BIOAVAILABILITY AND DISPOSITION OF THE BIOACTIVE FOOD COMPONENT D-LIMONENE, AND IMPLICATIONS FOR BREAST CANCER PREVENTION

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ABSTRACT:

d-Limonene is a monoterpene found in high concentration in citrus peel oil. Evidence from animal models and cell culture indicate that it has strong anti-cancer effects, particularly in mammary cancer models. Chapter 1; "D-LIMONENE: A BIOACTIVE FOOD COMPONENT FROM CITRUS AND EVIDENCE FOR A POTENTIAL ROLE IN BREAST CANCER PREVENTION AND TREATMENT" is a review paper accepted to Oncology Reviews. This review describes the evidence for *d*-limonene's anti-cancer mechanisms, bioavailability and safety, focusing on relevance to breast cancer prevention. Chapter 2; "ADIPOSE TISSUE ACCUMULATION OF D-LIMONENE WITH THE CONSUMPTION OF A LEMONADE PREPARATION RICH IN D-LIMONENE CONTENT" is published in Nutrition and Cancer journal and describes a phase I clinical trial in which participants consumed 40 oz of high-limonene lemonade daily. This study demonstrated that after 4 weeks of oral consumption of high-limonene lemonade, *d*-limonene deposits in high levels in adipose tissue. Chapter 3; "A CLINICAL BIOMARKER STUDY OF TOPICALLY APPLIED D-LIMONENE FOR BREAST CANCER PREVENTION" was submitted to Nutrition and Cancer journal. In this phase 0 clinical study, four weeks of a 10% *d*-limonene formulation resulted in minimal change in NAF and plasma biomarkers or *d*-limonene levels. Biomarkers in NAF and plasma, however, were significantly differently correlated with BMI and menopausal status, perhaps suggesting effect modifications. Chapter 4: "MOUSE MAMMARY TISSUE DISTRIBUTION OF D-LIMONENE AND PERILLIC ACID FOLLOWING ORAL AND TOPICAL D-LIMONENE ADMINISTRATION," was a study comparing *d*-limonene and perillic acid disposition after administration of 10% and 20% *d*-limonene in coconut oil in topical and

oral forms to SKH-1 mice. This study demonstrated that *d*-limonene deposits in high levels in mouse mammary tissue after both oral and topical administration short-term, but is largely cleared after 24 hours in this model. Perillic acid deposits in high levels in adipose after oral administration, and these high concentrations remained after 24 hours. Chapter 5: "IMPLICATIONS AND FUTURE DIRECTIONS" provides a summary of the key findings from these three projects and proposals for future research. The appendices provide results from smaller *d*-limonene projects, as well as extensions of the body of the dissertation work.

CHAPTER 1
D-LIMONENE: A BIOACTIVE FOOD COMPONENT FROM CITRUS AND EVIDENCE FOR A POTENTIAL ROLE IN BREAST CANCER PREVENTION AND TREATMENT.

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Abstract:

Although limited, observations from cell culture, animal and epidemiological studies support the presence of anti-cancer properties in citrus peel and the primary bioactive food constituent, *d*-limonene. Early evidence from animal models suggests that when ingested, *d*-limonene exhibits a wide spectrum of biologic activity including chemotherapeutic and chemopreventive effects. In some of these early models, an analogue of *d*-limonene, perillyl alcohol, demonstrated a more potent effect than limonene itself. Yet when perillyl alcohol advanced to clinical trials, several trials were ended early due to dose-limiting toxicities. Alternatively, oral *d*-limonene administration in humans is well-tolerated even at high doses supporting its investigation as a potential bioactive for cancer prevention. Though the exact mechanisms of action of *d*-limonene are unclear, immune modulation and anti-proliferative effects are commonly reported. Here we review the pre-clinical evidence for *d*-limonene's anti-cancer mechanisms, bioavailability and safety, as well as the evidence for anti-cancer effects in humans, focusing on studies relevant to its use in the prevention and treatment of breast cancer.

Introduction: The Bioactive Food Component, *d*-Limonene.

The National Institutes of Health (NIH) Office of Dietary Supplements defines bioactive food components (BAFC) as; "components or constituents in foods or dietary supplements, other than those needed to meet basic human nutritional needs, that are responsible for changes in health status"[1]. A number of compounds that meet this definition of BAFC are present in plant foods and recently reviewed in detail in an article by Kim *et al* [2]. This review will focus on *d*-limonene specifically, a well-tolerated, lipophilic constituent of citrus peel with potent *in vitro* and *in vivo* anti-cancer properties. *d*-Limonene belongs to a class of compounds called terpenoids which also includes; carvone, carveol, oleanic acid, ursolic acid, α- and β-carotene, lutein, lycopene, zeaxanthine, and cryptoxanthineis. Terpenoids as a group have shown great promise in terms of overall health promotion including anti-cancer, cardioprotective and antioxidant effects and a broad review covering the mechanisms behind these effects for each terpenoid sub-class has been written by Wagner and Elmadfa [3]. A broad overview of the anti-cancer activities only can be found in a review by Rabi and Bishayee [4]. In 1997, Gould reviewed the sub-class monoterpenes which includes limonene, perillyl alcohol, and carveol, and briefly presents the range of putative anti-cancer effects in preclinical models as evidence to move forward with these agents in phase I clinical trials [5]. Evidence regarding safety information for *d*-limonene for prevention and treatment of cancer as well as an emphasis on treatment for gallstones and gastro-esophageal reflux is reviewed by Sun [6]. This review describes the available evidence supporting *d*-limonene as the most promising monoterpene to develop as an anti-cancer agent,

particularly for the breast. Most of the evidence provided is specific to breast cancer, with some expansion into evidence derived from research using other cancer sites in order to demonstrate general anti-cancer activity such as antioxidant activity or immune-enhancing effects, or information on disposition and metabolism of *d*-limonene.

d-Limonene is the primary BAFC peel oil comprising 75% of lemon peel oil, 95% of orange peel oil, and 87% of mandarin peel oil [7]. In animal and cell culture models, *d*-limonene has been shown to prevent or delay the growth of a number of cancer types including lymphomas [8], mammary [9-12], gastric [13, 14], liver [15, 16], lung [17], and prostate cancer [18]. Like many BAFC, *d*-limonene exhibits a variety of chemopreventive properties. Table 1 summarizes the studies discussed in this review that demonstrate *d*-limonene action as an anti-cancer agent and the potential mechanism in each case. Most likely, *d*-limonene affects multiple anticancer pathways, an important quality for development of agents to prevent and treat breast cancer, since the mechanisms driving initiation and progression of different breast cancers are multifactorial [19]. Evidence of similar anti-cancer effects in humans is limited, but *d*-limonene has demonstrated some promising activity in pilot clinical trials, and it is well tolerated [20-22]. Chemically, *d*-limonene is non-polar, making it a very lipophilic compound and likely to deposit in fatty tissues, such as breast, after oral consumption (Fig 1). Thus, the pre-clinical evidence combined with *d*-limonene's structural character make it a likely candidate for development as a chemopreventive agent against select cancers, particularly cancers originating in tissues of high adiposity such as breast.

Perillyl Alcohol: a *d*-Limonene Analogue.

Some of the early work investigating the efficacy of *d*-limonene and other monoterpenes in cell culture and animal models of breast cancer demonstrated that perillyl alcohol had more potent anti-cancer effects than *d*-limonene itself. For example, perillyl alcohol has been shown to be a more potent inhibitor of the growth of mammary cancer cell lines T-47D, MCF-7, and MDA-MB-231 [23]. *In vivo* studies show similar differences in agent potency. For example, tumor inhibition in rats induced with the carcinogen dimethylbenz(a)anthracene (DMBA) was significantly higher with perillyl alcohol than limonene [24]. Perillyl alcohol has been shown to influence a number of cellular functions relevant to tumorigenesis including inhibition of post-translational isoprenylation of the oncoprotein ras [25], inhibition of angiogenesis [26], and modulation of the anti-tumor action of transforming growth factor-β (TGF-β) [27]. These pre-clinical studies provided the initial enthusiasm for perillyl alcohol as a more promising chemopreventive agent for development than *d*-limonene.

Based on this evidence perillyl alcohol advanced to phase I/II clinical trials as an oral agent for the treatment of breast cancer and other solid tumors. The results from these efforts were largely discouraging. In a Phase II trial of patients with metastatic breast cancer treated with perillyl alcohol four times daily at 1,200 – 1,500 mg per dose, there were no objective responses after 12 months, and recruitment was halted with 14 of the intended 40 patients due to poor drug tolerance [28]. Another study included patients with advanced solid tumors of different cancer sites including non-small cell lung cancer (N = 12), melanoma (N = 3), leiomyosarcoma of the uterus (N = 2), renal cell carcinoma

(N = 1), mesothelioma (N =1), Ewing's sarcoma (N = 1), and colon cancer (N = 1). All patients had received prior chemotherapy. Perillyl alcohol was administered at 4,800 mg/m^2 dose escalated to 11,200 mg/m^2 daily. Again, no anti-tumor activity was observed and the maximum tolerated dose was 8,400 mg/m^2 daily. Dose limiting toxicities were nausea and vomiting [29]. A third phase I trial in patients with solid malignancies (N = 17) demonstrated no objective tumor responses and dose-limiting toxicities of chronic nausea and fatigue as well as severe heart burn and vomiting with a maximum of 2,800 mg/m^2 perillyl alcohol [30].

Dose limiting toxicity to perillyl alcohol as an oral agent is influenced by the requirement for a high or frequent dosing schedule to maintain elevated plasma levels to achieve a therapeutic effect [30]. The requirement for high dosing is largely a function of perillyl alcohol's polar structure and rapid clearance in urine. The dose limiting toxicities and failure to achieve any anti-tumor activity *in vivo* largely led to a decline in interest in this agent in the treatment and prevention setting where the toxicities unacceptably outweigh in potential benefit for anti-cancer effects acting earlier in the preneoplasia state. In contrast, *d*-limonene is a hydrophilic compound with similar anti-cancer properties that exhibit distinct metabolism and disposition largely depositing in adipose tissue with low toxicity in the oral form [21, 31]. Table 2 summarizes our work demonstrating that after healthy adults consume 500 mg *d*-limonene daily from lemonade for four weeks, *d*-limonene adipose tissue levels accumulate to 120-fold that of plasma. Therefore, a lower dose of *d*-limonene may be sufficient to achieve comparable steady-state therapeutic levels at the tissue level compared to high doses of

perillyl alcohol without the associated toxicities. This may be particularly attractive for tumors arising in more fatty tissues like the breast.

The following is a summary of the available pre-clinical evidence that demonstrates *d*-limonene's mechanisms of effect in preclinical models with particular attention to the evidence pertinent to breast cancer. In light of the promising results from recent phase I/II clinical trials, there is a need to add to the original preclinical research to provide mechanistic evidence to guide future clinical trials.

Mechanisms of Chemopreventive Activity of *d*-Limonene

Immune modulation. In animal models, the strongest effect of *d*-limonene exposure involves direct modulation of primary immune function, which is an important mechanism in the early stages of many breast cancers [32]. Immunotherapy is currently under investigation as future breast cancer prevention and treatment option [33]. The first demonstration of *d*-limonene's effect on immune response was by Evans *et al*., who showed that BALB/c mice pre-treated with 10% *d*-limonene in a gastric tube have an increased primary and secondary antibody response to keyhole limpet hemocyanin (KLH) compared to the control [34]. In a more recent study, rats given 250 mg *d*-limonene/kg body weight for 8 consecutive days had significantly more alveolar macrophages than the control. Additionally, the phagocytic activity of these macrophages demonstrated a dose-dependent increase in response to *d*-limonene administration up to 1,000 mg/kg/d [35]. Both studies indicate direct effects on immune activation in response to *d*-limonene administration. Similarly, Raphael *et al* assessed the

immune-modulating activity of *d*-limonene showing that BALB/c mice receiving 5 intraperitoneal injections per day for 5 days of *d*-limonene (100 μmoles/Kg bodyweight/dose/animal) demonstrated a doubling in the total number of white blood cells (WBC) 9 days post administration [36]. The total number of bone marrow cells was significantly increased from baseline ($P < 0.001$), and this assessment was further supported by a non-significant increase in α-esterase positive cells for both the *d*-limonene-treated mice. Mice also demonstrated a 7-fold increase in total antibody production on day 12 as well as the 6-fold increase in antibody producing cells in the spleen on day 5. *d*-Limonene significantly reduced the inflamed paw size 24 hours after administration of the sensitizing agent compared to the control ($P < 0.001$), suggesting an anti-inflammatory role. Overall, the authors concluded that these results suggest that *d*-limonene is able to enhance immune responses without causing hypersensitivity [36].

Only one study has demonstrated an anti-cancer effect specific to an enhanced immune response for *d*-limonene. Toro-Arreola *et al* monitored immunity-related tumor reduction in female BALB/c albino mice inoculated with the lymphoma cell line, L-5178-Y after addition of either 10% or a 0% *d*-limonene added to their diet [8]. Tumor-bearing mice fed a *d*-limonene enriched diet had a significantly increased lifespan, up to 10 days longer ($P < 0.004$), than control mice fed standard chow. Mice fed *d*-limonene also had a largely decreased tumor burden by day 15 (*d*-limonene: 0.27g +/- 0.17; control: 0.65g +/- 0.18) and demonstrated enhancement of macrophage phagocytosis and microbicidal activity. Although not statistically significant, the *d*-limonene group showed a trend toward increased nitric oxide production in the peritoneal macrophages,

supporting a potential role for *d*-limonene in macrophage activation. These results, however, were not associated with any significant increase in T-lymphocyte proliferation or activity as compared to control mice [8].

None of these studies indicate a signaling mechanism that would explain the increase in immune response after *d*-limonene treatment. Host immune response is important for resistance to many types of cancer, including breast, and new cancer therapies that activate an immune response are in development [37, 38]. Because *d*-limonene has demonstrated immune-modulating effects specific to macrophage activation, it is important to determine whether this mechanism could explain *d*-limonene's effectiveness in pre-clinical mammary cancer models.

Role in modulating chemical carcinogenesis. In rodent mammary chemical carcinogenesis models, *d*-limonene's anticancer mechanisms appear to differ depending on the carcinogen. For example, a 5% *d*-limonene diet was effective in significantly reducing the number of N-nitroso-N-methylurea (NMU) – induced mammary tumors when fed during the promotion/progression stage ($P < 0.001$), but the same oral dose was not shown to be effective in arresting tumor growth when fed during the initiation stage of tumorigenesis [9]. In DMBA – induced mammary tumors, a 5% *d*-limonene diet significantly reduced the number of tumors at both initiation and promotion/progression stages [10]. In mouse mammary organ gland culture, *d*-limonene demonstrated 78% inhibition of DMBA-induced lesions at a concentration of 1×10^{-8} M, further supporting anti-initiation effects [39].

Both DMBA and NMU tumors are models of estrogen receptor and prolactin receptor positive human breast cancers and are dependent on estrogen for initiation and promotion [40]. Thus, it is possible that *d*-limonene's anticancer effect in these models could be through modulation of estrogen metabolism via cytochrome p450 (P450). Additionally, the NMU model is a direct carcinogen [41], while DMBA requires metabolic activation by P450, primarily by CYP1B1 [42]. *d*-Limonene may be inhibiting conversion of DMBA to it's carcinogenic form through P450 modulation, which would explain its lack of effect on NMU initiation. In cell culture, *d*-limonene has demonstrated some P450 inhibition [43], however, this is an understudied potential mechanism of action.

Anti-oxidant activity. More investigations have focused on the possibility that *d*-limonene is affecting initiation and progression events through the ability of its conjugated double bond structure to quench free radicals, thus alleviating cellular oxidative stress. In breast cancer models, increased oxidative stress can induce initiation of a tumor as well as promote tumor growth [44]. Therefore, agents that can reduce excess oxidative stress offer potential as anti-cancer agents for the breast. A study quantifying antioxidant ability of select citrus fruits found the peel of lemons and oranges to be three-fold more effective than the fruit as measured by xanthine-xanthine oxidase test, 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced superoxide (O_2-) suppression, and nitric oxide suppression [45]. While these estimates of antioxidant potential are crude, it suggests that those BAFC present in the peel of citrus, such as *d*-limonene, has

greater antioxidant capacity than the fruit. A study by Gerhauser *et al* assessed the antioxidant potential of several potential chemopreventive agents, including both ascorbic acid and *d*-limonene, each of which are present in citrus peel [46]. *d*-Limonene's oxygen radical absorbance capacity (ORAC) was measured at 0.9 μmol Trolox Equivalents per gram, which was about half that of ascorbic acid, suggesting that the most potent anti-oxidant capability in citrus peel may be due to other components in the peel such as ascorbic acid, rather than *d*-limonene itself. No data were presented on the combined antioxidant potential of these compounds, a factor of relevance to dietary intake of citrus fruit and its role in cancer prevention.

In a recent report by Roberto *et al*, *d*-limonene demonstrated peroxidase (Px) and catalase (CAT)-like activity in concentrations less than 50 μg/mL in lymphocyte culture, but this activity was not demonstrated at higher concentrations [47]. Superoxide dismutase (SOD)-like activity by *d*-limonene increased with increasing concentration of *d*-limonene up to 1,000 μg/mL. *d*-Limonene also demonstrated 1,1-diphenyl-2-picryl-hydrazyl free radical scavenging ability at concentrations up to 50 μg/mL, but again this effect was not present at higher concentrations. *d*-Limonene, at very high concentrations was also able to enhance the activity of SOD only and of CAT, and Px at 50 -100 μg/mL. Additionally *d*-limonene reduced the amount of H_2O_2 significantly at 10 – 50 μg/mL *d*-limonene, and this effect was lost at higher concentrations. Overall, this study suggests that *d*-limonene protects normal lymphocytes from oxidative stress at low concentrations with no associated change in cell viability [47]. In general, while *d*-limonene may act as

a low-level antioxidant overall, its anti-cancer properties are most likely attributable to other mechanisms of action.

Farnesyl transferase inhibition. A more widely supported mechanism to explain the chemopreventive activity of *d*-limonene is through inhibition of the prenylation of the monomeric G-protein, Ras which is involved in cell proliferation and migration. Ras is activated via protein prenylation and subsequent association with the cellular membrane. In many carcinomas, Ras is mutated. Preclinical data indicates that *d*-limonene and its metabolites modulate Ras prenylation via farnesyl transferase inhibition [11, 48, 49]. In normal Ras activation, the enzyme farnesyl transferase facilitates the addition of a farnesyl group to Ras, which, after a cascade of phosphorylation activates Ras, initiating the cellular signaling pathways for cell proliferation and migration. Mutated Ras is associated with aberrant cell proliferation and migration and a reduced ability to undergo apoptosis, all contributing to carcinogenesis [50]. Figure 2 (adapted from Brunner *et al*) [51] is an illustration displaying the steps needed for Ras activation and the target of farnesyl transferase inhibitors (FTIs), the enzyme farnesyl transferase, subsequently preventing aberrant Ras activation. A series of FTIs, including R115777, SCH66336, BMS-214662, and tipifarnib, have been developed for cancer therapy to inhibit this pathway. While these drugs likely target farnesyltransferase more specifically than *d*-limonene, clinical trials with these compounds have been halted because of dose-limiting toxicities such as nausea, vomiting, neurological complications, and even abnormal cardiac function [52,

53]. The toxicities observed in these trials may indicate that less potent FTIs, such as compounds like *d*-limonene, may be more beneficial overall.

For breast cancer specifically, FTIs are a less obvious therapy. Less than 5% of breast tumors contain a mutated Ras [54], however, modulating this enzyme still demonstrates beneficial effects for breast cancer therapy downstream or independent of Ras activation [55]. Because Ras modulates cell proliferation and differentiation, even if it is a case where Ras is not mutated, *d*-limonene may still modulate Ras via inhibition of the farnesyl transferase enzyme, thus modulate cell proliferation overall, slowing cancer growth. This effect was demonstrated in an NMU-induced mammary tumor model to assess *d*-limonene's efficacy against Ras-induced mammary carcinoma. Roughly half of NMU-induced mammary tumors contain a Ras mutation, thus it is an appropriate model to compare *d*-limonene's effectiveness against mammary tumors with or without a Ras mutation [11]. The investigators found that *d*-limonene supplementation at a dose of 5% daily for 2 weeks prior to NMU injection increased tumor latency from 83.5 days for the control rats to 135 days ($P = 0.04$) in the *d*-limonene treated rats. The total number of carcinomas decreased from 4 per animal in the control group to 1 in the *d*-limonene group ($P = 0.01$). Ras expression in the tumors did not differ across the two groups, with 49% of tumors in the control animals and 50% of tumors in the *d*-limonene supplemented animals presenting the Ras mutation. Therefore, while *d*-limonene had anti-tumor action against tumors overall, it was not preferentially acting against mutated Ras-specific tumors, suggesting that *d*-limonene was either still acting as a modulator of cell

proliferation either as an FTI, or else by either some other mechanism of anticarcinogenic activity [11].

An earlier study by the same group demonstrated that when rats were fed a 10% *d*-limonene diet, 68% of DMBA-induced ($P < 0.001$) and 96% of NMU-induced ($P < 0.001$) small and large tumors regressed completely compared to their pair-fed control [56]. Additionally, *d*-limonene prevented the occurrence of secondary tumors in 63% of DMBA-induced tumors and 100% of the NMU-induced. A dose-response analysis indicated that significant protection against primary tumor formation was observed only at doses of 7.5-10% *d*-limonene with continuous administration over a period of 11 weeks. These effects were observed without *d*-limonene toxicities even at the highest dose and continuous administration. Because a minimal histopathological analysis did not reveal either an immunological nor apoptotic protective effect, and the NMU group was more responsive to *d*-limonene treatment, the investigators concluded that protective effect of *d*-limonene administration must be from the inhibition of prenylated G-proteins [56].

There are other studies that demonstrate *d*-limonene treatment in mice or rats with NMU-initiated mammary tumors is effective [9, 56, 57], however, none of these studies further explore farnesyl transferase inhibition as a mechanism. Because of the evidence demonstrating *d*-limonene's effect in this type of cancer, its role as an FTI and modulator of the enzyme's downstream effects warrants further investigation.

TGF-β1 induction. *d*-Limonene has also demonstrated inhibition of mammary cancer in rats specific to transforming growth factor-β1 (TGF-β1**)** induction, concurrent with an increase in the mannose-6-phoshate/insulin like growth factor II (M6P/IGF-II) receptor [27]. Rats were administered 50 mg/kg DMBA to initiate mammary cancer. After development of an advanced mammary tumor ($\geq$ 1 g), rats were assigned to either a 10% *d*-limonene diet or control. In the limonene treated group, 87% of the advanced tumors demonstrated regression, and this was significantly greater than the 7% regression seen in the control ($P < 0.0001$). Tumors of limonene-fed rats demonstrated substantially increased intensity of immunohistochemical staining for both TGF-β1 and M6P/IGF-II receptor. mRNA levels of M6P/IGF-II receptor were also significantly increased ($P < 0.001$), but this was not observed for TGF-β1, indicating that the increase in immunohistochemical staining is most likely due to post-translational events [27]. While the role of TGF-β1 is paradoxical and is not well understood in relation to breast cancer [58], in this case, an increase in the protein and receptor in response to *d*-limonene administration was protective against mammary tumors.

Apoptosis. Limited evidence also indicates that in some cancers, *d*-limonene may be acting via apoptotic mechanisms. Agents that induce apoptosis have been effective in prevention tumor growth and metastasis in preclinical models of breast cancer [59, 60]. While, to date, limonene has not demonstrated apoptotic mechanisms in preclinical models for breast specifically, it has in other cancers. In a prostate cancer cell line, DU-145, Rabi *et al.* demonstrated that apoptosis was induced by combination

limonene and docetaxel treatment at clinically relevant levels of both drugs [18]. In a mouse model, BALB/c nude mice were injected with the gastric cancer cell line BGC-823 [13]. After 8 weeks of 10 mg/mL *d*-limonene administered in a saline solution, mice were sacrificed and tumors were analyzed. Limonene feeding was associated with significantly reduced ($P < 0.05$) gastric tumor size as well as metastasis to peritoneum and other organs ($P < 0.05$). Using terminal deoxynucleotidyl transferase dUTP nick end labeling, the investigators noticed that the untreated tumors contained significantly less apoptotic cells as compared to tumors in the *d*-limonene treated group ($P < 0.05$) suggesting that induction of apoptosis may also be a mechanism of action for *d*-limonene [13]. This evidence provides support for future preclinical models to investigate whether *d*-limonene also induces apoptosis in the context of breast cancer.

While these studies demonstrate *d*-limonene's strong chemotherapeutic activity in animal models, its biological mechanisms have not been fully explained. The metabolic activity and deposition of *d*-limonene upon oral consumption is also unclear. *d*-Limonene's major circulating metabolite is perillic acid [20]. Because perillic acid is water soluble and easily measured in blood serum, it had previously been used as a biomarker of *d*-limonene intake. Evidence from phase I clinical trials indicate that *d*-limonene is not completely metabolized upon oral consumption and may circulate at levels comparable to its metabolites [21]. Recent evidence from our lab demonstrates that *d*-limonene deposits at much higher concentrations in adipose than in plasma [61], thus measuring perillic acid in plasma as a surrogate for exposure most likely does not

reflect total *d*-limonene deposition in target tissues. The body of pre-clinical evidence has led to the initiation of phase I clinical trials to evaluate *d*-limonene's safety and efficacy for breast and skin cancer treatment and prevention.

d-Limonene Tolerability and Dosing Studies

In an early pilot study to begin assessment of *d*-limonene metabolism and tolerability in humans, 7 subjects were fed 100 mg/kg body weight *d*-limonene in custard, thought to be a clinically active dose. Their blood was drawn at 0, 4, and 24 hours. *d*-Limonene metabolites were then quantified using gas chromatography-mass spectrometry. It was determined that 100 mg/kg body weight could be consumed without gradable toxicity, and they identified the main circulating *d*-limonene metabolites to be perillic acid, dihydroperillic acid, and limonene-1,2-diol [20]. A recent pharmacokinetic study in 24 healthy Chinese males demonstrated that after a 300 mg oral dose, *d*-limonene circulates in blood serum at levels up to 40.1 to 327.4 ng/mL over a time-period of 12 hours [62]. *d*-Limonene metabolite levels were not measured in this study. Another study demonstrated that, in rats, after oral administration of 200 mg/kg *d*-limonene, oral bioavailability was estimated to be 43% [63]. Thus, there is ample evidence supporting *d*-limonene's safety and bioavailability after oral consumption, making it well posed for development as a chemopreventive agent.

