extraction21 by using rabbit antiserum against an estradiol-6-carboxymethylxime-BSA conjugate (EIR, Wurenlingen, Switzerland) and estradiol iodine 125 reagent DSL 4420 from the estradiol RIA kit DSL 4800 (Beckman Coulter [formerly known as DSL], Fullerton, CA). Sensitivity was 3 pmol/L by calculation from the 95% CIs of the zero standard. Plasma samples from both the anastrozole and letrozole cohorts were analyzed in the same laboratory.

RESULTS

Relationship of Plasma E2 With Tumor Gene Expression

Quantitative trait analysis (QTA) by Spearman correlation was used to identify 226 genes from the 25,386 filtered genes for which expression in tumor biopsies correlated with plasma E2 levels at a $P < .005$ (Table 1). Genes previously known to be associated with a transcriptional response to estrogen appeared to be overrepresented among the genes closely correlated with plasma E2. TFF1 expression correlated most highly with pretreatment plasma E2 levels (Fig 1A). GREB1 (gene regulated by estrogen in breast cancer), an early response gene in the estrogen receptor-regulated pathway,22,24 (Fig 1B) was the fifth-most positively correlated gene, whereas expression of the PGR, the gene encoding the progesterone receptor, was also significantly correlated ($P = .003$). Plasma E2 levels were significantly higher in patients with immunohistochemical (IHC) PgR-positive tumors than in those with PgR-negative tumors (medians, 35 and 23 pmol/L, respectively; $P = .002$). There was no significant relationship between plasma E2 and ER IHC levels. Plasma E2 did not correlate with either nodal status or tumor size ($P = .55$ and 0.52, respectively).

GSEA19 revealed that gene sets associated with the transcriptional response to estrogen deprivation in other published data sets were significantly enriched in the genes most tightly correlated with pre-treatment plasma E2 levels. In particular, the genes identified as regulated in vitro by E2 through ER-$\alpha$ and ER-$\beta$28 were statistically significantly enriched (q value, $< 0.2$; Table 2). Pathway analysis by using Ingenuity software on the 98 annotated genes positively correlated with plasma E2 levels ($P < .005$) and revealed a network showing a significant relationship between 29 of these genes (Fig 2).

For additional analysis, an index of estradiol-responsive genes was generated by averaging the expression of four key genes, which was denoted AvERG (ie, average estrogen-responsive genes). TFF1, GREB1, and PDZKI were selected for this index as the highest ranked genes in our analysis that had been responsive to estrogen deprivation with AIs in the two published clinical studies available to date22,23 and

![Graph A: TFF1 Expression vs Log2 Estradiol](image1)

![Graph B: GREB1 Expression vs Log2 Estradiol](image2)

![Graph C: AvERG vs Log2 Estradiol](image3)

![Graph D: AvERG vs Log2 Estradiol](image4)

**Fig 1.** Correlation of plasma estradiol and estrogen-responsive gene expression. (A) Plasma estradiol versus TFF1 expression in discovery data set. (B) Plasma estradiol versus GREB1 expression in discovery data set. (C) Plasma estradiol versus AvERG average estrogen-responsive genes; ie, mean of TFF1, GREB1, PDZKI, and PGR expression in discovery data set. (D) Plasma estradiol versus AvERG expression in independent validation data set.

<table>
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<th>Gene Set</th>
<th>Size of Gene Set</th>
<th>Normalised Enrichment Score</th>
<th>Nominal P</th>
<th>FDR q</th>
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Abbreviation: FDR, false discovery rate.

*Estradiol-related pathway.
in the key published in vitro study of estradiol-stimulated genes in breast cancer cells.\textsuperscript{10} PGR was included, as it has been identified as a marker of estrogenicity in numerous studies spanning 3 decades\textsuperscript{3} and is in routine clinical use. No other combination of genes was tested. The resultant index correlated with plasma E2 levels with $r = 0.51$ ($P < .0001$; Fig 1C).

