

Plasma Metabolomic Profiles of Breast Cancer Patients after Short-term Limonene Intervention

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Abstract

Limonene is a lipophilic monoterpene found in high levels in citrus peel. Limonene demonstrates anticancer properties in pre-clinical models with effects on multiple cellular targets at varying potency. While of interest as a cancer chemopreventive, the biologic activity of limonene in humans is poorly understood. We conducted metabolite profiling in 39 paired (pre/postintervention) plasma samples from early-stage breast cancer patients receiving limonene treatment (2 g QD) before surgical resection of their tumor. Metabolite profiling was conducted using ultra-performance liquid chromatography coupled to a linear trap quadrupole system and gas chromatography-mass spectrometry. Metabolites were identified by comparison of ion features in samples to a standard reference library. Pathway-based interpretation was conducted using the human metabolome database and the MetaCyc database. Of the 397 named metabolites identified,

72 changed significantly with limonene intervention. Class-based changes included significant decreases in adrenal steroids ($P < 0.01$), and significant increases in bile acids ($P \leq 0.05$) and multiple collagen breakdown products ($P < 0.001$). The pattern of changes also suggested alterations in glucose metabolism. There were 47 metabolites whose change with intervention was significantly correlated to a decrease in cyclin D1, a cell-cycle regulatory protein, in patient tumor tissues ($P \leq 0.05$). Here, oral administration of limonene resulted in significant changes in several metabolic pathways. Furthermore, pathway-based changes were related to the change in tissue level cyclin D1 expression. Future controlled clinical trials with limonene are necessary to determine the potential role and mechanisms of limonene in the breast cancer prevention setting. *Cancer Prev Res*; 8(1); 86–93. ©2014 AACR.

Introduction

Limonene is a monocyclic monoterpene and the major component in the essential oils of citrus fruits. Extensive preclinical evidence supports a number of anticancer properties of limonene with the most consistent being prevention in experimental models of mammary carcinogenesis. In carcinogen-induced rat mammary tumor models, limonene fed during the promotion/progression stage inhibits the development of tumors induced by 7,12-dimethylbenz(α)anthracene (DMBA) as well as tumors induced by *N*-methyl-*N*-nitrosourea (NMU; refs. 1, 2). Dietary feeding of limonene also inhibits the development of *ras* oncogene-induced mammary carcinomas in rats (3). In addition to demonstrating activity to inhibit tumor development, limonene has been investigated for chemotherapeutic activity. Oral feeding of limonene has been shown to induce a dose-dependent regres-

sion of DMBA- and NMU-induced mammary tumors without observable systemic toxicity (4, 5).

In rodent models of different solid tumors, limonene has been reported to exhibit effects on a number of the cancer hallmarks (i.e., proliferation, apoptosis, inflammation) with the exact mechanism of action unknown (6). Dietary feeding of limonene has also been shown to modulate carcinogen-metabolizing enzymes in rats, affecting the detoxification of chemical carcinogens (7). The ability of limonene to inhibit proliferation has been attributed to effects on isoprenylation of small proteins in the molecular weight range of 21,000 to 26,000 Da that include members of the Ras family of geranylpyrophosphate-binding proteins that regulate cell growth and are commonly deregulated in human cancers. Furthermore, several studies have reported changes in gene and protein expression in monoterpene-treated tumors undergoing regression (8). Of these, induction of mannose-6-phosphate/insulin-like growth factor II receptor and activation of the transforming growth factor β 1 (TGF β 1) signaling pathway are among the best described (9). More recently, Yoon and colleagues demonstrated that limonene inhibits prostaglandin E_2 production in macrophages (10) while d'Alesso showed that limonene exerts anti-inflammatory properties in preclinical models and in humans (11). Other preclinical evidence indicates that limonene may enhance immune response and act generally as an immune modulator (12).

Clinical development of monoterpenes has focused on the limonene analogue, perillyl alcohol in advanced cancer patients (13–17). The underwhelming results of these trials reduced enthusiasm for limonene development. Unlike perillyl alcohol, limonene distributes favorably to adipose tissue in rodents

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(18, 19) and humans (20) resulting in tissue levels comparable with the active concentrations in preclinical models. Favorable distribution of limonene to adipose tissue suggests that evaluation of limonene's potential clinical activity deserves further attention, particularly within the context of cancers arising from organs with high adiposity such as breast.

