Caffeine and Caffeic Acid Inhibit Growth and Modify Estrogen Receptor and Insulin-like Growth Factor I Receptor Levels in Human Breast Cancer

Ann H. Rosendahl1, Claire M. Perks2, Li Zeng2, Andrea Markkula1, Maria Simonsson1, Carsten Rose3, Christian Ingvar4, Jeff M.P. Holly2, and Helena Jernström1

Abstract

Purpose: Epidemiologic studies indicate that dietary factors, such as coffee, may influence breast cancer and modulate hormone receptor status. The purpose of this translational study was to investigate how coffee may affect breast cancer growth in relation to estrogen receptor-α (ER) status.

Experimental Design: The influence of coffee consumption on patient and tumor characteristics and disease-free survival was assessed in a population-based cohort of 1,090 patients with invasive primary breast cancer in Sweden. Cellular and molecular effects by the coffee constituents caffeine and caffeic acid were evaluated in ER+ (MCF-7) and ER− (MDA-MB-231) breast cancer cells.

Results: Moderate (2–4 cups/day) to high (≥5 cups/day) coffee intake was associated with smaller invasive primary tumors ($P_{\text{trend}} = 0.013$) and lower proportion of ER− tumors ($P_{\text{trend}} = 0.018$), compared with patients with low consumption (≤1 cup/day). Moderate to high consumption was associated with lower risk for breast cancer events in tamoxifen-treated patients with ER− tumors (adjusted HR, 0.51; 95% confidence interval, 0.26–0.97). Caffeine and caffeic acid suppressed the growth of ER+ (P ≤ 0.01) and ER− (P ≤ 0.03) cells. Caffeine significantly reduced ER and cyclin D1 abundance in ER+ cells. Caffeine also reduced the insulin-like growth factor I receptor (IGFIR) and pAkt levels in both ER+ and ER− cells. Together, these effects resulted in impaired cell-cycle progression and enhanced cell death.

Conclusions: The clinical and experimental findings demonstrate various anticancer properties of caffeine and caffeic acid against both ER+ and ER− breast cancer that may sensitize tumor cells to tamoxifen and reduce breast cancer growth. Clin Cancer Res; 21(8): 1877–87. © 2015 AACR.

Introduction

Breast cancer is the major cancer affecting women with a lifetime risk of more than 10% in the general population in Western countries. The prevalence is, however, much lower in Asia and many economically developing countries (1). However, Asian immigrants to the United States acquire the prevalence of that country within one generation, indicating a strong effect of environmental exposures. Although the majority of primary breast cancers in estrogen-responsive patients with endocrine treatment, many patients progress to estrogen-insensitive disease with poor prognosis (2, 3). A critical determinant of such progression is the loss of the estrogen receptor-α (ER), which is associated with a more aggressive tumor phenotype and the loss of sensitivity to endocrine therapies such as tamoxifen (3). ER can stimulate cellular proliferation via progesterone receptor (PgR) induction and transcriptional upregulation of cyclin D1, leading to enhanced cell-cycle progression, whereas ER loss has been associated with enhanced motility and metastatic abilities of breast cancer cells. Although loss of ER activity and expression has critical consequences for breast cancers, it generally occurs without evidence of ER gene mutation or deletion. Alterations in ER levels can be regulated at transcriptional or posttranscriptional stages, or through epigenetic mechanisms (4). Given that the prevalence of breast cancer varies between different geographic locations, potential modifiable environmental, dietary, and lifestyle determinants may also influence the ER status.

The insulin-like growth factor (IGF) family has been identified as an additional driver of breast cancer. The mitogenic and antiapoptotic signals are mediated via the IGF type I receptor (IGFIR) and its downstream targets MAPK–ERK or PI3K–Akt. The ER and IGFIR signaling systems are tightly linked and subject to feedback cross-talk. For example, estrogen and IGFs coregulate several genes that affect breast cancer outcome (5). In addition, hyperactive IGFIR–PI3K–Akt signaling has been implicated to contribute in the acquisition of resistance to endocrine therapy during breast cancer progression (6). Estrogen increases IGFIR mRNA and antiestrogens, like tamoxifen, can reduce serum IGF levels and IGF-mediated growth (7, 8). Conversely, blocking IGF actions can inhibit estrogen-mediated growth (9). Similar to ER

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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activity, various anthropometric, lifestyle, and dietary factors can influence the IGF system with impact on cancer development and progression.