Validation of Association Between Plasma E2 and AvERG Expression

To determine the validity of these observations, we tested the four key estradiol-dependent genes in an independent group of ER-positive breast cancer samples from postmenopausal patients before treatment in a phase II trial of neoadjuvant letrozole.\textsuperscript{15} Plasma taken at diagnosis and Agilent microarray measurements from tumors were available from 73 patients. Of the four classical estrogen-dependent genes identified in our discovery set (ie, TFF1, GREB1, PDZK1, and PGR) only PGR was not individually significantly correlated with plasma E2 ($P \leq .05$), whereas the AvERG correlated overall with $r = 0.32$ ($P = .006$; Fig 1D). The CIs for the correlation coefficients for PGR in the two sample sets overlapped markedly, which indicated no significant heterogeneity despite the lack of statistical significance in the validation set (initial samples: $r = 0.25$; 95% CI, 0.053 to 0.44; validation samples: $r = 0.17$; 95% CI = $-0.072$ to 0.39). Similarly, plasma E2 levels were higher in PgR-positive (by IHC) than PgR-negative occurrences in the validation set, but this was not statistically significant ($P = .17$).

Contribution of ESR1 Levels to AvERG Gene Expression

As E2 induces transcription primarily through ER-\(\alpha\) (ESR1) in breast carcinomas, we investigated the effect of ESR1 levels, as measured by cDNA microarray analysis, on AvERG. ESR1 expression was
correlated with AvERG in our original data set \((r = 0.27; P = .006)\) and not with plasma E2 levels \((r = 0.01; \text{not significant})\). On multivariable analysis, variation in plasma E2 and ESR1 were found to account for 27% and 10% of the variation in AvERG, respectively. The correlation of the AvERG with a normalized mean of plasma E2 and ESR1 was 0.61 \((P < .0001; \text{Fig 3A})\). Equivalent analysis of the validation samples found plasma E2 and ESR1 levels accounted for 18% and 15% of the variation in AvERG, respectively, with analysis that included ESR1 levels producing a correlation of \(r = 0.54\) \((P < .0001; \text{Fig 3B})\).

### Contribution of Cofactor Levels to AvERG Gene Expression

Coactivators and corepressors interact with the bound ER to modulate gene transcription. \(^{29}\) To assess the role that levels of the primary coactivators and corepressors have on estrogen-dependent gene expression, univariate analysis was undertaken with AvERG expression as the dependent variable and the following coregulators: NCOR1, NCOR2, NCOA1, NCOA2, and NCOA3 as potentially independent covariables. Significant associations were found with NCOR1 \((P = .002)\), NCOA1 \((P = .03)\), and NCOA3 \((P = .01)\). Inclusion of these three factors with plasma E2 and ESR1 in a multivariable analysis revealed log plasma E2 \((P < .001)\), ESR1 \((P = .001)\), and NCOR1 \((P = .007)\) to be independently significant with these factors, which explained 41% of the variation in AvERG expression. None of the cofactors were found to be significant on equivalent analysis of the independent data set, but the Agilent platform provided a lower dynamic range of expression values.

### Relationship of Pretreatment Plasma E2 and AvERG With Intrinsic Subtype Status

Intrinsic subtype was ascribable to 69 tumors: 35 luminal A, 17 luminal B, 12 HER2-like, and five basal-like tumors. AvERG and plasma E2 were significantly higher in luminal A tumors compared with the other sets, when considered together \((P = .008 \text{ and } .011, \text{respectively})\). Differences of similar magnitude were present between luminal A and B tumors when considered separately, but the significance levels were lower because of fewer numbers \((P = .097 \text{ and } .040, \text{respectively})\).

### Relationship of Pretreatment Plasma E2 With Ki67 Staining After 2 Weeks of Treatment With Anastrozole

We have previously reported that higher Ki67 expression after 2 weeks of endocrine therapy is significantly associated with lower recurrence-free survival. \(^{30}\) To determine whether pretreatment plasma estradiol and/or ESR1 levels affect this potential surrogate marker for outcome, we investigated the relationship between plasma E2 and the standardized mean of plasma E2 and ESR1 with the level of tumor Ki67 expression after 2 weeks of treatment. Plasma E2 and the standardized mean were both significantly correlated with 2-week Ki67, although this was borderline for the former \((r = -0.179; P = .05; \text{and } r = -0.389, P = .0005, \text{respectively; Fig 4})\).

