Therapeutics, Targets, and Chemical Biology

Cancer Research

NSAID Use Reduces Breast Cancer Recurrence in Overweight and Obese Women: Role of Prostaglandin–Aromatase Interactions

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Abstract

Obesity is associated with a worse breast cancer prognosis and elevated levels of inflammation, including greater cyclooxygenase-2 (COX-2) expression and activity in adipose-infiltrating macrophages. The product of this enzyme, the proinflammatory eicosanoid prostaglandin E2 (PGE2), stimulates adipose tissue aromatase expression and subsequent estrogen production, which could promote breast cancer progression. This study demonstrates that daily use of a nonsteroidal anti-inflammatory drug (NSAID), which inhibits COX-2 activity, is associated with reduced estrogen receptor α (ERα)–positive breast cancer recurrence in obese and overweight women. Retrospective review of data from ERα-positive patients with an average body mass index of >30 revealed that NSAID users had a 52% lower recurrence rate and a 28-month delay in time to recurrence. To examine the mechanisms that may be mediating this effect, we conducted *in vitro* studies that utilized sera from obese and normal-weight patients with breast cancer. Exposure to sera from obese patients stimulated greater macrophage COX-2 expression and PGE2 production. This was correlated with enhanced preadipocyte aromatase expression following incubation in conditioned media (CM) collected from the obese-patient, sera-exposed macrophages, an effect neutralized by COX-2 inhibition with celecoxib. In addition, CM from macrophage/preadipocyte cocultures exposed to sera from obese patients stimulated greater breast cancer cell ERα activity, proliferation, and migration compared with sera from normal-weight patients, and these differences were eliminated or reduced by the addition of an aromatase inhibitor during CM generation. Prospective studies designed to examine the clinical benefit of NSAID use in obese patients with breast cancer are warranted. *Cancer Res; 74(16); 4446–57. ©2014 AACR.*

Introduction

Obesity has become a significant global public health problem in the past 30 years (1). More than 35% of Americans have a body mass index (BMI) of $\geq$30 kg/m^2 (2), an alarming percentage given the association of obesity with an increased incidence of, and mortality from, numerous chronic diseases, including breast cancer. Several studies have demonstrated a link between obesity and a worse breast cancer prognosis in both premenopausal and postmenopausal women, including a prospective study of almost 500,000 women that found a progressive escalation in the risk of breast cancer mortality with each successive increase in BMI category (3). Other studies have established positive correlations between obesity and breast cancer recurrence (4), a shorter disease-free survival and lower rates of overall survival, independent of tumor stage at diagnosis (5–7).

Obesity is hypothesized to affect outcomes in postmenopausal, hormone-responsive patients with breast cancer negatively by promoting adipose tissue expression of aromatase, the rate-limiting enzyme in estradiol production. This theory has been supported by the results of clinical studies with anastrazole and letrozole showing a reduction in the efficacy of these aromatase inhibitors with increasing BMI (8, 9). Plasma estradiol and estrone sulfate levels in obese patients remain significantly elevated in comparison to nonobese patients following letrozole treatment (10), indicating that this reduced response rate may be related to suboptimal inhibition of obesity-associated aromatase activity. Given that aromatase inhibitors are prescribed at a fixed dose, it is possible that their decreased efficacy in obese women is because of underdosing. However, two phase III clinical trials of anastrozole found that a 10-fold increase in dose provided no additional overall benefit. It is likely that these trials included a number of obese women, suggesting that greater dosage will not solve the problem of obesity-related aromatase inhibitor resistance (11, 12).

Recent findings by Dannenberg and colleagues confirm the presence of elevated aromatase expression in the breast tissue of overweight and obese women (13, 14). Crucially, they have

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demonstrated high congruence between aromatase levels and local breast tissue inflammation, as measured by the number of crown-like structures (CLS-B). CLS-B are inflammatory foci composed of a necrotic adipocyte surrounded by macrophages, which produce excess amounts of several proinflammatory mediators. These include cyclooxygenase-2 (COX-2)–derived prostaglandin E2 (PGE2), a potent stimulant of aromatase expression in preadipocytes, the predominant site of aromatase expression within adipose tissue (13–15). This obesity–inflammation–aromatase axis may be a significant contributor to the reduced aromatase inhibitor response and increased mortality rate observed in obese postmenopausal patients. Collectively, these studies suggest that obese postmenopausal women with estrogen receptor α (ERα)-positive breast cancer may benefit from combining hormone therapy with a drug targeting the COX-2 pathway.

In this study, we retrospectively examined the association between nonsteroidal anti-inflammatory drug (NSAID) use and recurrence rate in a hormone-responsive breast cancer patient population to determine whether this COX-2 pathway-targeting drug group improves prognosis. NSAID use reduced the recurrence rate by approximately 50% and extended the disease-free survival by more than 2 years in our largely overweight/obese, postmenopausal population of women. Furthermore, to assess whether inflammation-associated preadipocyte aromatase expression may be responsible for this effect, we utilized an *in vitro* model of the obese patient's tumor microenvironment. Here we demonstrated that obesity-associated circulating factors enhance preadipocyte aromatase expression and estradiol production via their stimulation of macrophage COX-2 expression, resulting in greater breast cancer cell ERα activity, proliferation, and migration.

Materials and Methods

Subjects

Review of medical records dated between January 1, 1987, and January 12, 2011, from the Cancer Therapy and Research Center (CTRC) at the University of Texas Health Science Center at San Antonio (UTHSCSA) and the START Center for Cancer Care clinic in San Antonio, Texas, yielded a patient population of 440 women diagnosed with invasive, ERα-positive breast cancer. Exclusion criteria included diagnosis of carcinoma *in situ*, hormone therapy refusal, or noncompliance, unavailable treatment information, discontinuation of adjuvant therapy because of insurance issues or health problems, diagnosis of triple negative breast cancer, breast cancer metastasis at the time of diagnosis, unavailable diagnosis dates, and underweight status. The collection of patient data from these medical records was approved by the Institutional Review Boards (IRB) of UTHSCSA and START and conducted in accordance with the Declaration of Helsinki and good clinical practice. Informed consent was obtained from all patients before participation.

