Research Article

Cancer Prevention Research

Human Breast Tissue Disposition and Bioactivity of Limonene in Women with Early-Stage Breast Cancer

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Abstract

Limonene is a bioactive food component found in citrus peel oil that has shown chemopreventive and chemotherapeutic activities in preclinical studies. We conducted an open-label pilot clinical study to determine the human breast tissue disposition of limonene and its associated bioactivity. We recruited 43 women with newly diagnosed operable breast cancer electing to undergo surgical excision to take 2 grams of limonene daily for two to six weeks before surgery. Blood and breast tissue were collected to determine drug/metabolite concentrations and limonene-induced changes in systemic and tissue biomarkers of breast cancer risk or carcinogenesis. Limonene was found to preferentially concentrate in the breast tissue, reaching high tissue concentration (mean = 41.3 μg/g tissue), whereas the major active circulating metabolite, perillic acid, did not concentrate in the breast tissue. Limonene intervention resulted in a 22% reduction in cyclin D1 expression ($P = 0.002$) in tumor tissue but minimal changes in tissue Ki67 and cleaved caspase-3 expression. No significant changes in serum leptin, adiponectin, TGF-β1, insulin-like growth factor binding protein-3 (IGFBP-3), and interleukin-6 (IL-6) levels were observed following limonene intervention. There was a small but statistically significant postintervention increase in insulin-like growth factor I (IGF-I) levels. We conclude that limonene distributed extensively to human breast tissue and reduced breast tumor cyclin D1 expression that may lead to cell-cycle arrest and reduced cell proliferation. Furthermore, placebo-controlled clinical trials and translational research are warranted to establish limonene's role for breast cancer prevention or treatment. *Cancer Prev Res; 6(6); 577–84. ©2013 AACR.*

Introduction

Recent success in disease prevention among high-risk women with selective estrogen receptor (ER) modulators (SERM; refs. 1, 2) and aromatase inhibitors (3–5) has confirmed that breast cancer can be clinically targeted with chemopreventive agents before detectable disease. However, adoption of these agents in risk reduction for breast cancer in clinical practice for generally healthy women is still limited because of the side effects associated with these agents. Therefore, developing and testing chemopreventive agents that have minimal toxicity and higher tolerability for long-term usage in high risk, but otherwise healthy women, remains a major challenge. In addition, developing preventive agents with a broader spectrum of activity including action against ER-negative breast cancer remains a pressing issue.

Limonene, a monocyclic monoterpene, is a major component in the essential oils of citrus fruits. Limonene has exhibited chemopreventive activity against many cancer types with the most compelling results in mammary carcinogenesis models. In carcinogen-induced rat mammary carcinogenesis models, limonene fed during the promotion/progression stage inhibited the development of tumors induced by 7,12-dimethylbenz(a)anthracene (DMBA), which requires metabolic activation to its carcinogenic form, or tumors induced by N-methyl-N-nitrosourea (NMU), a directly acting carcinogen (6, 7). Dietary feeding of limonene also inhibited the development of *ras* oncogene-induced mammary carcinomas in rats (8). Limonene has also been shown to exert chemotherapeutic activity. Oral feeding of limonene resulted in significant regression of DMBA- or NMU-induced mammary carcinoma in a dose-dependent manner, without any observable systemic toxicity (9, 10).

Clinical development of monoterpenes has focused on a hydroxylated analog of limonene, perillyl alcohol. Multiple early-phase trials have been conducted with perillyl alcohol in advanced cancer patients (11–15) with a few reported cases of disease stabilization. It was concluded that clinical antitumor activity of perillyl alcohol is not likely to occur at safe doses. Perillyl alcohol undergoes extensive first-pass metabolism and is converted almost completely to 2 active but polar metabolites, perillic acid and dihydroperillic acid

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in humans (12–15). Because of their polarity, the tissue distribution of these metabolites may be limited, thus limiting their *in vivo* tissue activity. There is one reported phase I/II trial with limonene in patients with cancer with locally advanced or metastatic disease (16). The study reported a partial response in one patient with breast cancer and stabilization of the disease in 3 patients with colon cancer (16). Unlike perillyl alcohol, limonene is bioavailable in the systemic circulation in humans after oral administration (16). We, and others, have shown that limonene distributes favorably to adipose and mammary tissues in rodents (17, 18), likely due to its high lipophilicity. In addition, we have recently shown that limonene distributes extensively to adipose tissue in humans (19). Favorable distribution of limonene to the tissue may lead to better *in vivo* tissue bioactivity, suggesting that evaluation of the potential clinical activity of limonene deserves further attention.