### Relationship of Pretreatment Plasma E2 and AvERG With Clinical Response

We conducted an exploratory analysis to determine whether pretreatment plasma E2 and/or AvERG correlated with clinical response in the approximately 50% of the patients that received anastrozole alone for their complete courses of neoadjuvant therapy. In each occurrence, there was a trend toward higher levels in the complete and partial responders versus in those who showed stable
This study is the first to show a relationship between the expression of estrogen-dependent genes in ER-positive breast carcinomas and basal levels of estrogen. Although such results may not appear surprising, findings that intratumoral E2 levels are 10 to 20 times higher than those in plasma, and the absence of a reported correlation between levels in the two compartments, have led to the view that intratumoral estrogen synthesis has a greater influence on estrogen signaling than uptake from the circulation. The data presented in this article challenge that view and strongly suggest that differences in plasma E2 levels between patients have a significant influence on breast tumors. In particular, this influence may be important to consider in future genomic studies of ER-positive breast cancer, and this may extend to premenopausal women in whom the within-patient changes in E2 levels through the menstrual cycle are marked. Our data relating to Ki67 also suggest that E2 levels merit additional investigation in relation to their possible influence on disease outcome on AIs.

The strength of the relationship between AvERG and plasma E2 levels in this heterogeneous group of ER-positive tumors is remarkable. The relationship was replicated in an independent sample set with good statistical confidence, despite a smaller sample size. The low plasma levels of E2 in postmenopausal women measured in this study would be unquantifiable by most routinely used assays and may be one reason that this relationship has not been previously observed. Additional exploration should be conducted only with the use of such specialist assays. In a model system, proportional uptake of E2 from plasma was inversely related to plasma E2 concentrations. Our data do not allow us to determine whether this inverse relationship extends to the expression of estrogen-related genes.

The inclusion of ESR1 mRNA expression as a variable along with plasma E2 explained 37% of the variability in the AvERG. There is sufficient unexplained variability for intratumoral synthesis to make a meaningful contribution, but analytic imprecision and other factors also may be influential. One such potential influence examined in this study is the expression of nuclear coregulators. Additional exploratory modeling that allowed for an interaction between the five coregulators and ER explained 54% of the variability in AvERG (data not shown). These relationships were not confirmed in the validation set, possibly because of the lower dynamic range on the Agilent platform. These apparent relationships need additional study before they could be considered reliable.

The absence of a relationship between plasma E2 and ESR1 expression and the known effect of estrogen deprivation on the index genes suggest that the relationship between plasma E2 and these estrogen index genes is due to signaling influences rather than to the evolution of particularly estrogen-sensitive tumors as a result of higher E2 levels. As well as providing evidence for the importance of basal levels of E2 on breast cancer biology, the creation of AvERG provides a new index for understanding the clinical and biologic importance of other putative estrogenic influences. For example, a number of steroids thought of largely as androgens also have been found to have significant estrogenic activity in vitro, and it has been speculated that these may be particularly important in patients under treatment with AIs. They would not be expected to be influenced by such treatment; as a result, they may provide a possible resistance mechanism.

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Employment or Leadership Position: None Consultant or Advisory Role: Mitch Dowsett, AstraZeneca (C) Stock Ownership: Helen
Plasma Estradiol and Gene Expression in ER-Positive Breast Cancer

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## Table A1. Patients Contributing to Cofactor Analyses

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<tr>
<td>AvERG pre and post (difference), plasma E2, ESR1</td>
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<tr>
<td>AvERG, plasma E2, Ki67 2 week</td>
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<td>AvERG, ESR1, Ki67 2 week</td>
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<td>AvERG, plasma E2, ESR1, Ki67 2 week</td>
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<tr>
<td><strong>Independent data set</strong></td>
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<tr>
<td>AvERG, plasma E2, ESR1, and any cofactor</td>
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**NOTE.** Unless otherwise stated, analyses with subsets of the variables had the same number of patients as the analyses involving all the variables (eg, analysis in the study set of AvERG, plasma E2, and ESR1 only would have involved 97 patients).

Abbreviation: AvERG, average estrogen responsive genes (ie, mean of TFF1, GREB1, PDZK1, and PGR).