Study design and data collection

This exploratory study utilized retrospective data collected from patient charts. BMI was calculated and patients classified as normal weight (18.5–24.9 kg/m^2), overweight (25.0–29.9 kg/m^2), or obese ($\geq$30.0 kg/m^2). Perimenopausal women were classified as premenopausal because they receive tamoxifen as their first-line hormonal therapy (16, 17). Patients were designated as NSAID users if progress notes described daily use of aspirin, ibuprofen, celecoxib, naproxen, meloxicam, or another COX-2 inhibitor in the list of medications. Information on prediagnostic NSAID use was not available, and specific data on postdiagnostic NSAID dosage and length of use was not collected. Use of analgesics that do not target COX-2 as well as pro re nata (prn) COX-2 inhibitor use did not qualify for inclusion in the NSAID user group. Recurrence was defined as any local, contralateral, distant tumor, or metastasis of the primary breast cancer. Second primaries were not classified as recurrent breast cancer.

Serum samples

Serum was collected from 25 postmenopausal patients with breast cancer under an IRB approved biorepository collection protocol at the CTRC of UTHSCSA. The collection and use of these biological samples was conducted in accordance with the Declaration of Helsinki and good clinical practice. Informed consent was obtained before participation, and all samples and data were de-identified before release to maintain patient confidentiality. Serum was pooled according to two patient BMI categories, normal weight and obese. Serum from 5 normal-weight and 5 obese postmenopausal women, who were ineligible control subjects in the Polish Women's Health Study, was also utilized. The design of this study has been described elsewhere (18). This serum was not pooled.

Cell lines and reagents

The ERα-positive MCF-7 and T47D and ERα-negative MDA-MB-231 breast cancer cell lines (ATCC) were maintained in IMEM (GIBCO Life Technologies) supplemented with 10% fetal bovine serum (FBS). Preadipocytes isolated from women undergoing elective surgical procedures were a generous gift from Dr. Rong Li, UTHSCSA, and have been described previously (19). They were maintained in DMEM/F12 1:1 media (GIBCO Life Technologies) plus 10% FBS. U937 monocytes (ATCC) were cultured in RPMI supplemented with 10% FBS and matured to macrophages at the time of seeding for experimentation by incubating the cells in 10.0 ng/mL phorbol 12-myristate 13-acetate (TPA) for 48 hours. Testosterone, anastrozole, celecoxib, and TPA were purchased from Sigma.

Conditioned media

Macrophage-conditioned media (CM) was generated by seeding 4×10^5 U937 monocytes per well in 6-well plates, maturing them to macrophages with TPA, then serum-starving the cells for 6 hours. The macrophages were then exposed to 2% pooled or individual patient sera in serum-free media (SFM) for 1 hour, the sera removed and cells washed with phosphate buffered saline (PBS) followed by incubation in SFM for 24 hours. The CM was then collected and stored at −20°C for subsequent *in vitro* assays. To assess the impact of macrophage COX-2 inhibition, the macrophages were pretreated with 30 μmol/L celecoxib or vehicle for 1 hour before as well as during sera exposure. Macrophage/preadipocyte coculture CM was

generated by first maturing 2×10^5 U937 monocytes per well into macrophages on top of confluent preadipocytes seeded in 6-well plates. The coculture was serum-starved for 6 hours, exposed to a 2% pooled sera in SFM for 1 hour, then washed with PBS and incubated for 24 hours in SFM ± testosterone (100 nmol/L), anastrozole (1 μmol/L), and/or vehicle. The CM was then collected and stored at −20°C for subsequent *in vitro* assays. This CM was supplemented with 2% charcoal-stripped (CS)-FBS before use in all assays to provide a baseline level of the nutrients needed for cellular function, absent any hormones that would interfere with our evaluation of the effects of the estradiol produced by preadipocytes.

Quantitative RT-PCR

Macrophage *PTGS2* (COX-2) mRNA levels were measured following a 6-hour serum-starvation period (baseline), then a 1-hour exposure to 2% sera from obese or normal-weight patients in SFM, and after a subsequent 12 and 24 hours in SFM (following the removal of the sera). Preadipocyte *CYP19A1* (aromatase) mRNA levels were assessed following a 24-hour incubation in macrophage CM. MCF-7 and T47D *TFF1* (pS2) and *CCND1* (cyclin D1) mRNA levels were measured after a 24-hour incubation in macrophage/preadipocyte coculture CM supplemented with 2% CS-FBS. All cells were serum-starved for 6 hours before exposure to sera or CM. Total RNA was isolated using TRizol reagent (Invitrogen) and reverse transcribed with Promega's ImProm II Reverse Transcription System. The primer sequences are as follows: *PTGS2*: forward, 5′-CCCTTGGGTGTCAAAGGTAA-3′; reverse, 5′-GCCCTCGCTTATGATCTGTC-3′; *CYP19A1*: forward, 5′-GCCGAATCGAGAGCTGTAAT-3′; reverse, 5′-GAGAATTCATGCGAGTCTGGA-3′; *TFF1*: forward, 5′-GGTCGCCTTGGAGCAGA-3′; reverse, 5′-GGGCGAAGATCACCTTGTT-3′; *CCND1*: forward, 5′-TGGAGGTCTGCGAGGAACAGAA-3′; reverse, 5′-TGCAGGCGGCTCTTTTTCA-3′. The manufacturer's recommended cycling conditions for the QuantiFast SYBR Green PCR Kit (Qiagen) were used. Data shown represent the average of at least three independent experiments, with the exception of preadipocyte aromatase expression measured following culture in macrophage CM generated with the serum samples from the Polish Women's Health Study.

Fatty acid analysis

Serum total fatty acids were extracted and analyzed by gas chromatography as previously described (20).

PGE2 and estradiol concentrations

The concentration of PGE2 in macrophage CM and sera was measured using the PGE2 Parameter Assay Kit from R&D Systems. The estradiol concentration in macrophage/preadipocyte coculture CM and sera was analyzed with the Estradiol EIA Kit from Cayman Chemical.

ERE luciferase assay

The ERE luciferase assay was performed as described previously (21), with the MCF-7 and T47D cells exposed for 48 hours to the macrophage/preadipocyte coculture CM supplemented with 2% CS-FBS. Relative ERα activity was calculated by dividing the fluorescence value (standardized to renilla) from cells grown in each experimental condition by that from cells grown in CM generated with exposure to sera from patients of normal weight followed by incubation in SFM plus testosterone. Data shown represent the average of at least three independent experiments.