In recent years, mechanisms involved in mediating cancer chemopreventive effects of naturally derived plant polyphenols from green tea and coffee have been widely studied in several chemopreventive effects of naturally derived plant polyphenols progression.

In [82x128]blotting, the anti-ERα antibody (1D5) was obtained from Abcam; anti-IGFIRβ (C-20) was purchased from Santa Cruz Biotechnology; anti–phospho-AktSer473 (#4060), anti-Akt (#9272), anti–Bcl2 (#2860), anti–Bcl-xl (#2764), anti-cleaved caspase-7 (#9491), anti-cleaved PARP (#9541), anti-cyclin D1 (#2926), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, #3683) were purchased from Cell Signaling Technology Inc.

Materials and Methods

Reagents
All chemicals and reagents were purchased from Sigma-Aldrich unless stated otherwise. Cell culture media, penicillin–streptomycin, and FBS were purchased from Invitrogen. For immunoblotting, the anti-ERα antibody (1D5) was obtained from Abcam; anti-IGFIRβ (C-20) was purchased from Santa Cruz Biotechnology; anti–phospho-AktSer473 (#4060), anti-Akt (#9272), anti–Bcl2 (#2860), anti–Bcl-xl (#2764), anti-cleaved caspase-7 (#9491), anti-cleaved PARP (#9541), anti-cyclin D1 (#2926), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, #3683) were purchased from Cell Signaling Technology Inc.

Translational Relevance
Despite improved diagnostic and treatment modalities, breast cancer remains the leading cause of cancer-related death among women worldwide. An increased understanding of the clinical and mechanistic effects of modifiable components of our daily diet on breast cancer characteristics and prognosis could lead to better patient information for managing disease. This study reveals that increasing coffee consumption is associated with significantly smaller invasive breast tumor sizes, a lower proportion of ER− tumors, and improved disease-free survival among tamoxifen-treated women with ER+ breast cancer. Mechanistically, this study shows that predominantly caffeine, but also caffeic acid, mimics the actions of antiestrogens and modifies major growth regulatory pathways ER/cyclin D1 and IGFIR/pAkt, resulting in impaired cell-cycle progression and reduced cellular proliferation. Together, these translational findings provide a more comprehensive understanding of the clinical and mechanistic effects of these dietary factors that can modify breast cancer progression.

Study population
The present study is based on an extended cohort of 1,090 women, ages 24 to 99 years, diagnosed with primary invasive breast cancer between October 2002 and December 2012 at the Skane University Hospital (Lund, Sweden), without preoperative treatment and with no other cancer history within the past 10 years. This is an ongoing cohort (BC Blood study) and is now updated to include almost twice as many women as recently reported by our group (17). The inclusion and exclusion criteria were compliant to the REporting recommendations for tumor MARKer prognostic study (REMARK) guidelines (18). During the preoperative visit, body measurements of weight, height, waist and hip circumference, and breast volume were collected (as previously described; refs. 19, 20). Extensive questionnaire data with information on reproductive factors, the use of exogenous hormones or other medications, lifestyle factors, including smoking, alcohol, and coffee consumption, etc., were obtained from the cohort members at the preoperative visit. Information on tumor size, lymph node status, histologic grade, proliferative index (Ki67), and hormone receptor status (ER and PgR) were obtained through pathology reports and medical records. ER+ status was routinely determined until December 2009, using antibody clone 1D5 (DAKO), and from 2010 using clone SP1 (Ventana Medical Systems), as previously described (17, 21). Follow-up information of breast cancer recurrence or death was obtained from patient charts, pathology reports, the Regional Tumor Registry or the Population Registry. Written informed consents were obtained from all participating women and the study was approved by the local ethics committee at Lund University (LU 75-02, LU 37-08, LU 658-09, LU 58-12, LU 379-12, and LU 227-13).

Cell culture
The human breast cancer cell lines MCF-7, T47D (both ERα-positive) and MDA-MB-231 (ERα−) were purchased from and validated by ATCC-LGC Standards and maintained in DMEM supplemented with 10% FBS and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) in a humidified 5% CO2 atmosphere at 37°C. Tamoxifen-resistant (Tam-R) MCF-7 cells were kindly supplied by Julia Gee (Cardiff University, UK) and were derived and maintained as outlined previously (22). For in vitro experiments, cells were seeded in growth media for 24 hours followed by transfer to phenol red- and serum-free media (SFM; DMEM:Ham’s nutrient mix F-12 supplemented with BSA (0.2 mg/mL), sodium bicarbonate (1.2 mg/mL), transferrin (0.01 mg/mL), l-glutamine (2 mmol/L), and antibiotics as above) and dosed as specified below.