We have recently completed a presurgical study of limonene in women with newly diagnosed operable breast cancer to determine the breast tissue disposition of limonene and its associated bioactivity (21). In the completed trial, 2 g of oral limonene daily for 2 to 6 weeks resulted in low micromolar limonene concentrations in the breast tissue that was associated with a significant 22% reduction in cyclin D1 expression in tumor tissue (21). Overexpression of cyclin D1 promotes the transition of cells out of the G₁ and into the cell cycle (22) and is commonly overexpressed and deregulated early in breast tumorigenesis in humans (23, 24). To gain further insights into the *in vivo* activity of limonene and to identify blood correlates of limonene effect at the tissue level, we conducted an analysis of plasma metabolites using samples collected from our recently completed trial and correlated our results with cyclin D1 tissue level changes.

Materials and Methods

Clinical study

Details of the clinical study and main study findings are published elsewhere (21). Briefly, we accrued 43 women with newly diagnosed operable breast cancer to take 2 g of limonene daily for 2 to 6 weeks before voluntary surgical excision of their tumor. Forty women completed the intervention. Blood and breast tissue were collected to determine limonene and metabolite concentrations and limonene-induced changes in systemic and tissue biomarkers of breast cancer risk or carcinogenesis. Expression of the cell-cycle regulator (cyclin D1) in diagnostic and surgical tissue sections was assessed using immunohistochemistry (IHC). Positively stained nuclei were quantified using Aperio Spectrum (Aperio Technologies; ref. 25), and software performance was validated by a trained pathologist. After completion of the aims of the original trial cited above, 39 pairs of plasma samples collected before and after limonene intervention were available for metabolomic profiling.

Metabolomic profiling

Metabolomic studies were conducted at Metabolon Inc. on nontargeted platforms that enable relative quantitative analysis of a broad spectrum of molecules with a high degree of confidence (26). All samples were shipped on dry ice to Metabolon, Inc., were assigned unique identifiers to track samples during processing, and were stored at -80°C until processed. For sample preparation, proteins were precipitated from the plasma with methanol that contained standards to report extraction efficiency. The resulting supernatant was split into equal parts for analysis on the three platforms. Detailed descriptions of the instrumentation configurations and conditions, data acquisition, and software approaches for data handling, were previously described (26, 27). Briefly, samples destined for gas chromatography (GC)-mass spectrometry (MS) analysis were dried under vacuum desiccation for a minimum of 24 hours and then derivatized under nitrogen using bistrimethyl-silyl-trifluoroacetamide. Samples were analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer (Thermo Fisher Sci-

entific Inc.) using electron impact ionization. Ultra-performance liquid chromatography (UPLC)/MS-MS² was carried out using a Waters Acquity UPLC (Waters Corporation) coupled to a linear trap quadrupole MS (Thermo Fisher Scientific Inc.) equipped with an electrospray ionization source. Two separate UPLC/MS-MS² injections were performed on each sample: one optimized for positive ions and one for negative ions. Metabolites were identified by automated comparison of the ion features in the experimental samples to an in-house reference library composed of more than 2,400 authentic chemical standard entries that included retention time, molecular weight (*m/z*), preferred adducts, and in-source fragments as well as their associated MS-MS² spectra. This library allowed the rapid identification of metabolites in the experiment with high confidence.

Instrument variability was determined by calculating the median relative standard deviation (RSD) for the internal standards that were added to each sample before injection into the mass spectrometers and was found to be 6%. A homogenous pool containing a small amount of all study samples was included in the analysis as technical replicate samples. Overall process variability was determined by calculating the median RSD for all endogenous metabolites (i.e., noninstrument standards) present in the technical replicates and was found to be 12%.