Cell proliferation and migration

MCF-7, T47D, and MDA-MB-231 cell proliferation was measured using cell counting by hemocytometer following a 48-hour incubation in macrophage/preadipocyte coculture CM supplemented with 2% CS-FBS. The cells were seeded in 24-well plates at a density of 1×10^4 cells/well for MCF-7 and MDA-MB-231 cells and 1.5×10^4 cells/well for T47D cells. Migration of these same cell lines was assessed using the wound healing assay as previously described (22) following a 24-hour incubation in macrophage/preadipocyte coculture CM supplemented with 2% CS-FBS. For both experiments, the cells were serum-starved for 6 hours before treatment. Data shown represent the average of at least three independent experiments.

Statistical analyses

Patient data. The data were examined for normality. Duplicates were also analyzed to guarantee that one patient was not recorded twice in the event of referrals or transfer of care to the other clinic. Pearson χ^2 tests were used to analyze categorical variables of NSAID users and nonusers. A Student *t* test was used to examine mean differences in numerical variables. Time to recurrence was assessed by the Wilcox nonparametric test. Age at diagnosis and tumor stage were included as *a priori* confounding factors. Logistic regression was used to predict recurrence. A P value of <0.05 was considered significant. Statistical analyses were performed in R Foundation for Statistical Computing (version 2.10.1).

In vitro data. Differences between cells exposed to 2 different experimental conditions were measured using the Student *t* test. One-way ANOVA was used to analyze COX-2 expression. For experiments involving 2 independent variables, 2-way ANOVA was used. A P value of <0.05 was considered significant.

Results

Patient characteristics

The patient population consisted of 440 women with invasive, ERα-positive breast cancer. NSAID users did not significantly differ from nonusers by BMI category, tumor stage, hormone receptor status, tumor type, race, or type of surgery. Not surprisingly, NSAID users were more likely to be older, postmenopausal, and diabetic. The majority of patients in our population were overweight/obese (BMI $\geq$ 25 kg/m^2), such that the average BMI for both users and nonusers was >30 kg/m^2 (Table 1).

NSAID use is associated with a reduced risk of recurrence and longer disease-free survival

The recurrence rate in NSAID users was 52% lower than non-NSAID users [odds ratio (OR), 0.48; 95% confidence interval (CI), 0.22–0.98; Table 2]. After controlling for patient use of

Table 1. Descriptive characteristics of patients with breast cancer ($n = 440$)

Characteristics	NSAID nonusers ($n = 281$)	NSAID users ($n = 159$)	P
Age at diagnosis, years (mean ± SD)	55.5 ± 10.3	60.7 ± 10.7	<0.001
BMI, kg/m^2 (mean ± SD)	30.7 ± 6.21	31.9 ± 6.70	0.072
Time to recurrence, months (median)[a]	50.6	78.5	0.464
	N (%)		χ^2, P_{Trend}
Recurrence			
Yes	34 (12.1)	10 (6.3)	0.050
No	247 (87.9)	149 (93.7)	
BMI category			
Normal	50 (17.8)	25 (15.7)	0.132
Overweight	94 (33.4)	41 (25.8)	
Obese	137 (48.8)	93 (58.5)	
Menopausal status			
Premenopausal	110 (39.2)	32 (20.1)	<0.001
Postmenopausal	171 (60.8)	127 (79.9)	
Race			
Hispanic	134 (47.7)	74 (46.5)	0.135
White	111 (39.5)	57 (35.8)	
African-American	5 (1.8)	10 (6.3)	
Other	4 (1.4)	1 (0.7)	
Missing/unavailable	27 (9.6)	17 (10.7)	
Tumor stage			
I	101 (35.9)	60 (37.8)	0.389
II	107 (38.1)	64 (40.2)	
III	56 (19.9)	23 (14.5)	
Missing/unavailable	17 (6.1)	12 (7.5)	
Hormone receptor status			
ER^+/PR^+	246 (87.6)	133 (83.6)	0.255
ER^+/PR^-	35 (12.4)	26 (16.4)	
Histologic type			
Ductal	218 (77.6)	121 (76.1)	0.156
Lobular	33 (11.7)	21 (13.2)	
Mucinous	1 (0.4)	2 (1.2)	
Missing/unavailable	29 (10.3)	15 (9.5)	
Type of surgery			
Lumpectomy	128 (45.5)	72 (45.3)	0.405
Mastectomy	122 (43.4)	69 (43.4)	
Bilateral mastectomy	26 (9.3)	12 (7.5)	
Other excision[b]	5 (1.8)	6 (3.8)	
Adjuvant hormonal therapy			
Aromatase inhibitor	171 (60.9)	125 (78.6)	<0.001
Tamoxifen	110 (39.1)	34 (21.4)	
Type of NSAID[c]			
Aspirin	—	129 (81.1)	
Other	—	30 (18.9)	
Diabetes status			
Diabetic	56 (19.9)	57 (35.9)	<0.001
Not diabetic	225 (80.1)	102 (64.1)	
Diabetes drug			
Metformin	37 (66.1)	33 (57.9)	0.036
Other	19 (33.9)	24 (42.1)	

(Continued on the following page)

Table 1. Descriptive characteristics of patients with breast cancer ($n = 440$) (Cont'd)

Characteristics	NSAID nonusers ($n = 281$)	NSAID users ($n = 159$)	*P*
Omega-3 fatty acid use			
Yes	32 (11.4)	40 (25.1)	<0.001
No	249 (88.6)	119 (74.9)	
Statin use			
Yes	65 (23.1)	67 (42.1)	<0.001
No	216 (76.9)	92 (57.9)	

Abbreviation: PR, progesterone receptor.

[a]Wilcox nonparametric test used to analyze time to recurrence analysis in 44 patients who had a recurrence.

[b]Other excision includes segmentectomy and quadrantectomy.

[c]Calculations pertinent to the 159 patients classified as NSAID users.

statin drugs and omega 3 fatty acids, which also have anti-inflammatory effects, NSAID users' recurrence rate was still approximately half the rate of nonusers (OR, 0.52; 95% CI, 0.23–1.08). NSAID users remained disease-free for an average of 78.5 months, whereas nonusers averaged 50.6 months, a difference of more than 2 years (Table 1). Overweight/obese patients had a 1.86-fold higher risk of recurrence versus normal-weight patients when controlling for NSAID use and type of hormone therapy (OR, 1.86; 95% CI, 0.76–5.62; Table 2). Unfortunately, the small number of normal-weight patients, combined with their low rate of recurrence, precluded our ability to examine whether NSAID use was more effective in preventing recurrence in patients with an elevated BMI. However, despite the small sample size, we found that NSAID use was associated with a substantial reduction in recurrence rate in this predominantly overweight/obese postmenopausal patient population. Larger studies have observed more modest effects (23–25).

