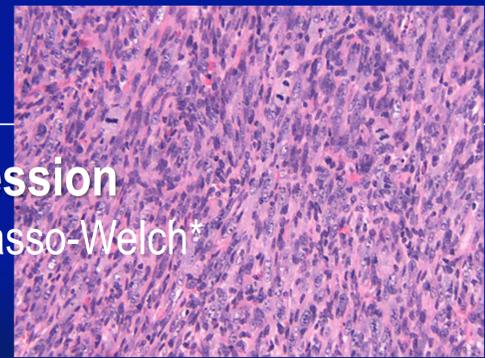


# Effects of Continuous Combined Oral Contraceptives on Mouse Mammary Gland Structure and Tumor Progression

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## Abstract

The use of cyclic oral contraceptives (OC) is associated with an overall increased risk of ER/PR negative breast cancer, particularly in current users, and women who start OC use early in life. The effect of **extended** (i.e., continuous) OC on mammary gland structure or breast cancer risk has not been evaluated. The purpose of the current study was to test the hypothesis that **extended OC provides protection against breast cancer**, by removing the hormone withdrawal phase associated with both cyclic OC and normal cycling. BALB/cJ mice were fed a fresh liquid diet daily containing no OC, or ethinyl estradiol and levonorgestrel, either continuously for 28 days, or as a cyclic regimen (three days OC, followed by one day basal liquid diet). After 28 days, 10 mice per group were sacrificed and mammary glands dissected for whole mount and histologic analysis. The extended OC group showed an average increase in epithelial density of 65% compared to control; the cyclic group was increased by 13%. Both OC regimens induced a more differentiated state, with a decrease in TEB frequency balanced by an increase in alveolar buds, and a trend towards increased ductal secretions. Cyclic OC increased Ki-67 staining in small mammary ducts (epithelium plus stroma). Both OC regimens increased the number of cells infiltrating the mammary adipose stroma. An additional 20 mice per group were injected orthotopically into the mammary fat pad with 10<sup>5</sup> ER negative syngeneic TM2H mammary epithelial tumor cells, and tracked for tumor latency, growth, and tumor burden. The growth of transplanted TM2H tumor cells showed a 25% increased latency, and a 30% decreased tumor burden in the mice receiving continuous OC, suggesting that the mammary environment of mice who had previously received continuous OC exposure was less favorable to growth of transplanted tumor cells than control. Once established, growth rate was unchanged by OC. Cyclic OC showed intermediate effects to decrease tumor growth.

## Introduction

Oral contraceptives (OC) provide long-lasting protection to decrease the frequency of ovarian cancer by 46% (for "ever-use" of OCs) and of uterine cancer by as much as 80% (after 10 years of OC use) [reviewed in (1,2)]. In contrast to OC's effects on endometrial and ovarian cancer risk, **OC usage has not been shown to be a preventative agent for breast cancer**. Although individual studies show varying risk, a recent meta-analysis calculates that overall, OC usage increases breast cancer risk by 1.24 (for current use), 1.16 (<4 years previous), or 1.07 (5-9 years previous) (3). For current users, risk is further increased when the age at first use was less than 20, suggesting that there may be an **OC-sensitive developmental window soon after the onset of puberty** (3). More than 44.5 million women in the US have been "ever-users" of oral contraception (4), and a **large proportion of OC users (54%) are between ages 15 and 19** (3). Breast cancer risk due to OC has thus far **only** been determined for a **cyclic-dosing regimen**, i.e., three weeks on, followed by one week of hormone withdrawal. **The effects of the recently FDA-approved continuous OC dosing regimen on breast cancer risk have not been evaluated**. Prior to its approval for general use in 2007, continuous OC was limited primarily to treating dysmenorrhea (5,6). Due to its increased efficacy in pregnancy prevention and complete relief of premenstrual symptoms, continuous OC dosing is increasingly prevalent, resulting in changing patterns of estrous cycling in a potentially enormous pool of young women (7-9).