We hypothesized that limonene would distribute extensively to human breast tissues and exert biologic activities that could lead to breast cancer prevention. To test this hypothesis, we conducted an open-label pilot study of limonene in women with a recent diagnosis of operable breast cancer electing to undergo excision surgery. The primary aim of the study was to determine the disposition of limonene in the breast tissue with secondary end points being measurement of modulation of tissue and systemic biomarkers related to breast cancer risk or carcinogenesis.

Materials and Methods

Study design

This study was an open-label, single-arm intervention trial. Patients with a diagnosis of early-stage breast cancer scheduled to undergo definitive surgery were recruited to receive short-term limonene intervention in a window period of 2 to 6 weeks before surgery; the duration of treatment was based on patient preferences for surgery scheduling. The primary objective was to determine the bioavailability of limonene in the breast tissue. The secondary objectives were to determine the effect of limonene intervention on markers of cell proliferation, apoptosis, and cell-cycle regulation in the breast tissue and on serum levels of insulin like growth factor I (IGF-I), IGF binding protein-3 (IGFBP-3), leptin, adiponectin, TGF-β1, and interleukin-6 (IL-6). This study was approved by the University of Arizona Institutional Review Board. Written informed consent was obtained from all participants.

Study drugs

A commercially available limonene product (Heartburn Free) was purchased from Enzymatic Therapy, Inc. This product is currently marketed as dietary supplement for heartburn relief. Each capsule contains 1 g of limonene with no other additives. Product quality assurance documents indicated that the limonene content conforms to the label and the product is stable for at least 36 months. The study product was stored at room temperature and protected from environmental extremes during the study conduct.

Study participants

Study participants were recruited from the University of Arizona Cancer Center, Breast Surgical Oncology Clinic (Tucson, AZ). Participants were more than 18 years of age, elected to undergo excision surgery for a recent diagnosis of operable breast cancer, had no clinical evidence of metastatic breast cancer, had an Eastern Cooperative Oncology Group performance status 0–1, and had normal hepatic, renal, and marrow function. Participants were excluded if they were on treatment for chemotherapy or radiotherapy, used selective ER modifiers or aromatase inhibitors within the past 3 months, had a history of other malignancies within the past 5 years excluding nonmelanoma skin cancer and cancers confined to organs with removal as only treatment, had participated in another clinical intervention trial within the past 3 months, had uncontrolled concurrent illness, were pregnant or breast-feeding, and used dietary supplement that contains large amounts of limonene within the past 3 months.

Study procedures

At the baseline visit, participants underwent the following procedures and assessments: weight, height, a blood sample for routine labs and for determination of baseline serum drug and biomarker levels, a complete medical history, concomitant medications, vital signs, and a urine pregnancy test, if applicable. Upon determination of eligibility (20 ± 8.4 days from diagnostic tumor biopsy), participants took 2 study capsules (2 g limonene) once daily with food for 2 to 6 weeks until the day before surgery and were instructed not to change their dietary habits and not to take any supplement that contains large amounts of limonene. Within 3 days before surgery, participants underwent the following procedures or assessments: a blood sample for routine labs and for determination of postintervention serum drug/metabolite and biomarker levels, concomitant medications, vital signs, a urine pregnancy test if applicable, and adverse event assessment. The National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0 was used for adverse event description and grading. At surgery, the surgical specimen was handled according to the Institution standard for pathology review, and a small section of adjacent grossly normal tissue was collected and flash-frozen for tissue drug/metabolite concentration determination. Formalin-fixed, paraffin-embedded tissue blocks from each subject's diagnostic core biopsy and surgical specimen were requested from the pathology department for tissue biomarker analysis. Participants were followed for 2 weeks after surgery to capture any study-related side effects.