Obesity stimulates preadipocyte aromatase expression and estradiol production via elevated macrophage COX-2 expression

We next sought to determine whether the NSAID-associated reduction in recurrence rate observed in the overweight/obese patient population may be because of the effect of these drugs on local aromatase expression. To this end, we utilized an *in vitro* model in which cultured cells were exposed to pooled sera samples from obese or normal-weight postmenopausal patients with breast cancer to mimic the tumor microenvironment of obese versus normal-weight women. The characteristics of the serum donors, including serum concentrations of various cytokines, adipokines, and growth factors, have been previously described (21). Following a 1 hour exposure to OB sera, COX-2 expression in cultured macrophages was a modest 24% higher than cells exposed to N sera (Fig. 1A). However, exposure to OB sera for 1 hour increased macrophage PGE2 production 5-fold versus N sera in the 24 hours following sera removal (Fig. 1B). Consequently, we assessed whether macrophage COX-2 expression may continue to increase during that 24-hour period following sera removal, finding that mRNA levels were 68% and 92% greater in OB versus N sera–exposed cells at 12 and 24 hours, respectively (Fig. 1C). Preadipocytes cultured in CM from macrophages exposed to OB versus N sera had 52% greater aromatase expression (Fig. 1D). Treatment of the macrophages with celecoxib during sera exposure neutralized the difference in preadipocyte aromatase expression, suggesting that OB sera–induced macrophage PGE2 production may be responsible for this effect. Preadipocyte aromatase expression was also measured following growth in CM from macrophages exposed to serum from 10 individual OB or N postmenopausal women, allowing us to examine whether similar results are obtained using nonpooled serum with the data averaged by BMI category. Serum from ineligible control subjects in the Polish Women's Health Study, a breast cancer case–control study that has been described previously (18), was used, enabling us to additionally determine whether our results are unique to sera obtained from a specific patient population. The average preadipocyte aromatase expression was over 2-fold greater following culture in macrophage CM generated with OB versus N subjects' sera (Fig. 1E), supporting the findings obtained using pooled sera from the CTRC patients. Finally, the OB sera–induced increase in aromatase expression was correlated with a 16-fold amplification in preadipocyte estradiol production in the presence of exogenous testosterone, the substrate for aromatase (Fig. 1F). The addition of the aromatase inhibitor anastrozole to the macrophage/preadipocyte coculture following sera exposure completely nullified the difference between OB and N, demonstrating that the increase in estradiol

Table 2. Logistic regression model to predict breast cancer recurrence

Predictors	OR (95% CI)	*P*
NSAID use		
Users	Reference	
Nonusers	0.48 (0.22–0.98)	0.05
BMI category		
Normal	Reference	
Overweight + obese	1.86 (0.76–5.62)	0.11

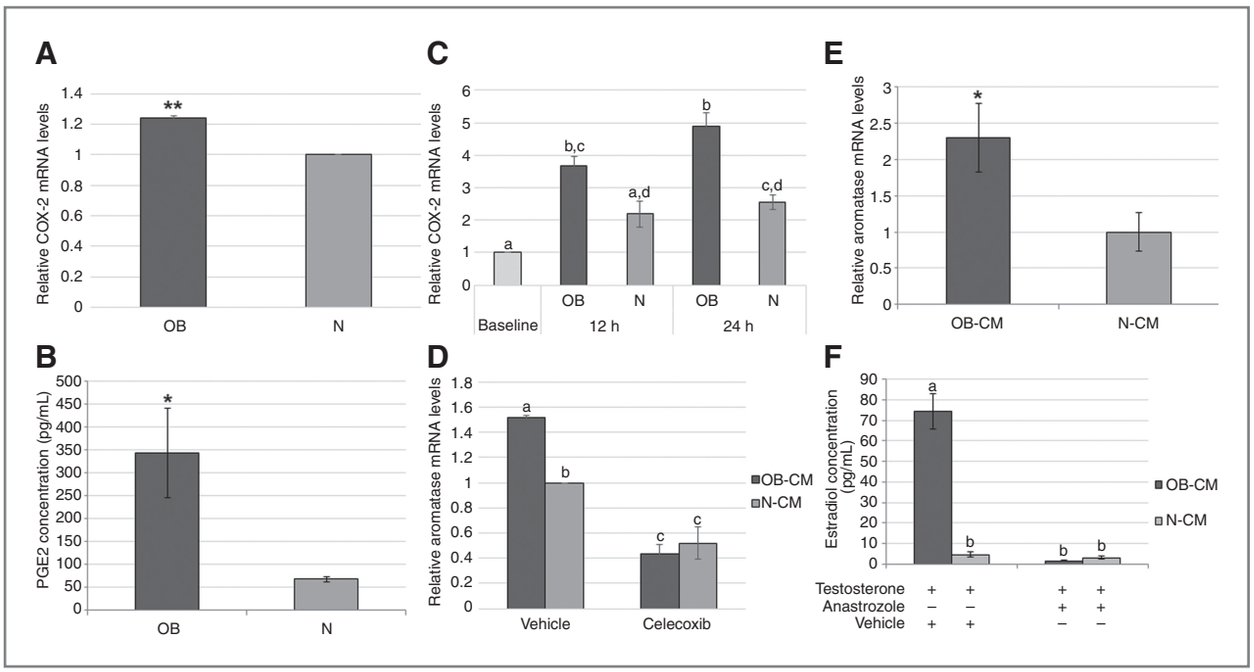

Figure 1. Obesity stimulates macrophage COX-2 expression, resulting in elevated preadipocyte aromatase expression and estradiol production. A, COX-2 expression in U937 cells matured to macrophages following 1 hour exposure to sera from obese (OB) patients versus sera from normal-weight (N) patients. B, PGE2 concentration in CM from macrophages following exposure to sera from OB or N patients. C, COX-2 expression in U937 cells (matured to macrophages) after a 6-hour serum starvation period (baseline), 12 hours after removal of the sera (OB and N, 12 h), and 24 hours after removal of the sera (OB and N, 24 h). D, aromatase expression in preadipocytes incubated for 24 hours in macrophage CM generated following patient sera exposure (OB-CM and N-CM) with vehicle or celecoxib treatment. E, the impact of macrophage CM on preadipocyte aromatase expression when individual patient serum samples were utilized for CM generation, with the results averaged according to patient BMI category. F, estradiol concentration in CM from a macrophage/preadipocyte cocultures exposed to patient sera (OB-CM and N-CM), then incubated in serum-free media with vehicle, testosterone, and/or anastrozole. Data shown represent the average of at least three independent experiments, with the exception of E. *, $P < 0.05$; **, $P < 0.01$ in comparison to N or N-CM; different letters indicate significant differences, $P < 0.05$.

production was solely because of the OB sera's effect on aromatase expression.