It has been suggested that any factor that interrupts regular cycling decreases lifelong breast cancer risk (10), possibly as a function of breast tissue "age" [reviewed by (2)]. **Patterns of estrous cycling are linked to breast cancer risk**. Late menarche and early menopause are inversely associated with risk of in situ and invasive breast cancer (10,11). The number of menstrual cycles a woman experiences in her lifetime contributes to cumulative breast cancer risk; women in nonindustrial societies have 1/3-1/4 the number of cycles over a lifetime and a significantly lower breast cancer incidence (1). Similarly, nulliparous women, who by definition have undergone a reproductive history of uninterrupted cycling, have a 20-40% higher risk of developing postmenopausal breast cancer (12). With each cycle, the breast undergoes limited expansive growth and angiogenesis, followed by regression and inflammatory remodeling during hormone withdrawal. Similar to the hypothesis that the risk of parity-associated breast cancer (type II PABC) arises from the extensive stromal remodeling that occurs during post-lactational involution [reviewed in (13)], it is likely that a **lifetime of "mini-involutions"** during normal estrous/menstrual cycling (or cyclic OC dosing) may contribute to breast cancer risk in nonparous women. We therefore predict that the removal of hormone withdrawal from the cycle by use of continuous OC dosing may therefore **decrease** breast cancer risk.

The current study examines (i) the effects of OC regimen on the microenvironment of the murine post-pubertal mammary gland, and (ii) the effects of post-pubertal OC exposure on subsequent tumor growth and progression, using the ER negative transplantable TM2H breast cancer cell line (14,15). We employ the innovative approach of a completely oral method of administration of the OC, avoiding the usually employed oral gavage, to avoid a potential introduction of stress as a cofactor during the prolonged length of the experiments. Mice receive a liquid diet supplemented +/- diluted OC (Amethyst, containing 90 µg levonorgestrel and 20 µg ethinyl estradiol). The mg/kg/day mouse dosage was calculated by normalization to surface area (mg/m<sup>2</sup>) (16). A 4-day cyclic regimen has been chosen to mimic the most common length of estrous cycling of regularly cycling rodents (17). Day 50 of age, shortly after the initiation of puberty, was chosen as the time point of beginning exposure because OC-associated increased breast cancer risk is associated with OC use initiated at an early age (15-19 years of age) (3).

## Materials and Methods

**Animals**  
BALB/cJ mice were purchased from Jackson Labs at 35 days of age. Mice were tattooed for individual identification 5-10 days after arrival. Mice were group housed and fed chow until initiation of liquid diets. Upon single housing, mice were provided with square nest-lets, wood shavings, and paper huts for warmth and stimulation. Animal rooms were air-conditioned and humidity controlled, with a light cycle of 12 h on and 12 h off. Animals were housed in accordance with the standards set by the NIH and the University at Buffalo Institutional Animal Care and Use Committee.

**Dietary model of OC exposure**  
Mice were group housed until 43 days of age, when they were singly housed for acclimation to liquid Lieber/DeCarli diets (Dyets, Inc., Cat #710027). Weights and dietary consumption were tracked for 7 days to empirically determine optimum volume consistently consumed. At day 50 of age, mice were distributed into three groups: Control (no OC), continuous OC (OC every day for 28d), or cyclic OC (OC for 3 days, followed by one day no OC, for 7 cycles (28 days)). Mice (~17g at the start) were fed 15 ml of liquid diets per day, +/- OC (diluted from Amethyst (Watson Pharmaceuticals)), to deliver 0.0041 mg/kg ethinyl estradiol and 0.0185 mg/kg levonorgestrel. Human equivalent dose was calculated by body surface area conversion (Reagan-Shaw, 2007). Liquid diets were delivered using capped graduated sipper feeding tubes (30 ml size, Dyets Inc., Cat #900012), attached to the cage by feeding tube holders (Dyets Cat #901100) as described (Tobias et al., 2012). Mice were individually housed throughout the 4 week feeding period.

**Experiment 1: Effects of OC on mammary gland structure**  
After 28d of feeding with either control (n=10), continuous (n=10) or cyclic (n=11) diets, mice were euthanized and sera were collected from the thoracic cavity. Contralateral abdominal mammary glands (MG4) were dissected for preparation of mammary whole mounts or paraffin embedding. MG 5 were removed and frozen for subsequent RNA and protein studies. Uterine horn and ovaries were dissected, weighed, and formalin fixed for paraffin embedding.

**Whole mount mammary gland analyses**  
Mammary gland whole mounts were prepared as previously described (Tobias et al., 2012). **Epithelial density** was assessed on whole mounts scanned using an Aperio under 20X objective. TIF images were imported into NIH Image J. After digitally removing the intramammary lymph node, the image was thresholded to include the ductal tree only, and epithelial density was calculated as % positive pixels over total mammary gland area.. **Frequency of terminal structures** was calculated by scoring all terminal structures visible at the epithelial/adipose interface as terminal ductules (TD): single small ducts with no terminal thickening; terminal end buds (TEB): club shaped structures; or alveolar buds (AB): two or more clustered buds with no terminal thickening.