Statistical analysis

Missing values for a given metabolite were imputed with the observed minimum detection value based on the assumption that they were below the limits of instrument detection sensitivity. All comparisons were performed using log-transformed data. All statistical analyses were performed using the "R" program v2.14.2 (R Foundation, <http://cran.r-project.org/>; ref. 28). Paired two sample *t* tests were used for all comparisons unless otherwise noted. The FDR method was used to correct for multiple comparisons; these estimated *Q*-values are reported (29). For convenience of data visualization, raw area counts for each biochemical were rescaled by dividing the value for a specific biochemical in each sample by the median value for that specific biochemical. Correlations changes in metabolites, represented as the posttreatment/pre-treatment ratio, were compared with posttreatment/pre-treatment changes in cyclin D1 using Spearman correlation. Pathway-based interpretation of biochemicals were constructed using MetaCyc (30) and the human metabolome database (31).

Results

Individual metabolite changes

Global plasma metabolomic profiling was conducted using a combination of high-throughput LC- and GC-based MS on a total of 39 paired plasma samples collected from women on a short-term oral limonene intervention before surgery. Patient, tumor, and treatment characteristics have been previously reported (21). A total of 397 named biochemicals were detected using a standard reference library. Table 1 summarizes metabolites that changed with limonene intervention. Because this is an effort to generate hypotheses for limonene's systemic activity, a $Q \leq 0.10$ after FDR correction was considered significant. All metabolites, however, with $P \leq 0.05$ are listed along with the fold change to capture pathway-based change. Of the identified biochemicals, plasma levels of 72 changed with $P \leq 0.05$ (42 rose and 30 fell); of these, 39 survived correction for false discovery given a tolerance of $Q \leq 0.10$ (19 rose and 20 fell). Of the metabolites that changed with

Table 1. Metabolites that changed after 2 to 6 weeks daily limonene intervention (72 metabolites $P \leq 0.05$; 39 metabolites $Q \leq 0.10$)

	Fold change	P	Q
Adrenal steroids			
Androsterone-S	0.75	<0.001	<0.001
Epiandrosterone-S	0.79	<0.001	<0.001
Pregnenolol disulfate	0.82	<0.001	0.01
4-androsten-3 β ,17 β diol disulfate 2	0.90	0.01	0.06
DHEA-S	0.93	0.02	0.12
Secondary bile acids			
Glycodeoxycholate	2.30	<0.01	0.02
Glycoursodeoxycholate	3.60	<0.01	0.03
Taurodeoxycholate	3.50	<0.01	0.03
Taurocholate	3.40	<0.01	0.03
Taurochenodeoxycholate sulfate	0.90	0.01	0.06
Glychenodeoxycholate	2.50	0.01	0.02
Glycocholate	2.60	0.02	0.10
Taurochenodeoxycholate	2.00	0.02	0.12
Deoxycholate	1.70	0.05	0.18
Glucose/energy metabolism			
Glucuronate	1.83	<0.01	0.03
Pyruvate	1.66	<0.01	0.03
Acetylcarnitine	0.89	<0.01	0.03
3-hydroxybutyrate (BHBA)	1.09	0.01	0.06
Fructose	1.56	0.02	0.10
Mannitol	4.87	0.03	0.14
Citrate	0.94	0.04	0.17
Malate	1.38	0.04	0.17
Collagen breakdown products			
Glycine	1.38	0.01	0.01
Proline	1.20	<0.001	0.01
Trans-4-hydroxyproline	1.70	<0.001	0.01
Pro-hydroxy-pro	1.40	0.02	0.11
Amino acids and metabolites			
Indolepropionate	2.60	<0.001	<0.001
Alanine	1.21	<0.001	0.02
3-phenylpropionate (hydrocinnamate)	1.95	<0.001	0.01
3-(4-hydroxyphenyl)lactate	0.87	<0.01	0.03
Kynurenate	0.87	<0.01	0.04
Phenylacetate	2.08	0.01	0.06
γ -glutamylisoleucine	0.91	0.01	0.07
α -hydroxybutyrate (AHB)	0.90	0.01	0.08
Phenylalanyltryptophan	0.92	0.01	0.08
Aspartate	0.93	0.02	0.12
N-acetylglycine	1.41	0.03	0.13
γ -glutamylalanine	1.15	0.03	0.13
α -hydroxyisovalerate	0.93	0.03	0.13
Aspartylphenylalanine	1.09	0.03	0.13
Isoleucylvaline	1.47	0.03	0.15
Phenylalanine	0.95	0.04	0.13
Isobutyrylcarnitine	1.48	0.05	0.18
Fatty acids			
Tetradecanedioate	0.84	<0.001	0.01
Hexadecanedioate	0.82	<0.001	0.02
Tetradecanedioate	0.84	<0.001	0.01
10-undecenoate (11:1n1)	0.86	<0.01	0.03
5-dodecenoate (12:1n7)	0.91	<0.01	0.04
Myristate (14:0)	0.94	0.01	0.09
Myristoleate (14:1n5)	1.21	0.01	0.09
3-hydroxydecanoate	0.93	0.01	0.09
Pentadecanoate (15:0)	0.94	0.02	0.10
Octadecanedioate	0.94	0.02	0.11
Eicosenoate (20:1n9 or 11)	1.06	0.04	0.17
Palmitoleate (16:1n7)	1.13	0.04	0.17
Eicosapentaenoate (EPA; 20:5n3)	0.97	0.05	0.18
Lysolipids			
2-myristoylglycerophosphocholine	2.30	0.01	0.06
1-oleoylglycerophosphoethanolamine	1.63	0.02	0.11