Cell proliferation assay
MCF-7, MDA-MB-231, T47D, and Tam-R cells were exposed to caffeine (0–5 mmol/L) or caffeic acid (0–50 µmol/L) with or without tamoxifen (1 µmol/L) for 48 hours in SFM. Cell proliferation was determined following [3H]thymidine incorporation as a measure of DNA synthesis for a period of 4 hours before the
end of the treatment protocol, as described in ref. 23. Total cell number and viability was assessed by trypan blue exclusion using a hemocytometer.

**Cell-cycle analysis**

Cells were dosed with caffeine (0–5 mmol/L) or caffeic acid (0–50 μmol/L) in SFM for 48 hours. Floating and adherent cells were collected, fixed in 70% cold ethanol, and incubated with PI/RNase buffer (BD Biosciences). Approximately $2 \times 10^6$ events (cells) were collected for each sample using a FACSCalibur (BD Biosciences) dual laser, flow cytometer with CellQuest Pro Software (BD Biosciences). Cell-cycle distribution was determined using FlowJo Software (TreeStar Inc.).

**Western immunoblotting**

Cells were exposed to caffeine (0–5 mmol/L) or caffeic acid (0–50 μmol/L) in SFM for 72 hours after which cell lysates were prepared and protein concentration determined, as described previously (24). Protein samples (50–60 μg/lane) were resolved by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% (w/v) milk in TBS Tween-20 (TBST) and probed with antibodies to Bcl-2, Bcl-xl, cleaved caspase-7, cleaved PARP, ERα, IGFRβ, pAkt, Akt, cyclin D1, or GAPDH as a loading control. Protein abundance was detected with horseradish peroxidase–specific secondary antibodies and visualized by SuperSignal West Extended Duration Substrate (Thermo Fisher Scientific) using the Alpha Innotech FluorChem FC2 imaging system and AlphaView version 3.0.3.0 software (ProteinSimple) or ImageJ software (NIH).

**Statistical analysis**

**Clinical data.** Baseline characteristics of patients with low ($\leq$1 cup/day), moderate (2–4 cups/day), or high ($\geq$5 cups/day) coffee consumption were compared as specified by using the Jonckheere–Terpstra test for continuous variables (not normally distributed) and linear-by-linear association test for categorical variables. In survival analyses, patients were followed from inclusion until first breast cancer recurrence, last follow-up, or death, whichever came first, before January 1, 2013. Of 1,090 women, 8 were excluded because of generalized disease within 3 months of diagnosis, leaving 1,082 women included in the survival analyses. Disease-free survival was estimated using the Kaplan–Meier method. The log-rank test was used for univariable survival analysis of the risk of early breast cancer recurrence in relation to coffee consumption. The influence of coffee consumption on prognosis was also analyzed using multivariable Cox regression to calculate HR with 95% confidence intervals (CI), adjusted for potential confounding factors; age (<$\leq$60 years), histologic grade 3 (yes/no), invasive tumor size ($\geq$20 mm, or muscular or skin involvement) and any axillary lymph node involvement (yes/no). Because few patients had an invasive tumor size $\geq$51 mm, or muscular or skin involvement, these patients were combined with the patients with invasive tumor sizes between 21 and 50 mm in the multivariable analyses. All statistical analyses were performed using SPSS Statistics 19.0 software (IBM). A P value of <0.05 was considered statistically significant. Because this was an exploratory study, nominal P values are presented without adjustment for multiple testing.

**Experimental data.** A minimum of three independent repeats were performed for each experiment. Proliferation data are expressed as means ± SE of triplicate wells. Densitometry measurements of Western blot data are expressed as means ± SE of at least three individual repeats. Statistical analyses were performed using the unpaired Student t test with Excel 2007 software. A P value of <0.05 was considered statistically significant.