Analysis of limonene and perillic acid levels in tissue and serum

Limonene and perillic acid levels in breast tissue and serum were analyzed by specific chromatography-based assays (20, 21). Briefly, limonene levels in serum and tissue were quantified using gas chromatography-mass spectrometry (GC-MS). Chromatographic separation of limonene

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and internal standard was achieved on a high resolution GC DB-5MS fused silica capillary column (Agilent Technologies, Santa Clara) with a temperature gradient. Limonene and internal standard were detected by selective ion monitoring for target ions 93 m/z and 107 m/z, respectively. Quantification of perillic acid was achieved using liquid chromatography-MS. Chromatographic separation was achieved on a BDS Hypersil C18 column (Phenomenex, Thermo Electron) and a linear gradient of 2 mobile phases. Perillic acid was detected by selective ion monitoring for target ion 165 m/z.

Immunohistochemistry for tissue biomarkers

Immunohistochemistry (IHC) assays were used to assess markers of cell proliferation (Ki67), apoptosis (cleaved caspase-3, CC3), and cell-cycle regulation (cyclin D1) in diagnostic and surgical tissue sections. A precision microtome was used to prepare 3 μm sections on coated slides for each specimen. The IHC was conducted on a Discovery XT Automated Immunostainer (VMSI - Ventana Medical Systems) using VMSI-validated reagents, including deparaffinization, antigen retrieval with a borate-EDTA buffer, primary antibody staining, detection and amplification, and hematoxylin counterstaining. A biotin-free 3,3′ diaminobenzidine (DAB) detection system was used for CC3 and cyclin D1 and a biotinylated–streptavidin–horseradish peroxidase and DAB system was used for Ki67.

For Ki67, mouse monoclonal antibody (clone: MIB-1, Dako) was diluted 1:100. Human tonsil carcinoma was used as a positive control. For CC3, anti-CC3 rabbit polyclonal antibody (Cell Signaling Technology #9661L) was diluted 1:8,000. Human tonsil carcinoma was used as a positive control. For cyclin D1, rabbit monoclonal antibody (clone: SR4-R, VMSI) in a prediluted dispenser was used. Human breast carcinoma was used as a positive control.

On the IHC slides, the tumor region was marked by an expert board-certified pathologist (R. Nagle), and the marker expression was quantified by Aperio Spectrum software (Aperio Technologies). The Ki67 and CC3 expression was assessed by percentage of positively stained nuclei. Cyclin D1 expression was assessed by the long score, which is calculated by percentage of positively stained nuclei multiplied by staining intensity (staining intensity was scored on a scale of 0–3, where 0 was no staining, 1 was weak, 2 was moderate, and 3 was strong). The Aperio IHC Nuclear Image Analysis software detects the nuclear staining for a target chromogen for the individual cells in selected regions and quantifies their intensity based on a validated algorithm (22).

Serum biomarker analysis

Leptin, adiponectin, IGF-I, IGFBP-3, TGF-β1, and IL-6 were all quantified using ELISA-based immunoassays (R&D Systems). Serum samples were diluted before analysis according to manufacturer instructions for leptin, IGF-I, TGF-β1, and IL-6, and were diluted 1:200 for the adiponectin assay. Assays were linear over the following concentration ranges; leptin: 15.6 pg/mL–1,000 pg/mL; adiponectin: 3.9–250 ng/mL; IGF-I: 0.094–6.0 ng/mL; IGFBP-3: 0.781–50 ng/mL, TGF-β1: 31.2–2,000 pg/mL; IL-6: 0.156–10 pg/mL. For each assay, baseline and postintervention samples of the same individual were analyzed in the same batch and each sample was analyzed in duplicate.