Obese patient serum contains higher arachidonic acid and saturated fatty acid levels

Given our finding that exposure to the OB versus N patient sera promotes greater macrophage COX-2 expression and PGE2 production, we next explored 2 possible mechanisms mediating this effect by profiling the fatty acid content of the sera. The concentration of arachidonic acid, the omega-6 fatty acid substrate utilized by COX-2 for PGE2 production, was significantly higher ($P < 0.001$) in the OB patient sera. The palmitate level as well as the total level of saturated fatty acids, which can stimulate macrophage COX-2 expression (15), were also higher ($P < 0.001$) in the OB patient sera (Table 3). We then measured PGE2 and estradiol levels in the patient sera, as systemic levels of these factors may also impact tumor progression, but there were no significant differences between OB and N (Table 3).

Obesity-associated preadipocyte aromatase expression promotes greater breast cancer cell ERα activity

After establishing that obesity-associated circulating factors stimulate preadipocyte aromatase expression via induction of macrophage COX-2 expression, we sought to determine whether the resulting elevation in estradiol promotes enhanced breast cancer cell ERα activity. ERα-positive breast cancer cells cultured in CM from OB versus N sera–exposed macrophage/preadipocyte cocultures exhibited greater ERα activity, as measured by ERE luciferase assay, with 29% and 42% differences in MCF-7 and T47D

Table 3. Serum fatty acid, PGE2, and estradiol concentrations

	Obese	Normal weight
Fatty acids (nmol/mL)		
Arachidonic acid	420.6 (1.30)[a]	391.3 (2.06)
Palmitate	2,205 (0.993)[a]	1,545 (6.95)
Total SFA	3,413 (8.29)[a]	2,617 (13.2)
PGE2 (pg/mL)	705.8 (151.8)	711.4 (142.3)
Estradiol (pg/mL)	9.39 (1.09)	9.75 (1.73)

Abbreviation: SFA, saturated fatty acids.
[a], $P < 1 \times 10^{-3}$ in comparison to normal weight. Standard error of the mean shown in parentheses.

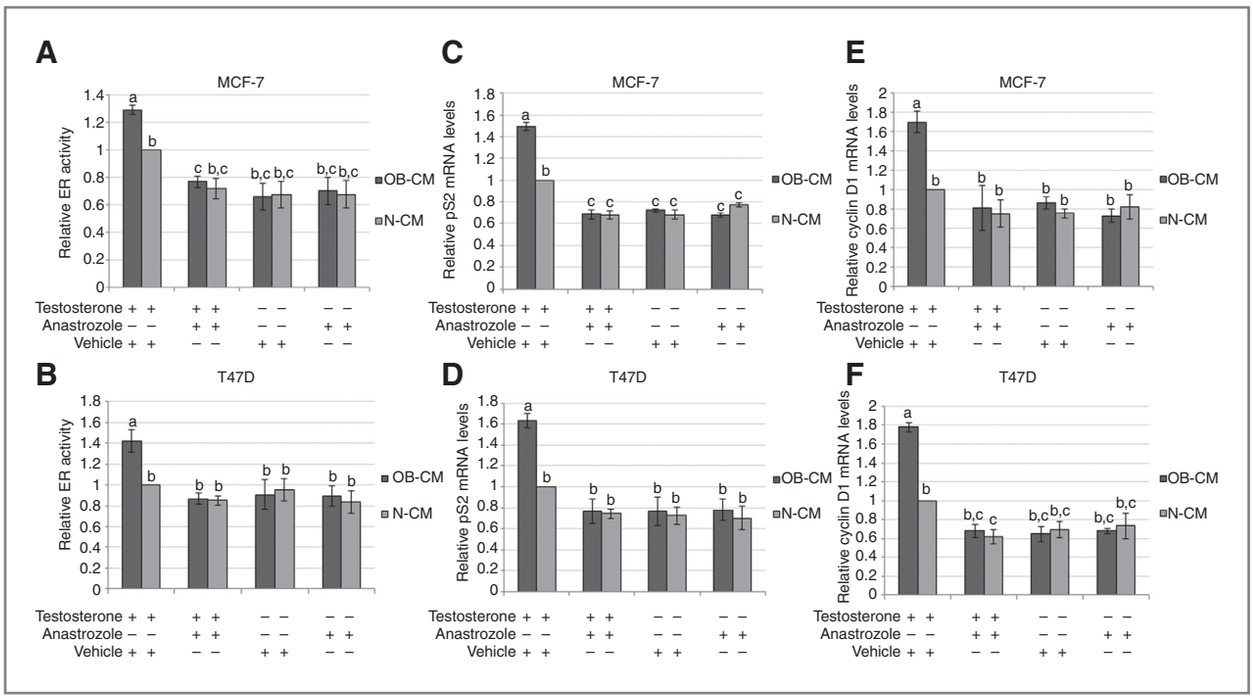

Figure 2. Obesity-associated preadipocyte aromatase expression promotes greater breast cancer cell ERα activity. MCF-7 and T47D breast cancer cell ERα activity after culture in macrophage/preadipocyte coculture CM, as measured by ERE luciferase reporter (A and B) and qPCR analysis of pS2 (C and D) and cyclin D1 (E and F) expression. Experimental conditions include CM from cocultures exposed to sera from obese or normal-weight patients (OB-CM and N-CM), followed by incubation in serum-free media with testosterone plus vehicle, testosterone plus anastrozole, vehicle alone, or anastrozole alone. Data shown represent the average of at least three independent experiments. Different letters indicate significant differences, $P < 0.05$.