**Results**

**Associations between tumor size, ER status, and coffee consumption among women with breast cancer**

To evaluate the impact of coffee consumption on patient and tumor characteristics among women with breast cancer, coffee consumption was categorized into low ($\leq$1 cup/day), moderate (2–4 cups/day), or high ($\geq$5 cups/day), whereas no further definition of a cup was specified. Patient and tumor characteristics according to coffee consumption for the 1,090 patients included in the study are shown in Table 1. Women that were younger at their first full-term pregnancy showed a trend of drinking moderate to high amounts of coffee daily. In addition, the proportion of current smokers was higher with increasing coffee consumption, whereas the proportion of alcohol abstainers was higher with decreasing coffee consumption. Interestingly, moderate to high coffee consumption was associated with significantly smaller invasive tumor sizes ($P_{\text{trend}} = 0.013$), whereas concomitantly lower proportion of ER$^+$ tumors and conversely higher proportion of ER$^-$ tumors ($P_{\text{trend}} = 0.018$). After stratification according to ER status (Table 2), increasing coffee consumption remained significantly associated with smaller invasive tumor sizes among women with ER$^+$ tumors ($P_{\text{trend}} = 0.009$), whereas no significant association was found among ER$^-$ tumors ($P_{\text{trend}} = 0.512$). However, a nonsignificant trend toward lower tumor proliferation measured by the Ki67 index ($P_{\text{trend}} = 0.092$) was observed with increasing coffee intake among women with ER$^+$ tumors. These results suggest that coffee may have growth-suppressing properties on breast cancer cells and that ER$^+$ tumors may be more sensitive to the cellular effects by coffee.

**Effects of caffeine and caffeic acid on ER$^+$ and ER$^-$ breast cancer cell growth and viability**

Given the clinical observations of smaller breast tumor sizes and lower frequency of ER$^+$ tumors with increasing coffee consumption, we next examined how two major coffee constituents may affect breast cancer growth in relation to ER status at the cellular level. Increasing doses of caffeine significantly suppressed the proliferation and total cell number of both ER$^+$ MCF-7 and ER$^-$ MDA-MB-231 (Fig. 1A) breast cancer cells, with a maximum inhibition at 5 mmol/L, compared with control ($P < 0.01$). At 1 mmol/L caffeine, the proliferation was reduced by 80% in MCF-7 and 60% in MDA-MB-231 cells ($P < 0.01$) and by 40% in MDA-MB-231 cells ($P = 0.054$). Similarly, caffeic acid reduced both MCF-7 and MDA-MB-231 cell growth, although to a lesser extent than caffeine (Fig. 1A). A concomitant increase in cell death was observed for MCF-7, but not for MDA-MB-231 (Fig. 1A). The response to caffeine and caffeic acid were identical in the Tam-R cells as with the parental MCF-7 cells (Fig. 1B). The caffeine-induced cell death in MCF-7 was associated with reduced prosurvival Bcl-xl levels whereas increased active caspase-7 and cleaved PARP, a signature event of apoptosis (Fig. 1C and Supplementary Fig. S1). These results demonstrate that caffeine, but also caffeic acid, suppress the proliferation of human breast cancer cells, and suggest that...
Table 1. Patient and tumor characteristics of the 1,090 patients in relation to daily coffee consumption (cups/day)