To date, the only study in which cancer patients were administered *d*-limonene was a Phase I study conducted by Vingushin *et al*, where 32 adults with locally advanced metastatic solid tumors were given oral doses of *d*-limonene ranging from 0.5- 12 g/m^2

[21]. In these patients, peak plasma levels of *d*-limonene measured by liquid chromatography-mass spectrometry (LC-MS) circulated at levels very close to its metabolites (Table 3). In terms of chemoprevention, one breast cancer patient, of ten, demonstrated a reduction in tumor size in response to 8 g/m^2 per day dosing schedule. There were three colorectal cancer patients with no measurable tumor progression that received doses 0.5-1 g/m^2 per day. These effects were seen with few reports of adverse events and while *d*-limonene did not cause tumor regression, it did slow progression and possibly prevent metastasis. In two breast cancer patients, *d*-limonene and the primary metabolite perillic acid were found to preferentially deposit mammary tumors compared to serum levels, this same preferential distribution to the tumor was not seen in the other metabolites (Table 4). Because *d*-limonene seems to preferentially deposit in breast tumors compared to plasma levels, this preliminary data suggests that it has potential for biological activity in mammary tissue [21].

In order for *d*-limonene to be developed as a chemopreventive for breast cancer specifically, it is necessary to determine if it deposits in high enough levels in the breast to modulate the microenvironment. One preclinical study in rats has assessed total *d*-limonene metabolite levels in adipose, mammary, other tissues and plasma were assessed indirectly using radioactivity after oral administration. While they did not assess each metabolite individually, at all time-points, parent compound with metabolite concentration was higher in mammary and adipose tissue than in the plasma (Table 5) [64].

In a Phase I clinical study conducted at our institution to assess tolerability and *d*-limonene deposition, healthy adults consumed 40 oz of lemonade made with the peel, daily for four weeks. Because each patient prepared their lemonade individually, *d*-limonene levels ranged from 400-600 mg. There was a significant increase in *d*-limonene levels in the fat biopsies after 4 weeks of lemonade intervention ($P = 0.009$); initial levels ranged from non-detectable to 7.79 μM and post-intervention levels ranged from 53.6 to 294 μM. Plasma *d*-limonene levels increased moderately from initial levels ranging from 0.35 to 0.72 μM to post intervention levels of 0.54 to 1.65 μM ($P = 0.016$). Post-intervention adipose *d*-limonene levels were 40-170 times higher than plasma *d*-limonene levels and this was also significant ($P = 0.009$). Our results support the hypothesis that *d*-limonene accumulates in adipose tissue after oral dosing and also indicate the need for additional studies of *d*-limonene for chemoprevention in tissues like the breast that are comprised of a significant fat fraction. Patient hematology and blood chemistry remained normal through the intervention [31]. Taken together, these preliminary results demonstrate that *d*-limonene is available in circulation for deposition in adipose tissue. Additionally, the primary target for *d*-limonene seems to be fatty tissue, thus it may have a unique role in breast cancer prevention. More research is necessary to determine if *d*-limonene is depositing near the breast duct, where 80% of breast cancer originates [65].

Citrus Peel Consumption and Chemoprevention

In terms of chemopreventive activity, the only studies to date to assess citrus peel intake specifically and cancer prevention come from an analyses of the Southeastern

Arizona Health Study by Hakim *et al*, a study conducted from 1994 and 1996 that recruited adult cases of non-melanoma skin cancer from the Arizona Skin Cancer Registry [66]. In Arizona, there is a significantly higher incidence of non-melanoma skin cancer compared to other U.S. regions, of which most cases are due to over-exposure to the sun. In the first study, self-reported average daily intake of one tablespoon (equal to 6 g) of citrus peel was associated with a 34% reduced risk of skin cancer (OR = 0.66, 95% CI = 0.45 – 0.95) in a sample population of 470 women (242 cases and 228 controls) residing in Arizona [67]. Given that *d*-limonene comprises roughly 75% of the 0.7 mL of lemon peel oil extracted from a 60 g lemon peel, there is about 50 mg of *d*-limonene present in a tablespoon of lemon peel. Therefore, based on this study, it appears that 50 mg per day would be the minimal daily intake of *d*-limonene to assert chemopreventive activity in relation to skin cancer risk reduction. Intakes of 0.5 - 8 g/m^2 /day *d*-limonene, the equivalent of 289 – 4,624 mg *d*-limonene per day for an average adult, were well tolerated in Phase I/II therapeutic clinical trials [21]. Thus, it is possible to achieve dosage levels of *d*-limonene that have relevance in terms of cancer prevention without toxicity.

The second study by this group was a case-control to assess the joint effects of black tea and citrus peel intake in relation to squamous cell carcinoma (SCC) risk [66]. Subjects (N=234) for this case-control study were again selected from the Southeastern Arizona Health Study. All subjects were $\geq$ 30 years of age with no history of other cancer and no incidence of metastatic SCC. Control subjects (N=216) were randomly selected from throughout the Tucson Metropolitan Area. All study participants

completed questionnaires describing their citrus and black tea intake. Total combined citrus peel and black tea intake was associated with a 78% decreased risk (OR=0.22; CI=0.10-0.51) for non-melanoma skin cancer. Black tea alone was associated with a 40% decreased risk, but this was not significant (OR = 0.60; CI = 0.30-1.23). Citrus peel intake, however, was associated with a 70% decreased risk (OR = 0.30; 0.13-0.72) [66]. These studies provide preliminary evidence in humans that there exist biologically active components in the peel of citrus that may have a protective effect against select cancers, especially those occurring within or adjacent to fatty tissues such as skin and breast given the lipophilic nature of the putative citrus peel anti-carcinogens such as limonene.

Citrus Fruit Intake and Cancer Prevention

American trials associating citrus consumption and breast cancer risk have largely concluded that there is a lack of protective associations in American epidemiological studies [68-70]. The low exposure to *d*-limonene in the American diet may explain some of the inconsistency in demonstrating significant reductions in cancer risk related to citrus intake across studies. Estimates from the Continuing Survey of Food Intakes by Individuals suggest that American consume less than an average of 31 g citrus daily [71], whereas according to the European Prospective Investigation into Cancer and Nutrition cohort, Greek men and women consume 273.0 and 241.9 g of total fruit and 51.1 g and 45.1 g of citrus daily [72]. This level of intake of citrus has also been shown to be significantly protective against breast cancer in China (OR = 0.65; 95% CI = 0.48 – 0.88) for citrus fruits (*P* for trend < 0.001)) [73]. A prospective investigation involving 22,043

adults residing in Greece found a 24% decrease in overall cancer death rate in association with habitual intake of the Mediterranean diet [74]. Overall, these data suggest that a Mediterranean diet, high in citrus and citrus peel, offers unique cancer preventive potential in comparison to other diets throughout the world including the Western diet [75].

A study conducted by Hakim *et al* assessed the *d*-limonene concentration of common commercial juices as well as lemonade freshly prepared in the Mediterranean style, which is made with the entire fruit, including the peel [76]. A standard store-bought container of lemonade purchased in the U.S. contained roughly 3 mg/L *d*-limonene. Commercially available orange juice samples ranged in *d*-limonene content between 20-73 mg/L depending on the container type, with concentrations greater in cans and concentrate than in glass or plastic storage containers. Preparation of lemonade using a Mediterranean recipe, which includes 2 whole lemons/L, found the *d*-limonene content to be much higher, with an average of over 1,000 mg *d*-limonene per liter [76]. If studies assessing citrus intake include citrus from commercial juices, which are low in *d*-limonene content as well as other potential BAFC, this may partially explain the null results for an anti-cancer citrus association observed in Western countries. While it is unclear which specific aspects of the Mediterranean diet affords this protection, *d*-limonene is a common constituent and is generally consumed as fresh citrus peel or from lemonade made from whole lemons.

Conclusion

The evidence demonstrating *d*-limonene's anticarcinogenic activity combined with its lipophillic nature makes *d*-limonene well-posed as a chemotherapeutic agent for breast cancer. Because *d*-limonene most likely affects multiple pathways, it has strong potential for development as a chemopreventive agent, particularly for cancers such as breast with multifactorial etiologies. While pharmaceutical agents can be developed for a more specific target than BAFC, this may not guarantee their efficacy in overall anti-cancer activity. Currently, there have not been enough published mechanistic investigations within the context of breast cancer specifically in order to determine the population that would benefit best from limonene supplementation. *d*-Limonene's mechanisms that have been demonstrated in other cancer models such as antioxidant activity, apoptotic effects and immune modulation should be investigated within a breast model. Additionally, because limonene deposits in high levels in adipose tissue and has demonstrated lipolytic effects [7], it is possible that *d*-limonene affects fatty acid mobilization and metabolism; future research using techniques in metabolomics should investigate this mechanism and potential implications for breast cancer. Although oral administration of *d*-limonene has been the focus of this review, unpublished data in our lab also indicates that limonene deposits in high levels in mouse mammary glands after topical application. It is also being investigated as a penetration enhancer for percutaneous absorption of tamoxifen in breast cancer models [77]. Future studies investigating limonene's potential as a topical agent either alone or in combination with a more potent chemotherapeutic drug should also be conducted. Because *d*-limonene may be easily incorporated into the diet, via the consumption of citrus peel, this would allow

for a simple, cost-effective strategy for low dose exposure to chempreventive actions that may be beneficial in primary prevention of select cancers. More robust investigations into the anti-cancer mechanisms of action of *d*-limonene in humans within the context of breast cancer will be required to advance its use as a dietary chemopreventive or therapeutic agent.

Table 1: Mechanisms Identified for *d*-Limonene Anti-Cancer Activity

Proposed Mechanism	**Author**	**Title**	**Tumor Status**	**Model System**	***d*-Limonene Effect**
Immune modulation					
	Evans	*Modulation of immune responses in mice by d-limonene*	Healthy model	BALB/c mice	Increased primary and secondary antibody response to keyhole limpet hemocyanin.
	Hamada	*Distribution and immune responses resulting from oral administration of d-limonene in rats*	Healthy model	Wistar-Furth Female Rats	Increased alveolar macrophage production and phagocytic activity.
	Raphael	*Immunomodulatory activity of naturally occurring monoterpenes carvone, limonene, and perillic acid*	Healthy model	BALB/c mice	Doubling in the total number of white blood cells (WBC), significantly increased total number of bone marrow cells was from baseline ($P < 0.001$), and a 7-fold increase in total antibody production.
	Del Toro-Arreola	*Effect of d-limonene on immune response in BALB/c mice with lymphoma*	Lymphoma	BALB/c mice	*E*nhancement of macrophage phagocytosis and microbicidal activity as well as a significantly increased lifespan ($P<0.004$) and decreased tumor burden.
Anti-oxidant activity					
	Murakami	*Suppressive effects of citrus fruits on free radical generation and nobiletin, an anti-inflammatory polymethoxyflavonoid.*	Healthy models	Human promyelocytic HL-60 cells and macrophage RAW264.7 cells	Peels from lemons and oranges were found to be three-fold more effective antioxidant than the fruit.
	Gerhauser	*Mechanism-based in vitro screening of potential cancer chemopreventive agents.*	Liver cancer	Hepa1c1c7 murine hepatoma cell culture	*d*-Limonene's oxygen radical absorbance capacity (ORAC) was measured to be 0.9 μmol TE per gram.
	Roberto	*Antioxidant Activity of Limonene on Normal Murine Lymphocytes: Relation to H_2O_2 Modulatin and Cell Proliferation*	Lymphoma	Lymphocytes	Low concentrations seem to protect normal lymphocytes from oxidative stress.

Farnesyl transferase inhibition					
	Haag	*Limonene-induced regression of mammary carcinomas*	Mammary Tumors	Wistar-Furth Female Rats	68% of DMBA-induced ($P < 0.001$) and 96% of NMU-induced ($P < 0.001$) tumors regressed completely. 63% of DMBA-induced tumors and 100% of the NMU-induced secondary tumors were prevented.
	Gould	*Limonene chemoprevention of mammary carcinoma induction following direct in situ transfer of v-Ha-ras*	Mammary Tumors	Wistar-Furth Female Rats	Overall anti-tumor action was not preferentially acting against mutated Ras-specific tumors.
TGF-β1 induction					
	Jirtle	*Increased Mannose 6 Phosphate/Insulin-like Growth Factor II Receptor and Transforming Growth Factor β Levels during Monoterpene-induced Regression of Mammary Tumors*	Mammary Tumors	Fischer 344 Female Rats	Increased intensity of immunohistochemical staining for both TGF-β1 and M6P/IGF-II receptor ($P < 0.001$) concurrent with significant regression of mammary tumors (87%).
Apoptosis					
	Lu	*Inhibition of growth and metastasis of human gastric cancer implanted in nude mice by d-limonene.*	Gastric cancer	BALB/c nude mice	Tumors in treated group showed large concentrations of apoptotic cells ($P < 0.05$).
	Ravi	*d-Limonene sensitizes docetaxel-induced cytotoxicity in human prostate cancer cells: generation of reactive oxygen species and induction of apoptosis.*	Prostate Cancer	Prostate Carcinoma DU-145 and Normal Prostate Epithelial PZ-HPV-7 cells	Caspase-mediated apoptosis was induced in the cancer cell line only.

Table 2: Average *d*-limonene levels determined using gas chromatography-mass spectrometry in time-matched adipose and plasma samples collected from healthy adults after a single dose of high-limonene lemonade consumption and after 4 weeks of daily consumption.

	Adipose *d*-limonene concentration, μmol/L[1]	Plasma *d*-limonene concentration, μmol/L[2]	Adipose/plasma *d*-limonene ratio[2]
Initial			
Mean (SD)	3.79 (3.26)[5]	0.48 (0.21)	7.2 (7.4)
Range	ND – 7.79	0.35 – 0.72	ND – 19.2
Post-4 wk			
Mean (SD)	137 (87.2)[3,6]	1.12 (0.42)[4]	123.0 (46.3)
Range	53.6 – 293.9	0.54 – 1.65	54.0 – 216

[1] n = 7
[2] n = 6
[3] Significantly higher than the initial adipose samples ($P = 0.009$)
[4] Significantly higher than the initial plasma samples ($P = 0.016$)
[5] Initial adipose and plasma levels were not significantly different ($P = 0.33$)
[6] Post-4 wk intervention adipose levels were significantly higher than plasma levels ($P = 0.009$)

Table 3: Peak plasma levels of *d*-limonene and its metabolites on day 1 from a phase I study in 32 patients with various locally advanced metastatic solid tumors (0.5 – 8 g/mg^2/day *d*-limonene MTD)[a]

Compound	Peak plasma levels (μM)[b]
d-Limonene	10.8 ± 6.7
perillic acid	20.7 ± 13.2
dihydroperillic acid	16.6 ± 7.9
limonene 1,2-diol	11.1 ± 6.1
uroterpenol	14.3 ± 1.5

[a] **Table adapted from Vigushin *et al.*[14]**
[b] ***d*-Limonene and metabolite levels in plasma were determined using LC-MS**

Table 4: Intratumoral levels of *d*-limonene and its metabolites in two breast cancer patients[a]

	Patient A	**Patient B**
Compound	**Tissue/Plasma[a]**	**Tissue/Plasma[b]**
d-limonene	5.52	1.91
perillic acid	1.45	0.14
dihydroperillic acid	0.74	0.67
limonene 1,2-diol	0.42	0.17
uroterpenol	1.7	2.3

[a] Table adapted from Vigushin *et al.*[14]
[b] *d*-Limonene and metabolite levels were determined using LC-MS.

Table 5: Limonene and its metabolites concentration in tissues/plasma[a]

Time (hr) after dosing	Adipose/ Plasma[a]	Mammary/ Plasma[b]
1	3.45	1.77
2	3.40	1.97
4	7.15	4.69
12	5.90	5.00
24	6.86	5.34

[a] Table adapted from Crowell *et al.*[44]
[b]Ratios based on radioactivity of *d*-limonene and metabolites, each metabolite was not evaluated separately.

Figure 1. Chemical structure of *d*-limonene ($C_{10}H_{16}$).

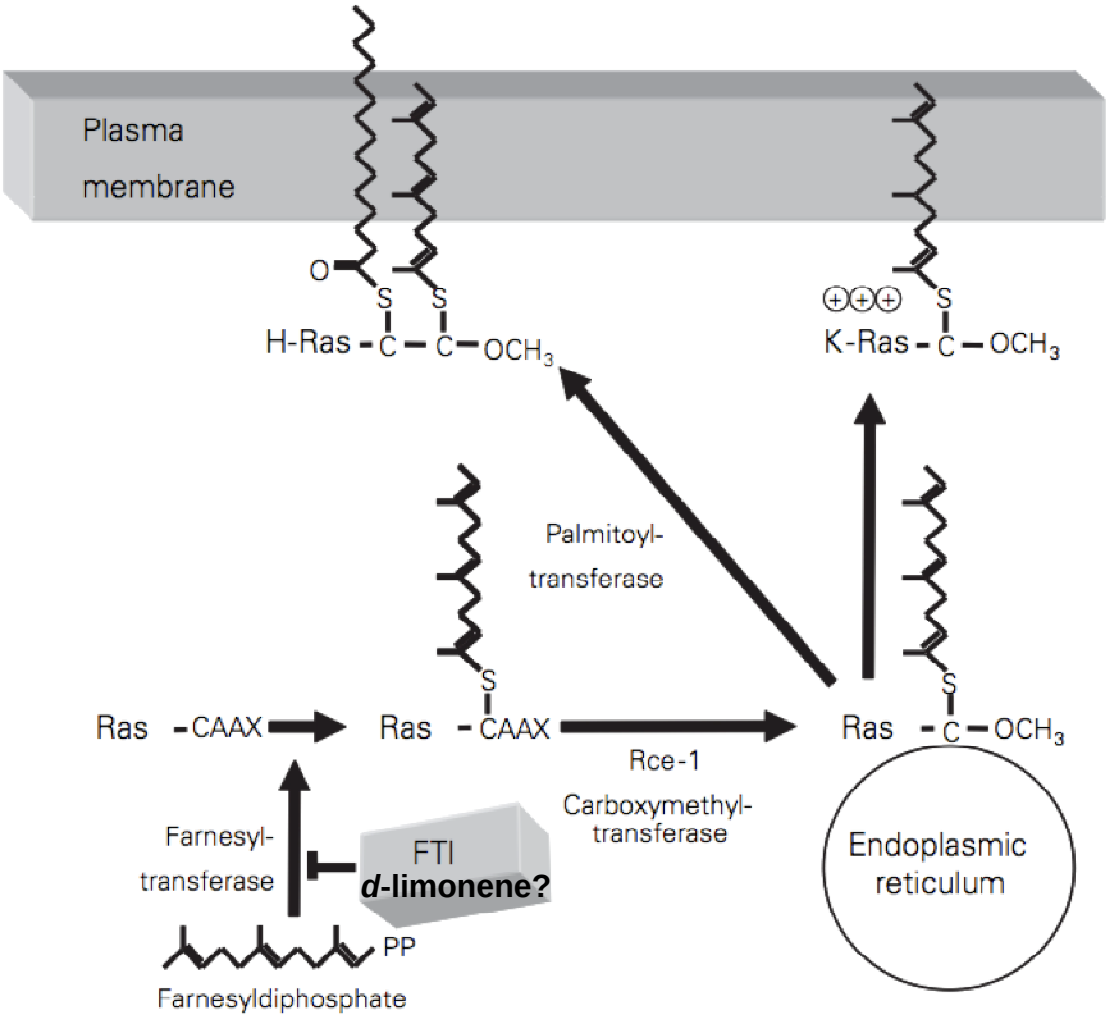

Figure 2: Ras postranslational modification and transformation. Ras must be bound to the cell membrane to have transforming activity. Oncogenic Ras proteins lose their transforming activity when attachment to the plasma membrane is blocked by farnesyltransferase inhibitors (FTIs). *d*-Limonene potentially acts as a less-potent FTI. After prenylation, the next steps in posttranslational modification are AAX proteolysis by Rce1 and then α-carboxymethylation of farnesylated cysteine residues by isoprenylcysteine carboxyl methyltransferase. Cysteine palmitoylation sites are contained in H-Ras, N-Ras, and K-Ras4A or a polylysine domain in K-Ras4B as depicted by positive charges in the figure. H-Ras is shown as an example of the three palmitoylated forms. (Figure and text adapted from Brunner *et al.*)[37]

CHAPTER 2:
ADIPOSE TISSUE ACCUMULATION OF *D*-LIMONENE WITH THE CONSUMPTION OF A LEMONADE PREPARATION RICH IN *D*-LIMONENE CONTENT

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Abstract

d-Limonene is a bioactive food component found in high concentration in citrus peel oil with anti-cancer effects in preclinical studies of mammary carcinogenesis. Extrapolation of preclinical data to human cancer is limited, in part, by inadequate information on the oral bioavailability and tissue disposition of *d*-limonene in humans. As a fat-soluble compound, *d*-limonene is more likely to deposit in fatty tissues such as the breast. To assess disposition of *d*-limonene in humans, we conducted a pilot study of oral *d*-limonene-rich lemonade. Following a one-week washout period devoid of citrus, healthy adults consumed 40 oz of freshly prepared lemonade, containing 500-600 mg *d*-limonene, daily for 4 weeks. On the first and last consumption days, blood and buttock fat biopsy were collected. Matched pre- and post-intervention fat biopsies (n = 7), and matched pre- and post-intervention plasma samples (n = 6) were analyzed for *d*-limonene levels using gas chromatography-mass spectrometry. There was a significant increase in *d*-limonene levels in the fat biopsies after 4 weeks ($P = 0.009$); initial levels ranged from non-detectable to 7.79 μmol/kg-tissue and post-intervention levels ranged from 53.6 to 294μmol/kg-tissue. Plasma *d*-limonene levels increased from 0.35 to 0.72 μmol/L initially to post intervention levels of 0.54 to 1.65 μmol/L ($P = 0.016$). Post-intervention adipose *d*-limonene levels were 51.0 – 195 times higher than plasma levels ($P = 0.009$). Our results demonstrate accumulation of *d*-limonene in adipose tissue after oral dosing and support additional studies of *d*-limonene for chemoprevention in tissues like the breast that are comprised of a significant fat fraction.

Introduction

Epidemiological evidence suggests that diets high in fruits and vegetables may be protective against certain cancers [78]. This protective effect has been attributed to a number of compounds found in food that have pharmacological effects, called bioactive food components (BAFC) [79]. *d*-Limonene is a BAFC commonly found in high concentration in citrus peel oil. In animal models, *d*-limonene and structural analogs demonstrate strong chemopreventive effects in lymphomas, mammary, gastric, liver, and lung cancers [8, 12, 13, 56, 80]. Of these cancers, the preclinical evidence is strongest for a potential chemopreventive role in mammary carcinogenesis. For example, rats fed a 5% *d*-limonene diet before introduction of nitrosomethylurea (NMU) demonstrated significantly reduced tumor size and tumor number compared to the control diet [9, 57]. In rats with 7,12-dimethylbenz(a)anthracene (DMBA) induced mammary cancer, *d*-limonene diets as low as 0.1 and 0.01% were effective in significantly increasing tumor latency [81, 82]. In mouse mammary gland organ culture, *d*-limonene demonstrated 78% inhibition of initiation of DMBA-induced tumors at a concentration of 1 x 10-8 mol/L [39]; a concentration achievable with oral dosing. While the anti-cancer mechanism of *d*-limonene is unclear, inhibition of the isoprenylation of a number of small GTPase proteins that have involvement in cell proliferation and motility, such as Ras and Rho, have been implicated [11, 49].

Despite the body of evidence demonstrating *d*-limonene's anticancer effects, research investigating its disposition in humans is limited. *d*-Limonene is bioavailable in humans after oral administration. The two major circulating metabolites of limonene are

perillic acid and dihydroperillic acid [20]. In a trial conducted in advanced cancer patients, peak plasma levels of limonene, perillic acid, and dihydroperillic acid were found to be around 10-12, 20-37, and 11-16 μmol/L, respectively, at the maximum tolerated dose (8 g/m^2) [21]. Perillic acid and dihydroperillic acid have been shown to be more potent inhibitors of protein isoprenylation than limonene, and perillic acid is also a more potent inhibitor of cell growth [23, 83]. However, *d*-limonene is likely to have a favorable tissue distribution for exerting biological activities due to its high lipophilicity while the tissue distribution of the oxygenated metabolites may be limited by their polarity.

Data from animal studies suggest that *d*-limonene might distribute preferentially into anatomical sites rich in fatty tissue such as breast. In female rats given an oral dose of radiolabeled *d*-limonene, the radioactivity concentrates in adipose and mammary tissues suggesting accumulation of *d*-limonene and/or its derived metabolites [83]. In the cancer therapeutic trial cited earlier [21], limonene and its metabolites were identified and quantified in metastatic lymph node tissue collected from two breast cancer patients receiving limonene 8 g/m^2 per day for 21 days. The intratumoral *d*-limonene levels were found to exceed the corresponding plasma levels by 1.9 and 5.5 fold whereas most metabolites of limonene were trace constituents in tissue with tissue-to-plasma ratios of limonene 4-10 times higher than its major metabolite, perillic acid [21]. Based on these limited data, we hypothesize that *d*-limonene has favorable tissue distribution and will distribute extensively to anatomical sites rich in fatty tissue. To test this hypothesis, we

conducted a pilot study to determine the systemic and adipose tissue disposition of *d*-limonene following consumption of limonene-rich lemonade.

Materials and Methods

Study Participants. Seven healthy adults participated in this pilot study, six females and one male. Eligible participants had to be willing to consume 40 oz of lemonade daily. The average age was 29 years old (range, 18 - 38 years) and the average body mass index was 25.4 kg/m^2 (range, 22.4 – 31.5 kg/m^2). All participants were in general good health, were not pregnant or breast-feeding, had normal liver and renal function, and had no incidence of cancer within the last 5 years or other serious or chronic diseases. The study was approved by the University of Arizona Human Subjects Committee and written consent was obtained from all participants.