Statistical methods

For each of the biomarkers, descriptive statistics (i.e., median and interquartile range (IQR) were conducted on the measurements at baseline and the changes from baseline to postintervention. The distributions for some of the biomarkers were not symmetrical. Therefore, a 2-sided signed rank test was conducted to test whether the changes from baseline to postintervention are significantly different from zero and a 2-sided Wilcoxon rank-sum test was conducted to test whether the baseline levels and the changes are significantly different between groups (e.g., ER− vs. ER+). Significance level was set at 5% for all tests.

We have chosen not to adjust these analyses for multiple comparisons as our goal was to identify the biomarkers for subsequent studies.

Results

Between Aug 19, 2009 and March 2, 2011, 59 women were consented to the study. Fifteen did not meet final eligibility and one withdrew consent. Forty-three women initiated the limonene intervention, 3 terminated the intervention early due to adverse events, 1 due to heartburn, 1 because of mild nausea and vomiting, and 1 because of abdominal bloating and cramping as well as heartburn. In total, 40 women completed the intervention. The mean treatment duration was 21.5 ± 8.8 days for women completing the intervention. Table 1 summarizes the baseline subject and tumor characteristics for women completing the intervention. The average age was 58.3 ± 11.0 years and body mass index (BMI) was 30.8 ± 7.6 kg/m^2. There were 14 and 26 pre- and postmenopausal women, respectively. Tumors were on average 13.2 ± 10.7 mm.

Limonene and perillic acid concentrations in pre- and postintervention serum samples and postintervention breast tissue were determined using specific chromatographic assays. Limonene and perillic acid were not detectible in preintervention serum. We did not collect flash-frozen tissue from our participants at baseline to determine preintervention tissue limonene concentration because this would have imposed an additional invasive procedure. Figure 1 illustrates postintervention limonene and perillic acid levels in serum and breast tissue. The mean serum limonene and perillic acid concentration was 0.07 ± 0.09 and 0.65 ± 1.13 μg/mL plasma (0.48 ± 0.67 and 3.89 ± 6.81 μmol/L), respectively. The mean breast tissue limonene and perillic acid concentration was 41.3 ± 49.9 and 0.57 ± 1.48 μg/g tissue (332.3 ± 336.1 and 5.72 ± 10.28 μmol/L), respectively. Average dietary exposure to limonene is estimated to be 0.12 mg/kg/day for the U.S. population with additional trace amounts from environmental exposure (23), however, whether this

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Table 1. Demographics and tumor characteristics for participants completing the limonene intervention ($N = 40$)

Age, y	58.5 ± 18.5[a]
BMI, kg/m^2	28.9 ± 8.7
Menopausal status (pre/post)	
Premenopausal	14
Postmenopausal	26
Tumor size, mm	13.2 ± 10.7
Tumor stage (IS/T1/T2)	
IS	13
T1	24
T2	3
Node positive, *n* (%)	10 (25%)
ER	
Positive[b]	29
Negative	7
NA[c]	4
PR	
Positive[b]	22
Negative	14
NA[c]	4

[a]Median ± IQR.
[b]>5%.
[c]Not available.

results in high cumulative breast disposition of limonene is unknown. Flash-frozen breast tissue from 17 women who did not participate in the limonene intervention trial was analyzed to assess basal limonene tissue levels. Of these 17, there were 10 patients with invasive ductile carcinoma, 1 with DCIS, and 6 with benign disease. The average age of these women was 56.7 ± 12.0 years, which was not significantly different from our participants. Low levels of limonene were detected in these samples, averaging 0.08 ± 0.13 μg/g, confirming that the high tissue limonene concentration after the limonene intervention is derived from the intervention agent.