<table>
<thead>
<tr>
<th>Patient characteristics, median (IQR)</th>
<th>All (n = 1,090)</th>
<th>Low (0–1) (n = 202)</th>
<th>Moderate (2–4) (n = 666)</th>
<th>High (5+) (n = 220)</th>
<th>Ptest</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis, y</td>
<td>61.2 (52.1–68.1)</td>
<td>59.4 (48.6–67.8)</td>
<td>62.7 (55.0–69.3)</td>
<td>58.6 (49.5–65.6)</td>
<td>&gt;0.3a</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>69.0 (62.0–78.0)</td>
<td>68.0 (60.0–74.6)</td>
<td>70.0 (62.0–79.0)</td>
<td>69.2 (61.0–79.0)</td>
<td>&gt;0.3a</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.65 (1.62–1.70)</td>
<td>1.65 (1.61–1.69)</td>
<td>1.65 (1.62–1.70)</td>
<td>1.67 (1.63–1.70)</td>
<td>&gt;0.3a</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.1 (22.5–28.4)</td>
<td>24.5 (21.9–27.7)</td>
<td>25.2 (22.8–28.6)</td>
<td>25.0 (22.6–27.9)</td>
<td>&gt;0.3a</td>
</tr>
<tr>
<td>Waist-to-hip ratio</td>
<td>0.86 (0.81–0.91)</td>
<td>0.86 (0.81–0.90)</td>
<td>0.86 (0.81–0.90)</td>
<td>0.87 (0.82–0.91)</td>
<td>&gt;0.3a</td>
</tr>
<tr>
<td>Total breast volume (mL)</td>
<td>1,000 (650–1,500)</td>
<td>925 (350–1,463)</td>
<td>1,000 (700–1,600)</td>
<td>950 (600–1,400)</td>
<td>&gt;0.3a</td>
</tr>
<tr>
<td>Age at first full-term pregnancy (y)</td>
<td>25.0 (22.0–28.0)</td>
<td>26.0 (25.0–29.0)</td>
<td>24.0 (20.0–28.0)</td>
<td>24.0 (20.0–28.0)</td>
<td>0.009b</td>
</tr>
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<td>Current smoker, n (%)</td>
<td>219 (20.1%)</td>
<td>18 (8.9%)</td>
<td>119 (17.8%)</td>
<td>82 (37.3%)</td>
<td>&lt;0.001b</td>
</tr>
<tr>
<td>Alcohol abstainer, n (%)</td>
<td>116 (10.6%)</td>
<td>33 (16.3%)</td>
<td>70 (10.5%)</td>
<td>13 (5.9%)</td>
<td>0.001b</td>
</tr>
<tr>
<td>Tumor characteristics, n (%)</td>
<td></td>
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<td></td>
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<tr>
<td>Invasive tumor size (pT)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>785 (72.0%)</td>
<td>134 (66.3%)</td>
<td>485 (72.6%)</td>
<td>166 (75.5%)</td>
<td>0.013b</td>
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<td>2</td>
<td>287 (26.3%)</td>
<td>60 (29.7%)</td>
<td>175 (26.2%)</td>
<td>52 (23.6%)</td>
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<td>3</td>
<td>16 (1.5%)</td>
<td>8 (4.0%)</td>
<td>6 (0.9%)</td>
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<td>4</td>
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<td>0</td>
<td>2 (0.3%)</td>
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<tr>
<td>Node status</td>
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<td></td>
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<tr>
<td>0</td>
<td>668 (61.4%)</td>
<td>116 (57.7%)</td>
<td>419 (62.8%)</td>
<td>133 (60.5%)</td>
<td>&gt;0.3a</td>
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<td>1–3</td>
<td>326 (30.0%)</td>
<td>65 (32.3%)</td>
<td>192 (28.8%)</td>
<td>69 (34.1%)</td>
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<tr>
<td>4–4</td>
<td>94 (8.6%)</td>
<td>20 (10.0%)</td>
<td>56 (8.4%)</td>
<td>18 (8.2%)</td>
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<td>Histologic grade</td>
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<tr>
<td>1</td>
<td>266 (24.4%)</td>
<td>43 (21.3%)</td>
<td>167 (25.0%)</td>
<td>56 (25.5%)</td>
<td>&gt;0.3a</td>
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<td>2</td>
<td>540 (49.6%)</td>
<td>110 (54.5%)</td>
<td>324 (48.6%)</td>
<td>106 (48.2%)</td>
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<tr>
<td>3</td>
<td>283 (26.0%)</td>
<td>49 (24.3%)</td>
<td>176 (26.4%)</td>
<td>58 (26.4%)</td>
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<tr>
<td>Ki67</td>
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<tr>
<td>Negative/low (&lt;20%)</td>
<td>300 (63.0%)</td>
<td>63 (63.0%)</td>
<td>173 (62.0%)</td>
<td>64 (66.0%)</td>
<td>&gt;0.3a</td>
</tr>
<tr>
<td>High (&gt;20%)</td>
<td>176 (37.0%)</td>
<td>37 (37.0%)</td>
<td>106 (38.0%)</td>
<td>33 (34.0%)</td>
<td></td>
</tr>
<tr>
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<td>614</td>
<td>102</td>
<td>189</td>
<td>123</td>
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<tr>
<td>Hormone receptor status</td>
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<tr>
<td>ER⁺</td>
<td>956 (87.9%)</td>
<td>185 (91.6%)</td>
<td>586 (88.1%)</td>
<td>185 (84.1%)</td>
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<tr>
<td>ER⁻</td>
<td>131 (12.1%)</td>
<td>17 (8.4%)</td>
<td>79 (11.9%)</td>
<td>35 (15.9%)</td>
<td></td>
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<tr>
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<td>3</td>
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<tr>
<td>PgR⁺</td>
<td>774 (71.2%)</td>
<td>139 (68.8%)</td>
<td>483 (72.6%)</td>
<td>152 (69.1%)</td>
<td>&gt;0.3a</td>
</tr>
<tr>
<td>PgR⁻</td>
<td>313 (28.8%)</td>
<td>63 (31.2%)</td>
<td>182 (27.4%)</td>
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<td>0</td>
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<tr>
<td>ER⁺ PgR⁺</td>
<td>767 (70.6%)</td>
<td>138 (68.3%)</td>
<td>479 (72%)</td>
<td>150 (68.2%)</td>
<td>&gt;0.5a</td>
</tr>
<tr>
<td>ER⁺ PgR⁻</td>
<td>189 (17.4%)</td>
<td>47 (23.5%)</td>
<td>107 (16.1%)</td>
<td>35 (15.9%)</td>
<td>0.052b</td>
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<td>ER⁻ PgR⁺</td>
<td>124 (11.4%)</td>
<td>16 (7.9%)</td>
<td>75 (11.3%)</td>
<td>33 (15.0%)</td>
<td>0.022b</td>
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<tr>
<td>ER⁻ PgR⁻</td>
<td>7 (0.6%)</td>
<td>1 (0.5%)</td>
<td>4 (0.6%)</td>
<td>2 (0.9%)</td>
<td>&gt;0.3a</td>
</tr>
<tr>
<td>Missing (n)</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