Study Design and Intervention. This study was a single arm 4-week lemonade feeding trial conducted among healthy, free-living adults. Prior to baseline evaluation, participants were instructed to consume at least 3 glasses of water per day for 1 week. During this time, participants were also required to refrain from any citrus or citrus products. At their baseline visit, participants were given breakfast with high *d*-limonene lemonade. The lemonade was freshly prepared by blending 2 whole lemons (with peel) with 40 oz (1,182 mL) of water. Six hours after the lemonade consumption, a blood sample and a needle fat biopsy from the buttock were collected. Participants were then provided with a one-week supply of fresh lemons and instructions for the daily lemonade preparation. Subjects were required to prepare 40 oz of lemonade fresh daily and

consumed the preparation each day in a single dose with food for 4 weeks. Participants returned weekly for safety and adherence evaluation and brought samples of their homemade lemonade for *d*-limonene content analysis to determine individual *d*-limonene intakes. At the end of week 4, participants returned to the clinic and consumed breakfast with 40 oz of lemonade prepared fresh in the clinic. Six hours after the lemonade consumption, a second blood sample and a repeat fat biopsy from the buttock were collected. In addition, a fasting blood sample was collected before and after 4 weeks of daily lemonade intervention for a complete blood count with differential leukocyte count and a comprehensive blood chemistry analysis for evaluation of safety. Lemonade aliquots, plasma and buttocks adipose biopsy samples were stored at -80^{o}C until *d*-limonene analysis.

Plasma *d*-limonene analysis. Plasma *d*-limonene concentrations were determined using a published assay [84] with minor modifications. Briefly, plasma samples or plasma calibration standards (100 μL) were mixed with an equal volume of the internal standard solution (1.2 mg/L of perillyl aldehyde in 100% acetonitrile) to precipitate the plasma proteins. After vortexing and centrifugation, the supernatant was removed and mixed with 100 μL hexane for *d*-limonene extraction. This mixture was then vortexed and centrifuged. One microliter of the hexane layer was injected into the GC-MS system with a splitless injection at 220^{o}C. Chromatographic separation of *d*-limonene and internal standard was achieved on a high resolution GC DB-5MS fused silica capillary column with an initial oven temperature set at 60^{o}C and increased to 140^{o}C at 20^{o}C per min. The mass spectrometer source was set at 280^{o}C with the mass

analyte analyzer set at selective ion monitoring with the prominent masses of each analyte. *d*-Limonene and the internal standard elute at approximately 9 and 14 minutes, respectively. Calibration curves were constructed by plotting *d*-limonene to internal standard peak area ratios against the *d*-limonene concentration. The assay is linear over the *d*-limonene concentration range of 1 to 250 ng/mL. The inter- and intra-day variation for the assay was <10%. Extraction recovery of both *d*-limonene and internal standard were > 80%.

Adipose *d*-limonene analysis. Adipose *d*-limonene concentrations were analyzed according to a method developed in our laboratory [61]. Briefly, adipose biopsies were weighed and incubated at 37^{o}C in a water bath for 2.5 hours with 200 µL of 30% potassium hydroxide and 1 mL ethanol to induce saponification. Calibration standards, prepared by spiking different concentrations of limonene to a fixed amount of pork fat, were saponified using the same procedure. After cooling to room temperature, 3 mL hexane, 1 mL purified H_20, and 30 µL internal standard solution (9.5 mg/L of perillyl aldehyde in methanol) were added to the saponified samples and calibration standards. Samples were vortexed and then centrifuged at room temperature. The hexane layer was then removed and concentrated to 0.3 mL under a stream of nitrogen on a pre-frozen metal block. One microliter of the concentrate was injected into the GC-MS system with a splitless injection at 220^{o}C. Chromatographic separation of *d*-limonene and internal standard was achieved on a high resolution GC DB-5MS fused silica capillary column with an initial oven temperature held at 70^{o}C for 10 minutes, ramping 15^{o}C/minute up to 300^{o}C and held for 5 minutes. The mass spectrometer source temperature was set to

250^{o}C with the mass analyzer set to selected ion monitoring mode with a positive polarity for the prominent masses of each analyte. Limonene and the internal standard elute at approximately 8 and 15 minutes, respectively. Calibration curves were constructed by plotting *d*-limonene to internal standard peak area ratios against the amount of *d*-limonene spiked. The assay is linear between 79.0 – 2,529 ng *d*-limonene. Within and between day assay variations were < 10%. Extraction recovery of both *d*-limonene and internal standard were > 80%.

Lemonade *d*-limonene analysis. The analysis of *d*-limonene content in the lemonade was performed using a reversed-phase HPLC procedure as previously described [85]. An aliquot of the lemonade was mixed and diluted with the mobile phase before injecting into the HPLC. Chromatographic separation was achieved using a Supelco LC-ABZ column (150 X 4.6 mm, Supelco, Bellefonte, PA), and a mobile phase consisted of acetonitrile and sodium acetate buffer [25 mM (pH 5.0)] in the ratio of 70:30. The flow rate of the mobile phase was 1.1 mL/min. The column eluent was monitored with a UV detector at a wavelength of 230 nm. *d*-Limonene contents were quantified using calibration curves prepared with *d*-limonene standards diluted with the mobile phase. The calibration curve was linear over the concentration range of 0.5 to 100 mg/mL.

Data analysis. The differences between patient-matched pre- and post-intervention adipose *d*-limonene levels were compared using a paired, two-tailed, student's t-test. A paired, two-tailed, student's t-test was also used to compare patient-matched pre- and post-intervention plasma *d*-limonene levels. The differences between

time-matched plasma and adipose *d*-limonene levels were also compared using a paired, two-tailed, t-test. A P value < 0.05 was considered statistically significant. Pearson's correlation was used to determine if *d*-limonene juice levels were associated with *d*-limonene deposition in adipose and/or plasma and was also used to determine if initial or post-intervention serum levels were correlated to the time-matched adipose samples.

Results

Analysis of the *d*-limonene content of repeat lemonade samples showed *d*-limonene levels ranging between 480 – 790 mg *d*-limonene per 40 oz of lemonade. Compliance was determined by the return of unused lemons and was 100%. Assuming participants consumed the full 40 oz of lemonade per day, the average levels measured in the weekly lemonade samples for each patient would represent their *d*-limonene consumption. The consumption of study lemonade was well tolerated with no clinically significant changes in hematology or blood chemistry. Consuming the lemonade with a meal alleviated gastrointestinal distress and maximized *d*-limonene absorption.

Figure 3 shows the relative *d*-limonene adipose concentrations after a single dose and again after repeat daily dosing of high *d*-limonene lemonade for 4 weeks for individual study subjects (n = 7). As illustrated in the figure, *d*-limonene concentrations were low or un-detectable in the adipose tissue 6 hours after the initial lemonade consumption for all subjects, and greatly increased after 4 weeks of daily consumption. Table 6 summarizes the average and range of *d*-limonene concentrations of all adipose samples collected after the initial single dose of high *d*-limonene lemonade and after 4

weeks of daily consumption. Data are presented in molar concentrations for comparisons of concentrations used in prior studies. Initial adipose *d*-limonene concentrations ranged from not detectable to 7.79 μmol/kg-tissue (mean = 3.79 μmol/kg). On average, post intervention adipose *d*-limonene levels increased 44-fold to 53.6 – 294 μmol/kg-tissue (mean = 137 μmol/kg) and this was statistically significantly higher than the initial levels ($P = 0.009$).

Figure 4 shows the change in plasma *d*-limonene concentration for the individual study subjects (n = 6) after the single-dose and subject-matched post repeated lemonade dosing daily for 4 weeks. All subjects demonstrated measurable *d*-limonene in plasma 6 hours after the initial lemonade consumption although at concentrations in the low μmol/L range. As shown in Table 6, initial plasma levels were 0.35 – 0.72 μmol/L (mean = 0.48 μmol/L) and this was not significantly different from initial adipose levels ($P = 0.157$). There was a small but statistically significant increase in post-intervention plasma *d*-limonene levels ($P = 0.016$) with post-intervention levels ranging from 0.54 to 1.65 μmol/L (mean = 1.12 μmol/L). We compared the adipose and plasma *d*-limonene concentrations by assuming that the adipose tissue has a density of 0.9 g/ml (kg/L). Post-intervention adipose *d*-limonene levels were found to be significantly higher than the post-intervention plasma *d*-limonene concentrations ($P = 0.009$) with an adipose-to-plasma concentration ratio of 51 to 195 (mean = 111).

There was a positive correlation between the average amount of *d*-limonene measured in the study lemonade and the post-intervention (4 week) adipose *d*-limonene concentration ($\rho = 0.91$; $P = 0.003$; Figure 5). There was no significant correlation

between average measured *d*-limonene content in lemonade and change in *d*-limonene deposition in adipose, plasma *d*-limonene levels at either time-point, or adipose-to-plasma *d*-limonene ratios. Importantly, *d*-limonene levels in adipose and plasma were not correlated at either the initial feeding ($\rho = 0.26$; $P = 0.30$) or after four weeks of repeat daily dosing ($\rho = 0.31$; $P = 0.87$). There were no significant associations between body mass index (BMI) and *d*-limonene final adipose or plasma levels or changes in *d*-limonene deposition from baseline. Nevertheless, these exploratory correlative analyses would need to be interpreted with cautions due to the small sample size.

Discussion

The primary objective of this pilot feeding study was to determine whether *d*-limonene partitions extensively to human adipose tissue after oral consumption. Among 7 healthy adults consuming high-limonene lemonade, *d*-limonene did distribute preferentially to adipose tissue as compared to plasma. After the single dose of lemonade, adipose *d*-limonene levels were 0 – 20 (mean 7.6) fold higher compared to time-matched plasma samples. The post-intervention adipose *d*-limonene levels ranged from 51 to 195 (mean 111) times of that in corresponding plasma samples. The data suggest significant deposition of *d*-limonene in adipose as compared to plasma and wide individual variability in exposure with lemonade feeding. Our findings are consistent with data from animal studies. A study by Crowell *et al* showed that in female rats, *d*-limonene and its derived metabolites (non-specific assay) depot in anatomical sites rich in fatty tissue [83]. In that study, a single dose of 1g/kg *d*-limonene resulted in peak adipose

tissue levels of *d*-limonene/metabolite were 6.6 times greater than those achieved in plasma whereas the peak level in mammary tissue was 5 times greater than the plasma levels. While the *d*-limonene exposure was much higher in these rodents than the current study, the adipose-to-plasma ratio deposition pattern is comparable to the current study where we found that the adipose biopsy *d*-limonene levels following the initial dose were on average 7.6 times higher than the plasma levels. In addition to the preferential distribution to the adipose tissue, we showed that *d*-limonene accumulates extensively in adipose tissue following repeated dosing. On average, there was a 44-fold increase in post intervention adipose *d*-limonene levels. This extent of accumulation of *d*-limonene was not observed in plasma samples (2.6-fold increase was observed in plasma). Based on general pharmacokinetic principles, the 2.6-fold increase in plasma *d*-limonene concentration following repeated dosing is not unexpected when the agent is administered approximately every elimination half-life. The differential accumulation of adipose and plasma *d*-limonene suggests that measurement of circulating *d*-limonene levels in humans may significantly underestimate the *d*-limonene concentrations in adipose tissue.

Another important finding of our study is that consumption of dietary lemonade made from citrus peel gave rise to high *d*-limonene concentrations in human adipose tissue. An average adipose tissue *d*-limonene concentration of 137 μmol/kg was achieved from consuming two whole lemons, or on average of 575 mg *d*-limonene daily. These pilot data suggest that *d*-limonene concentrations might reach biologically relevant levels with a dietary lemonade intervention because *d*-limonene at a concentration of 1 x

10^{-8} mol/L demonstrated 78% inhibition of initiation of DMBA-induced tumors in mouse mammary gland organ culture [39]. However, *d*-limonene concentrations in the low mmol/L range were required to inhibit mammary tumor growth in cell culture experiments [86] and to inhibit G protein prenylation [87, 88]. Intervention with higher doses of oral *d*-limonene products may be required to achieve low mmol/L tissue concentration and thus modulate these specific chemopreventive responses.

Our data provide indirect evidence that daily lemonade interventions could result in *d*-limonene deposition at biologically relevant levels in other high adipose tissue besides the buttocks, such as the breast. It is of interest that the most compelling antitumor activity of *d*-limonene has been observed in models of mammary carcinogenesis [9, 10, 39] as compared to other tumor sites. Adipose tissue is an active organ producing and secreting adipokines and other growth hormones [89], which might have direct or indirect effects on mammary carcinogenesis through endocrine-, paracrine- and autocrine-mediated pathways [90] as well as through effects on the tumor microenvironment [65, 91, 92]. Further studies are needed to determine whether *d*-limonene would affect the expression and secretion of adipose derived cytokines and hormones in response to a daily high *d*-limonene lemonade intervention.

In this study, we showed that daily dietary lemonade intervention was met with high adherence and was well tolerated. Additionally, high *d*-limonene intake for 4 weeks did not affect body weight, blood chemistry or hematology. Study participants consumed between 480 – 790 mg (mean = 574 mg) of *d*-limonene each day. These levels are much higher than commercially available lemonade or orange juice, which contain roughly 3

mg/L and 20-73 mg/L *d*-limonene, respectively [93]. It is worth noting that the amount consumed was strongly correlated with the adipose *d*-limonene concentration, but was not correlated with the plasma concentration. Additionally, adipose to plasma ratios were not correlated with *d*-limonene consumption after either the initial feeding or after repeat daily dosing. Our study further illustrates the importance of measuring agent levels in the target tissue (or surrogate target) because plasma concentrations may not always reflect the target tissue distribution and accumulation. Our data suggests that *d*-limonene may accumulate in the breast, given the high adiposity of breast tissue. Further research is needed to determine the effects of *d*-limonene on the expression and secretion of adipose derived cytokines and hormones and its effects in breast tissue and thus its potential as a cancer preventive agent.

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Table 6: Average *d*-limonene levels determined in time-matched adipose and plasma samples collected after a single dose of high-limonene lemonade consumption and after 4 weeks of daily consumption.

	Adipose *d*-limonene concentration, μmol/kg[1]	Plasma *d*-limonene concentration, μmol/L[2]	Adipose/plasma *d*-limonene ratio[2,3]	Post-4 wk/ initial adipose *d*-limonene ratio[1]	Post- 4wk/ initial plasma *d*-limonene ratio[2]
Initial					
Mean (SD)	3.79 (3.26)[6]	0.48 (0.21)	7.6 (7.5)	-	-
Range	ND – 7.79	0.35 – 0.72	ND – 20.0	-	-
Post-4 wk					
Mean (SD)	137 (87.2)[4,7]	1.12 (0.42)[5]	111.0 (51.5)	44.5 (29.3)	2.6 (1.1)
Range	53.6 – 293.9	0.54 – 1.65	51.0 – 195	8.6-89.3	1.1 – 4.2

[1] n = 7
[2] n = 6
[3] Calculated by assuming the fat density of 0.9 g/ml (kg/L)
[4] Significantly higher than the initial adipose samples ($P = 0.009$)
[5] Significantly higher than the initial plasma samples ($P = 0.016$)
[6] Initial adipose and plasma levels were not significantly different ($P = 0.157$)
[7] Post-4 wk intervention adipose levels were significantly higher than plasma levels ($P = 0.009$)

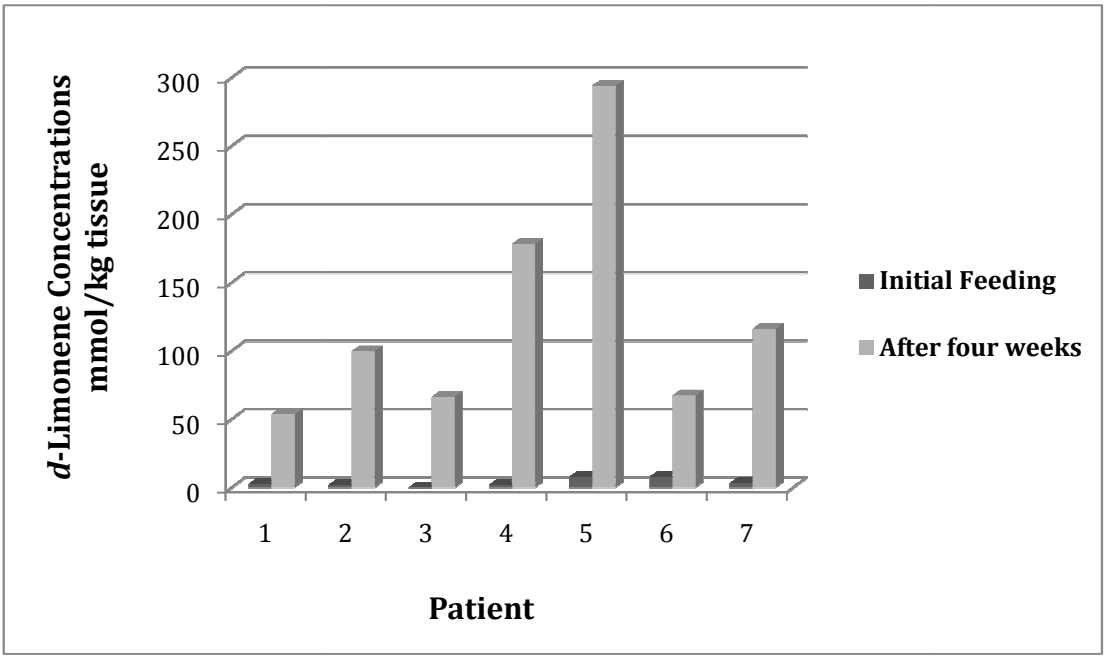

Figure 3: Individual subject *d*-limonene concentrations in matched needle buttock biopsies 6 hours after initial high-limonene lemonade consumption and after four weeks of repeat daily dosing.

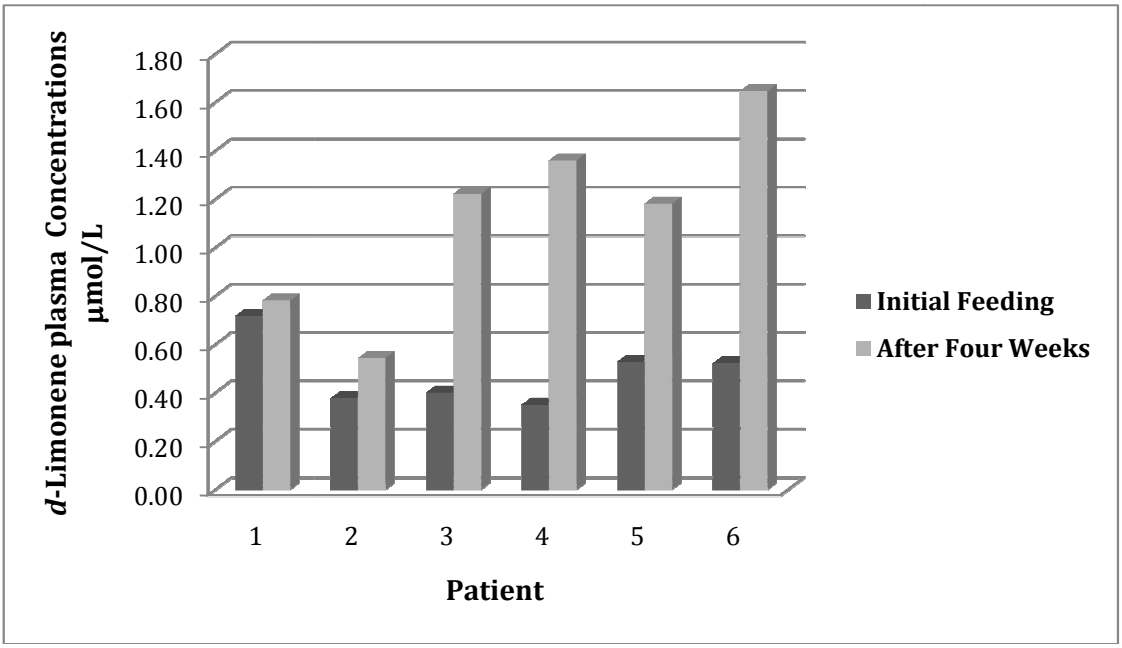

Figure 4: Individual subject *d*-limonene concentrations in matched plasma samples after initial high-limonene lemonade feeding and after four weeks of repeat daily dosing (500 mg *d*-limonene/day).

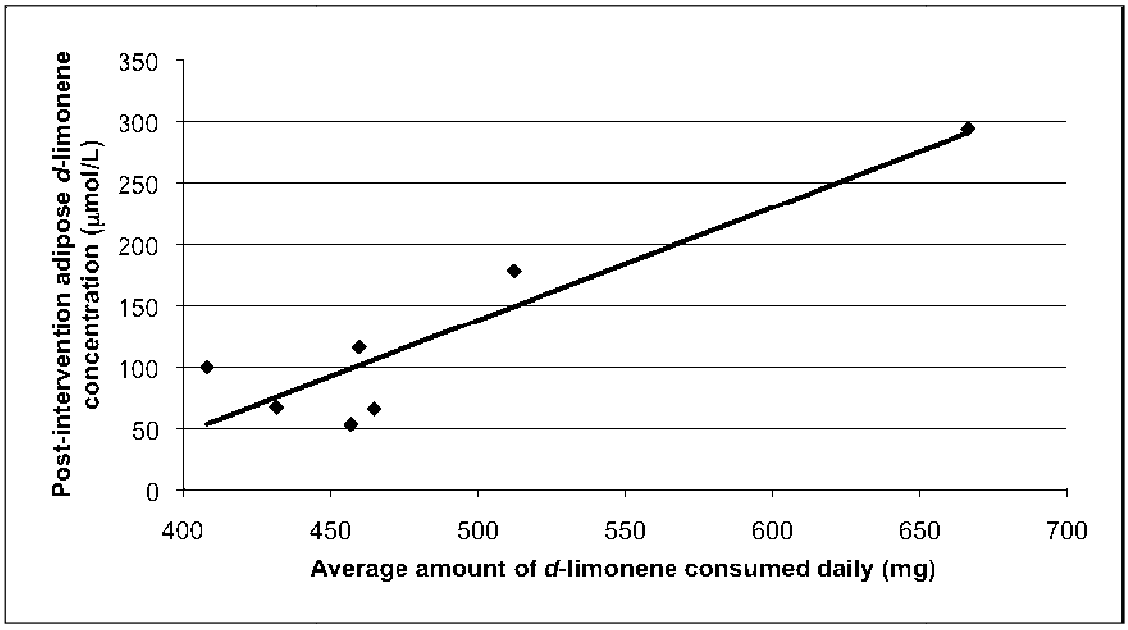

Figure 5: Correlation between average amount of *d*-limonene consumption from high-limonene lemonade and adipose *d*-limonene concentration after 4 weeks of daily feeding. (R^2 = 0.84; ρ = 0.91; *P* = 0.003)

CHAPTER 3
A CLINICAL BIOMARKER STUDY OF TOPICALLY APPLIED *D*-LIMONENE FOR BREAST CANCER PREVENTION.

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Abstract:

Background: *d*-Limonene has demonstrated anti-cancer effects in preclinical models of mammary carcinogenesis. We conducted an early phase biomarker study to assess breast tissue bioavailability of topically applied *d*-limonene. **Methods:** Forty-three healthy women applied a massage oil (10% orange oil) containing *d*-limonene to their breast daily for four weeks. Pre and post-intervention nipple aspirate fluid (NAF) and plasma samples were collected for determination of *d*-limonene concentrations and the expression of epidermal growth factor (EGF), transforming growth factor-beta 1 (TGF-β1), and adiponectin. **Results:** Repeated application of the massage oil formula was well tolerated, but did not significantly change NAF or plasma *d*-limonene levels or biomarker expression. NAF and plasma biomarker levels were differentially associated with BMI and menopausal status. NAF biomarker levels were not correlated to BMI whereas plasma EGF, TGF-β1 and adiponectin levels in postmenopausal women were all negatively correlated with BMI. Subgroup analyses demonstrated that plasma EGF and TGF-β1 levels significantly decreased in healthy-weight postmenopausal women post-intervention ($P = 0.0081$ and $P = 0.0002$ respectively). **Conclusions:** Topical application of *d*-limonene was well tolerated, but did not result in measurable *d*-limonene in NAF or plasma. Significant associations between the *d*-limonene response biomarkers, BMI, and menopausal status appear to limit the detectability of potential subgroup drug effects. **Impact:** This study demonstrates the acceptability and tolerability of topically applied cancer-prevention candidates to the breast and highlights effects of menopausal status and BMI on NAF and plasma biomarkers.

Introduction:

Despite advances in treatment, breast cancer is still the leading cause of cancer death in women, and in 2009 there were still about 192,370 new cases of invasive breast cancer and about 62,280 new cases of carcinoma in situ in the United States (American Cancer Society) [94]. Recent success in disease reduction among high-risk women with selective estrogen receptor modulators (SERMs) such as tamoxifen and raloxifene [95-100] has confirmed that breast cancer can be clinically targeted with chemopreventive agents prior to 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 unacceptable side effects. Recently, the potential role of aromatase inhibitors (AIs) in preventing breast cancer has been inferred from adjuvant trial data involving reduction of contralateral breast cancer risk by up to 70–80% of estrogen receptor-positive (ER+) breast cancers [101-104]. Based on these promising data, the role of AIs in breast cancer prevention is being evaluated. However, AI treatment is also associated with multiple side effects and poor drug adherence in women with cancer, which would limit its acceptability for long-term use as a prevention agent. Therefore, identification of chemopreventive agents that have less toxicity and higher tolerability for chronic use in high risk, but otherwise healthy women, remains an unmet need. In addition, developing preventive agents with broader spectrum activity including action against ER- negative breast cancer is desirable.

One putative breast cancer prevention agent is *d*-limonene, a monocyclic monoterpene and major component of the essential oils of citrus fruits. Oral

administration has been associated with diverse biological activities, including antitumor activity [105], with the most compelling results in mammary carcinogenesis models [11, 56, 106, 107]. In rats, a 5% *d*-limonene diet is effective in preventing initiation of 7,12-dimethylbenz(α)anthracene (DMBA)-induced mammary cancer [10] and promotion of both DMBA and N-methyl-N-nitrosourea (NMU)-induced tumors [9, 10].

The oral doses given in these animal studies translate to a high human dose that may not be feasible in humans over the long-term as a prevention strategy. *In vitro* studies have demonstrated that *d*-limonene can penetrate both the epidermis and dermal layers of the skin [108]. It has also been explored as an enhancer of the percutaneous absorption of pharmaceutical drugs, including tamoxifen [109, 110]. In addition, limonene distributes preferentially to adipose tissue after oral consumption in both humans [111] and rodents [64]. With these unique characteristics, limonene applied topically to the breast is likely to be absorbed percutaneously and accumulate in the breast tissue since the normal breast is mainly composed of adipose tissue.