Figure 2 illustrates tissue marker expression from 29 limonene intervention participants who had sufficient residual tumor in both the diagnostic and surgical tissue sections for biomarker evaluation. Cyclin D1 expression decreased significantly from a long score of 107.1 ± 79.4 in the diagnostic biopsy collected before the limonene intervention to 79.7 ± 70.7 in the surgical specimen collected after the limonene intervention ($P = 0.002$). There were 6 participants on hormone replacement therapy (HRT) at study entry, 3 of whom discontinued the HRT at the start of the intervention. These 3 women had a large decrease in cyclin D1 expression (69.2% decrease); however, there was still a statistically significant overall decrease in cyclin D1 after excluding those participants who discontinued HRT from the analysis ($P = 0.018$). Minimum changes were observed in Ki67 expression (5.44 ± 8.0% vs. 6.68 ± 14.1%) and CC3 expression (1.85 ± 3.16% vs. 1.61 ± 2.35%). Current or past hormonal therapy and/or oral contraceptive use did not seem to be related to tissue biomarker expression at study entry (data not shown). We also conducted exploratory subgroup analyses based on menopausal status, ER and progesterone receptor (PR) status, and BMI. There were no significant postintervention changes in Ki67 or CC3 in the subgroup analyses (data not shown). Table 2 summarizes the exploratory subgroup analyses for cyclin D1. Cyclin D1 expression decreased significantly ($P = 0.007$) in postmenopausal women but was unchanged in premenopausal women. Cyclin D1 decreased significantly in ER-positive women ($P = 0.006$). A decrease in cyclin D1 was also observed in ER-negative women; however, this change was not statistically significant ($P = 0.125$), probably due to the small sample size. Cyclin D1 decreased significantly in both PR-positive and negative women. Cyclin D1 decreased significantly in women with BMI less than or equal to the median BMI of 28.94 kg/m^2 ($P = 0.003$) but did not change significantly in women with BMI greater than the median BMI.

Table 3 summarizes the serum biomarker data from 39 participants who had both the pre- and postintervention serum samples. Baseline serum biomarker levels are reported as medians (±IQR) and were as follows; leptin: 31.76 (±19.99) ng/mL; adiponectin: 9,103 (±7,137) ng/mL; IGF-I: 62.11 (±18.50) ng/mL; IGFBP-3: 1,903

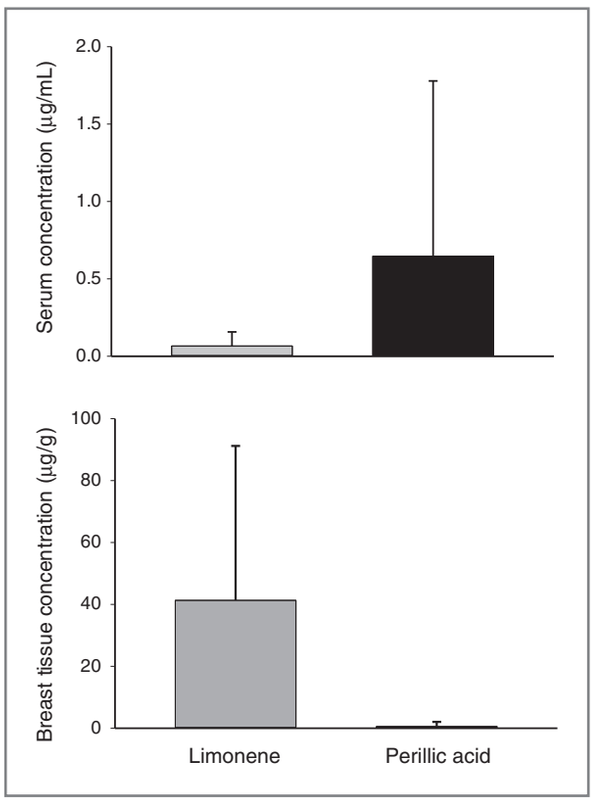

Figure 1. Serum and breast tissue levels of limonene and its major circulating metabolite, perillic acid, after 2 to 6 weeks 2 g oral limonene administration daily.