cells, respectively (Fig. 2A and B). Coculture treatment with anastrozole after sera exposure significantly decreased the OB-induced ERα activity in both cell lines, eliminating the difference between OB versus N CM-induced breast cancer cell ERα activity. OB and N coculture CM produced without testosterone stimulated MCF-7 and T47D cell ERα activity that was statistically equivalent to that induced by CM from cocultures treated with testosterone and anastrozole. Similar results were obtained when the CM was generated with anastrozole treatment but no testosterone. This shows that the presence of testosterone during coculture CM generation is required for the OB-induced elevation in breast cancer cell ERα activity, further demonstrating that this effect is because of preadipocyte aromatase activity. We utilized pS2 and cyclin D1 expression as additional measures of ERα activity, obtaining analogous results. Following growth in OB versus N CM, pS2 expression was 49% higher in MCF-7 cells and 63% greater in T47D cells (Fig. 2C and D) whereas cyclin D1 expression was elevated by 70% in MCF-7 cells and 78% in T47D cells (Fig. 2E and F). Coculture treatment with anastrozole neutralized these differences in pS2 and cyclin D1 expression in both cell lines and, as seen with the ERE luciferase assays, CM generated without testosterone stimulated equivalent expression. Together, these results demonstrate that the obesity-associated, COX-2-induced increase in preadipocyte aromatase expression and estradiol production can enhance breast cancer cell ERα activity.

Breast cancer cell proliferation and migration are induced by obesity-associated preadipocyte aromatase expression

To determine whether the obesity-associated, COX-2–induced elevation in preadipocyte aromatase expression and breast cancer cell ERα activity could result in enhanced disease progression, we assessed the impact of the macrophage/preadipocyte coculture CM on breast cancer cell proliferation and migration. Culturing MCF-7 and T47D cells in OB versus N CM generated with exogenous testosterone increased proliferation by 59% and 55%, respectively (Fig. 3A and B). Treatment of the coculture with anastrozole during CM generation eliminated the difference between OB and N, demonstrating that the OB CM's elevated estradiol concentration is responsible for its effect on cell proliferation. This conclusion is further supported by the lack of any difference in proliferation levels in MDA-MB-231 cells, an ERα-negative breast cancer cell line, following culture in the 4 CM conditions (Fig. 3C). A similar trend was seen when we examined the impact of the coculture CM on ERα-positive breast cancer cell migration. OB CM stimulated 57% more MCF-7 and 46% greater T47D cell migration in comparison to N CM (Fig. 4A and B). This effect was neutralized in the T47D cells by treatment of the coculture with anastrozole during CM production. However, although aromatase inhibition significantly decreased OB-induced MCF-7 cell migration, these cells still migrated farther than those cultured in N CM with anastrozole. Intriguingly, the migration of MDA-MB-231 cells was also significantly enhanced by

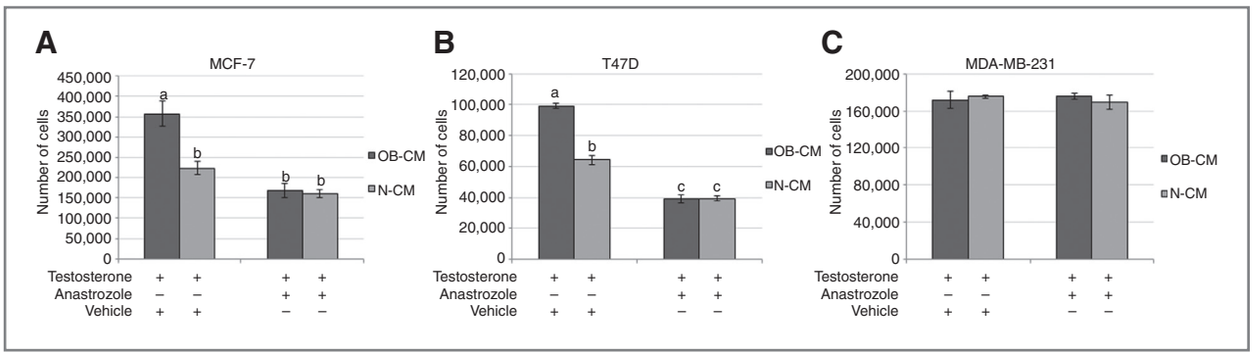

Figure 3. ERα-positive breast cancer cell proliferation is induced by obesity-associated preadipocyte aromatase expression. MCF-7 (A), T47D (B), and MDA-MB-231 (C) breast cancer cell proliferation in response to culture in macrophage/preadipocyte coculture CM. Experimental conditions include CM from cocultures exposed to sera from obese or normal-weight patients (OB-CM and N-CM) followed by incubation in serum-free media with testosterone plus vehicle or testosterone plus anastrozole. Data shown represent the average of at least three independent experiments. Different letters indicate significant differences, $P < 0.05$.

culture in OB versus N CM (Fig. 4C). Consistent with this cell line's ERα-negative status, anastrozole treatment during CM generation did not affect the CM's impact on migration. Overall, these findings strongly suggest that obesity-associated circulating factors may promote ERα-positive breast cancer progression via stimulation of macrophage COX-2 expression and the subsequent increase in preadipocyte aromatase expression.

Discussion

Suboptimal pharmacologic aromatase inhibition in postmenopausal patients with ERα-positive breast cancer has detrimental consequences to clinical outcome. COX-2 is a critical node for the convergence of various upstream pathways of inflammation, including IL1, IL6, and TNFα signaling (26, 27), and it seems to be a key mediator of biologic processes affecting treatment failure, such as PGE2 synthesis and the resulting aromatase expression and estrogen production. Our study is the first to specifically examine the molecular mechanisms that may mediate the impact of daily NSAID use on recurrence rate and time to disease progression in patients with invasive breast cancer receiving adjuvant endocrine therapy. We have demonstrated that the NSAID users in this patient population had a 52% lower recurrence rate. A similar trend was seen after controlling for patient age and tumor stage at diagnosis, although the strength of the association was reduced (data not shown). This is not surprising given that late-stage tumors, as well as the aggressive tumors that disproportionately develop in younger women, seem less likely to significantly benefit from the modest effects of this drug group. NSAID users also remained disease-free for more than 2 years longer than nonusers, a difference that was not statistically significant but may be a clinically relevant variance in outcome.