*aThe Jonckheere–Terpstra test.

*bThe linear-by-linear association test.

ER⁺ tumors may be more sensitive to the growth inhibiting properties of the coffee constituents.

Coffee constituents impair the cell-cycle progression in breast cancer cells

Having demonstrated that caffeine and caffceic acid suppressed the proliferation and viability of breast cancer cells, we further assessed their effect on cell-cycle distribution. Increasing doses of caffeine resulted in impaired G1 to S transition in both ER⁺ MCF-7 (P ≤ 0.003; Fig. 2A and C) and ER⁻ MDA-MB-231 (P ≤ 0.001; Fig. 2B and D) cells, with an accumulation of cells in G0–G1, a subsequent reduction of cells in S and G2–M phases and enhanced cell death. Although caffceic acid reduced MCF-7 and MDA-MB-231 cell densities (Fig. 2A and B), only minor modulations of the cell-cycle distribution by caffceic acid were observed in the ER⁺ (Fig. 2A and C) as well as ER⁻ cells (Fig. 2B and D).

Caffeine and caffceic acid alter hormone receptor levels and downstream effectors in breast cancer cells

The role of coffee constituents in modulating molecular mechanisms with impact on breast cancer cell growth in relation to ER status was further investigated. Exposure to caffeine significantly reduced the ER abundance in ER⁺ MCF-7 cells. In fact, 5 mmol/L caffeine almost completely abolished the ER levels.
Impact of Coffee on Breast Cancer

(Fig. 3A). Although an approximately 50% reduction of MCF-7 proliferation was obtained by caffeic acid treatment, the effect on ER abundance was less pronounced (Fig. 3A). The ER suppression was associated with a concomitant cyclin D1 decrease, and no detectable levels remained after 5 mmol/L caffeine exposure (Fig. 3A). In contrast, caffeine exposure resulted in reexpression of ER expression to the endogenous ER levels seen in untreated MCF-7 cells.

Caffeine exposure was further associated with a 70% and a 25% reduction in Akt phosphorylation was found for both cell types following caffeic acid exposure (Fig. 3B). However, caffeine did not under these conditions retranscribe ER protein expression to the endogenous ER levels seen in untreated MCF-7 cells.

Caffeine exposure was also associated with a 70% and a 25% reduction in IGFIR abundance in MCF-7 (Fig. 3B) and MDA-MB-231 cells (data not shown). In vitro data show that caffeine and caffeic acid attenuated the growth of predominantly ER tumors among women with breast cancer. Moreover, in the present study, mechanistic in vitro data show that the response to the coffee constituents were maintained and additive to the inhibitory effects of tamoxifen and caffeine or caffeic acid showed that the response to the coffee constituents were maintained and additive to the inhibitory effects of tamoxifen in both ER+ MCF-7 and T47D breast cancer cells (data not shown).