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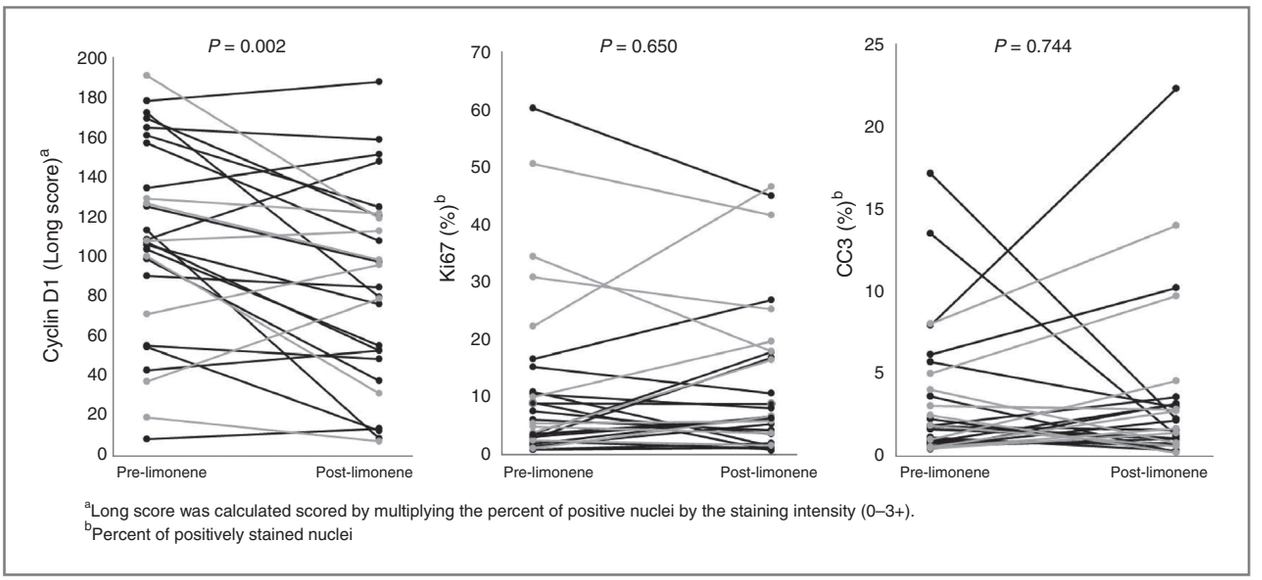

Figure 2. Cyclin D1, Ki67, and Cleaved Caspase 3 (CC3) as measured at baseline (pre-limonene) and at surgery (post-limonene). Gray lines represent data from premenopausal women and black lines represent data from postmenopausal women.

(±739) ng/mL; IGF-I/IGFBP-3 ratio: 0.033 (±0.010); TGF-β1: 38,799 (±10,4952) pg/mL; IL-6; 2.10 (±2.26) pg/mL. IGF-I and the IGF-I/IGFBP-3 ratio were both significantly increased as a result of the intervention ($P = 0.009$ and 0.02, respectively).

Table 4 lists possibly or probably related adverse events from all women who initiated limonene intervention. Events were primarily related to gastrointestinal symptoms. The most commonly observed symptom was described as "citrus burps" and was experienced by 56% of the participants. Diarrhea was also experienced by 28% of the participants and nausea by 16%. Events were mostly mild and were not dose limiting.

Discussion

In this single-arm, open-label trial of limonene in patients with early-stage breast cancer, we found that taking 2 g of limonene daily for 2 to 6 weeks gave rise to high breast tissue limonene concentrations. An average breast tissue limonene concentration of 41.3 μg/g tissue (332.3 μmol/L)

Table 2. Changes in cyclin D1 expression, stratified by ER/PR status, menopausal status, and BMI at baseline

Cyclin D1	Baseline expression	Change	P^b
Premenopausal ($N = 10$)	85.5 ± 84.4[a]	−9.6 ± 32.9	0.232
Postmenopausal ($N = 19$)	108.8 ± 70.5	−29.8 ± 54.2	0.007
P^c	0.271	0.455	
ER(−; $N = 5$)[d]	42.2 ± 79.3	−13.3 ± 16.1	0.125
ER(+; $N = 23$)	108.1 ± 89.7	−27.7 ± 53.8	0.006
P	0.051	1.000	
PR(−; $N = 8$)[d]	76.9 ± 76.2	−12.7 ± 28.1	0.039
PR(+; $N = 20$)	110.9 ± 82.1	−31.8 ± 54.0	0.012
P	0.055	0.706	
BMI ≤ median[e] ($N = 16$)	106.5 ± 80.9	−32.81 ± 52.6	0.003
BMI > median ($N = 13$)	108.1 ± 79.4	−5.8 ± 22.9	0.497
P	0.914	0.273	

[a]Median ± IQR.
[b]Derived from signed rank test.
[c]Derived from Wilcoxon rank sum test.
[d]Negative ≤5% expression.
[e]Median of 28.94 kg/m^2.