This report summarizes the findings from an early phase biomarker study conducted to evaluate the breast tissue bioavailability of topically applied *d*-limonene using nipple aspirate fluid (NAF) as a surrogate specimen. The study also determined the effect of topical *d*-limonene application on potential secretable protein biomarkers, EGF, TGF-β1, and adiponectin that may be associated with *d*-limonene activity or breast cancer risk.

Methods:

Study Participants.

We recruited women who were 18-65 years of age, had good performance status, had normal organ and marrow function, were willing to use adequate contraception, were willing to avoid citrus products throughout the study, and had both breasts intact. Participants were excluded if they were pregnant or breast feeding, had invasive cancers within the past 5 years, participated in another clinical intervention trial within the past 3 months, had uncontrolled severe metabolic disorders or other serious acute or chronic diseases, were unable to produce NAF, had known allergic or sensitive reactions to skin care products, citrus or coconut oil, or had ongoing skin disorders such as eczema and psoriasis. The study was approved by the University of Arizona Human Subjects Committee and written consent was obtained from all participants.

Study Agent.

Limonene-containing essential oils are already in use in aromatherapy massage with no reported clinical toxicity, we have therefore used such preparations as a topical application approach in this exploratory clinical study. Organic orange essential oil (NOW Foods, Bloomingdale, IL, USA) was selected as the source of limonene. This product was reported to be prepared by cold compression of peel and cuticles of organically grown orange. We analyzed the limonene content of this product by GC-MS and found that it contained 93% limonene. We selected the fractionated coconut oil (From Nature with Love, Oxford, CT, USA) as the base oil to dilute the orange oil for

massage application. Fractionated coconut oil was reported to contain only medium-chain triglycerides, which were isolated through the hydrolysis of pure coconut oil and then fractionated by steam distillation to isolate the medium-chain triglycerides.

Study Intervention.

At the initial clinic visit, study participants were screened for NAF productivity. The breast was first cleaned with an apricot scrub and then NAF production was stimulated by breast massage combined with application of a warm, wet towel. Those who were successful in producing NAF from at least one breast continued with the study. Those who were not successful were offered to return and attempt NAF collection again. Those who were unable to produce NAF after repeated attempts were excluded from the study.

Participants who were able to produce NAF had a blood sample collected for complete blood count (CBC) with differential and a blood chemistry panel. A urine pregnancy test was performed for women who were not surgically sterile or were less than one year post-menopausal. A complete medical history was obtained. Height (by subject report), weight, blood pressure, pulse, and temperature measurements were obtained.

Eligible participants underwent a minimum of 4 weeks of washout in which they were required to avoid consumption and use of citrus and citrus-containing products

including skin care products. They were provided with a daily diary for recording any adverse events.

After completion of the washout period, study participants visited the clinic for baseline sample collection. A urine pregnancy test was repeated for fertile women. A positive pregnancy test excluded the subject from continuing study participation. NAF was collected into small capillary tubes and then immediately diluted in phosphate buffered saline (1:10). Blood (7 mL) was collected into a Vacutainer tube containing sodium heparin and centrifuged at 1,000 x g for plasma separation. Plasma and diluted NAF were stored at -80^{o} C prior to analysis. Following baseline sample collection, study participants underwent their first massage session in the clinic. The massage oil was prepared fresh by blending 3 drops of organic orange oil (containing 0.14 g of limonene) with 1.35 mL fractionated coconut oil to give a final orange oil concentration of 10%. Participants were instructed to wear surgical gloves and use their fingertips to massage the blended massage oil to the breasts in circular fashion with slight pressure, massaging each breast for 5 minutes. Participants were advised to avoid areola and nipples. Participants were provided with massage oil supplies which consist of pre-aliquoted fractionated coconut oil for daily use, aliquots of orange oil, droppers, blending dishes, and gloves for once daily massage application for 4 weeks. Participants were instructed that the massage application could be performed after showering or bathing and should be at least 8 hours before next showering/bathing. Participants were advised to avoid exposing their breasts to direct sunlight or a tanning bed throughout the intervention

period and were provided with a daily diary for recording any adverse events. Participants were instructed to record adverse events and time of massage application on the daily diary.

Following the 4-week intervention, participants returned the day after their last massage application. Blood and NAF were collected and processed as they were at baseline. Additional blood samples were collected for post-intervention CBC and blood chemistry. Massage oil supplies and diary were examined to evaluate compliance. Participants were instructed to continue to record adverse events for two weeks after the massage intervention before they were taken off study.

Sample Analysis.

Plasma and NAF limonene analysis: Plasma and NAF limonene concentrations were determined using a published gas chromatography-mass spectrometry (GC-MS) assay [84] with minor modifications. Briefly, plasma and NAF samples (100 μl) were mixed with an equal volume of the internal standard solution (1.2 μg/ml of perillyl aldehyde in 100% acetonitrile) to precipitate the plasma and NAF proteins. After vortexing and centrifugation, the supernatant was removed and mixed with 100 μl hexane. This mixture was then vortexed and centrifuged for 10 minutes at 1,400 x g at room temperature. One microliter of the hexane layer was injected into the GC-MS system with a splitless injection at 220^{o}C. Chromatographic separation of limonene and internal standard was achieved on a high resolution GC DB-5MS fused silica capillary

column with an initial oven temperature set at 60^{o} C and increased to 140^{o} C at 20^{o} C per min. The mass spectrometer source was set at 280^{o} C with the mass analyte analyzer set at selective ion monitoring with the prominent masses of each analyte. The mass spectrometer source was set at 280^{o} C with the mass analyte analyzer set at selective ion monitoring for target ions 93 *m/z* and 107 *m/z* for limonene and internal standard, respectively.

Plasma and NAF biomarker analysis. NAF and plasma EGF, TGF-β1, and adiponectin, were measured using ELISA based immunoassays (R&D Systems, Minneapolis, MN, USA). Plasma samples were diluted prior to the analysis according to the manufacturer instructions for EGF and TGF-β1, and were diluted 1:200 for adiponectin assay. NAF samples were further diluted 1:100 – 400 for EGF, 1:20 – 40 for TGF-β1 and 1:3 – 1:20 for adiponectin. Assays were linear over the concentration range of 3.9 - 250 pg/mL, 31.2 – 2,000 pg/mL, and 3.9 - 250 ng/mL for EGF, TGF-β1, and adiponectin, respectively. For each assay, baseline and post-intervention samples of the same individual were analyzed in the same batch and each sample was analyzed in duplicate.

Statistical Analysis.

The signed rank test was used to compare the paired data, including the comparison between pre- (post-) intervention *d*-limonene NAF levels and pre- (post-) intervention *d*-limonene plasma levels since they were measured in the same subject.

The Wilcoxon rank sum test was used to compare the two independent samples. Specifically, comparisons between pre- and post-intervention *d*-limonene NAF and plasma levels as well as for differences between pre- and post-intervention protein biomarker levels were conducted using the signed rank tests. After separating the data by BMI, the signed rank tests were also used to compare protein biomarker levels in both NAF and plasma pre to post-intervention within the healthy and overweight subgroups separately. Comparisons between premenopausal and postmenopausal in protein biomarker levels were conducted using the Wilcoxon rank sum test. Spearman correlation coefficients were derived to determine correlations between BMI and NAF or plasma protein biomarker levels. A *P*-value of < 0.05 was considered statistically significant.

Results:

Participants and tolerability of topically applied *d*-limonene. A total of eighty-eight women were consented, of which 43 met the eligibility criteria. Of those ineligible, 42 women were not able to produce adequate NAF (≤ 2 μL), one was unwilling to refrain from citrus, and one had abnormal hepatic enzymes. One woman withdrew due to intolerance to the citrus fumes. Overall, 43 eligible participants completed the study intervention; 16 premenopausal and 27 postmenopausal. The average age of the participants who completed the intervention was 51.7 years old (range 23 - 66) and the average body mass index was 26.2 kg/m^2 (range 18.7 – 36.2 kg/m^2). Table 7 presents the demographic data for those participants completing the intervention. Four weeks of daily topical application of *d*-limonene containing massage oil was well tolerated and met

with high compliance. Three postmenopausal women developed a rash in the sun-exposed area of the application site. The orange oil preparation used in these women was analyzed by GC-MS and found to have increased levels of an oxygenated *d*-limonene by-product (limonene oxide), which has been previously associated with skin irritation [112]. The rash was mild and did not limit daily activities, and all three women completed the study without dose reduction. Subsequent participants were supplied with a new batch of orange oil and no further participants experienced similar rashes. Two women complained of tingling at the application site, but it was temporary and they also completed the study without dose reduction. In addition, the massage intervention did not result in any changes in hematology measurements and blood chemistry.

Plasma and NAF *d*-limonene concentrations. Participants produced a wide range of NAF volume (3 – 50 μL). For 28 participants there was insufficient sample volume to analyze all three biomarkers. Of these participants, we have selected to analyze the *d*-limonene concentration in NAF and matched plasma samples from 10 individuals who had sufficient NAF yield for all endpoint analysis. Figure 6 illustrates the *d*-limonene levels in NAF and plasma from baseline to post-intervention. Data are presented as: median (mean $\pm$ SD). *d*-Limonene levels were 11.35 (13.32 $\pm$ 5.91) ng/mL at baseline and did not change after 4 weeks of topical massage application ($P = 0.38$). Plasma *d*-limonene concentrations were 1.46 (2.53 $\pm$ 3.27) ng/mL at baseline and also did not change after 4 weeks of topical massage application ($P = 0.77$). Interestingly, NAF *d*-limonene levels were significantly higher than the time-matched plasma ($P < 0.01$).

Plasma and NAF Biomarker Levels and Response to Topical *d*-Limonene.

The total protein concentration in NAF varied widely among the study participants (13 – 101 mg/mL) suggesting potential variation in individual protein expression. Thus, all NAF protein biomarker measurements were normalized by the total protein concentration. Total, there were 34 EGF, 37 TGF-β, and 28 adiponectin pairs with time-matched plasma analyzed. Table 8 summarizes the pre and post-intervention NAF biomarker levels in all women and stratified by menopausal status. Data is presented as: median (mean $\pm$ SD). Baseline NAF levels of EGF, TGF-β1 and adiponectin levels in all women combined were; 1,027 (3,957 $\pm$ 4,610) ng/g, 409 (901 $\pm$ 1,853) ng/g, and 13.4 (23.1 + 26.1) μg/g respectively. Baseline NAF levels of EGF, TGF-β1 and adiponectin in premenopausal women were; 8,671 (7,759 $\pm$ 2,854) ng/g, 923 (2,079 $\pm$ 3,027) ng/g, and 8.3 (11.6 $\pm$ 9.1) μg/g protein, respectively. Baseline NAF levels of EGF, TGF-β1 and adiponectin in postmenopausal women were 702 (2,532 $\pm$ 4,354) ng/g, 277 (361 $\pm$ 357) ng/g, and 15.0 (26.3 $\pm$ 28.5) μg/g protein, respectively. Postmenopausal women had significantly lower baseline NAF EGF ($P = 0.0022$) and TGF-β1 ($P < 0.0001$) levels than premenopausal women. Adiponectin levels were non-significantly higher by two-fold higher in postmenopausal women ($P = 0.0880$), most likely because of the very small sample size of premenopausal women. Neither premenopausal nor postmenopausal women demonstrated significant changes in NAF EGF, TGF-β1, or adiponectin following 4 weeks of topical application of *d*-limonene containing massage oil.

Table 9 presents the baseline and post-intervention plasma levels of EGF, TGF-β and adiponectin in all women and stratified by menopausal status. In all women

combined there were 43 matched plasma pairs for all biomarkers with 16 of these from premenopausal women and 27 from postmenopausal women. Data is presented as: median (mean $\pm$ SD). Pre-intervention plasma levels of EGF, TGF-β1 and adiponectin in all women were 21.5 (26.3 $\pm$ 21.1) pg/mL, 9.39 (9.92 $\pm$ 3.77) ng/mL, and 10.93 (11.99 $\pm$ 5.74) μg/mL respectively. Pre-intervention plasma levels of EGF, TGF-β1 and adiponectin in premenopausal women were 22.0 (34.1 $\pm$ 29.0) pg/mL, 9.31 (9.92 $\pm$ 3.76) ng/mL, and 9.10 (9.67 $\pm$ 4.19) μg/mL, respectively. Pre-intervention plasma levels of EGF, TGF-β1 and adiponectin in postmenopausal women were 22.5 (21.6 $\pm$ 13.5) pg/mL, 9.39 (10.49 $\pm$ 3.13) ng/mL, and 12.03 (13.37 $\pm$ 6.14) μg/mL, respectively. EGF and TGF-β1 levels were not different between pre and post-menopausal women (P = 0.2891 and P = 0.5097 respectively). Postmenopausal women had higher adiponectin levels than premenopausal women, but this difference did not reach statistical significance (P = 0.0607). None of the pre-intervention plasma biomarker levels were statistically significantly correlated to NAF levels (data not shown). Neither premenopausal nor postmenopausal women demonstrated significant changes in plasma EGF, TGF-β1, or adiponectin following 4 weeks of topical application of *d*-limonene containing massage oil.

In order to characterize trends in the data, we have examined the correlation of baseline levels of EGF, TGF-β1, and adiponectin with BMI (Table 10). In the premenopausal women, BMI was not significantly correlated with NAF EGF (P = 0.6059; n = 9), TGF-β1 (P = 0.5015; n = 11), or adiponectin (P = 0.5441; n = 6). Plasma EGF and TGF-β1 were not significantly associated with BMI in premenopausal women

(P = 0.3446; n = 16 and P = 0.1130; n = 16, respectively). Plasma adiponectin was significantly negatively associated with BMI (P = 0.0165; n = 16) in premenopausal women. In the postmenopausal women, NAF EGF, TGF-β1, and adiponectin were not significantly correlated with BMI (P = 0.7852; n = 25, P = 0.6831; n = 26, P = 0.5012; n = 23, respectively). Plasma EGF, TGF-β1 and adiponectin were all significantly negatively correlated with BMI (P = 0.0027; n = 27, P < 0.0001; n = 27, P = 0.0458; n = 27, respectively) in postmenopausal women.

Because of the correlations observed between plasma biomarkers and BMI, we have re-evaluated the intervention effect on plasma biomarkers based on menopausal status and stratified by "healthy-weight" (BMI $\leq$ 25) and "overweight" (BMI > 25) subgroups (Table 11). There were no underweight women in this study (BMI $\leq$ 18.5). There were 8 healthy-weight premenopausal women, 8 overweight premenopausal women, 13 healthy-weight postmenopausal women, and 14 overweight postmenopausal women with enough sample volume for the paired analysis of all three biomarkers. Data are expressed as: median (mean $\pm$ SD). Baseline plasma levels of EGF [healthy-weight: 16.2 (24.0 $\pm$ 21.7) pg/mL, overweight: 37.8 (44.2 $\pm$ 33.0) pg/mL] and TGF-β1 [healthy-weight: 8.14 (8.66 $\pm$ 2.79) ng/mL, overweight: 11.09 (11.18 $\pm$ 4.15) ng/mL] in premenopausal women were in general higher in the overweight than the healthy-weight women, but there were no statistically significant intervention-related changes. Conversely, adiponectin levels in premenopausal women were in general lower in the overweight than the healthy-weight women [healthy-weight: 11.41 (11.23 $\pm$ 4.95) μg/mL,

overweight: 7.53 (8.06 ± 2.67) μg/mL]. Adiponectin was not changed following the intervention in either group.

All baseline plasma biomarker levels in postmenopausal women were on average higher in the healthy-weight women but were not statistically significantly different [EGF healthy-weight: 26.7 (27.8 ± 13.9) pg/mL, overweight: 11.4 (16.0 ± 10.7) pg/mL; TGF-β1 healthy-weight: 11.32 (12.13 ± 2.62) ng/mL, overweight: 8.22 (8.97 ± 2.85) ng/mL; adiponectin healthy-weight 16.51 (15.79 ± 6.56) μg/mL, overweight: 10.61 (11.12 ± 4.95) μg/mL]. Plasma EGF levels in postmenopausal healthy-weight women decreased 36% post-intervention, and this was statistically significant ($P = 0.0081$). Conversely, EGF levels increased by 74% in overweight postmenopausal women, but this did not reach statistical significance ($P = 0.1531$). TGF-β1 plasma levels in postmenopausal healthy-weight women decreased 28% from pre- to post-intervention and this was statistically significant ($P = 0.0002$). There was an increase in TGF-β1 in the postmenopausal overweight women of 19%, however, this was not statistically significant ($P = 0.1937$). The *d*-limonene intervention had no effect on plasma adiponectin levels in either the healthy ($P = 0.2163$) or overweight ($P = 1.0000$) women.

Further analyses indicate that the two-way interaction between BMI, menopausal status, on the plasma biomarkers EGF ($P = 0.0353$) and TGF-β1 ($P = 0.0097$) were statistically significant.

Discussion:

The concept of topical application of chemopreventive agents to the breast provides potential for localized drug delivery with minimal systemic side effects. This rationale has initiated the development of the chemotherapeutic drug, tamoxifen, as a topical agent [113, 114]. Topical application of a naturally available agent, like *d*-limonene, through a lotion or cream also could provide a low-risk, cost-effective breast cancer prevention strategy. Our study showed that topical application of *d*-limonene containing massage oil did not change the NAF or plasma *d*-limonene concentration, suggesting limited percutaneous absorption of the topically applied agent. However, it is also plausible that the topically applied *d*-limonene would deposit in the fatty breast tissue with minimal secretion to NAF or systemic circulation because *d*-limonene preferentially distributes to adipose tissue showing a high adipose-to-plasma concentration ratio [111].

Because of concerns of limited secretion of *d*-limonene from adipose tissue, we measured the effect of topical *d*-limonene application on secretable protein biomarkers that may be associated with *d*-limonene activity or breast cancer risk; EGF, TGF-β1, and adiponectin. NAF EGF levels in healthy pre and postmenopausal women were similar in our study to those previously reported [115-117]. NAF EGF levels in the previous study were significantly correlated with estradiol and estradiol precursors and suggested as a possible growth factor mediator of hormone associated breast cancer risk [115]. Preclinical studies support tumor-promoting effects of EGF including induction of cell motility [118]. Boccardo *et al.* demonstrated that in women who eventually develop

breast cancer, EGF cyst levels were on average twice as high as EGF levels in breast cyst fluid of women who did not develop breast cancer [119]. Our findings of lower EGF levels in the postmenopausal women are consistent with a positive effect of hormone levels, however, NAF EGF levels were unchanged in the overall group following four weeks of topical *d*-limonene administration. Similarly, EGF levels in plasma were also unchanged after four weeks of topical limonene intervention in the entire sample. In subgroup analysis, a significant reduction in plasma EGF was seen in postmenopausal healthy-weight women after four weeks of *d*-limonene intervention. Because EGF is an estrogen-response growth factor [120], it is possible that an intervention effect was observed in the leaner, postmenopausal group because of the lack of a confounding effect of estrogen. Nevertheless, these results should be interpreted with caution due to limited sample size.

In the present study, we also determined NAF and plasma TGF-β1 levels. To our knowledge, this is the first study to identify TGF-β1 in NAF. TGF-β1 is understood to have a dual role in terms of cancer development; in the presence of cancers with disturbances in the signaling pathway, TGF-β1 serves as a tumor promoter and high tumor levels are found in metastasis [58, 121, 122]. In healthy individuals, however, TGF-β1's primary function is to limit epithelial proliferation, as well as to regulate cell cycle and apoptotic pathways, therefore halting pre-malignant growth [58]. Pre-clinical evidence suggests that *d*-limonene's anti-cancer mechanism could be through modulation of TGF-β1. An early study conducted by Jirtle *et al* demonstrated that rats with stable mammary tumors given a 10% *d*-limonene diet had 87% regression of the tumors; in both

the early and late regressing tumors, and TGF-β1 was on average increased 2-fold [27]. Nevertheless, in this study, NAF and plasma TGF-β1 levels were not changed after topical *d*-limonene application. In subgroup analysis, plasma TGF-β1 was significantly reduced in postmenopausal healthy-weight women following topical *d*-limonene intervention. The data further suggest that an intervention effect may only be observed in a subgroup that has minimum confounding effect from other endogenous, perhaps hormonal, factors.

NAF and plasma adiponectin levels were also assessed in our study. To our knowledge, this is also the first study to identify adiponectin in NAF, although, it has been quantified in breast milk [123]. Adiponectin is an adipokine primarily secreted from adipose tissue, and is negatively correlated with breast cancer risk (42, 43). It's roles include lipid and glucose metabolism as well as vascular inflammatory processes [124], and elevated levels are indicative of enhanced immunity [125]. Preclinical evidence indicates that *d*-limonene's inhibitory effects on mammary carcinogenesis may also be related to its immune enhancing effects [8, 34-36]. In addition, preclinical data from our lab suggests that *d*-limonene is able to reverse TNF-α induced suppression of adiponectin secretion from 3T3-L1 adipocytes (unpublished data). In this study, however, 4 weeks of topical *d*-limonene application to the breast had no effect on adiponectin levels in NAF or plasma in all women combined or when women were separated by BMI and menopausal status.

Interestingly, our data suggest that the circulating EGF, TGF-β1, and adiponectin are regulated differently from the NAF protein expression. No correlation was observed

between plasma and NAF protein expression. In relation to menopausal status, NAF EGF and TGF-β1 levels in postmenopausal women were significantly lower than those in premenopausal women, while menopausal status did not seem to effect expression of these proteins in plasma. Adiponectin levels in postmenopausal women were non-significantly higher than those in premenopausal women in both NAF and plasma. There were also disparities in the correlation of NAF and plasma proteins with BMI. NAF protein levels were not correlated to BMI whereas plasma EGF and TGF-β1 levels in postmenopausal women correlated negatively with BMI. Plasma adiponectin in both pre and postmenopausal women correlated negatively with BMI. Further analyses indicated a statistically significant two-way interaction between BMI and menopausal status on the biomarkers, EGF and TGF-β1 in plasma only. These data suggest that the underlying biological factors associated with BMI and menopausal status may affect biomarker levels and that circulating protein expression may not necessarily reflect the microenvironment in the breast.

Overall, this early phase clinical trial demonstrates that four weeks of topical *d*-limonene administration in the form of massage oil is safe and well tolerated in healthy women. The massage oil application did not result in consistent changes in plasma and NAF *d*-limonene levels and had minimal effect on plasma and NAF EGF, TGF-β1, and adiponectin. Menopausal status and BMI appeared to influence the plasma and/or NAF expression of these proteins. Subgroup analysis showed a significant reduction of plasma EGF and TGF-β1 in 'healthy-weight' postmenopausal women following 4 weeks of *d*-limonene application, suggesting that the potential *d*-limonene effect in other subgroups

could be masked by overwhelming endogenous factors. To continue the evaluation of topical *d*-limonene for breast cancer prevention, additional studies would be needed to evaluate different doses and/or formulations to increase the systemic and breast tissue exposure to the studied agent.

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Table 7: Demographics for participants completing the topical *d*-limonene intervention

Premenopausal Women	16
Postmenopausal Women	27
Age	54 (51.7 $\pm$ 9.8)[a]
Body Mass Index (kg/m^2)	25.1 (26.2 $\pm$ 4.8)[a]
Race/Ethnicity: *n* (%)	
Caucasian	37 (86.0)
Black	2 (4.7)
Native American	2 (4.7)
Unknown	2 (4.7)

[a]median (mean $\pm$ SD)

Table 8: NAF[a] pre- and post-4-week topical *d*-limonene intervention biomarker levels[b]

	Baseline	**Post-Intervention**	**Baseline vs Post-Intervention (*P*-value)**
EGF (ng/g PT)			
All Women (n = 34)	1,027 (3,957 ± 4,610)[c]	2,266 (3,922 ± 4,458)	0.7333
Premenopausal (n = 9)	8,671 (7,759 ± 2,854)	5,335 (7,251 ± 4,116)	0.8203
Postmenopausal (n = 25)	702 (2,532 ± 4,354)	682 (2,674 ± 3,976)	0.4354
Pre vs Postmenopausal (*P*-value)	0.0022	0.0041	
TGF-β1 (ng/g PT)			
All Women (n = 37)	409 (901 ± 1,853)	310 (1,091 ± 2,575)	1.000
Premenopausal (n = 11)	923 (2,079 ± 3,027)	649 (2,709 ± 4,250)	0.8984
Postmenopausal (n = 26)	277 (361 ± 357)	244 (351 ± 357)	0.9779
Pre vs Postmenopausal (*P*-value)	<0.0001	0.0012	
Adiponectin (μg/g PT)			
All Women (n = 28)	13.4 (23.1 ± 26.1)	17.3 (23.5 ± 25.2)	0.6733
Premenopausal (n = 5)	8.3 (11.6 ± 9.1)	7.6 (9.6 ± 5.8)	0.5625
Postmenopausal (n = 23)	15.0 (26.3 ± 28.5)	22.1 (27.3 ± 27.2)	0.4780
Pre vs Postmenopausal (*P*-value)	0.0880	0.0065	

[a]NAF: Nipple Aspirate Fluid

[b]*P*-values derived from nonparametric tests, Wilcoxon rank sum test for two independent samples (i.e. pre- vs. post-menopausal) and signed rank test for the paired data (i.e. baseline vs. post intervention).

[c]median (mean ± SD)

Table 9: Plasma pre- and post-4-week topical *d*-limonene intervention biomarker levels[a]

	Pre-Intervention	Post-Intervention	Baseline vs Post-Intervention (*P*-value)
EGF (pg/mL)			
All Women (n = 43)	21.5 (26.3 ± 21.1)[b]	16.7 (23.8 ± 15.0)	0.6517
Premenopausal (n = 16)	22.0 (34.1 ± 29.0)	17.6 (28.3 ± 19.1)	0.8999
Postmenopausal (n = 27)	22.5 (21.6 ± 13.5)	16.7 (21.2 ± 11.6)	0.6909
Pre vs Postmenopausal (*P*-value)	0.2891	0.2642	
TGF-β (ng/mL)			
All Women (n = 43)	9.39 (10.28 ± 3.30)	8.28 (9.39 ± 3.58)	0.1530
Premenopausal (n = 16)	9.31 (9.92 ± 3.77)	7.51 (8.78 ± 3.45)	0.3225
Postmenopausal (n = 27)	9.39 (10.49 ± 3.13)	8.54 (9.76 ± 3.67)	0.3841
Pre vs Postmenopausal (*P*-value)	0.5097	0.3532	
Adiponectin (μg/mL)			
All Women (n = 43)	10.93 (11.99 ± 5.74)	11.40 (12.45 + 6.33)	0.1473
Premenopausal (n = 16)	9.10 (9.67 ± 4.19)	9.39 (10.16 ± 5.34)	0.2114
Postmenopausal (n = 27)	12.03 (13.37 ± 6.14)	13.96 (13.80 ± 6.57)	0.3914
Pre vs Postmenopausal (*P*-value)	0.0607	0.0939	

[a] *P*-values derived from nonparametric tests, Wilcoxon rank sum test for two independent samples (i.e. pre- vs. post-menopausal) and signed rank test for the paired data (i.e. baseline vs. post intervention).