### Discussion

A growing body of literature indicates that modifiable dietary or lifestyle factors, such as coffee, may have a protective role against the risk or progression of breast cancer (14, 15, 17). In the present translational study, we demonstrate an association between increasing coffee consumption and significantly smaller invasive tumor sizes, whereas concomitantly lower proportion of ER+ tumors among women with breast cancer. Moreover, in the present study, mechanistic in vitro data show that the response to the coffee constituents were maintained and additive to the inhibitory effects of tamoxifen in both ER+ MCF-7 and T47D breast cancer cells (data not shown).

<table>
<thead>
<tr>
<th>Table 2. Selected tumor characteristics of 1,087 patients stratified by ER status in relation to daily coffee consumption (cups/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ER+ tumors (n = 956)</strong></td>
</tr>
<tr>
<td><strong>ER status</strong></td>
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<tr>
<td>Invasive tumor size (pT)</td>
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<td>4</td>
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<tr>
<td><strong>Ki67</strong></td>
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<tr>
<td>Negative/low (&lt;20%)</td>
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<tr>
<td>High (&gt;20%)</td>
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<tr>
<td>Missing (n)</td>
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<tr>
<td><strong>ER+ tumors (n = 131)</strong></td>
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<tr>
<td><strong>ER status</strong></td>
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<tr>
<td>Invasive tumor size (pT)</td>
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<td>High (&gt;20%)</td>
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<td>Missing (n)</td>
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</tbody>
</table>

Information on ER status was missing for 3 patients.

The linear-by-linear association test.
mixture of bioactive components, and there are several plausible biologic pathways whereby coffee might alter breast cancer risk or progression. Caffeine and the polyphenol constituents have been suggested to contribute to its anticancer activities. However, various coffee constituents may differentially affect ER$^+$ and ER$^-$ breast cancer subtypes. The present findings demonstrate smaller invasive tumor sizes, whereas concomitant lower proportion of ER$^+$ tumors and consequently higher proportion of ER$^-$ tumors with increasing coffee consumption. These findings from almost twice as many women, confirm the previous report from a subset of the present cohort (17). When stratifying according to ER status, the association between increasing coffee consumption and smaller invasive tumor size was only observed in ER$^+$ tumors, suggesting that coffee suppresses breast tumor growth, and that ER$^+$ tumors may be more sensitive to these effects. A previous report demonstrated a protective effect of caffeine intake on both ER$^+$ and ER$^-$ breast cancer risk in the Nurses' Health Study, although only significant for the ER$^+$ group (25). Although at the same time, no association with caffeine intake and overall breast cancer risk in the Women's Health Study was reported, the

Figure 1. Caffeine or caffeic acid reduces human breast cancer cell growth in vitro. ER$^+$ (MCF-7), ER$^-$ (MDA-MB-231), and Tam-R breast cancer cells were treated with the indicated concentrations of caffeine (Caff) or caffeic acid (CaA) in SFM for 48 (A and B) or 72 hours (C). Proliferation (A and B) was assessed using $[^3]$H]thymidine incorporation as a measurement of DNA synthesis. Total cell number and cell death were assessed by cell counting (A). The graphs represent the mean ± SE of three (A) or five (B) determinations; *, P < 0.05; **, P < 0.01; ***, P < 0.001, the Student t test. Total protein (C) was extracted and analyzed by immunoblotting for the expression of Bcl-2, Bcl-xL, cleaved caspase-7, and cleaved PARP. GAPDH is shown as loading control. One representative image from at least three independent experiments is shown.
The proportion of ER<sup>+</sup> tumors was decreased and the proportion ER<sup>-</sup> tumors increased (29). The shifted ER proportions are in line with both the clinical and experimental data in the present study.