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Table 3. Changes in serum biomarkers ($N = 39$)[a]

	Baseline	Change	P^b
Leptin, ng/mL	31.76 ± 19.99	−0.38 ± 9.11	0.870
Adiponectin, ng/mL	9,103.1 ± 7137	−167.6 ± 2665	0.795
IGF-I, ng/mL	61.34 ± 31.45	5.34 ± 16.59	0.005
IGFBP-3, ng/mL	1,902 ± 739	39.8 ± 371.5	0.349
IGF-I/IGFBP-3	0.031 ± 0.009	0.002 ± 0.009	0.020
TGF-β1, pg/mL	38,799 ± 10,492	−1,134.4 ± 5128	0.153
IL-6, pg/mL	2.10 ± 2.26	−0.18 ± 1.04	0.089

[a]Median ± IQR.
[b]Derived from signed rank test.

was observed with several participants reaching low mg/g tissue concentrations. However, the major circulating metabolite, perillic acid, was observed in tissue concentrations one hundredth that of limonene. Preferential concentration of limonene but not its major circulating metabolite, perillic acid, in the breast tissue is consistent with our hypothesis. Our data suggests that circulating drug levels may not be reflective of the target tissue drug concentrations. Importantly, our data provided target tissue drug levels that should be considered for future translational research on monoterpenes. *In vitro* studies have shown that low mmol/L levels of limonene resulted in inhibition of mammary tumor cell growth (24) and G protein prenylation (25, 26).

Our study also showed that limonene intervention resulted in a significant reduction in breast tumor cyclin D1 expression but had minimal effects on markers of cell proliferation and apoptosis. Interestingly, discontinuation of HRT in 3 participants was related to a striking decrease in cyclin D1 in the postintervention specimens. Baseline cyclin D1 expression was not related to HRT use; however, and several participants not on HRT at baseline had a decrease in cyclin D1 expression similar to that of those who discontinued HRT use. Cyclin D1 expression also seemed to be related to clinical factors, such as menopausal status and ER/PR status, but not BMI. Cyclin D1 plays a critical role in regulating cell-cycle progression. Overexpression of cyclin D1 accelerates passage through the G_1 phase of the cell cycle (27), suggesting that increased expression may lead to the loss of normal regulatory constraints and confer a growth advantage. Cyclin D1 is overexpressed in hyperplasia and intraductal carcinoma of the breast (28, 29), suggestive of its importance in the earliest stages of carcinogenesis. Reduction in cyclin D1 expression could lead to cell-cycle arrest at the G_1 phase, resulting in inhibition of tumor cell proliferation. In human breast cancer cells, cyclin D1 expression is modulated in response to mitogens, growth factors, and antiestrogens, with changes in cyclin D1 expression preceding changes in cell proliferation (30). In addition, limonene has been shown to inhibit proliferation through a cyclin D1-dependent mechanism in breast cancer cell lines (31). It is important to note that observed changes in cyclin D1 expression in this single-arm trial could be related to differences in tissue collection and processing procedures for the biopsy and surgical specimens. Therefore, limonene-induced changes in cyclin D1 expression should be validated in future placebo-controlled trials.