[b] median (mean ± SD)

Table 10: Association between pre-intervention biomarker levels and BMI: Regression analysis

EGF	**Spearman Correlation**	***P* - value**
NAF		
Premenopausal (n = 9)	-0.2000	0.6059
Postmenopausal (n = 25)	0.0587	0.7852
Plasma		
Premenopausal (n = 16)	0.2529	0.3446
Postmenopausal (n = 27)	-0.5539	0.0027
TGF-β		
NAF		
Premenopausal (n = 11)	-0.2273	0.5015
Postmenopausal (n = 26)	-0.0879	0.6831
Plasma		
Premenopausal (n = 16)	0.4118	0.1130
Postmenopausal (n = 27)	-0.6999	<0.0001
Adiponectin		
NAF		
Premenopausal (n = 6)	-0.3143	0.5441
Postmenopausal (n = 23)	0.1514	0.5012
Plasma		
Premenopausal (n = 16)	-0.5882	0.0165
Postmenopausal (n = 27)	-0.3875	0.0458

Table 11: Pre-intervention to post-intervention plasma biomarkers separated by healthy-weight and overweight[a]

	Healthy-weight (BMI < 25)				Overweight (BMI >25)			
Biomarker	**Baseline**	**Post-Intervention**	**Post/ Pre Ratio**	***P*-Value**	**Baseline**	**Post-Intervention**	**Post/ Pre Ratio**	***P*-Value**
EGF (pg/mL):								
Premenopausal	16.2 (24.0 ± 21.7)[b,c]	24.5 (29.2 ± 22.2)	1.22	0.5469	37.8 (44.2 ± 33.0)[d]	17.6 (27.3 ± 16.9)	0.62	0.4609
Postmenopausal	26.7 (27.8 ± 13.9)[e]	8.8 (17.7 ± 8.75)	0.64	0.0081	11.4 (16.0 ± 10.7)[f]	24.6 (27.9 ± 23.5)	1.744	0.1531
TGF-β (ng/mL):								
Premenopausal	8.14 (8.66 ± 2.79)[c]	7.70 (8.49 ± 2.76)	0.98	0.9453	11.09 (11.18 ± 4.15)[d]	7.49 (9.07 ± 4.15)	0.81	0.3125
Postmenopausal	11.32 (12.13 ± 2.62)[e]	8.32 (8.71 ± 2.83)	0.72	0.0002	8.22 (8.97 ± 2.85)[f]	10.96 (10.64 ± 4.30)	1.186	0.1937
Adiponectin (μg/mL):								
Premenopausal	11.41 (11.23 ± 4.95)[c]	12.19 (11.99 ± 5.89)	1.07	0.3828	7.53 (8.06 ± 2.67)[d]	6.39 (8.15 ± 4.37)	1.01	0.5469
Postmenopausal	16.51 (15.79 ± 6.56)[e]	18.69 (16.74 ± 6.90)	1.06	0.2163	10.61 (11.12 ± 4.95)[f]	10.66 (11.08 ± 5.08)	0.996	1.0000

[a] *P*-values derived from the signed rank test.
[b] median (mean ± SD)
[c] n = 8 for healthy-weight premenopausal women
[d] n = 8 for overweight premenopausal women
[e] n = 13 for healthy-weight postmenopausal women
[f] n = 14 for overweight postmenopausal women

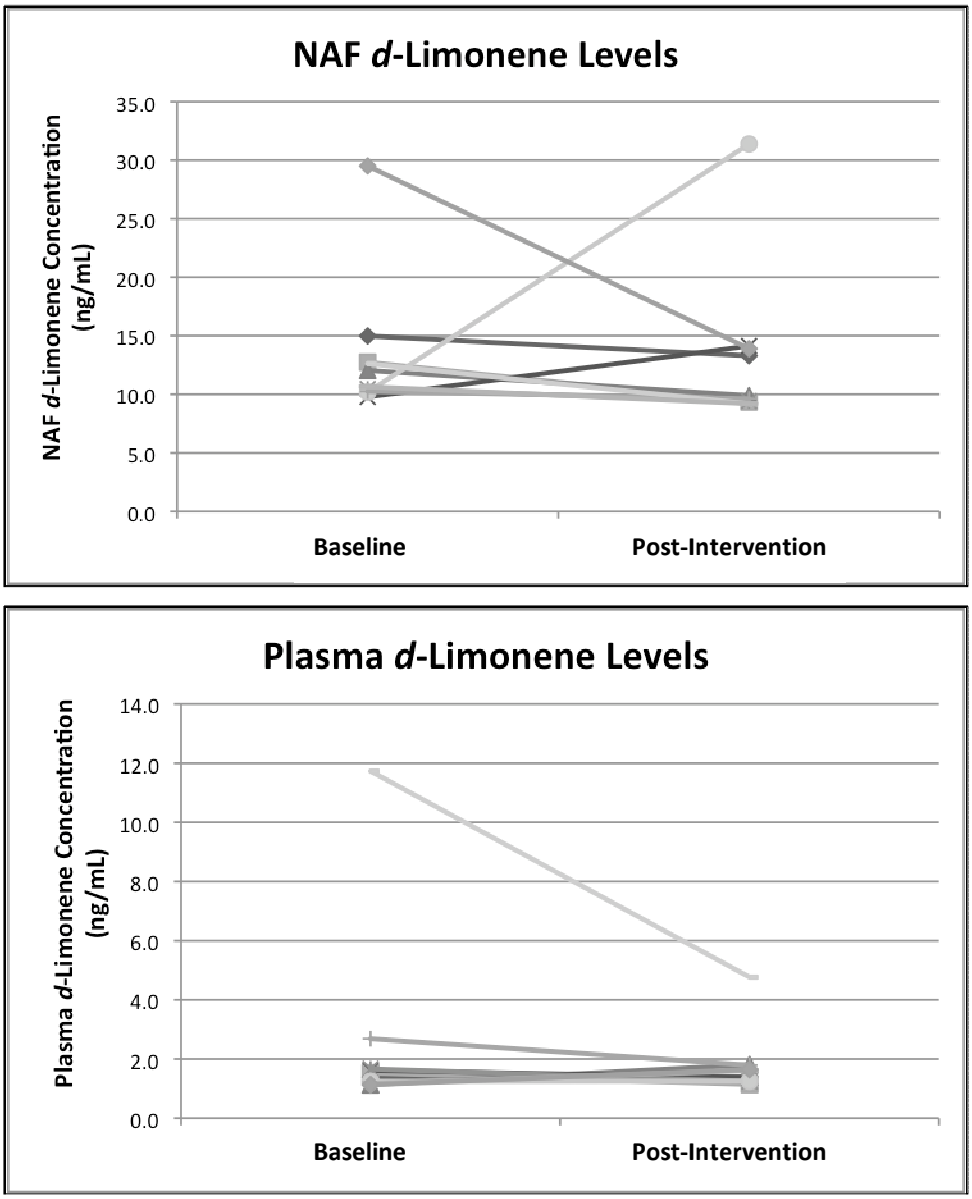

Figure 6: *d*-Limonene levels collected before and after 4 weeks of topical application of *d*-limonene containing massage oil

CHAPTER 4:
MOUSE MAMMARY TISSUE DISTRIBUTION OF D-LIMONENE AND PERILLIC ACID FOLLOWING ORAL AND TOPICAL D-LIMONENE ADMINISTRATION

Abstract

The bioactive food component, *d*-limonene, is a monoterpene found in high concentration in citrus peel oil, and it has demonstrated potent anti-cancer effects in pre-clinical models of breast cancer. However, the effective oral dose translates to a human dose that may not be feasible for chronic dosing. To evaluate the potential of topical *d*-limonene application as an alternative route of administration, we conducted a disposition study in SKH-1 hairless mice to compare the mammary tissue distribution of *d*-limonene and its major active metabolite, perillic acid, after oral and topical *d*-limonene administration. Groups of SKH-1 hairless mice received daily oral or topical administration of 10 or 20% orange oil in coconut oil (0.3 and 0.6 g *d*-limonene per kg bw) or coconut oil for 4 weeks. Topical administration was applied to the skin covering the mammary fat pads. Plasma, mammary fat pads (MFP), and neck fat (NF) were collected at 4 and 24 hrs after the last *d*-limonene administration. Topical and oral administration of *d*-limonene resulted in similar levels of *d*-limonene in MFP and NF, suggesting equivalent target tissue disposition. The MFP and NF *d*-limonene levels were 27 – 51 μM 4 hours after the final dose and declined to 0.1 – 0.4 μM 24 hours after the last dose. Plasma *d*-limonene concentrations were low after topical and oral *d*-limonene administration with levels similar to those receiving the vehicle controls. In the MFP and NF, perillic acid concentrations were 32 – 628 μM 4 hrs after the final oral dose with comparable levels in plasma. Tissue perillic acid levels sustained at 59 – 850 μM, while systemic perillic acid was mostly cleared 24 hours after the final oral dose. Topical *d*-limonene treatment did not result in significant or measurable perillic acid levels in MFP

and NF. We conclude that topical and oral orange oil administration resulted in similar target tissue disposition of *d*-limonene but the oral route gave rise to high levels of perillic acid in the target tissue which would lead to greater biological activity than topical *d*-limonene application.

Introduction

New chemopreventive agents with low toxicities are needed for the prevention of breast cancer. The bioactive food component, *d*-limonene (Figure 7) has exhibited chemopreventive activity against many cancer types with the most compelling results in mammary carcinogenesis models. *d*-Limonene fed during the promotion/progression stage inhibits the development of tumors induced by 7,12-dimethylbenz(a)anthracene (DMBA) or tumors induced by *N*-methyl-*N*-nitrosourea (NMU) [9, 10, 56]. Dietary feeding of *d*-limonene also inhibits the development of *ras* oncogene-induced mammary carcinomas in rats [11]. *d*-Limonene has also been shown to exert chemotherapeutic activity. Oral feeding of *d*-limonene results in significant regression of DMBA- or NMU-induced mammary carcinoma in a dose dependent manner [56, 107], without any observable systemic toxicity.

The levels of *d*-limonene administered in these preclinical studies, however, translate to a human oral dose that may not be feasible for long-term use. *In vitro* studies have demonstrated that topically applied *d*-limonene can penetrate both the epidermis and dermal layers of the skin [108]. *d*-Limonene has also been explored as an enhancer of the percutaneous absorption of pharmaceutical drugs, including tamoxifen [109, 110]. In

principle, transdermal drug delivery offers several benefits over oral administration including improved bioavailability to the target tissue for certain conditions, an ability to offer a low-level consistent delivery of drug, and less systemic toxicity [126]. Transdermal delivery also avoids hepatic/intestinal first-pass metabolism [126]. It is unknown, however, if topical application of *d*-limonene to a target organ will result in high enough local deposition to reach levels that would elicit an anti-cancer effect.

In order to evaluate the potential of topical route of *d*-limonene administration as an alternative route of administration, we conducted a disposition study in mice to compare the distribution of *d*-limonene and its major active metabolite, perillic acid, to mammary fat pads after oral or topical administration of *d*-limonene.

Materials and methods

Chemicals

d-Limonene, perillyl aldehyde, perillic acid, and salicylic acid were purchased from Sigma Chemicals (St. Louis, MO, USA). Coconut oil was purchased from From Nature With Love (Oxford , CT, USA). Certified organic orange oil was purchased from NOW Foods (Bloomingdale, IL, USA). HPLC grade dichloromethane and methanol, 200 proof ethanol, acetonitrile and tert-butyl methyl ether (TBME) were obtained from EM Science (Gibbstown, NJ, USA). HPLC grade hexane was purchased from Honeywell Burdick & Jackson (Morristown, NJ, USA). ACS certified potassium hydroxide was purchased from Fisher Scientific (Pittsburg, PA, USA). Potassium chloride was purchased from Sigma-Aldrich (St. Loius, MO, USA) and phosphoric acid

was purchased from Mallinckrodt Inc. (Hazelwood, MO, USA). Deionized (DI) water was purified by a Barnstead Nanopure Infinity water purifier (Barnstead International, Dubuque, IA, USA). Surfasil siliconizing solution and RD5-35 were purchased from Thermo Scientific (Rockford, IL USA). Pork fat was purchased from a local market for calibration curves.

Animals

Pathogen-free hairless mice (female SKH-1 mice, 4-5 weeks of age, 19.5 – 22.3 g) were purchased from Charles River Laboratories (Wilmington, MA, USA). These mice were housed in temperature and light-controlled rooms and were given water and feed *ad libitum*.

Study Design

Mice were divided into seven experimental groups (N = 8 per group); untreated control, topical control receiving only coconut oil, oral control receiving only coconut oil, topical treatment of 10% orange oil in coconut oil, topical treatment of 20% orange oil in coconut oil, oral treatment of 10% orange oil in coconut oil, and oral treatment of 20% orange oil in coconut oil (Figure 2). For topical treatment, 80 μL (40 μL each to the left and right sides) of the mixed oil or coconut oil were applied to the skin directly above the mammary pad. An Elizabethan collar was placed on the mice for 20 min after the topical application to prevent the mice from licking the topically applied oil. For oral administration, 80 μL of the mixed oil or coconut oil were administered via oral gavage.

The *d*-limonene content in the orange oil was determined by gas chromatography mass spectrometry (GC-MS) and found to be 93%. The dose of *d*-limonene administered was determined to be 0.3 and 0.6 g/kg for the 10% and 20% mixed oil, respectively. Orange oil and coconut oil were chosen for dosing because they are safe for human use as topical massage oil and for oral intake, allowing for further clinical testing. The administration was carried out daily for four weeks. At the end of the four-week administration, half of the mice from each treatment group were sacrificed 4 hours after the last application/dosing, and half after 24 hours. Blood was collected via cardiac puncture and plasma separated. Mammary pads (MFP) on the left side and fat on the back of the neck (NF) were collected for *d*-limonene and perillic acid analysis. Animal experiments were performed according to ethical guidelines established by the University Institutional Animal Care and Use Committee.

Sample processing

Preparation of standards. *d*-Limonene stock (0.84 g/mL) is purchased commercially and stored at 4^{o}C. For analysis of *d*-limonene in plasma and fat, *d*-limonene calibration working standards were prepared fresh daily by serially diluting the stock in methanol to a concentration range of 0.13 to 100 μg/mL. For analysis of perillic acid in plasma, perillic acid stock solution was prepared fresh weekly in tert-butyl methyl ether (TBME) (2 mg/mL) and was stored at 4^{o}C. Perillic acid working standards were prepared daily by diluting the stock in TBME to a concentration range of 3.9 to 250 μg/mL. For analysis of perillic acid in the fat, perillic acid stock was prepared fresh

weekly in methanol (2 mg/mL) and were stored at 4°C prior to use. Perillic acid calibration working standards were prepared fresh daily by serially diluting the stock in methanol to a concentration range of 15.6 to 200 μg/mL.

***d*-Limonene and Perillic Acid Analysis in Plasma.** Plasma *d*-limonene concentrations were determined using a published assay [84] with minor modifications. Briefly, plasma samples (50 μL) were mixed with an equal volume of the internal standard solution (1.2 μg/mL of perillyl aldehyde in 100% acetonitrile) to precipitate the plasma proteins. After vortexing and centrifugation, the supernatant was removed and mixed with 100 μl hexane for *d*-limonene extraction. This mixture was then vortexed and centrifuged for 10 minutes at 1,400 x g at room temperature. One microliter of the organic layer was injected into the GC-MS system with a splitless injection at 220^{o}C. The GC-MS system consisted of a TRACE™ GC with a PVT injector and a Finnigan TRACE DSQ mass spectrometer (Thermo Electron Corporation, San Jose, CA, USA). Chromatographic separation of *d*-limonene and internal standard was achieved on a high resolution GC DB-5MS fused silica capillary column (30 m × 0.247 mm, I.D: 0.25 μm, Agilent Technologies, Santa Clara, CA, USA) with an initial oven temperature set at 60^{o}C and increased to 140^{o}C at 20^{o}C per min and then held for 4 min. The mass spectrometer source was set at 250^{o}C with the mass analyte analyzer set at selective ion monitoring for m/z 93 and 121 for *d*-limonene and internal standard respectively. *d*-Limonene and the internal standard elute at approximately 9 and 14 minutes,

respectively. The curve was linear over a plasma concentration range of 5 ng/mL to 2,000 ng/mL.

For analysis of perillic acid in plasma, plasma samples (50 μL) were mixed with an equal volume of the internal standard solution (1.2 μg/mL of perillyl aldehyde in 100% acetonitrile) to precipitate the plasma proteins, and acidified with 50 μL of 0.2 M phosphoric acid and 25 μL of 1.0 M potassium chloride. Perillic acid and internal standard were extracted with 140 μL TBME. Samples were vortexed and centrifuged for 10 minutes at 1,400 x g at room temperature. One microliter of the organic layer was injected into the GC-MS system with a splitless injection at 220^{o}C. Chromatographic separation of perillic acid and internal standard was achieved on a high resolution GC DB-5MS fused silica capillary column with an initial oven temperature set at 60^{o}C and increased to 140^{o}C at 10^{o}C/ min, then increased to 275^{o}C at 30^{o}C/min and held for 2 minutes. The mass spectrometer source was set at 250^{o}C with the mass analyzer set at selective ion monitoring to monitored for *m/z* 121 for both perillic acid and perillyl aldehyde. Perillic acid acid and the internal standard elute at approximately 10 and 11.5 minutes, respectively. The curve was linear over a plasma concentration range of 781 – 50,000 ng/mL.

***d*-Limonene and perillic acid analysis in fatty tissues.** Mammary gland and neck fat *d*-limonene concentrations were analyzed according to a method developed in our laboratory with minor modifications [61]. Fat was weighed and incubated at 37^{o}C in a water bath for 2.5 hours with 30 μL of 30% potassium hydroxide and 100 μL ethanol to induce saponification. After cooling to room temperature, 300 μL hexane, 50 μL purified

H_20, and 10 µL internal standard solution (1.2 µg/mL of perillyl aldehyde in methanol) were added. Samples were vortexed and then centrifuged for 10 minutes at 1,400 x g at room temperature. The organic layer was then removed and 1 µL was injected into the GC-MS system with a splitless injection at 220^{o}C with a Restek siltek glass injector liner. Initial oven temperature was set at 60^{o}C and held for 6 min, then ramped 20^{o}C/min to 140^{o}C and held for 4.5 min, then ramped again 20^{o}C/min up to 300^{o}C and held for 5 min. Calibration curves were linear over the range of 6.25 – 2,000 ng *d*-limonene.

The remaining solution following the removal of hexane was used for perillic acid analysis. To the remaining solution, 45 µL of potassium chloride, 45 µL 2 M phosphoric acid, 10 µL internal standard (2 µg/mL salicylic acid in methanol) and 400 µL of TBME were added for extraction. Samples were then vortexed and centrifuged for 10 minutes at 1,400 x g at room temperature. The organic layer was then removed and evaporated to dryness under a stream of nitrogen and reconstituted with 200 µL methanol. Samples were then vortexed and centrifuged for 10 minutes at 1,400 x g at room temperature and 20 µL of the clear supernatant was injected into the LC-MS system. The LC-mass spectrometry system consisted of a Surveyor HPLC system and a TSQ Quantum triple quadrupole mass spectrometer (Thermo Electron, San Jose, CA, USA). Chromatographic separation of perillic acid and the internal standard was achieved on a BDS Hypersil C18 column (150 mm L x 2.10 mm ID, 5µ Phenomenex, Thermo Electron, San Jose, CA, USA) with a BDS Hypersil C18 guard column (10 x 2.00 mm ID, 5µm Phenomenex) and a gradient of two mobile phases. Mobile phase A consisted of 0.1% glacial acetic acid in water, and mobile phase B consisted of 100% methanol. The following linear gradient

elution program was used: 90% A for 7 min; 25% A for 8 min; and 10% A for 0.1 min. The column was re-equilibrated with 90% A for 5 min before the next injection. The flow rate was maintained at 0.3 ml/min. Column effluent was diverted to waste from 0 to 3.6 min and from 12.03 minutes to re-equilibration. The mass spectrometric analysis was performed with the electro-spray ionization interface operated in negative ion mode. The analyte and the internal standard were measured by selected ion monitoring. The most abundant ion for the analyte and internal standard were 165.00 and 136.55 respectively. Calibration curves were linear over the range of 125 – 2,000 ng perillic acid.

Statistical Analysis

The Wilcoxon rank sum test was used to compare the two independent samples. Specifically, oral vs topical administration of *d*-limonene levels in MFP, NF, and plasma were compared, as well as 10% vs 20% treatment groups. The same comparisons were conducted for perillic acid levels. Levels of *d*-limonene and perillic acid in plasma vs levels in fat were also compared. Finally, *d*-limonene vs perillic acid levels were compared within the MFP, NF, and plasma of each treatment group. A *P*-value of < 0.05 was considered statistically significant.

Results

***d*-Limonene levels in mammary MFP, NF, and plasma.** Table 12 presents MFP, NF, and plasma *d*-limonene levels from each treatment group. Two mice in the 10% orally treated group and one in the 20% topically treated died due to infection

unrelated to the treatment, and could not be replaced. Thus, these were not included in the analysis. Two untreated control mice also became infected near the end of the study, *d*-limonene and perillic acid levels were not affected so were still included in the analysis. In the MFP, there was no difference in *d*-limonene levels between topical and oral administration, and there was also no difference between 10% and 20% formulations. At 4 hrs after the last dose, *d*-limonene levels were 7,257 $\pm$ 5,140, 6,743 $\pm$ 2,000, 6,963 $\pm$ 2,174, 17,465 $\pm$ 13,701 ng/g for 10% topical, 10% oral, 20% topical, 20% oral, respectively. At 24 hrs after the last application, *d*-limonene levels were 10.48 $\pm$ 12.11, 37.68 $\pm$ 10.3, 21.60 $\pm$ 19.88, 41.73 $\pm$ 33.60 ng/g for 10% topical, 10% oral, 20% topical, 20% oral, respectively. Levels at 4 hours after final *d*-limonene application, however, were statistically significantly higher in every treatment group compared to those at 24 hours ($P < 0.05$, Figure 9).

In the NF, the same pattern was observed, there was no difference in *d*-limonene levels between topical and oral *d*-limonene, and there was also no difference between 10% and 20% formulations. Similarly, levels at 4 hours after final *d*-limonene application were statistically significantly higher in every treatment group than those at 24 hours ($P < 0.05$, Figure 9). In addition, matched MFP and NF samples did not differ significantly from each other.

Controls contained low detectable levels of *d*-limonene in plasma. Oral or topical *d*-limonene at 10% or 20% dose level did not result in significantly increased plasma *d*-limonene at either 4 or 24 hours after the last administration. At 4 hours after the last dose, *d*-limonene levels in the MFP and NF were statistically significantly higher than

those in matched plasma; on average 1,000-fold higher ($P < 0.05$). At 24 hours after final *d*-limonene application, however, most fat *d*-limonene levels were not statistically different from that in plasma.

Perillic acid levels in MFP, NF, and plasma. Table 13 summarizes perillic acid levels of in MFP, NF, and plasma. Perillic acid levels in MFP were statistically significantly higher following oral *d*-limonene dosing than those after topical treatment ($P < 0.05$). At 4 hrs after the last administration, perillic acid levels were 33,517 $\pm$ 45,758 vs. 451 $\pm$ 903 ng/g after 10% oral vs. topical dosing and 5,923 $\pm$ 3,176 vs. 265 $\pm$ 530 ng/g after 20% oral vs. topical dosing. At 24 hrs after the last administration, perillic acid levels were 26,057 $\pm$ 20,608 and 10,917 $\pm$ 7,023 ng/g after 10 and 20% oral dosing, and the levels were below the limit of quantification after 10% and 20% topical dosing. For statistical purposes, measures that were BLQ were treated as zero. Perillic acid levels in NF demonstrated a similar pattern to the MFP.

Perillic acid plasma levels were statistically significantly higher in the orally treated groups than in the topically treated groups 4 hours after the last administration ($P < 0.05$). Plasma perillic acid levels were 79,523 $\pm$ 52,582 vs. 573 $\pm$ 387 ng/mL for 10% oral vs. topical dosing; 20,503 $\pm$ 9,022 vs. 516 $\pm$ 348 ng/mL for 20% oral vs. topical dosing. Plasma levels of perillic acid at 24 hours after last *d*-limonene topical treatment were not statistically significantly different from the orally treated groups; 587 $\pm$ 189 vs. 573 $\pm$ 389 ng/mL for 10% oral vs. topical dosing; 1,334 $\pm$ 890 vs. 409 $\pm$ 356 ng/mL for 20% oral vs. topical administration. Plasma levels of perillic acid at 24 hours after last *d*-

limonene oral treatment were non-significantly lower than at 4 hours ($P < 0.10$) but were not different between the two collection times after topical treatment.

At four hours post final *d*-limonene treatment, MFP and NF perillic acid levels were not statistically significantly different from plasma in most groups (Figure 10). In the 10% orally treated mice, MFP/plasma ratios were 36.7 $\pm$ 19.76 and NF/plasma ratios were 217 $\pm$ 145, but fat were not statistically significantly higher than plasma, most likely due to the very small sample size. In the 20% oral treatment groups, both MFP and NF perillic acid levels were statistically significantly higher than plasma 24 hours after final *d*-limonene application; MFP/plasma 9.03 $\pm$ 8.05, NF/plasma 15.85 $\pm$ 19.19 ($P < 0.05$).