Similar results have been obtained in some larger prospective studies examining NSAID use after breast cancer diagnosis. Holmes and colleagues (23) showed that aspirin use 6 to 7 days/week was associated with a significant reduction in the risk of recurrence (RR, 0.57; 95% CI, 0.39–0.82). Utilizing the Nurses' Health Study's substantial pool of subjects, the authors found no change in these results after stratifying by BMI, menopausal status, and ER status. In an analysis of postmenopausal breast cancer patient outcomes, Blair and colleagues (25) observed that any amount of regular NSAID use was correlated with a lower risk of breast cancer death (HR, 0.64; 95% CI, 0.39–1.05). Adjustment for ERα status, but not BMI category, increased the HR and reduced the statistical significance of this association. In contrast with these studies, the recurrence rate in a population of pre- and postmenopausal patients was not decreased by aspirin use $\geq$3 days/week (RR, 1.09; 95% CI, 0.74–1.61), but was affected by use of ibuprofen (RR, 0.56; 95% CI, 0.32–0.98; ref. 24). Controlling for BMI, menopausal status, and ERα did not alter these results.

The variability in design and patient population among these studies makes any comparison with our results difficult. Cumulatively, they seem to indicate that NSAID use may be an effective addition to adjuvant breast cancer treatment, regardless of BMI, ERα, or menopausal status. However, the association between NSAID use and a 50% lower disease recurrence in our study, despite a relatively small patient population, led us to hypothesize that the overwhelming prevalence of overweight/obesity among this population may have increased the NSAID benefit. Our patient population was also largely postmenopausal and included only hormone responsive patients. Because obesity and overweight status are associated with higher PGE2 levels and aromatase expression in female breast tissue (13, 14), it seems likely that the efficacy of COX-2 inhibitors in a postmenopausal, ERα-positive patient population would increase with greater adiposity. Perhaps the lack of variation in effect among BMI categories in previous studies is because of their failure to stratify the data by BMI, ERα, and menopausal status simultaneously. This question could potentially be addressed by previous trials assessing the clinical benefit of a celecoxib/aromatase inhibitor combination treatment for postmenopausal, hormone-responsive breast cancer. Most of these studies showed a modest benefit with at least 3 months of combination treatment, including trends toward more clinical complete response, longer duration of clinical benefit, and greater progression-free survival (28–30).

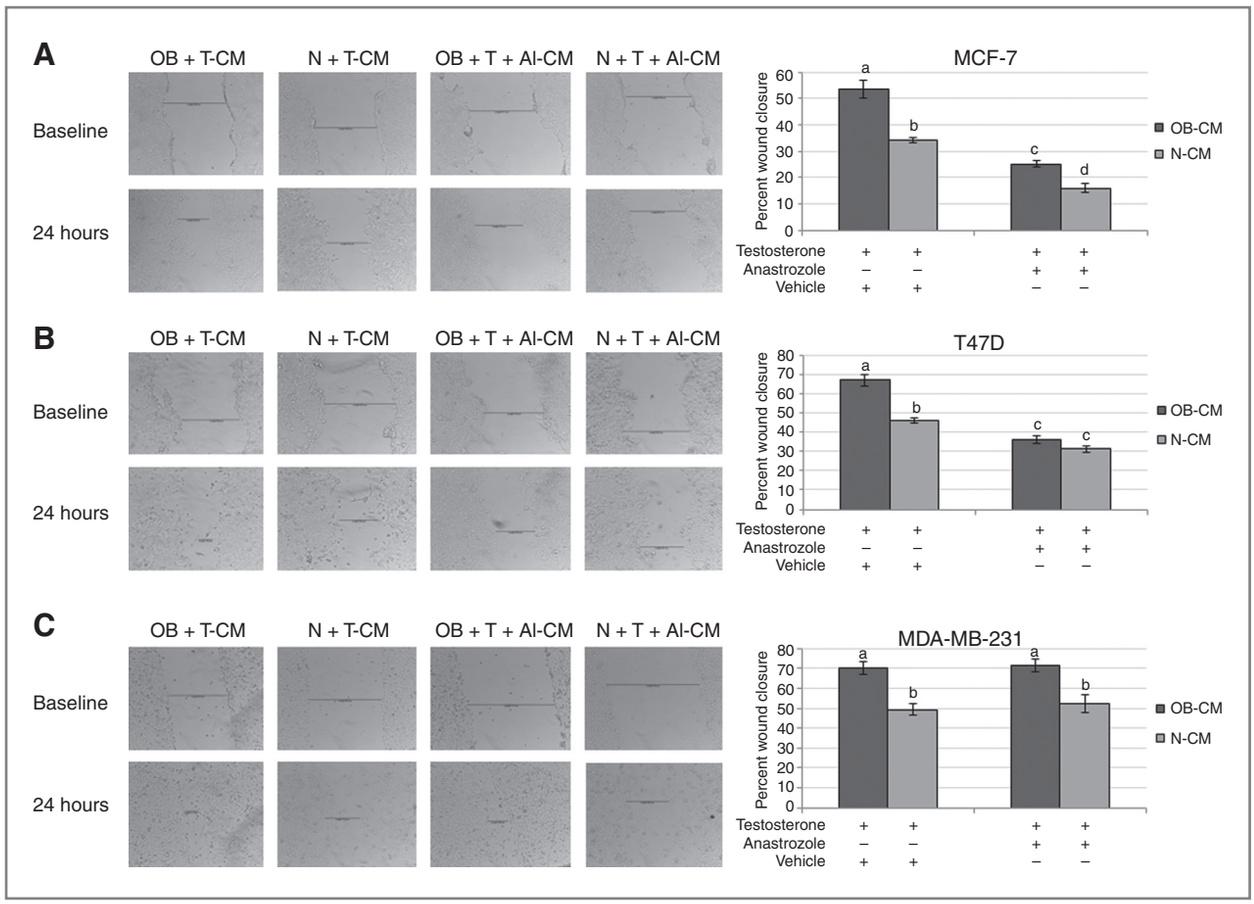

Figure 4. Obesity-associated preadipocyte aromatase expression promotes breast cancer cell migration. MCF-7 (A), T47D (B), and MDA-MB-231 (C) breast cancer cell migration during incubation in macrophage/preadipocyte coculture CM. Experimental conditions include CM from cocultures exposed to sera from obese or normal-weight patients (OB-CM and N-CM) followed by culture in serum-free media with testosterone plus vehicle (OB + T-CM, N + T-CM) or testosterone plus anastrozole (OB + T + AI-CM, N + T + AI-CM). Data shown represent the average of at least three independent experiments. Different letters indicate significant differences, $P < 0.05$.

Unfortunately, none of these trials analyzed the treatment benefit by BMI category, so it is impossible to determine from this data whether overweight/obese women in this patient population are more likely to benefit from COX-2 inhibition. To our knowledge, no one has examined the efficacy of a COX-2 inhibitor/aromatase inhibitor combination in animal models of obesity and mammary carcinogenesis either.