We did not observe a concurrent decrease in the proliferation marker Ki67 with cyclin D1. It is likely that cyclin D1 is an earlier response indicator for short-term limonene intervention and a longer intervention may be necessary to observe any changes in the proliferation marker, Ki67. Furthermore, our baseline Ki67 measurement was on average 11.6%, which may be too low to observe any changes. Smith and colleagues and Dowsett and colleagues reported higher Ki67 expression for breast cancers on neoadjuvant therapy (32, 33); our lower value probably represents selection bias in that we specifically excluded from our study patients treated with neoadjuvant therapy and less

Table 4. Summary of possibly or probably related adverse events from all participants who initiated limonene intervention ($N = 43$)[a]

	n (%)	
Adverse event	**Grade 1**	**Grade 2**
Citrus burps	23 (53.4)	1 (2.3)
Diarrhea	8 (18.6)	4 (9.3)
Nausea	6 (14.0)	1 (2.3)
Flatulence	7 (16.3)	0
Heartburn	1 (2.3)	4 (9.3)
Cramping	0	4 (9.3)
Vomiting	0	3 (7.0)
Taste disturbance	2 (4.7)	0
Bloating	0	1 (2.3)
Constipation	1 (2.3)	0
Dry mouth	1 (2.3)	0
Pruritis	1 (2.3)	0
Hypokalemia	1 (2.3)	0
Hypochloremia	1 (2.3)	0

[a]All events were mild or moderate grade.

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aggressive disease. In future, placebo-controlled trials of limonene are conducted with Ki67 as a primary end point, recruitment for participants with more advanced disease could be considered.

We assessed the limonene effect on serum biomarkers that have been shown to be associated with breast cancer risk and could potentially be modulated by limonene intervention. One of the better-characterized limonene-associated bioactivities in preclinical studies is induction of mannose-6-phosphate/insulin-like growth factor II receptor and activation of the TGF-β1 signaling pathway (34). Therefore, we assessed the serum levels of TGF-β1 and IGF-axis. IL-6 is a proinflammatory cytokine, which seems to be implicated in the promotion of breast cancer (35). In macrophage cell culture, limonene suppressed IL-6 production in a dose-dependent manner (36). Limonene has also shown immunomodulating effects in rodents (37–39) and lymphoma animal models (40). However, serum biomarkers assessed in our study were in general not changed with limonene treatment. There was a small but statistically significant overall increase in IGF-I and IGF-I/IGFBP ratio. The clinical significance of such changes is not known. Longer treatment duration may be necessary to see an intervention effect on these systemic biomarkers.

The study used the "presurgical" or "window-of-opportunity" study design because a small piece of breast tissue can be collected for direct measurement of limonene concentration in the target tissue without subjecting women to additional invasive tissue acquisition procedures. In addition, tissue sections from diagnostic biopsy and surgical specimen can be requested from pathology for tissue biomarker evaluation. We did not incorporate a placebo arm in this pilot study because the primary end point of the trial is limonene bioavailability in breast tissue and the contribution from nonintervention limonene exposure to the primary end point is minimum compared with the contribution from the intervention dose. A potential limitation of this design is the short intervention duration. Nevertheless, 2 to 6 weeks of limonene intervention gave rise to high breast tissue limonene concentrations. However, the intervention duration may be too short to observe changes in tissue and serum biomarkers.

Overall, this study showed that limonene preferentially concentrated in breast tissue compared with plasma. Perillic acid did not readily concentrate in breast tissue; this differential disposition of these monoterpenes indicates that perillic acid most likely has limited bioactivity in the target tissue. Limonene intervention in patients with early-stage breast cancer was safe and well tolerated. Short-term limonene intervention resulted in a significant decrease in cyclin D1 expression in tumor tissue but resulted in minimal changes in tissue Ki67, CC3 or systemic biomarkers. Further randomized, placebo-controlled trials are warranted to determine limonene's potential role in prevention or treatment of breast cancer.

Disclosure of Potential Conflicts of Interest

J.E. Lang has honoraria from speakers' bureau of Genomic Health and Aptiv Solutions and is a consultant/advisory board member of Guidepoint Global. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.E. Lang, M. Ley, H-H. Sherry Chow
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.A. Miller, R. Nagle, C.-H. Hsu, P.A. Thompson, C. Cordova, H-H. Sherry Chow
Writing, review, and/or revision of the manuscript: J.A. Miller, J.E. Lang, P.A. Thompson, A. Waer, H-H. Sherry Chow
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Cordova
Study supervision: J.E. Lang, H-H. Sherry Chow

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