***d*-Limonene compared to perillic acid disposition.** Table 14 displays the perillic acid to *d*-limonene ratios in fat and plasma after oral *d*-limonene administration. At 4 hours after the final *d*-limonene treatment, plasma perillic acid was statistically significantly higher than *d*-limonene in both the 10% and 20% oral treatment groups with a perillic acid/limonene ratio of 14,160 $\pm$ 19,498 and 7,124 $\pm$ 2,175, respectively ($P < 0.05$). At 24 hours after oral treatment, PA was 128 $\pm$ 26.78 times ($P = 0.13$) and 542 $\pm$ 190 times ($P < 0.05$) higher than *d*-limonene levels for the 10 and 20% dose, respectively. In the MFP and NF, there were no statistically significant differences between PA and *d*-limonene levels for the 10% dose at 4 and 24 hrs after the last dose. For the 20% dose, the PA levels were significantly lower than *d*-limonene levels in MFP and NF at 4 hrs after the last dose but were significantly higher than *d*-limonene levels 24 hrs after the last dose.

Discussion.

In this study, both oral and topical administration of 10% and 20% *d*-limonene in coconut oil (0.3 g/kg and 0.6 g/kg daily) resulted in limonene levels from around 7,000 to 17,000 ng/g (equivalent to 40 – 115 μM) in both the mammary fat pad and the neck fat, suggesting similar tissue bioavailability of the parent compound from both routes. The doses used in this study, 0.3 and 0.6 g/kg extrapolate to human equivalent doses of 1,459 mg and 2,919 mg for a 60 kg individual. In humans, 500 mg daily of oral *d*-limonene resulted in 0.14 μM concentrations in fat buttock biopsies taken 6 hours after the final *d*-limonene dose [127]. Crowell *et al* demonstrated that radiolabeled *d*-limonene and metabolites (non-specific assay) deposit in levels up to 6 mM in rat mammary glands and 9.33 mM in adipose four hours after a 1g/kg dose oral *d*-limonene; after 24 hours, these levels were reduced by half [83]. In our study, fat levels of *d*-limonene were largely cleared after 24 hours, with levels 1,000-fold lower (in the nM range) in all fatty sites in all treatment groups. Our results suggest that the adipose tissue levels maintained in Crowell's study could be from *d*-limonene metabolites, rather than the parent compound.

d-Limonene levels at both time-points in the NF were similar to MFP even after topical *d*-limonene application. Schafer and Schafer measured dermal absorption of *d*-limonene via total radioactivity and found peak plasma levels after 10 minutes [128]. Thus, it is likely that after topical application *d*-limonene reached peak levels in the MFP tissue quickly. Additionally, all depots of high adiposity in the mouse are connected by the lymphatic system [129], and *d*-limonene was measured up to 0.12 mM levels in the thoracic duct lymph fluid in rats after oral administration [35]. Therefore, it is likely that

after dermal absorption to the MFP, *d*-limonene is transported via the lymphatic to all sites of high adiposity, including NF.

Consistent with its high lipophilicity and previous findings in humans and rodents, *d*-limonene demonstrated preferential adipose-to-plasma tissue distribution. *d*-Limonene plasma concentrations were on average between 2.03 – 11.05 ng/mL (0.015 – 0.08 nM), with adipose tissue levels 1,000-fold higher. Other studies conducted in rats report higher circulating *d*-limonene levels; *d*-limonene reached 90 μM at four hours after a single oral dose at 1 g/kg [24] and 0.021 μM levels in serum 3 hours after a single oral dose of 1 g/kg [35]. In a pharmacokinetic study in humans, peak plasma *d*-limonene levels up to 2.4 μM were achieved between 1-3 hours after oral administration of 300 mg *d*-limonene [84]. Crowel *et al* also indicated preferential adipose to plasma disposition of total *d*-limonene and metabolites in rats, with levels 6-fold higher in adipose than in plasma at 4 hr after oral dosing of radiolabeled *d*-limonene [83]. It is possible that *d*-limonene is metabolized more quickly in mice than rats, which would explain the much higher adipose-to-plasma ratio than what was found in the previous study.

d-Limonene's major metabolite is perillic acid, and this is the first study to directly quantify perillic acid in adipose tissue. Orally administered *d*-limonene is readily taken up by the gastrointestinal tract and is rapidly converted to perillic acid by the liver [130]. In rats, 75 – 95% of *d*-limonene and metabolites are cleared in urine within 2 – 3 days after an 800 mg/kg dose, with the most clearance within 24 hours [130]. In this study, there were high plasma levels of perillic acid after oral *d*-limonene administration but not after topical application, to be expected considering the difference in *d*-limonene

absorption and hepatic first-pass metabolism between routes of administration. In a pharmacokinetic study in humans, perillic acid peak levels were on average 6.2 μM within about 1 hour after oral administration of a 500 mg *d*-limonene dose and then by 24 hours were cleared [85]. In another study in cancer patients administered 10g/m^2 *d*-limonene dose, perillic acid levels remained in the μM levels for several hours, but were cleared after 24 hours [21]. Similarly, in the current study, oral *d*-limonene administration resulted in high perillic acid concentrations in plasma four hours after final *d*-limonene dose, up to 50 μg/mL (0.30 mM) levels and the levels were 100 – 1,000 times lower 24 hours after the final dose.

In the topically treated mice, plasma perillic acid levels remained low and unchanged between the 4 and 24 hr time-points. Similarly, perillic acid levels in MFP and NF in these groups were BLQ. Because *d*-limonene is a volatile compound, some of the topically applied compound could have been lost as vapor, despite rapid dermal uptake. Additionally, the persistent and low levels in plasma could be because topically applied *d*-limonene takes longer to reach circulation and to undergo hepatic metabolism. Alternatively, the low perillic acid plasma levels, particularly at 4 hours, could be attributed to oral ingestion of *d*-limonene from normal mouse grooming patterns since protective collars were only on the mice receiving topically applied *d*-limonene for twenty minutes after application.

Because of its polar chemical structure, it was not expected that perillic acid would accumulate in high levels in adipose tissue. After oral administration, however, perillic acid accumulated to levels comparable to that of *d*-limonene in the MFP. These

levels persisted even though the plasma levels declined at 24 hr post dose, suggesting a binding component in MFP. In this study, after 24 hours in the 20% oral treatment group, perillic acid accumulated to 11.7 μg/g (63 μM) in MFP (5.5 times that of plasma) and 16.1 μg/g (87 μM) in NF (7.8 times that of plasma). In the 10% oral treatment group, after 24 hours, perillic acid levels were 26 μg/g (140 μM) in MFP (36.7 times that of plasma) and 157 μg/g (850 μM) in NF (217 times that of plasma). Thus, it is possible perillic acid binds to components in adipose tissue resulting in slow tissue clearance.

The *d*-limonene/perillic acid levels achieved in MFP in our study could potentially have an anti-cancer effect. One study demonstrated that *d*-limonene was effective at inhibiting tumor growth by 78% in mouse mammary gland organ culture at levels of 10 nM [39]. In most cell culture work, however, low mM levels of *d*-limonene were needed to elicit an anti-cancer effect [86-88]. Because *d*-limonene is cleared from both adipose and plasma, in order to maintain levels that would be effective in cancer prevention, daily administration may be necessary. Other fat-soluble compound such carotenoids, also demonstrate a rapid turnover from adipose tissue, and do not seem to reflect long-term intake [131, 132]. Similarly, vitamin E, previously thought to accumulate in adipose tissue over time [133], undergoes constant turnover from the fat and levels are tightly regulated with minimal accumulation [134].

Barden *et al* assessed perillic acid and *d*-limonene's effect in various breast cancer cell lines. In the T47-D cell breast cancer cell line, 10 μM of perillic acid inhibited proliferation 17% while 3 mM resulted in 90% inhibition. In the MCF-7 breast cancer cell line growth was inhibited 16% at 50 μM perillic acid and by 66% at a concentration

of 3 mM [23]. Our study indicates that oral administration of 10% or 20% *d*-limonene results in adipose tissue levels of perillic acid capable of exerting an anti-cancer effect, and those levels are maintained for at least 24 hours. In Bardon's study, *d*-limonene elicited a 15% decrease in the growth rate of T-47D cells at a concentration of 3 mM [23]. Because perillic acid seems to remain in adipose tissue for an extended period of time, the cancer preventive and therapeutic activity observed with 5% or 10% dietary *d*-limonene may attribute to both perillic acid and the parent drug [11, 56, 106, 107]. To our knowledge there are no studies assessing the synergistic effect of *d*-limonene and perillic acid in a cell culture cancer model.

In summary, topical and oral orange oil administration resulted in similar target tissue disposition of *d*-limonene. Oral but not topical administration gave rise to high levels of perillic acid in mammary fat pads. This suggests that topical administration in mixed oil is likely to exert less activity than the oral administration because perillic acid has been shown to exert more potent biological activity than *d*-limonene. Future research should assess the potential synergistic effects of *d*-limonene and perillic acid in mammary cancer models and the potential of evaluating topical formulations that could enhance target tissue delivery of the parent compound.

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Table 12: *d*-Limonene levels separated by time of sacrifice

	Plasma (ng/mL)	MFP[a] (ng/g)	Neck Fat (ng/g)	MFP[b] /Plasma ratio	Neck Fat[b] /Plasma ratio
4 hours after final *d*-limonene treatment					
Untreated Control (N = 4)	7.19 ± 5.47[c]	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Topical Control (N = 4)	1.31 ± 1.49	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Oral Control (N = 4)	1.46 ± 0.85	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
10% Topical (N = 4)	2.03 ± 1.69	7,258 ± 5,140[d,e,f]	4,018 ± 3,268[d,e,f]	4,104 ± 4,183[d,e,f]	1,540 ± 789[d,e,f]
20% Topical (N = 4)	2.55 ± 1.08	6,963 ± 2,174[d,e,f]	7,782 ± 2,133[d,e,f]	3,063 ± 2,286[d,e,f]	3,207 ± 1,651[d,e,f]
10% Oral (N = 4)	11.05 ± 6.88	6,743 ± 2,000[d,e,f]	7,474 ± 3,802[d,e,f]	1,434 ± 1,194[d,e,f]	1,345 ± 3,422[d,e,f]
20% Oral (N = 4)	3.04 ± 1.21	17,465 ± 13,701[d,e,f,g]	11,247 ± 4,301[d,e,f]	4,828 ± 2,516[d,e,f]	3,684 ± 1,520[d,e,f]
24 hours after final *d*-limonene treatment					
Untreated Control (N = 4)	3.58 ± 2.29[c]	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Topical Control (N = 4)	5.53 ± 7.88	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Oral Control (N = 4)	9.06 ± 6.96	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
10% Topical (N = 4)	3.94 ± 6.21	10.48 ± 12.11	12.38 ± 24.76	3.45 ± 5.97	7.99 ± 13.31
20% Topical (N = 3)	2.00 ± 0.49	21.6 ± 19.88[d]	20.94 ± 18.13[d]	8.47 ± 8.55	8.27 ± 7.17
10% Oral (N = 2)	4.54 ± 0.53	37.68 ± 10.3	53.03 ± 2.55	7.63 ± 2.92	10.61 ± 1.73
20% Oral (N = 4)	2.24 ± 1.30	41.7 ± 33.60[d,f]	44.99 ± 52.32	15.81 ± 8.25	17.59 ± 24.79

[a] MFP: Mammary Fat Pad

[b] Values corrected for density of fat (0.9 g/mL)

[c] Mean ± SD

[d] Significantly higher than corresponding control ($P < 0.05$)

[e] Significantly higher than levels in mice sacrificed 24 hours after final *d*-limonene treatment ($P < 0.05$)

[f] Value corrected for density is significantly higher than plasma ($P < 0.05$)

[g] N = 3 for 20% MFP

Table 13: Perillic acid levels separated by time of sacrifice

	Plasma (ng/mL)	MFP[a] (ng/g)	Neck Fat (ng/g)	MFP[b] /Plasma ratio	Neck Fat[b] /Plasma ratio
4 hours after final *d*-limonene treatment					
Untreated Control (N = 4)	0.00 ± 0.00 [c]	0.00 ± 0.00	0.00 ± 0.00	-	-
Topical Control (N = 4)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	-	-
Oral Control (N = 4)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	-	-
10% Topical (N = 4)	194 ± 387	451 ± 903	0.00 ± 0.00	0.52 ± 1.05	0.00 ± 0.00
20% Topical (N = 4)	515 ± 348	265 ± 530	0.00 ± 0.00	0.32 ± 0.65	0.00 ± 0.00
10% Oral (N = 4)	79,522± 52,582[d,e,f]	33,517 ± 45,758[d,f]	115,921 ± 206,946[d,f]	0.69 ± 0.74	1.77 ± 2.90
20% Oral (N = 4)	20,503 ± 9,022[d,e,f]	5,923 ± 3,176 [d,f,g,h]	7,754 ± 4,369[d,f,g]	0.34 ± 0.08[e]	0.48 ± 0.51[e]
24 hours after final *d*-limonene treatment					
Untreated Control (N = 4)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	-	-
Topical Control (N = 4)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	-	-
Oral Control (N = 4)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	-	-
10% Topical (N = 4)	573 ± 389	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20% Topical (N = 3)	408 ± 356	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
10% Oral (N = 2)	587 ± 189[d]	26,057 ± 20,608[d]	157,029 ± 140,152[d]	36.7 ± 19.76	217 ± 145
20% Oral (N = 4)	1,334 ± 890[d]	10,971± 7,023[d,g]	26,001± 309,536[d,g]	9.03 ± 8.05	15.85 ± 19.19

[a] MFP: Mammary Fat Pad

[b] Values corrected for density of fat (0.9 g/mL)

[c] Mean ± SD

[d] Significantly higher than corresponding control ($P < 0.05$)

[e] Significantly different than in mice sacrificed 24 hours after final *d*-limonene treatment ($P < 0.05$)

[f] Significantly higher than the corresponding percent topical *d*-limonene treatment ($P < 0.05$)

[g] Value corrected for density is significantly different than plasma ($P < 0.05$)

[h] N = 3 for 20% MFP

Table 14: *d*-Limonene compared to perillic acid Levels in oral treatment groups

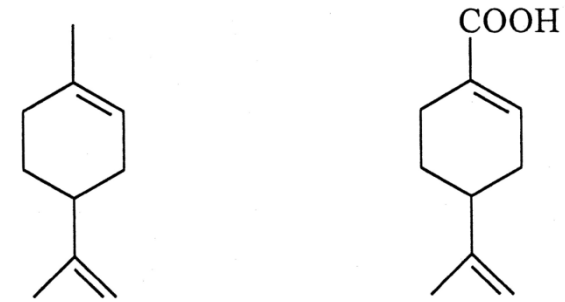
	Plasma	MFP[a]	Neck Fat
4 Hour Sacrifice	**PA[b]/*d*-limonene ratio**	**PA/*d*-limonene ratio**	**PA/*d*-limonene ratio**
10% Oral (N = 4)	14,160 ± 19,498[c,d]	2.22 ± 6.90	2.35 ± 32.18
20% Oral (N = 4)	7,124 ± 2,175[d]	0.28 ± 0.59[d]	0.49 ± 0.66[d]
24 Hour Sacrifice			
10% Oral (N = 2)	128 ± 26.78	796 ± 765	3,028 ± 2,788
20% Oral (N = 4)	542 ± 190[d]	230 ± 361[d]	1,742 ± 1,749[d]

[a] MFP: Mammary Fat Pad

[b] PA: Perillic Acid

[c] Mean ± SD

[d] PA statistically significantly different than *d*-limonene ($P < 0.05$)



d-Limonene Perillic Acid

Figure 7: Chemical structures of *d*-limonene and perillic acid.

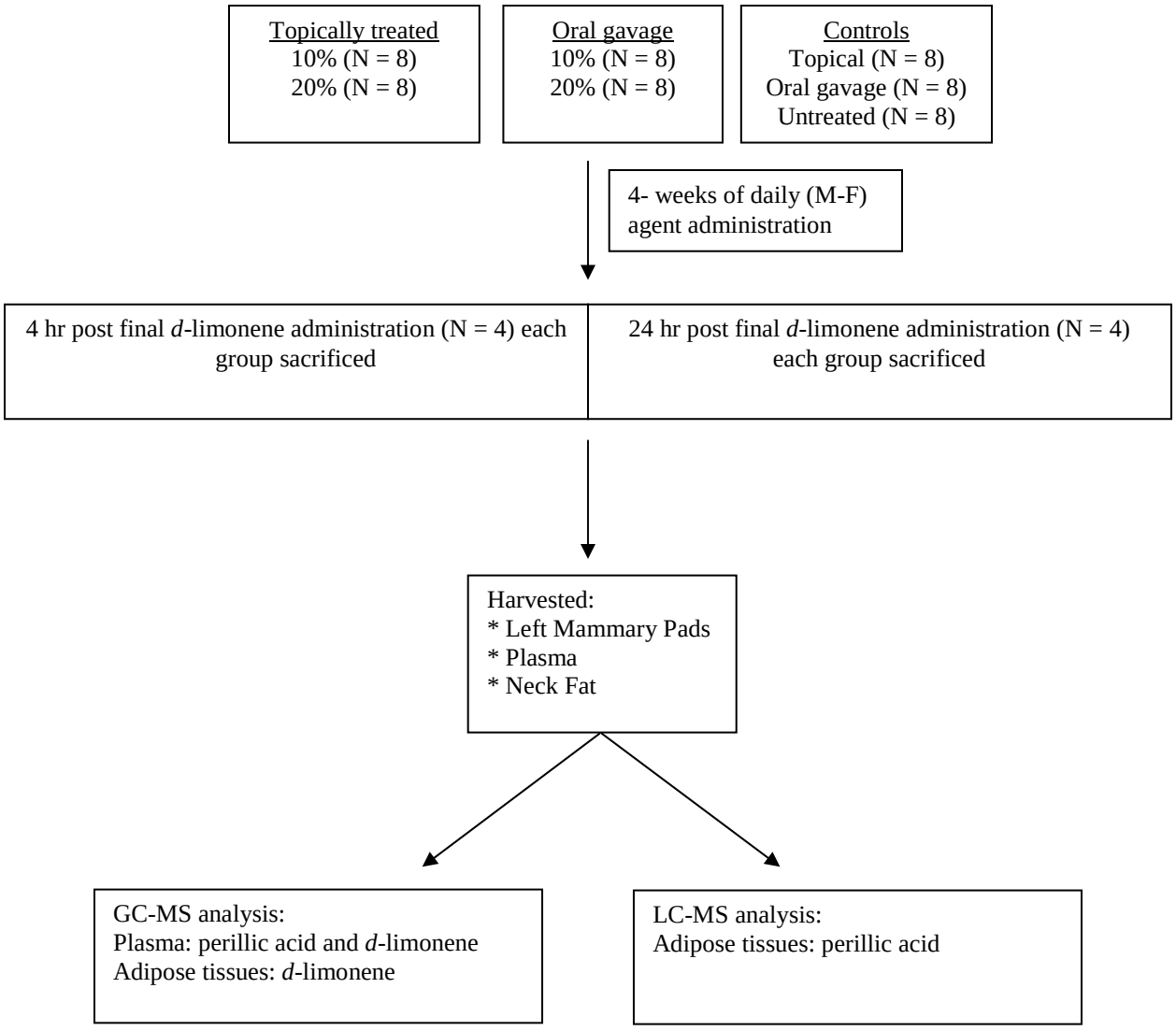


Figure 8: Schematic for administration of topical and oral *d*-limonene to SKH-1, 4-5 week old, hairless mice.

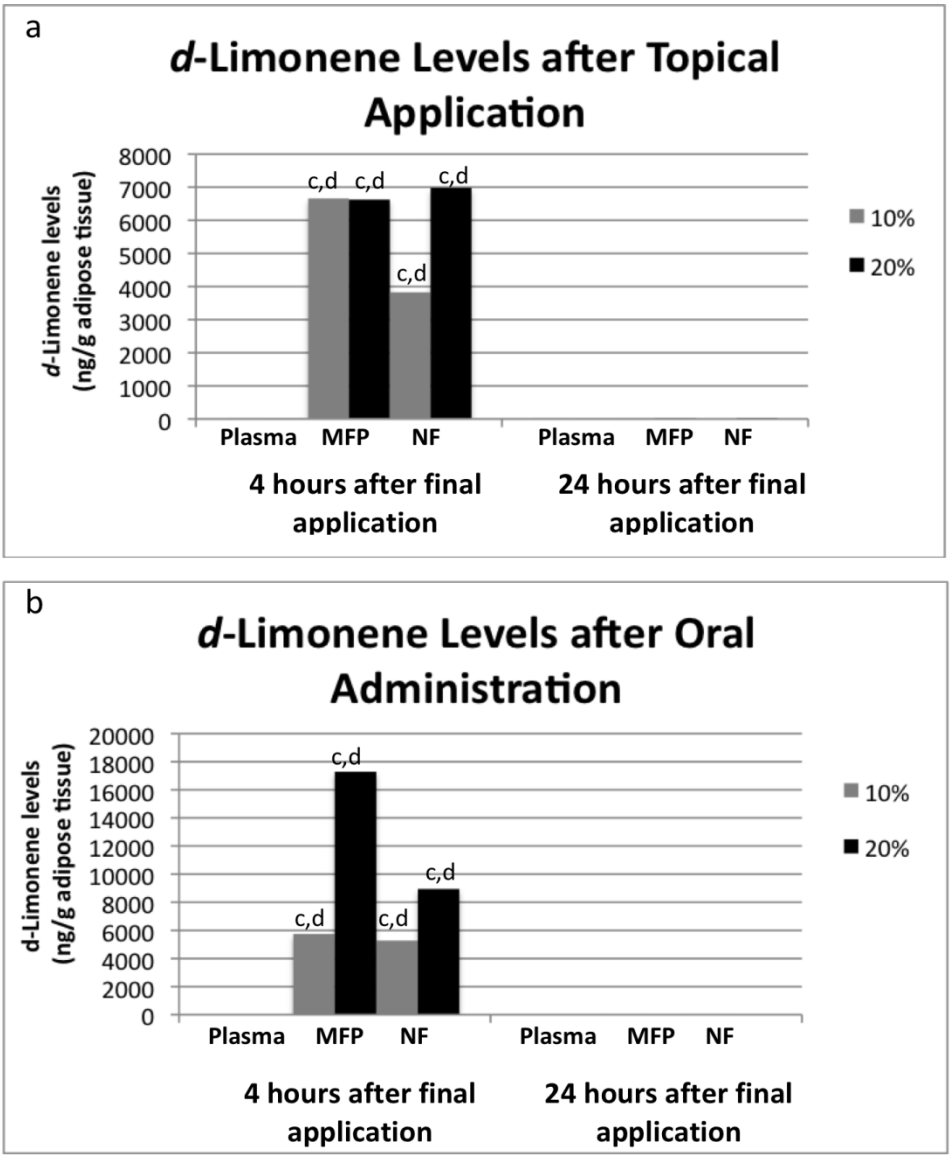


Figure 9: *d*-Limonene levels (ng/g adipose tissue) in plasma, mammary fat pad (MFP), and neck fat (NF) after either (a) topical or (b) oral administration of either 10% or 20% *d*-limonene in coconut oil. (c) Significantly higher than levels in mice sacrificed 24 hours after final *d*-limonene application ($P < 0.05$). (d) significantly higher than plasma ($P < 0.05$).

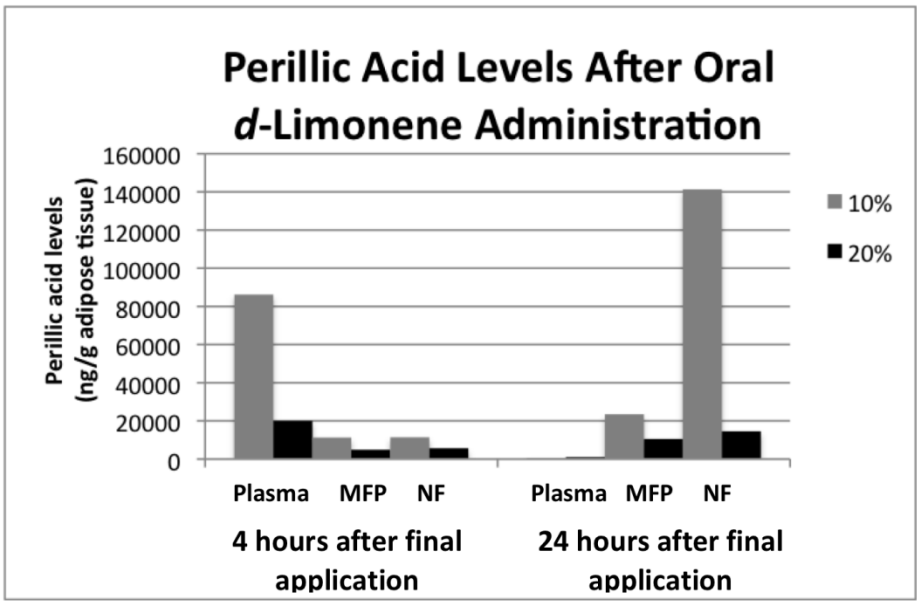


Figure 10: Perillic acid levels in plasma, mammary fat pad (MFP) and neck fat (NF) after oral administration of either 10% or 20% *d*-limonene in coconut oil.

CHAPTER 5:
IMPLICATIONS AND FUTURE DIRECTIONS

I. Summary of Dissertation Work

The strongest conclusion that can be drawn from this dissertation work is that *d*-limonene deposits in high levels in adipose tissue after either topical or oral administration. Oral dosing of *d*-limonene from high-limonene lemonade achieved *d*-limonene adipose concentrations in humans comparable to the levels that elicited an anti-cancer effect in preclinical models. Similar peak *d*-limonene levels were seen in the adipose of SKH-1 mice after either topical or oral administration. Oral *d*-limonene administration resulted in very high levels of *d*-limonene's major metabolite perillic acid in adipose tissues and in plasma, indicating extensive *d*-limonene metabolism in this model. Future work should focus on quantification of perillic acid in clinical samples. Most likely adipose tissue levels in humans will be comparable to human peak plasma levels (about 10-fold lower than peak plasma levels in mice).