(Continued on the following column)

Table 1. Metabolites that changed after 2 to 6 weeks daily limonene intervention (72 metabolites $P \leq 0.05$; 39 metabolites $Q \leq 0.10$) (Cont'd)

	Fold change	P	Q
1-heptadecanoylglycerophosphocholine	3.63	0.02	0.12
2-stearoylglycerophosphocholine	2.80	0.03	0.14
1-eicosadienoylglycerophosphocholine	2.68	0.04	0.17
2-palmitoylglycerophosphocholine	3.78	0.05	0.18
Other			
Homostachydrine	1.50	<0.01	0.04
Trigonelline (N'-methylnicotinate)	1.20	0.01	0.05
N4-acetylcytidine	0.89	0.01	0.06
1-methylxanthine	1.61	0.01	0.09
Hypoxanthine	0.91	0.01	0.10
5-acetyl-2-pyridinecarboxylic acid	0.92	0.02	0.10
Ascorbate	15.23	0.02	0.12
D-Histidyl-D-tryptophyl-D- α -glutamyl-L-seryl-L-alanyl-L-serylleucylleucine (HWESASXX)	0.94	0.02	0.12
L-urobilin	1.02	0.02	0.12
Inositol 1-phosphate (IIP)	1.44	0.04	0.17

limonene treatment, several could be grouped as specific classes of metabolites or along metabolic pathways as described below ($Q \leq 0.10$).

- i Adrenal/gonadal steroids: A number of the sulfated steroids produced by the adrenal gland and gonads and sulfated by liver sulfotransferases were affected by oral administration of limonene. Specifically, there were significant decreases in androsterone sulfate, epiandrosterone sulfate, 4-androsten-3 β ,17 β -diol disulfate 2, and pregnenolol disulfate.
- ii Bile acids: Multiple bile acids that were identified by this analysis increased with limonene intervention. Significant increases were observed for glycodeoxycholate, glycoursodeoxycholate, taurodeoxycholate, taurocholate, and deoxycholate. Conversely, there was a significant decrease in taurochenodeoxycholate sulfate.
- iii Collagen breakdown products: The collagen components glycine, proline, and 4-hydroxyproline were all significantly increased in plasma post-limonene intervention.
- iv Glucose/energy metabolism:
 - Elevations of the terminal product of the glycolysis pathway, pyruvate, fructose of the sorbitol pathway, and glucuronate were observed post-limonene intervention.
 - Ketones are formed using acetyl-CoA generated by fatty acid oxidation; there was a small but statistically significant increase in the ketone, β -hydroxybutyrate.
 - Acetylcarnitine, derived from acetyl-CoA, was significantly decreased.
- v Amino acids and oxidative products: Following the limonene intervention, statistically significant increases in glycine, indolepropionate, alanine, 3-phenylpropionate, and phenylacetate were observed, whereas 3-(4-hydroxyphenyl)lactate, kynurenine, gamma-glutamylisoleucine, alpha-hydroxybutyrate, and phenylalanyltryptophan, were significantly decreased.
- vi Fatty acids: There was also general decrease in plasma short- and medium-chain dicarboxylic fatty acids; significant decreases were observed in tetradecanedioate, hexadecanedioate, 10-undecenoate, 5-dodecenoate, myristate, 3-hydroxydecanoate, and pentadecanoate. Conversely, there was a slight but significant increase in myristoleate.