Given that obesity has been associated with a worse breast cancer prognosis (3–7) as well as a reduced response to aromatase inhibitor therapy among postmenopausal, ERα-positive patients (8, 9), determination of whether this specific population will benefit from COX-2 inhibition is an important question. Dannenberg and colleagues have demonstrated an obesity-associated, COX-2-induced elevation in preadipocyte aromatase expression using both preclinical models and patient breast tissue (13–15). Our aim was to confirm this phenomenon, using an *in vitro* model of the obese patient's breast tumor microenvironment, as well as determine whether this increased preadipocyte aromatase expression can promote greater epithelial cell ERα activity and subsequent proliferation and migration, 2 *in vitro* measures of cancer progression. We show that exposure to sera from obese postmenopausal women stimulates significantly greater macrophage COX-2 expression and a 5-fold increase in PGE2 production. Saturated fatty acids can promote COX-2 expression and PGE2 production in cultured macrophages (15, 31), and the increased lipolysis that accompanies obesity results in a higher concentration of circulating free fatty acids, particularly palmitate (32–34). We confirmed that the obese patient sera contains significantly higher levels of palmitate, total saturated fatty acids, and arachidonic acid, the omega-6 fatty acid substrate utilized by COX-2 to produce PGE2. Consequently, this difference in macrophage COX-2 expression and PGE2 production may be because of elevated levels of these free fatty acids in the obese patient sera. There is also some evidence that the inflammatory cytokines IL6 and TNFα, found in higher concentrations in our obese patient serum samples (21), can induce COX-2 expression (27, 35, 36). This suggests that more than one mechanism may be responsible for the obesity-associated upregulation of macrophage COX-2 expression and PGE2 production seen in this study.

Given our *in vitro* results, it may seem surprising that we found no difference between the OB and N patient sera in PGE2 and estradiol concentrations. However, PGE2 has a short half-life, so urinary concentrations of the stable end metabolite of PGE2 catabolism (PGE-M) are typically used to assess systemic PGE2 levels (37). In fact, although an elevated BMI has been associated with increased PGE-M levels (38), we are not aware of any studies demonstrating higher circulating PGE2 levels with obesity. In contrast, the lack of difference in serum estradiol levels by BMI does not agree with the literature, as researchers have generally found that obese postmenopausal women have higher estradiol levels (39–41). However, of the 25 women that provided the sera for this study, 9 obese patients and 1 normal-weight patient were receiving an aromatase inhibitor (21), which may have masked any difference in estradiol levels between the groups.

Taken together, our *in vitro* data provide compelling evidence that an obesity-associated increase in macrophage COX-2 expression promotes greater preadipocyte aromatase expression and estradiol production, resulting in an elevation in breast cancer cell ERα activity, proliferation, and migration. Neutralization of the obesity-induced elevation in ERα activity and cell proliferation by the addition of aromatase inhibitor treatment validates our hypothesis that these effects are because of preadipocyte aromatase expression. However, although incubation of the ERα-negative MDA-MB-231 cells in OB versus N macrophage/preadipocyte coculture CM did not differentially affect cell proliferation, it did stimulate significantly greater cell migration. In addition, OB CM continued to promote more extensive MCF-7 cell migration in comparison to N CM with aromatase inhibition during CM generation, although anastrozole did significantly decrease the OB CM-induced migration. These results indicate that, although locally produced estradiol clearly plays a role in mediating obesity-associated breast cancer cell migration, one or more additional signaling molecules produced by macrophages and/or preadipocytes is involved in this effect. Macrophage-derived PGE2 is one possibility, as it is known to promote breast cancer cell migration (42, 43). Several studies have concluded that it also stimulates breast cancer cell proliferation based on the ability of COX-2 inhibitors to hinder proliferation (44, 45). However, we saw no differential proliferation in the MDA-MB-231 cells following incubation in OB versus N CM, and Robertson and colleagues (46) demonstrated that treatment of MDA-MB-231 cells with exogenous PGE2 does not increase proliferation. The role of additional locally produced signaling molecules in the link between obesity-associated inflammation and breast cancer progression is an area that deserves further exploration in future studies.

This study focused on exploring how COX-2 inhibition via NSAID use could reduce breast cancer recurrence in obese and overweight women. However, a large majority of the NSAID users in our study were taking aspirin, which preferentially inhibits the COX-1 enzyme over COX-2 (47). Although most researchers consider suppression of COX-2 activity to be the predominant mechanism by which aspirin reduces cancer risk and progression, a few have examined the role of COX-1 in tumorigenesis. Hwang and colleagues (48) found that a majority of breast tumor samples overexpress COX-1, whereas Jeong and colleagues (49) demonstrated that a selective COX-1 inhibitor reduces MCF-7 cell growth. Others have shown that the combined inhibition of COX-1 and COX-2 produces a significantly greater suppression of breast cancer cell growth than either alone (50). Obesity has not been linked to increased COX-1 expression or activity, though, so we did not pursue an examination of this enzyme as part of our study.

Our current investigation strongly suggests that local estradiol production, induced by macrophage COX-2 activity, may be a key mediator in the link between obesity and postmenopausal, hormone-responsive breast cancer progression. This conclusion is supported by the clinical observation of a 52% lower recurrence rate and 28-month extension in time to recurrence with NSAID use in a largely overweight/obese and postmenopausal patient population with ERα-positive disease. Collectively, our results provide a strong rationale for further studies about the clinical benefit of aromatase inhibitor/COX-2 inhibitor combination treatment for obese, postmenopausal patients with breast cancer.

Disclosure of Potential Conflicts of Interest

M. Beeram has received speakers' bureau honoraria from Genentech, Inc. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L.W. Bowers, I.X.F. Maximo, A.J. Brenner, M. Beeram, S.D. Hursting, R.R. Tekmal, C.A. Jolly, L.A. deGraffenried

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L.W. Bowers, I.X.F. Maximo, R.R. Tekmal, C.A. Jolly, L.A. deGraffenried

Writing, review, and/or revision of the manuscript: L.W. Bowers, I.X.F. Maximo, A.J. Brenner, M. Beeram, S.D. Hursting, R.S. Price, C.A. Jolly, L.A. deGraffenried

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L.A. deGraffenried

Study supervision: M. Beeram, L.A. deGraffenried

Other (fatty acid analysis, data interpretation, and writing of the methods and results for these data): C.A. Jolly

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