Because topical drug administration is frequently associated with less toxicity, it was important to assess *d*-limonene's systemic and local effects after topical application to the breast. In women that applied topical 10% *d*-limonene to the breast for four weeks, *d*-limonene did not affect agent biomarkers; EGF, TGF-β or adiponectin in either NAF or plasma in all women combined. However, plasma levels of EGF and TGF-β in normal weight, postmenopausal women were significantly decreased. Given the chronic low circulating perillic acid levels were observed after dermal absorption of *d*-limonene in the mouse study, this significant decrease in plasma biomarkers may indicate circulating perillic acid activity. Further, in cell culture models, perillic acid has more potent anti-cancer activity than *d*-limonene. Both are active, however, and the potential synergistic

effects of the parent compound and metabolite within a physiological system are not understood.

While there was not a significant overall intervention effect on biomarker levels in the topical limonene trial, there were significant differences in these biomarker levels in NAF compared to plasma. Future clinical trials focusing on the development of anti-cancer agents for the breast should carefully consider differences in local and systemic levels of cancer biomarkers when determining agent efficacy. Additionally, because oral dosing typically results in more extensive metabolism than topical, mode of administration should be carefully considered. Finally, menopausal status and BMI seem to influence agent biomarkers and may be potential confounders of agent efficacy.

II. Chemopreventive Agents in Clinical Practice.

In order for any chemopreventive agent to gain acceptance in clinical trial it must meet several criteria; 1) have described disposition, metabolism, and bioavailability, 2) have defined mechanisms and/or targets, 3) demonstrate likelihood of efficacy in epidemiological and preclinical studies, 4) be affordable, 5) be easily administered, and 6) have no toxicities. This dissertation has described most of the preclinical and epidemiological work that has led to the investigation of *d*-limonene as an anti-cancer agent. This dissertation research also has contributed to the understanding of *d*-limonene's disposition and bioavailability. However, considerable work still needs to be accomplished in order for it to be accepted in clinical practice as a chemopreventive

agent. The following sections describe some of the potential future areas of investigation for *d*-limonene.

III. Potential Mechanisms

a. Aromatase inhibition. Because *d*-limonene does deposit in high levels in adipose tissue, one potential mechanism of action is inhibition of the phase I metabolizing enzyme, aromatase. *d*-Limonene is effective at the initiation and progression stages of DMBA-induced carcinogenesis [10, 39] and only the progression stage of NMU-induced [9]. Because DMBA requires metabolic activation by P450 to become carcinogenic, and NMU is a direct-acting carcinogen [41], this may be indirect evidence of P450-interaction by *d*-limonene. Out of the cytochrome P450 enzymes studied, CYP1B2 and CYP2C are induced by oral *d*-limonene [135-137]. Thus it is possible that *d*-limonene interacts with the P450 enzyme, aromatase, subsequently lowering the levels of circulating estrogen. In the topical limonene study, it was hypothesized that if *d*-limonene did affect estrogen levels, the estrogen-response protein, EGF, would act as a surrogate of this effect. While *d*-limonene had no effect on NAF EGF levels, the small changes in the plasma of healthy weight post-menopausal women warrant further investigation of this mechanism and other potential P450 interactions.

b. Modulation of the breast microenvironment. Because *d*-limonene deposits in high levels in fatty tissues, the local breast environment is likely affected. There is little preclinical research investigating modulation of markers produced by adipose tissue by *d*-limonene and/or perillic acid. **APPENDIX C** is a table of preliminary results from a

cell culture study with 3T3-L1 adipocytes. Adiponectin is primarily produced by adipose tissue and can indicate an organ's ability to elicit an immune response [125], important in preventing cancer at initiation. In this small study, *d*-limonene was able to partially recover adiponectin levels after administration of an inflammatory agent, TNF-α. The effect of *d*-limonene and perillic acid on other potential secretable proteins from adipose tissue involved in chronic inflammation, immune response, and cancer progression should also be explored.

c. Lipolytic effects. Another possible implication of *d*-limonene deposition in adipose tissue is that it may modulate fatty acid metabolism. A study measuring the bioactive compound-induced breakdown of olive oil into oleic acid and glycerol indicated that *d*-limonene may have lipolytic activity [7]. Future studies should be conducted in animal models to determine if *d*-limonene affects fatty acid motility and metabolism in humans.

IV. Toxicities

a. Targeted agents. As with many bioactive food components with multiple modes of action, early phase clinical trials indicate that limonene has limited to no toxicities. More targeted chemopreventive agents, such as aromatase inhibitors (AI's) and selective estrogen receptor modulators (SERMs), have been largely abandoned because these compounds pose increased risk of cardiovascular events [138]. Toxicity risk is likely to depend greatly on the individual health profile of the patient. For example, in a chemoprevention trial, the nonsteroidal anti-inflammatory drug (NSAID),

sulindac, was investiged in low, medium, and high-risk patients for cardiovascular disease [139]. Sulindac increased cardiovascular events overall, but when high-risk participants were excluded; there was no difference between treatment and placebo. Thus, sulindac exasperated the cardiovascular condition only those who were at risk at baseline. This trial supports the idea that if a complete individualized risk profile can be established, it is more likely appropriate chemopreventive agents will be administered.

b. Adipose deposition. Although *d*-limonene deposits in high levels in adipose tissue, the SKH-1 mouse model indicates that it is cleared, and it most likely does not pose the same risk for chronic toxicities like fat-soluble vitamins, A, D, E, and K which can accumulate over time when administered in high levels [140]. No acute toxicities were observed after topical or oral *d*-limonene as were observed with oral administration of its polar analog, perillyl alcohol [28-30]. After a renal toxicity specific to $\alpha_{2\upsilon}$-globulin produced by male rats was identified, there was extensive research to evaluate *d*-limonene's safety; this research demonstrated that there is minimal to no risk for humans in terms of renal toxicity [141]. Subchronic studies indicate no renal toxicities up to 1 g *d*-limonene per kg body weight for 6 months.

c. Metabolic interactions. Future research should also investigate the implications of *d*-limonene metabolism by P450 after oral administration in terms of potential hepatic toxicity and/or nutrient-drug interactions. Examples such as Chow *et al*'s work which demonstrates that gram levels of resveratrol significantly inhibit CYP enzymes [142] and Lambert *et al*'s work which demonstrates that epigallocatechin-3-gallate can cause hepatotoxicity [143] indicate that natural compounds should be

researched and administered with the same precautions as pharmaceutical drugs. A well-written review by Colato outlines several botanicals that seem to have interactions with P450 enzymes and therefore potentially have significant drug interactions [144]. In the absence of pharmaceutical drugs, early phase clinical trials indicate that *d*-limonene is safe in healthy adults, however, research future clinical trials should investigate potential drug interactions.

V. Mode of Administration

a. Chronic dosing. Another important characteristic of chemopreventive agents is the potential for long-term administration. According to Hakim *et al*, 50 mg of *d*-limonene from 1 Tbs lemon peel (roughly 0.71 mg/kg for the average adult) was sufficient to significantly reduce skin cancer risk. This amount could easily be achieved with chronic oral dosing. According to the Flavor and Extract Manufacturers Association, the average daily intakes of limonene in the US are 0.27 mg/kg daily. Therefore, the levels of limonene capable of eliciting a chemopreventive effect are close to the range of normal daily exposure, and minimal supplementation would be necessary. Topical application may be easier to chronically administer as a cream or oil, however, it is unknown what level of topical dosing, if any, would have a chemopreventive effect. For both oral and topical administration, optimal formulations based on individual need should be developed.

b. Topical vs oral. From the current work, there is still not a clear answer as to whether topical or oral *d*-limonene administration would be more effective for

chemoprevention. An advantage of topical administration is avoidance of first pass metabolism and thus any associated potential toxicities and drug interactions [145]. Topical application also offers potential for *d*-limonene to be administered concomitantly with a more specific chemotherapeutic like tamoxifen, and potentially lower the dose of the more potent drug. However, topical application of *d*-limonene results in lower circulating levels of perillic acid, the more potent anti-cancer agent, than oral administration. Additionally, *d*-limonene degradation forms oxidized metabolites, which are skin irritants when applied topically. Addition of *d*-limonene to a commercial cream or oil would be applied with bare hands, reducing the quantity of drug delivered to the application site. Further research is necessary to determine the optimal mode of administration.

VI. Future Directions: Application of the "Omics."

a. Identification of high-risk patients. Currently, risk for breast cancer is most commonly assessed using the Gail Model, which takes into account a woman's age, family history of breast cancer, breast density, previous cancer and age at menarche [146]. Screening is accomplished primarily through mammography. Other than presence of BRCA1 or BRCA2 mutations, however, there are no clinically accepted molecular markers of risk. In the future, personalized health care will depend on the identification of non-invasively obtained molecular markers. Identifying otherwise healthy people who are at increased risk continues to be a challenge, but application of throughput

technologies such as the “omics” will help identify those patients that will benefit from a chemopreventive agent.

b. Genomics and proteomics. Despite the growing evidence supporting *d*-limonene’s role as a chemopreventive agent, research cannot effectively move forward until a mechanism has been described. Most likely *d*-limonene affects multiple pathways involved in cancer initiation and progression. In an attempt to identify up or down-regulated proteins in NAF from the topical limonene study, a MudPIT protein profile of one patient pre and post-intervention were obtained (**APPENDIX D**). MudPIT protein profiling is expensive, however, and only identifies the most abundant proteins and does not account for post-translational modifications that may be important in determining a protein’s functionality. DNA and/or RNA micro-arrays are also useful for high throughput analyses of tissues to determine up or down-regulation of multiple pathways after administration of a chemopreventive agent. Use of proteomic and genomic techniques in combination can determine if a bioactive food component like *d*-limonene is interacting at the genome as either a transcription factor, like vitamin D [147] or resulting in epigenetic changes, like methylation by methionine and vitamins B6, B12, and folate [148].

c. Metabolomics. Because bioactive food components like *d*-limonene likely modulate several metabolic pathways, metabolomic profiling after administration of such compounds to determine their efficacy and to gain deeper understanding of their mechanisms is a logical step in the development of these BAFC and others as chemopreventive agents. The “metabolome” represents the network of metabolic

substrates and products, lipids, small peptides, vitamins and other cofactors from both host and the symbiotic microbiota [149]. Characterization of metabolomic profiles in urine or plasma from high-risk individuals would provide a non-invasive means to assess efficacy of administration of chemo-preventive agents like *d*-limonene by measuring both global changes in that profile as well as specific pre-identified biomarkers. Early metabolomic work has been successful in identifying cancer biomarkers. Nam *et al* was able to identify 4 candid biomarkers in urine using GC-MS that distinguished breast cancer patients with a similar sensitivity to mammography [150]. Odunsi was able to correctly identify 97% of epithelial ovarian cancer patients from the controls using NMR [151]. In addition, biomarkers in urine can be quantified to verify either compliance or overall intake, early work has already been successful in identifying biomarkers to assess onion [152] and soy intake [153]. As technology improves and predictive markers are identified, metabolomic profiling has the potential to be used to monitor early response to treatments, determining either toxicity or efficacy of a treatment plan.

d. Future risk identification. Assessment of a risk-profile for individuals in combination with an understanding of how those potential chemopreventives modulate that risk is necessary. Bioactive food components hold great potential as chemopreventive agents because many can be administered over the long-term with little toxicity. Because bioactive food components like *d*-limonene affect multiple pathways, they are ideal compounds prevention of cancers of multi-factorial etiologies such as breast. Experimental populations for *d*-limonene administration include women who are immunocompromised, overweight women, post-menopausal women, or breast cancer

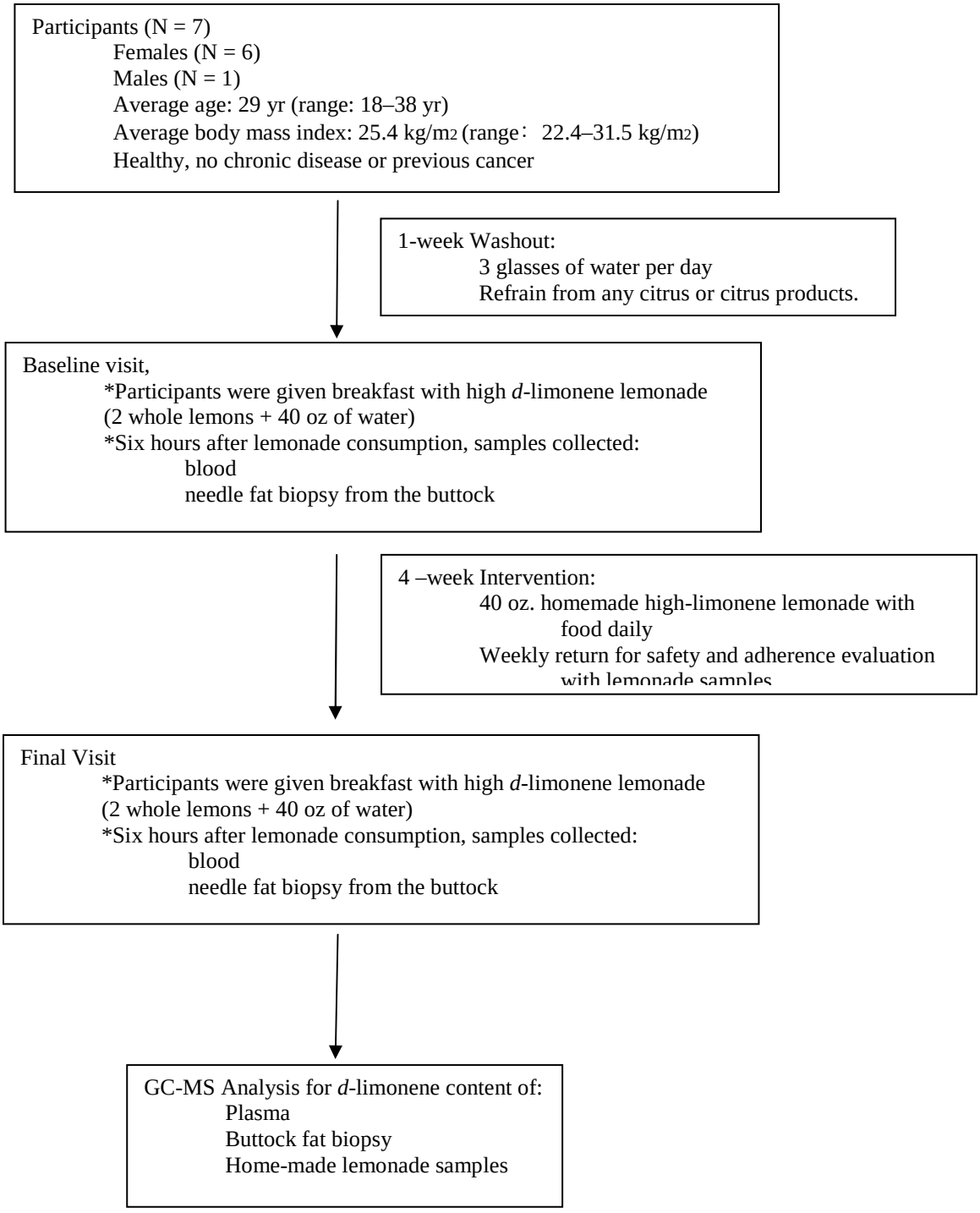
survivors to reduce risk of recurrence. The efficacy of limonene as a chemopreventive agent against other cancers that originate near areas of high adiposity such as skin, hepatic, ovarian, and colon cancers should also be explored. With the application of high throughput profiling techniques, "at risk" women will be easier to identify, as well as identifying the most appropriate chemopreventive to reduce that risk. Most likely *d*-limonene will be most effective within specific population with a specific disease risk profile.

VII. Conclusion:

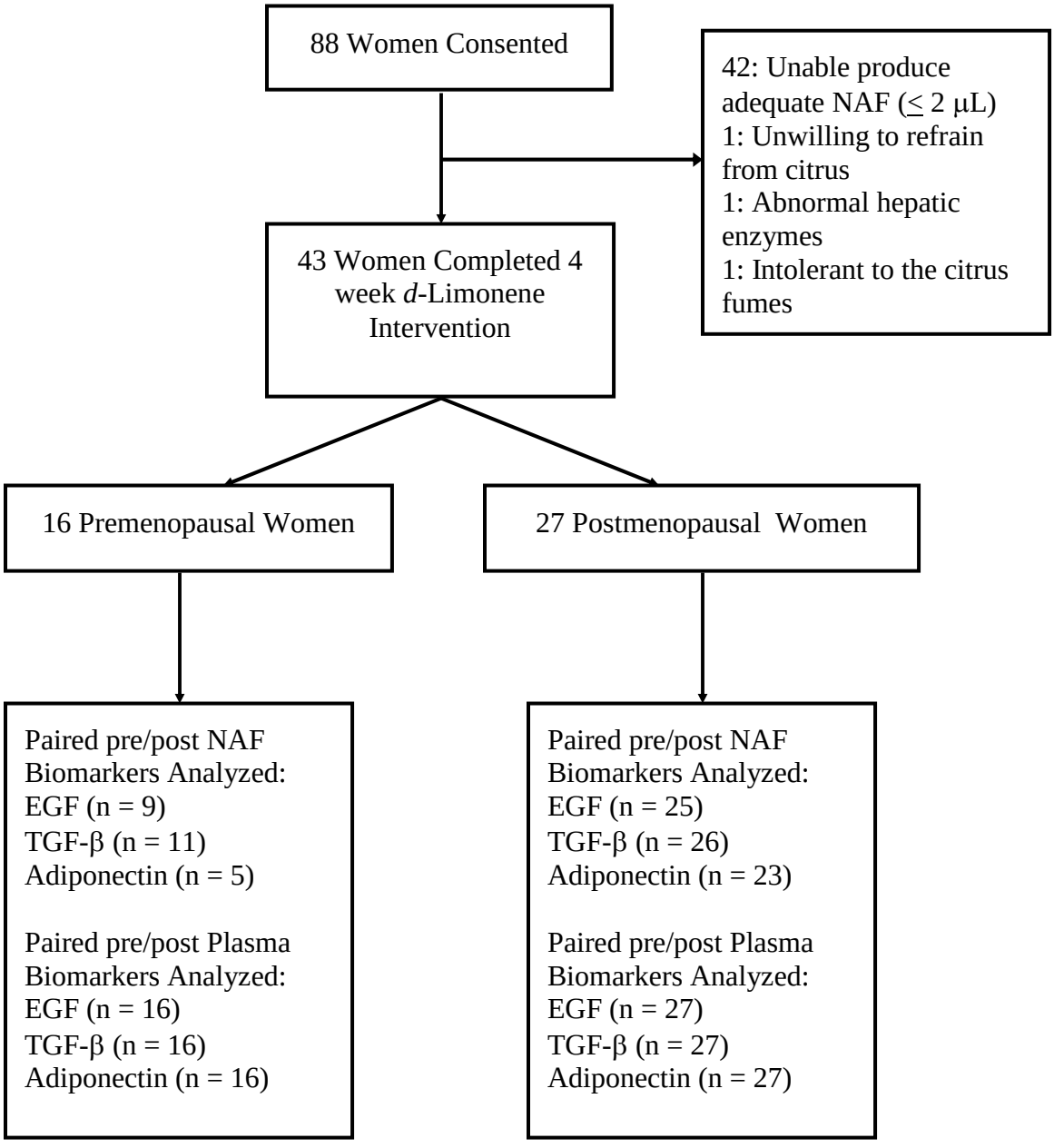
d-Limonene has immense potential as a chemopreventive agent for the breast. This dissertation work demonstrates that *d*-limonene deposits in high levels in adipose tissue after both topical and oral administration, and that the more potent anti-cancer compound, perillic acid also deposits in high levels in adipose tissue after oral administration. This work also supports previous research that indicates proteins expressed within the microenvironment of the breast are very different than plasma, and are influenced by weight and menopausal status. Although *d*-limonene's mechanism has yet to be elucidated, future development of this agent for breast cancer prevention remains promising.

APPENDIX A:

CONSORT DIAGRAM FOR ADIPOSE TISSUE ACCUMULATION OF *D*-LIMONENE WITH THE CONSUMPTION OF A LEMONADE PREPARATION RICH IN *D*-LIMONENE CONTENT



APPENDIX B:
CONSORT DIAGRAM FOR A CLINICAL BIOMARKER STUDY OF TOPICALLY APPLIED *D*-LIMONENE FOR BREAST CANCER PREVENTION



APPENDIX C:

RECOVERY OF ADIPONECTIN AFTER *D*-LIMONENE ADMINISTRATION TO TNF-α TREATED 3T3-L1 ADIPOCYTES

Treatment	Adiponectin[a] (ng/mL)	Total protein[b] (mg/mL)	Corrected Concentration (ug/g)
None[c]	113.91	15.62	7.29
None	234.46	31.14	7.53
d-limonene[d]	97.60	46.66	2.09
d-limonene	92.77	62.19	1.49
TNF-α[e]	26.07	77.71	0.34
TNF-α	30.05	93.23	0.32
d-limonene + TNF-α	170.78	108.75	1.57
d-limonene + TNF-α	186.57	124.27	1.50

[a]Adiponectin was measured using an ELISA kit. [b]Total protein was measured using BioRad protein assay (Hercules, CA). [c]None except dimethyl sulfoxide (DMSO) standard cell culture media treatment. [d]10 μM *d*-limonene used for both the limonene treatment alone and in combination with TNF-α. [e]10 nM TNF-αused for both TNF-α alone and in combination with *d*-limonene. *d*-Limonene was able to partially recover adiponectin after treatment with TNF-α, an inflammatory agent. Adiponectin has been identified as a marker for inflammatory status, with higher levels indicating lower inflammation. This provides preliminary evidence indicating that *d*-limonene might act directly within adipocytes either to relieve inflammatory stress in general or to directly recover adiponectin.

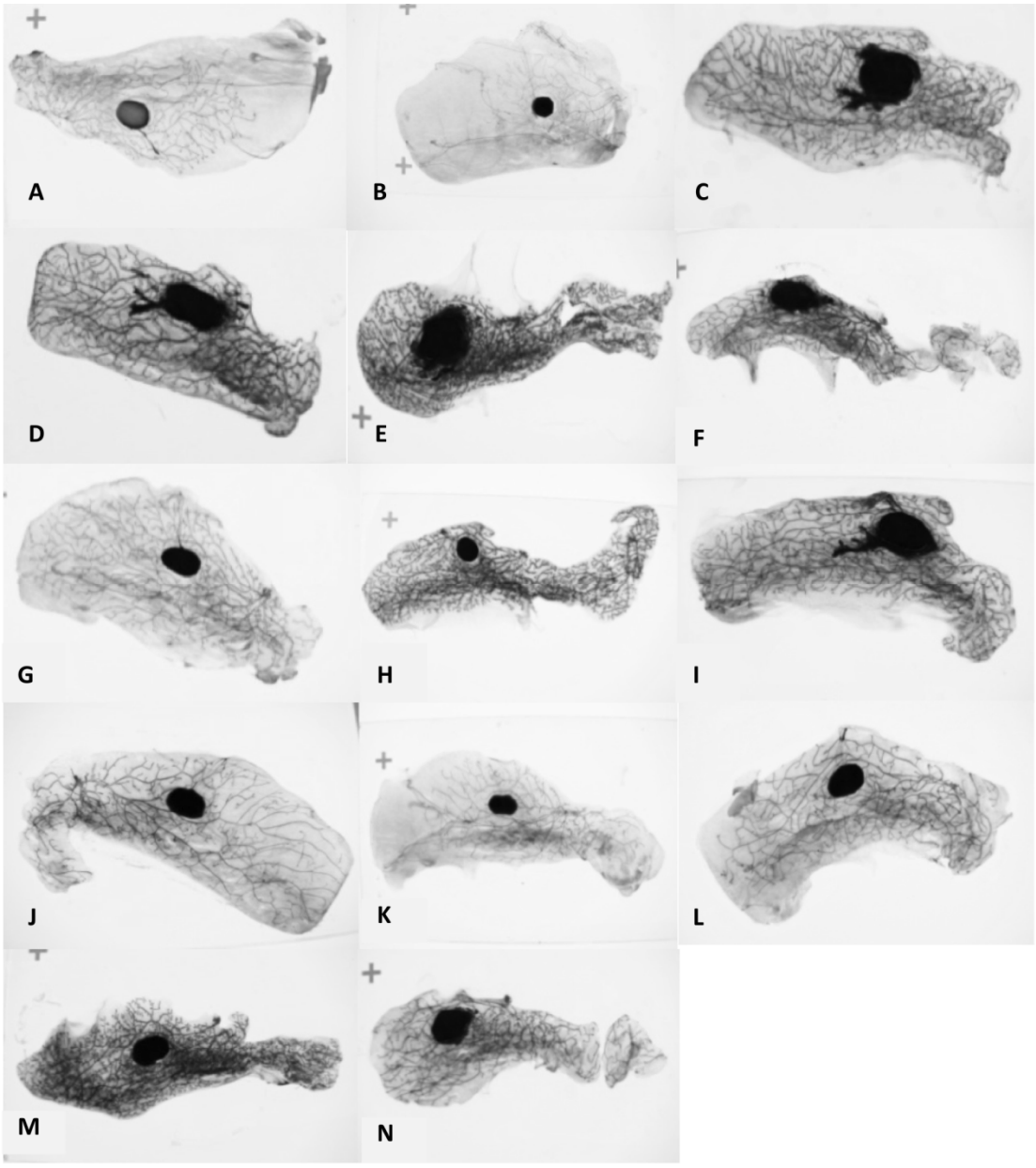
APPENDIX D:

TOPICAL ADMINISTRATION OF A 10% OR 20% *D*-LIMONENE OIL TO THE MOUSE MAMMARY GLAND

Mouse Topical Limonene Study			
	Mammary Pad	**Subcutaneous Fat**	**Skin**
	(ng/g fat)	**(ng/g fat)**	**(ng/g skin)**
Right 34-R2 control	ND[a]	ND	ND
Left 34-R2 control	ND	ND	ND
Right 35 -R1 10%	991.90	372.34	ND
Left 35-R1 10%	631.83	406.08	ND
Right 35-R2 10%	327.77	ND	ND
Left 35-R2 10%	347.11	ND	ND
Right 35 L1 10%	504.96	Missing	Missing
Left 35-L1 10%	363.90	Missing	Missing
Right 36-00 20%	2197.14	649.90	16.91
Left 36-00 20%	2521.80	331.37	77.48
Right 36-R2 20%	174.47	313.11	15.28
Left 36-R2 20%	79.07	497.99	18.12
Right 36-L1 20%	2783.22	342.41	32.52
Left 36-L1 20%	3088.39	432.50	27.47
Right 36-R1 20%	4251.41	638.30	33.98
Left 36-R1 20%	4097.19	307.63	37.01

[a]ND: Not Detectable. A topical formulation of orange oil (93% limonene) combined with coconut oil was applied to the right side only of the skin above the mammary glands of SK1 hairless mice. In general there was no difference between the right and left sides in any tissue, however, application was not precise, and so it is not clear whether the similarity is due to actual transport of limonene through systemic circulation, or to application. *d*-Limonene was on average 10-fold higher in the mammary glands than in the subcutaneous fat, and 100-fold higher in mammary glands than in skin. This small study indicates that both 10% and 20% *d*-limonene formulations can be percutaneously absorbed and result in deposition in high levels in adipose tissue.