Table 2. Metabolites whose change is significantly correlated to the change in tissue Cyclin D1 expression (47 metabolites $P \leq 0.05$; 23 metabolites $Q \leq 0.10$)

	<i>P</i>	<i>Q</i>	<i>R</i> ²
Lysolipids			
1-Palmitoylglycerophosphocholine	<0.001	0.01	0.62
1-Palmitoleoylglycerophosphocholine	<0.001	0.02	0.59
1-Stearoylglycerophosphocholine	<0.001	0.01	0.61
2-Stearoylglycerophosphocholine	<0.001	0.01	0.61
1-Oleoylglycerophosphocholine	<0.001	0.01	0.62
1-Eicosadienoylglycerophosphocholine	<0.001	0.01	0.63
2-Oleoylglycerophosphocholine	<0.01	0.05	0.55
2-Linoleoylglycerophosphocholine	0.01	0.09	0.51
1-Eicosatrienoylglycerophosphocholine	0.01	0.09	0.50
1-Heptadecanoylglycerophosphocholine	0.01	0.09	0.49
1-Palmitoylplasménylethanolamine	0.02	0.14	0.42
Acylcarnitines			
Palmitoylcarnitine	<0.001	0.01	0.63
Oleoylcarnitine	<0.001	0.02	0.59
Decanoylcarnitine	0.01	0.10	0.47
Butyrylcarnitine	0.02	0.14	0.43
Acetylcarnitine	0.02	0.14	0.43
Amino acids and metabolites			
Indolelactate	0.01	0.09	0.49
Kynurenine	0.01	0.10	0.47
α -hydroxyisocaproate	0.01	0.10	0.47
Phenylalanine	0.01	0.10	0.47
Serine	0.01	0.11	0.46
Phenol sulfate	0.02	0.12	0.45
3-indoxyl sulfate	0.02	0.14	0.43
Alanine	0.02	0.14	0.43
Asparagine	0.03	0.16	0.41
Aspartate	0.04	0.17	0.39
γ -glutamylvaline	0.04	0.17	0.39
Symmetric and asymmetric dimethylarginine (SDMA + ADMA)	0.04	0.18	0.39
Glucose/energy metabolism			
1,5-Anhydroglucitol (1,5-AG)	0.01	0.09	0.50
Fatty acids			
3-Carboxy-4-methyl-5-propyl-2-furanpropanoate (CMPF)	0.01	0.03	0.57
15-Methylpalmitate	0.01	0.10	0.48
<i>cis</i> -Vaccenate (18:1n7)	0.02	0.12	0.45
Palmitoyl sphingomyelin	0.02	0.14	0.42
Adrenate (22:4n6)	0.03	0.16	0.41
17-Methylstearate	0.03	0.17	0.40
Palmitate, methyl ester	0.04	0.17	0.39
Oleate (18:1n9)	0.04	0.19	0.38
Other			
Billirubin (E,E)	<0.001	0.01	0.65
N1-Methyladenosine	<0.01	0.05	0.53
Quinate	0.01	0.09	0.49
2-Ethylhexanoate	0.02	0.14	0.43
Pregnendiol disulfate	0.02	0.15	0.42
Erythritol	0.03	0.16	0.41
Billirubin (Z,Z)	0.03	0.17	0.40
Urate	0.04	0.17	0.39
Cholesterol	0.04	0.19	0.38
α -Tocopherol	0.04	0.19	0.38

vii Lysolipids: There was a general increase in all detected lysoglycerophosphorylcholines (lysoGPCs) with limonene treatment. However, only 2-myristoylglycerophosphocholine was significantly changed after FDR correction.

Metabolite changes correlate with tissue-level cyclin D1 changes

Because an important need for prevention studies is to have biomarkers of intervention effect that can be obtained noninva-

sively, we investigated those metabolites whose change correlated with the change in tissue level expression of cyclin D1 (as expressed by percent positively stained nuclei). Table 2 presents the 47 metabolites whose change from pre- to postintervention was correlated with the change in cyclin D1, of these 23 were significant after FDR correction. As a class, lysoglycerophosphocholines and acylcarnitines increased with the decrease cyclin D1 expression, with the exception of acetylcarnitine which decreased ($R^2 > 0.4$). Changes in amino acids as well as fatty acids were also significantly correlated with changes in cyclin D1 expression.