Several studies have described caffeine and caffeic acid as anticarcinogenic agents in various cell culture and in vivo models (30, 31). For instance, in hepatocellular carcinoma and epidermal JB6 cells, caffeine attenuated cell growth via induction of G<sub>0</sub>–G<sub>1</sub> cell-cycle arrest (32, 33). Furthermore, caffeine-mediated inhibition of metastatic potential have been demonstrated for melanoma cells (34). Caffeine has also been shown to inhibit skin cancer in mice by enhancing UVB-induced apoptosis (35). The experimental doses used in the present study are equivalent to previous...
A  
MCF-7 (ER+)

ER  
Cyclin D1  
IGFIR  
pAkt  
Akt  
GAPDH

B  
MDA-MB-231 (ER-)

ER  
Cyclin D1  
IGFIR  
pAkt  
Akt  
GAPDH

Relative ER levels (% of control)  

Caff (mmol/L)  
CaA (μmol/L)  

Relative cyclin D1 levels (% of control)  

Caff (mmol/L)  
CaA (μmol/L)  

Relative IGFIR levels (% of control)  

Caff (mmol/L)  
CaA (μmol/L)  

Relative pAkt levels (% of control)  

Caff (mmol/L)  
CaA (μmol/L)  

Relative Akt levels (% of control)  

Caff (mmol/L)  
CaA (μmol/L)  

Relative GAPDH levels (% of control)  

Caff (mmol/L)  
CaA (μmol/L)
The adjusted HR was 0.51 (95% CI, 0.24 to a lesser extent caffeic acid, inhibited the proliferation of ER
in vitro
physiologic achievable systemic doses. Translating experimental
in vitro
cells was associated with a concomitant cyclin D1 reduction, impaired G1 to S cell-cycle transition, reduced growth, and induction of apoptosis. This is potentially important as cyclin D1 gene amplification and protein overexpression is associated with impaired tamoxifen response (43), and if coffee constituents modify cyclin D1 levels this may potentially augment tamoxifen treatment response. However, cyclin D1 appears to have contrasting effects in ER⁺ and ER⁻ cells. Although overexpression of cyclin D1 may promote treatment resistance and a metastatic phenotype in ER⁻ cells, it appears to decrease migration and mammosphere formation in ER⁺ breast cancer cells (43–45). Such contrasting effects may explain the divergent regulation of ER and cyclin D1 by caffeine in ER⁺ versus ER⁻ cells, both resulting in growth inhibition.

In addition to modulating ER and cyclin D1 levels, caffeine treatment dose-dependently downregulated the growth and anti-apoptotic IGFIR levels with subsequent impaired AktSer473 phosphorylation in both MCF-7 and MDA-MB-231 cells, which may partially contribute to the reduced cell proliferation observed. This is to our knowledge, the first study to show a direct effect of caffeine on IGFIR regulation. Previously, inhibition of IGFIR signaling by green tea polyphenols as measured by reduced pAkt and pErk1/2, has been shown to reduce prostate cancer progression and invasion in the TRAMP mouse model (11). The IGF and
estrogen growth regulatory pathways are tightly linked and compensatory mechanisms by the IGFIR have been suggested to mediate cell proliferation in tamoxifen-resistant tumors (46). Therapies targeting IGFIR may, thus, be of clinical benefit for treatment of such cancers. The association between increasing coffee consumption and reduced risk of early breast cancer recurrence among tamoxifen-treated women with ER+ tumors in the present study may mechanistically be linked to the altered ER and impaired IGFIR signaling by enhanced exposure to the coffee constituents. This could sensitize the tumor cells to further inhibitory effects by tamoxifen with improved clinical response rate. These findings are consistent with and support the previous report showing an association between increasing coffee consumption and significantly increased disease-free survival among tamoxifen-treated women with ER+ tumors, in a subset of the present cohort (17). The present cell work demonstrates that caffeine and caffeic acid have inhibitory effects that are at least additive with the effects of tamoxifen. It appears that the inhibitory effects of caffeine and caffeic acid on ER+ cells are maintained and not diminished when the ER is either inactive or effectively blocked. Furthermore, it is possible that coffee consumption could alter the pharmacokinetics of tamoxifen via, for example, CYP1A2 or CYP2C8 (17), but that would require examination in an appropriate in vivo model.

In summary, this study shows inhibitory effects by caffeine and caffeic acid on breast cancer cell growth. These results are in line with previous epidemiologic reports demonstrating protective roles of coffee constituents and the risk or progression of breast cancer. The present findings enhance the general understanding of how modifiable factors present in our daily diet, such as coffee constituents, may alter ER status, IGFIR levels, and contribute to reduced growth of ER+ and ER– breast cancer cells.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**References**


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**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** A.H. Rosendahl, A. Markkula, M. Simonsson, C. Ingvar, J.M.P. Holly, H. Jernstrom

**Study supervision:** A.H. Rosendahl, C. Ingvar, H. Jernstrom

**Other (Pl of the BC Blood study):** H. Jernstrom

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