APPENDIX E:
MOUSE MAMMARY GLAND WHOLE MOUNTS OF AFTER FOUR WEEKS OF EITHER TOPICAL OR ORAL ADMINISTRATION OF 10% OR 20% *D*-LIMONENE



Whole mount analysis of adult virgin mice. A, B, Untreated controls. C, D, Topical controls. E,F, Oral controls. G, H, 10% Topical limonene treatments. I, J, 10% Oral limonene treatments. K, L 20% Topical limonene treatments. M, N, 20% oral limonene treatments. There is a large degree of variation in glandular development across groups. Most likely this is due to variations in age; mice were 8-9 weeks old. Exact birth dates are unknown. The differences in glandular development could be solely due to age differences. A treatment effect is unlikely because variation in ductile development is not consistent with treatment.

APPENDIX F:

PROTEOMIC PROFILE OF NIPPLE ASPIRATE FLUID FROM A POST-MENOPAUSAL WOMAN PRE- AND POST-FOUR WEEKS OF TOPICAL ADMINISTRATION OF A 10% *D*-LIMONENE OIL

Probability Legend:
over 95%
80% to 94%
50% to 79%
20% to 49%
0% to 19%

#	Visible?	Starred?	Bio View: Identified Proteins (209)	Accession Number	Molecular Weight	Protein Grouping Ambiguity	Fold Change (Fold Change)	332 JM_209357_332	333 JM_209357_333
1	☑	☆	cDNA FLJ78440, highly similar to Human lactoferrin	IPI00298860	78 kDa	★	1.1	5392	4274
2	☑	☆	Putative uncharacterized protein ALB	IPI00022434	72 kDa	★	0.9	3469	3605
3	☑	☆	SNC66 protein	IPI00383164	54 kDa	★	0.8	2459	2640
4	☑	☆	Polymeric immunoglobulin receptor	IPI00004573	83 kDa	★	1.0	3284	2949
5	☑	☆	Putative uncharacterized protein	IPI00550731	26 kDa	★	0.8	820	887
6	☑	☆	Apolipoprotein D	IPI00006662	21 kDa	★	0.6	663	963
7	☑	☆	cDNA FLJ35730 fis, clone TESTI2003131, highly similar to ALPHA-1-ANTICHYMOTRYPSIN	IPI00550991	51 kDa		1.0	871	829
8	☑	☆	IGL@ protein	IPI00154742	25 kDa	★	1.2	699	524
9	☑	☆	Prolactin-inducible protein	IPI00022974	17 kDa		1.0	560	499
10	☑	☆	alpha-2-glycoprotein 1, zinc precursor	IPI00166729	34 kDa		1.7	637	337
11	☑	☆	Alpha-lactalbumin	IPI00019524	16 kDa		2.0	620	280
12	☑	☆	CDNA FLJ25298 fis, clone STM07683, highly similar to Protein Tro alpha1 H,myeloma	IPI00386524	54 kDa	★	0.9	1197	1167
13	☑	☆	Isoform 1 of Clusterin	IPI00291262	52 kDa		0.7	204	260
14	☑	☆	Putative uncharacterized protein DKFZp686C02220 (Fragment)	IPI00423461	54 kDa	★	0.7	846	1021
15	☑	☆	Serotransferrin	IPI00022463	77 kDa	★	1.4	257	166
16	☑	☆	Immunoglobulin J chain	IPI00178926	18 kDa		1.2	188	142
17	☑	☆	Complement C4-A	IPI00032258	193 kDa	★	1.6	258	142
18	☑	☆	Lysozyme C	IPI00019038	17 kDa		1.0	97	88
19	☑	☆	Ig mu heavy chain disease protein	IPI00385264	43 kDa	★	0.5	81	159
20	☑	☆	Immunoglobulin heavy chain variant (Fragment)	IPI00940245	45 kDa	★	0.8	795	957
21	☑	☆	carboxyl ester lipase precursor	IPI00099670	80 kDa		1.4	139	91
22	☑	☆	Isoform 1 of Alpha-1-antitrypsin	IPI00553177	47 kDa	★	0.7	47	65
23	☑	☆	Isoform 2 of Retinoic acid receptor responder protein 1	IPI00410240	33 kDa		0.6	48	74
24	☑	☆	Ig kappa chain V-III region HAH	IPI00030205	14 kDa	★	0.8	67	75
25	☑	☆	IGL@ protein	IPI00658130	25 kDa	★	1.5	507	303
26	☑	☆	Kappa-casein	IPI00294773	20 kDa		0.9	72	71
27	☑	☆	cDNA FLJ50830, highly similar to Serum albumin	IPI00908876	60 kDa	★	0.8	1403	1589
28	☑	☆	Keratin, type II cytoskeletal 1	IPI00220327	66 kDa	★	3.0	95	29
29	☑	☆	Putative uncharacterized protein DKFZp686N02209	IPI00384938	53 kDa	★	1.0	58	53
30	☑	☆	Beta-2-microglobulin	IPI00004656	14 kDa		0.4	35	75
31	☑	☆	Isoform 1 of Vitamin D-binding protein	IPI00555812	53 kDa		0.8	36	41
32	☑	☆	Complement C3 (Fragment)	IPI00783987	187 kDa	★	1.2	43	33
33	☑	☆	IGL@ protein	IPI00719373	23 kDa	★	2.5	366	131
34	☑	☆	PR domain containing 1, with ZNF domain isoform 2	IPI00385453	77 kDa		0.5		10
35	☑	☆	Ig kappa chain V-I region AU	IPI00003111	13 kDa	★	1.7	58	30
36	☑	☆	Keratin, type I cytoskeletal 9	IPI00019359	62 kDa	★	1.0	44	40
37	☑	☆	cDNA FLJ14473 fis, clone MAMMA1001080, highly similar to Homo sapiens SNC73 protei...	IPI00386879	53 kDa	★	0.9	1187	
38	☑	☆	Xanthine dehydrogenase/oxidase	IPI00244391	146 kDa	★	4.3	43	9
39	☑	☆	Galectin-3-binding protein	IPI00023673	65 kDa		0.9	33	33
40	☑	☆	Isoform 1 of Obscurin	IPI00288940	868 kDa		1.6	16	9
41	☑	☆	Isoform 2 of Alpha-S1-casein	IPI00216119	22 kDa		2.4	32	12
42	☑	☆	Monocyte differentiation antigen CD14	IPI00029260	40 kDa		0.9	31	33
43	☑	☆	Transcobalamin-1	IPI00299729	48 kDa		3.7	37	9
44	☑	☆	Single chain Fv (Fragment)	IPI00007899	12 kDa	★	1.4	8	5
45	☑	☆	FLJ00385 protein (Fragment)	IPI00168728	56 kDa	★	1.9	48	23
46	☑	☆	Coiled-coil domain-containing protein 85A	IPI00289837	60 kDa		0.4	2	
47	☑	☆	Lactadherin	IPI00002236	43 kDa		7.1	47	
48	☑	☆	Keratin, type II cytoskeletal 2 epidermal	IPI00021304	66 kDa	★	3.3	44	12
49	☑	☆	Rheumatoid factor D5 light chain (Fragment)	IPI00816799	13 kDa	★	5.7	19	3
50	☑	☆	Follicular dendritic cell secreted peptide	IPI00168905	10 kDa		1.4	25	16
51	☑	☆	Putative uncharacterized protein IGHM	IPI00892870	52 kDa	★	0.8	40	46
52	☑	☆	Olfactomedin-4	IPI00022255	57 kDa		3.6	32	8
53	☑	☆	Keratin, type I cytoskeletal 10	IPI00009865	59 kDa	★	27	28	
54	☑	☆	Isoform 2 of Titin	IPI00023283	3806 kDa		2.7	6	2
55	☑	☆	Ig lambda chain V-II region NEI	IPI00382424	12 kDa		0.2		4
56	☑	☆	Beta-casein	IPI00010849	25 kDa		2.8	31	
57	☑	☆	ALK tyrosine kinase receptor	IPI00395632	176 kDa		3.3	11	
58	☑	☆	Neutrophil gelatinase-associated lipocalin	IPI00299547	23 kDa		0.2	6	26
59	☑	☆	Plasma protease C1 inhibitor	IPI00291866	55 kDa		2.2	12	5
60	☑	☆	Myosin-reactive immunoglobulin light chain variable region (Fragment)	IPI00384397	13 kDa	★	2.2	29	12
61	☑	☆	Isoform 4 of Guanine nucleotide exchange factor DBS	IPI00297139	112 kDa		4.8	5	
62	☑	☆	cDNA FLJ59350	IPI00165125	88 kDa		8.6	9	
63	☑	☆	Insulin-like growth factor-binding protein 2	IPI00297284	35 kDa		0.2		20
64	☑	☆	Aminopeptidase N	IPI00221224	110 kDa		2.5	14	5
65	☑	☆	Chitinase-3-like protein 1	IPI00002147	43 kDa		0.6	4	6
66	☑	☆	Ig lambda chain V region 4A	IPI00022890	12 kDa		0.8	10	11
67	☑	☆	Isoform 2 of Sushi domain-containing protein 1	IPI00478989	85 kDa		1.6	14	
68	☑	☆	Uteroglobin	IPI00006705	10 kDa		0.9	12	12
69	☑	☆	Prostasin	IPI00329538	36 kDa		1.8	6	3
70	☑	☆	Leucine-rich alpha-2-glycoprotein	IPI00022417	38 kDa		0.5	5	9
71	☑	☆	Putative uncharacterized protein	IPI00887169	25 kDa	★	1.6	510	
72	☑	☆	cDNA FLJ54442, highly similar to Scavenger receptor cysteine-rich type 1 protein M160	IPI00026975	160 kDa		1.2		7
73	☑	☆	Immunoglobulin lambda-like polypeptide 1	IPI00013438	23 kDa	★	0.9		9
74	☑	☆	Conserved hypothetical protein	IPI00743777	27 kDa		4.5	5	
75	☑	☆	Putative uncharacterized protein KIF26B	IPI00397820	185 kDa		3.6	8	
76	☑	☆	Protein S100-A9	IPI00027462	13 kDa		9.1	10	
77	☑	☆	Huntingtin	IPI00002335	348 kDa		0.2	4	
78	☑	☆	Ig lambda chain V-II region BUR	IPI00003947	12 kDa	★	1.2	18	
79	☑	☆	xin actin-binding repeat containing 2 isoform 1	IPI00550232	402 kDa		1.9	2	
80	☑	☆	Myosin-reactive immunoglobulin kappa chain variable region (Fragment)	IPI00384401	12 kDa	★	3.8	4	

80	☑	☆	Myosin-reactive immunoglobulin kappa chain variable region (Fragment)	IPI00384401	12 kDa	★	3.8	4	
81	☑	☆	Isoform 1 of Gelsolin	IPI00026314	86 kDa		0.2		5
82	☑	☆	Butyrophilin subfamily 1 member A1	IPI00013210	59 kDa		4.5	10	2
83	☑	☆	Ig kappa chain V-IV region JI	IPI00386132	15 kDa		13	14	
84	☑	☆	Putative uncharacterized protein ENSP00000392404 (Fragment)	IPI00942103	32 kDa		0.9	3	3
85	☑	☆	Putative interleukin-17 receptor E-like	IPI00413812	38 kDa		2.3	28	
86	☑	☆	complement component 4B preproprotein	IPI00418163	193 kDa	★	1.0	129	120
87	☑	☆	Transcriptional activator protein Pur-beta	IPI00045051	33 kDa		0.9	2	
88	☑	☆	Isoform 1 of Lysosomal-trafficking regulator	IPI00796450	429 kDa		2.7	3	
89	☑	☆	Prominin-1	IPI00012540	97 kDa		0.2		5
90	☑	☆	Putative uncharacterized protein DKFZp686P15220	IPI00645363	52 kDa	★	3.2	28	
91	☑	☆	Ig kappa chain V-I region EU	IPI00387026	12 kDa	★	7.2	8	
92	☑	☆	Alpha-2-HS-glycoprotein	IPI00953689	39 kDa		4.5	10	2
93	☑	☆	laminin alpha 2 subunit isoform b precursor	IPI00218725	344 kDa		1.2	4	3
94	☑	☆	vacuolar protein sorting 13C protein isoform 2B	IPI00412216	408 kDa		1.8	2	
95	☑	☆	MMAA protein	IPI00217023	47 kDa		1.8	2	
96	☑	☆	MYC binding protein 2	IPI00289776	510 kDa		0.2		4
97	☑	☆	Uncharacterized protein C2orf16	IPI00470912	224 kDa		0.9	2	
98	☑	☆	Protein S100-A8	IPI00007047	11 kDa		2.3	5	
99	☑	☆	Isoform 1 of GON-4-like protein	IPI00375803	249 kDa		0.2		4
100	☑	☆	Actin, cytoplasmic 1	IPI00021439	42 kDa	★	5.7	6	
101	☑	☆	Cubilin	IPI00160130	399 kDa		0.2		6
102	☑	☆	Cadherin-like 23	IPI00216560	370 kDa		7.6	8	
103	☑	☆	Putative uncharacterized protein DKFZp686J11235 (Fragment)	IPI00426060	54 kDa	★	1100	1161	
104	☑	☆	Isoform 5 of Dynamin-1-like protein	IPI00037283	79 kDa		6.7	7	
105	☑	☆	Isoform B of Collagen alpha-6(IV) chain	IPI00472200	164 kDa		3.6	4	
106	☑	☆	immunoglobulin-like and fibronectin type III domain containing 1	IPI00328885	384 kDa		1.8	2	
107	☑	☆	DMXL2 protein	IPI00152542	340 kDa		0.5		2
108	☑	☆	Putative uncharacterized protein IGHA2	IPI00829711	20 kDa	★	470	495	
109	☑	☆	Leucine-rich repeat-containing protein KIAA0644	IPI00006556	89 kDa		2.9	3	
110	☑	☆	Ceruloplasmin	IPI00017601	122 kDa		2.9	3	
111	☑	☆	Hemopexin	IPI00022488	52 kDa		1.8	2	
112	☑	☆	Zinc finger protein 469	IPI00644680	410 kDa		0.3		3
113	☑	☆	Isoform 1 of Putative uncharacterized protein C1orf133	IPI00787296	16 kDa		0.2		5
114	☑	☆	Apolipoprotein B-100	IPI00022229	516 kDa		0.4		5
115	☑	☆	Isoform 1 of Rho/Cdc42/Rac GTPase-activating protein RICS	IPI00787743	231 kDa		7.2	8	
116	☑	☆	Isoform 1 of Transcription elongation factor SPT5	IPI00298058	121 kDa		1.2	9	
117	☑	☆	Protein bassoon	IPI00020153	416 kDa	★	0.3		3
118	☑	☆	hypothetical protein XP_002342815	IPI00936081	55 kDa		1.9	2	
119	☑	☆	Isoform 1 of Collagen alpha-3(VI) chain	IPI00022200	344 kDa		0.5		2
120	☑	☆	CD59 glycoprotein	IPI00011302	14 kDa		4.5	5	
121	☑	☆	Isoform 1 of Protein AHNAK2	IPI00856045	617 kDa		0.3		3
122	☑	☆	Laminin subunit alpha-1	IPI00375294	337 kDa		0.5		2
123	☑	☆	VH3 protein (Fragment)	IPI00383732	16 kDa	★	6.7	7	
124	☑	☆	Isoform 1 of Hepatocyte growth factor receptor	IPI00029273	156 kDa		0.3		3
125	☑	☆	DNA excision repair protein ERCC-6	IPI00414779	168 kDa		0.5		4
126	☑	☆	SH2 domain-containing adapter protein B	IPI00017578	65 kDa		1.8	2	
127	☑	☆	Mucin-16	IPI00103552	2353 kDa		0.5		2
128	☑	☆	Hemicentin 1	IPI00045512	614 kDa		0.5		2
129	☑	☆	Isoform 2 of Serine-protein kinase ATM	IPI00289986	196 kDa		0.6		3
130	☑	☆	Ankyrin repeat domain-containing protein 11	IPI00914930	298 kDa		0.3		3
131	☑	☆	Isoform 1 of Immunoglobulin superfamily member 10	IPI00183913	291 kDa	★	1.9	2	
132	☑	☆	B-cell receptor-associated protein 31	IPI00218200	28 kDa		0.5		2
133	☑	☆	Isoform 2 of NADPH oxidase activator 1	IPI00216835	52 kDa		5.7	6	
134	☑	☆	hypothetical protein LOC158358	IPI00216990	228 kDa		1.9	2	
135	☑	☆	Isoform 1 of DNA mismatch repair protein Mlh3	IPI00005811	164 kDa		1.9	2	
136	☑	☆	Collagen alpha-2(V) chain	IPI00739099	145 kDa		0.5		2
137	☑	☆	Isoform 1 of Collagen alpha-1(XXII) chain	IPI00303152	161 kDa		2.7	3	
138	☑	☆	1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase beta-3	IPI00010400	139 kDa		0.5		2
139	☑	☆	Isoform 1 of Amiloride-sensitive cation channel 2, neuronal	IPI00293398	65 kDa		1.9	2	
140	☑	☆	Isoform 4 of Nesprin-1	IPI00247295	1005 kDa		3.8	4	
141	☑	☆	Isoform 1 of Collagen alpha-1(II) chain	IPI00186460	134 kDa		0.3		3
142	☑	☆	18 kDa protein	IPI00913961	18 kDa		1.9	2	
143	☑	☆	Glyceraldehyde-3-phosphate dehydrogenase	IPI00219018	36 kDa		3.8	4	
144	☑	☆	Isoform 1 of Zinc finger protein 608	IPI00166972	162 kDa		3.8	4	
145	☑	☆	Isoform 1 of Serine palmitoyltransferase 3	IPI00794843	62 kDa		7.6	8	
146	☑	☆	Heat shock 70 kDa protein 4	IPI00002966	94 kDa		0.2		6
147	☑	☆	Isoform 1 of Hydrocephalus-inducing protein homolog	IPI00647188	576 kDa		0.3		3
148	☑	☆	Isoform 1 of UPF0378 protein KIAA0100	IPI00373894	254 kDa		0.3		3
149	☑	☆	Beta-2-glycoprotein 1	IPI00298828	38 kDa		1.8	2	
150	☑	☆	Isoform 2 of Alcohol dehydrogenase 4	IPI00218899	43 kDa		2.9	3	
151	☑	☆	Alpha-tectorin	IPI00023051	240 kDa		1.8	2	
152	☑	☆	Isoform 1 of Spectrin beta chain, brain 3	IPI00018829	289 kDa		1.4	3	
153	☑	☆	Isoform 1 of Chromodomain-helicase-DNA-binding protein 7	IPI00794880	336 kDa		0.3		3
154	☑	☆	Cathepsin D	IPI00011229	45 kDa		2.9	3	
155	☑	☆	Apolipoprotein A-I	IPI00021841	31 kDa		2.9	3	
156	☑	☆	Isoform 1 of Tudor domain-containing protein 5	IPI00219978	116 kDa		2.9	3	
157	☑	☆	Zinc finger protein 217	IPI00025310	115 kDa		0.9	3	
158	☑	☆	Transcription initiation factor TFIID subunit 4	IPI00413755	110 kDa		0.5		2
159	☑	☆	Biorientation of chromosomes in cell division protein 1-like	IPI00797574	330 kDa		0.5		2
160	☑	☆	Isoform 5 of Protein transport protein Sec16A	IPI00031242	236 kDa		1.9	2	
161	☑	☆	Isoform 1 of Transmembrane and coiled-coil domain-containing protein 3	IPI00016665	76 kDa		0.5		2
162	☑	☆	Plexin-A1	IPI00552671	211 kDa		0.5		2
163	☑	☆	Myosin-7	IPI00025880	223 kDa		0.5		2
164	☑	☆	Poliovirus receptor-related protein 4	IPI00043992	55 kDa		0.5		2
165	☑	☆	Isoform 1 of Collagen alpha-1(XVI) chain	IPI00400935	158 kDa		2.9	3	
166	☑	☆	Isoform 2 of Nuclear receptor coactivator 3	IPI00220079	154 kDa		1.9	2	
167	☑	☆	Neurogenic locus notch homolog protein 3	IPI00029819	244 kDa		0.5		2
168	☑	☆	Isoform 1 of Mitogen-activated protein kinase kinase kinase 7-interacting protein 3	IPI00166840	79 kDa		0.5		2
169	☑	☆	Isoform 1 of Chaperone activity of bc1 complex-like, mitochondrial	IPI00176469	72 kDa		0.5		2
170	☑	☆	Tyrosine-protein kinase SgK269	IPI00737545	193 kDa		0.2		4
171	☑	☆	cut-like 2	IPI00022370	162 kDa		0.2		6
172	☑	☆	Isoform 2 of Transcription factor HIVEP3	IPI00328089	259 kDa		1.9	2	
173	☑	☆	26S protease regulatory subunit 8	IPI00023919	46 kDa		2.9	3	
174	☑	☆	Filaggrin	IPI00026256	435 kDa		1.4	3	
175	☑	☆	Leucine-rich repeat and fibronectin type-III domain-containing protein 6	IPI00289849	90 kDa		0.5		2
176	☑	☆	Leucine-rich repeat-containing protein 15	IPI00152871	64 kDa		1.8	2	
177	☑	☆	437 kDa protein	IPI00744732	437 kDa		1.8	2	
178	☑	☆	Uncharacterized protein C22orf29	IPI00472049	39 kDa		0.5		2
179	☑	☆	Cleavage and polyadenylation specificity factor subunit 1	IPI00026219	161 kDa		1.8	2	

180	☑	☆	Isoform 3 of HEAT repeat-containing protein 7A	IPI00386045	67 kDa	0.3		3
181	☑	☆	Isoform 2 of UNC93-like protein MFSD11	IPI00010329	43 kDa	1.9	2	
182	☑	☆	Isoform 2 of UPF0518 protein FAM160A2	IPI00149375	107 kDa	0.5		2
183	☑	☆	Isoform 1 of CD177 antigen	IPI00297444	46 kDa	0.3		3
184	☑	☆	SH3 multiple domains 1	IPI00400923	122 kDa	0.5		2
185	☑	☆	Zinc finger protein 646	IPI00004419	201 kDa	1.9	2	
186	☑	☆	Isoform 1 of Laminin subunit alpha-4	IPI00329482	203 kDa	0.5		2
187	☑	☆	Isoform 1 of Phosphoglucomutase-like protein 5	IPI00014852	62 kDa	0.5		2
188	☑	☆	Xylosyltransferase 1	IPI00183487	108 kDa	0.5		2
189	☑	☆	Isoform 1 of F-box only protein 24	IPI00219503	65 kDa	0.2		4
190	☑	☆	hypothetical protein XP_002347295	IPI00937428	20 kDa	0.5		2
191	☑	☆	Isoform 2 of Histone-lysine N-methyltransferase, H3 lysine-79 specific	IPI00289034	185 kDa	0.2		4
192	☑	☆	Isoform 2 of Serine/threonine-protein phosphatase 6 regulatory ankyrin repeat subuni...	IPI00167419	108 kDa	0.5		2
193	☑	☆	Isoform 1 of Apolipoprotein B-100 receptor	IPI00399183	115 kDa	2.9	3	
194	☑	☆	Fatty-acid amide hydrolase 2	IPI00043550	58 kDa	0.3		3
195	☑	☆	Isoform 1 of Dynein heavy chain 10, axonemal	IPI00784869	515 kDa	0.5		2
196	☑	☆	Putative uncharacterized protein SLC15A4	IPI00434466	56 kDa	0.6		3
197	☑	☆	Isoform 1 of AT-rich interactive domain-containing protein 1B	IPI00015404	236 kDa	1.9	2	
198	☑	☆	Receptor-type tyrosine-protein phosphatase eta precursor	IPI00290328	152 kDa	1.9	2	
199	☑	☆	Mucin-12	IPI00455957	558 kDa	1.9	2	
200	☑	☆	Isoform 1 of Transcription termination factor 2	IPI00290812	130 kDa	0.5		2
201	☑	☆	Methylcytosine dioxygenase TET1	IPI00303112	235 kDa	0.9		2
202	☑	☆	Isoform 1 of Ral GTPase-activating protein beta subunit	IPI00409601	167 kDa	0.5		2
203	☑	☆	Isoform 1 of Protocadherin Fat 4	IPI00852735	543 kDa	1.9	2	
204	☑	☆	PR domain zinc finger protein 13	IPI00480010	75 kDa	0.5		2
205	☑	☆	Interferon-induced very large GTPase 1	IPI00854845	279 kDa	1.9	2	
206	☑	☆	Isoform 1 of Vacuolar protein sorting-associated protein 13B	IPI00376439	449 kDa	1.9	2	
207	☑	☆	Probable G-protein coupled receptor 88	IPI00004926	40 kDa	0.5		2
208	☑	☆	TRAF-interacting protein	IPI00306611	53 kDa	0.5		2
209	☑	☆	Organic anion transporter LST-3b	IPI00243451	76 kDa	0.5		2

This output is from a MudPIT protein analysis from one post-menopausal woman chosen because there were large changes in her NAF levels of EGF, TGF-β, and adiponectin. Column 1 lists the number of proteins, generally in order of highest abundance to lowest. Column 2 indicates whether the protein was visible in the instrument. Column 3 is simply a marking tool for the user. Column 4 is the protein name. Column 5 is the accession number, which allows the protein to be searched on proteomic databases. Column 6 indicates the weight of the protein in kilodaltons (kDa). Column 7 indicates the fold-change, in this case pre to post-intervention. Columns 8 indicates the pre-intervention abundance, with the value indicating the number of times the instrument saw the peptide. Column 9 is abundance for each protein post-intervention. Overall there were major changes in some keratins and other structural proteins, some uncharacterized proteins, and a few milk proteins such as alpha-lactalbumin. There was not enough literature supporting the involvement of any of the significantly affected proteins in cancer, thus no more protein profiles were obtained.

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