Discussion

This study is the first to apply metabolomics to a presurgical trial of the bioactive food component limonene. In addition, this is one of the first to correlate plasma metabolomics to a putative breast tumor drug response biomarker. Among the individual metabolites that changed with limonene intervention, there were several consistent biochemical pathway-based alterations. The most striking included a general decrease in sulfated adrenal/gonadal steroids, increases in bile acids and collagen breakdown products, and changes in energy metabolism.

The ability of limonene to reduce circulating adrenal steroid levels could be an important contribution to its overall chemopreventive and oncostatic properties. Dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEA-S) are released into the circulation as inactive hormone precursors and taken up by specific tissues, such as breast, for the local conversion into androgen and estrogen sex steroids where they have a proliferative effect (32); DHEA-S was nonsignificantly reduced in our study. In two nested case-control clinical trials, women in the highest versus lowest quintiles of plasma levels of DHEA-S and androgens had greater risk of developing breast cancer (33, 34).

Conversely, almost all detected glycine- and taurine-conjugated secondary bile acids were elevated post-limonene intervention as was nonconjugated deoxycholate. Taurocholate sulfate, however, was an exception and was significantly decreased, possibly due to nonspecific effects of limonene on sulfation since all detected sulfated adrenal steroids were decreased postintervention. Bile acids act as emulsifying agents to aid the absorption of dietary fats as well as help eliminate hepatic wastes and cholesterol. They also act as signaling molecules and metabolic regulators through the farnesoid X receptor (FXR; ref. 35). The FXR is expressed in breast ductal epithelial cells, human breast cancer cell lines (36), and normal breast tissue (37). Therefore, although the changes observed here most likely reflect liver metabolism, systemic changes in the bile acid profile could also have an effect on breast tissue.

On the basis of an overall pattern of changes, it appeared that transfer of glucose-derived carbons to the mitochondria was restricted by limonene and/or that carbons were removed from the tricarboxylic acid (TCA) cycle, in the form of malate, to be converted into glucose via the gluconeogenesis pathway (modeled in Fig. 1). Pyruvate, a terminal product of glycolysis, was significantly elevated in plasma following limonene intervention, as was fructose of the sorbitol pathway, suggesting restricted entry or usage of glucose-derived carbons by the TCA cycle in the mitochondria. A shift in energy metabolism to preferential use of fatty acid incorporation into the TCA cycle is also supported by a small but significant increase in β -hydroxybutyrate which is increased during oxidation of fatty acids (38). Acetylcarnitine is

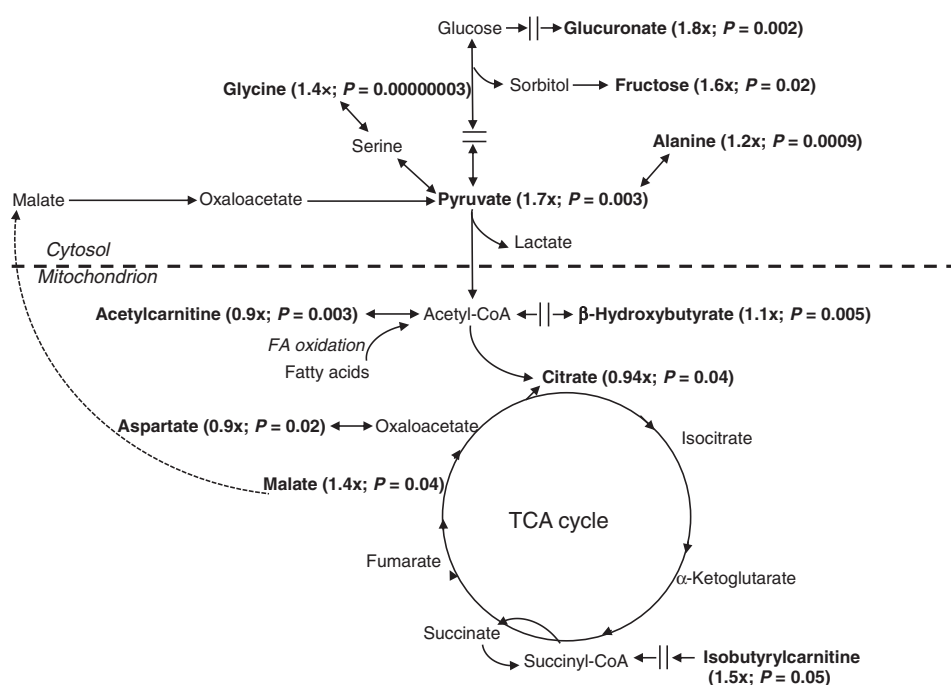


Figure 1. Metabolites that changed significantly from pre- to postintervention are in bold with their fold change and *P* value indicated. The pattern of changes supports restricted entry or usage of glucose-derived carbons with preferential incorporation of fatty acids into the TCA cycle.

used in the transport of fatty acids into the mitochondria (39), and was slightly but significantly decreased. In addition, isobutyrylcarnitine, which is a product of the acyl-CoA dehydrogenases (a group of mitochondrial enzymes involved in the metabolism of fatty acids or branched-chain amino acids; ref. 40), was increased 1.5-fold. Acetyl-CoA represents a major entry point of carbons derived from the oxidation of glucose, several amino acids, and fatty acids into the TCA cycle and is also a the precursor for cytosolic fatty acid synthesis (39). Amino acids and peptides also incorporate into energetic pathways at several entry points (41), specific changes are indicated in the figure.

After oral limonene intervention, there were also elevations in markers indicative of collagen remodeling or degradation, glycine, proline, hydroxyproline, and proline-hydroxyproline (42). Collagen is a major component of the extracellular matrix that serves as connective tissue that fills the interstitial space between cells (43). Breast cancer tissues have been reported to have a significant decrease in collagen and an accompanying increase in collagen degradative enzyme activity (44). Conversely, percent breast density is the strongest known, nonfamilial breast cancer risk factor, with the dense portion being primarily composed of collagen (45). It has also been recently proposed that proline acts as a "stress substrate" with increased levels indicating PPAR- γ upregulation and anticancer activity linked to increased apoptosis (46). Therefore, it will be important to determine the tissue source(s) of the collagen breakdown in this study to determine whether it represents a protective effect of limonene.

Preclinical studies have shown that limonene causes apoptosis in multiple cell types through indirect mechanisms (47, 48). Although several mechanisms may explain limonene's concerted effect, increased apoptosis in multiple cell types may potentially explain many of the observed metabolic changes in conjunction with the observed decrease in cyclin D1 in the breast tissue (49). The hypothesized effect is modeled in Fig. 2. Limonene has also been shown to inhibit proliferation through

a cyclin D1-dependent mechanism in breast cancer cell lines (50). We have previously demonstrated that oral limonene deposits in breast tissue (21) and in other tissues with high adiposity (20). Apoptosis of adipocytes and other cell types would release the main component of cellular membranes, phosphatidylcholines (51), which are metabolized to lysoGPC (52) which were significantly increased with limonene intervention. In addition, palmitoylcarnitine increases with apoptosis (53) consistent with our observed effect. Furthermore, changes in plasma levels of 11 lysoGPCs were highly negatively correlated with the change in cyclin D1 expression in tumor tissue (R^2 values ranging from 0.42 to 0.63).

Apoptosis of adipocytes (or lipolysis, another limonene effect; ref. 54) would also increase circulating lipids and cholesterol. The observed increase in bile acids could be a response to the increased lipids, given that this is their normal physiologic role (55). An increase in available lipids increases fatty acid oxidation for energy over glucose uptake (56), which is consistent with the observation that postintervention metabolite profiles indicate glucose shuttling into alternative pathways. Long-chain acylcarnitines, which are intermediates of fatty acid import into the mitochondria via the carnitine-palmitoyltransferase system (57) and can also be an indication of mitochondrial fatty acid oxidation, were positively correlated with the change of cyclin D1 levels.

Although the primary endpoint of the completed clinical trial was disposition of limonene to the breast tissue and plasma (21), here, we used metabolomic profiling to generate hypotheses about limonene's systemic anticancer effects. A limitation of this study is that our sample size was too small to relate changes in the metabolome to breast tumor pathology. Another limitation is that assessment of cyclin D1 by IHC is semiquantitative. Although an automated count of positive nuclei via Aperio is considered more quantitative than a pathologist score, the automation is limited by the quality of staining. Also, because this study was not

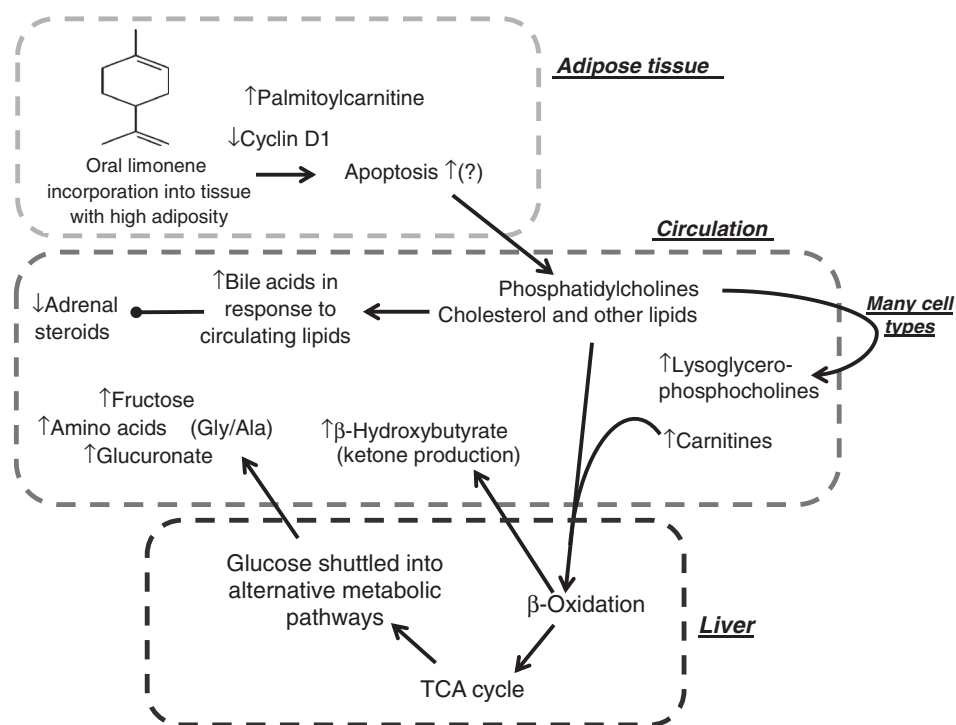


Figure 2.

The up and down arrows indicate observed changes with limonene intervention. Bold arrows indicate the hypothesized chain of events; limonene causes apoptosis in multiple cells types and is known to deposit in adipose tissue (top box), this leads to the hypothesized altered metabolism in the liver (bottom box) and may explain the observed changes in metabolites in circulation (middle box). Gly, glycine; Ala, alanine.

originally designed with metabolomics as a primary endpoint, fasting status and timing of blood draw and surgery were not controlled. Thus, we are unable to relate changes in metabolites to blood or tissue levels of limonene. When the statistical analysis was controlled for fasting status, however, the observed pre to post-intervention changes remained statistically significant. The concerted change of multiple biochemicals associated with each of these pathways strengthens the confidence in the results. In this study, the limonene treatment duration ranged from 2 to 6 weeks, governed by the surgery schedule. Analysis of the data showed that duration of limonene treatment did not affect the observed metabolite changes (data not shown).

This study supports the hypothesis that limonene's activity is likely through a general systemic effect rather than through a specific target. The finding that changes in expression of cyclin D1 in the tumor tissue were significantly inversely related to several acylcarnitines, lysoglycerophosphocholines, and amino acids suggests that surrogate markers of limonene effect are detectable in plasma. The overall pattern of energy changes in addition to independent markers following limonene treatment are consistent with the range of anticancer effects in preclinical models and support further research with limonene in the breast cancer prevention settings that are designed with metabolomic analyses as a primary outcome.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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