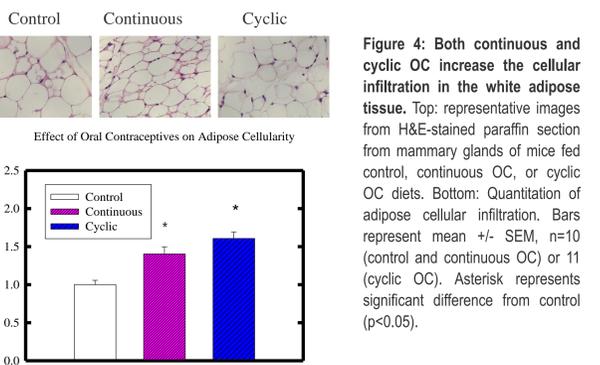
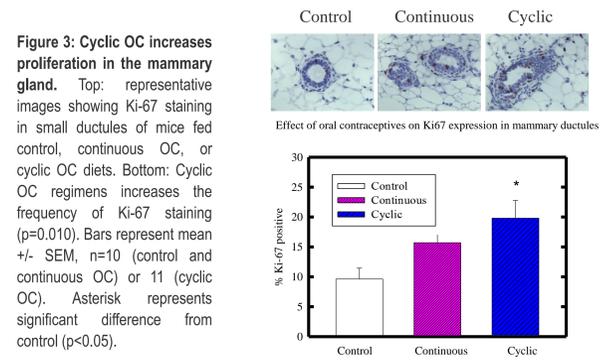
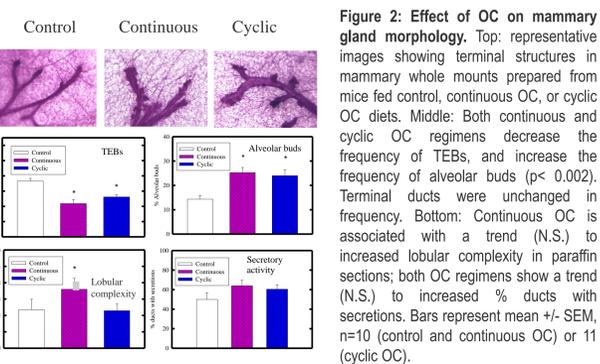
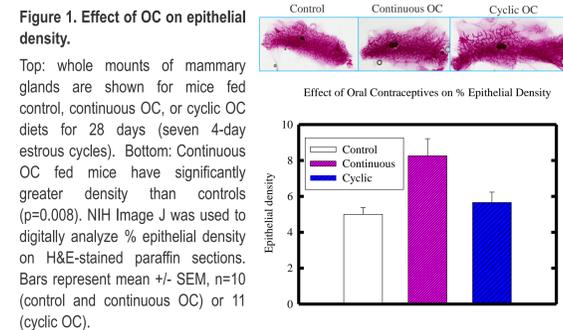
**Histology and Immunohistochemistry**  
Paraffin sections were assessed for the following: (1) **Alveolar complexity**: The % of ductular fields containing 2 or more ductules in an H&E stained, 10 20X-objective ductular fields per mouse; (2) **Secretory differentiation**: The percentage of ducts containing H&E stained secretions, 10 20X objective fields, per mouse; (3) **Epithelial proliferation**: The % of Ki-67 positive epithelial nuclei were scored in ten 20X fields per mouse; (4) **Adipose inflammatory recruitment**: # of H&E stained nuclei per 20X field, 10 fields per mouse; (5) **Mast cell per ductule**: Number of May-Grunwald-Giemsa stained mast cells per ductule in ten 20X objective fields per mouse.

**Experiment 2: Tumor studies**  
After 28d of liquid diets, control (n=22), continuous OC (n=20) and cyclic OC (n=20) mice were injected orthotopically into the fat pad of MG4 with 10<sup>5</sup> syngeneic TM2H breast cancer cells in 50 µl of PBS. TM2H hormone independent breast epithelial cells were obtained from Dr. Dan Medina (Baylor University). Mice were serially tracked by palpation every other day. When the largest tumor's diameter was 1 cm, mice were sacrificed by cervical dislocation, followed by cutting of the diaphragm. Tumors were recorded for size, and placed into 3.7% phosphate buffered formalin for paraffin embedding. Lungs were removed and wet weight was recorded for comparison of tumor growth between dietary groups. Lungs were fixed in zinc formalin (cat # 57012F, Richard Allen Scientific, Kalamazoo, MI), for subsequent evaluation of metastatic frequency in serial sections.

**Tumor data recorded**  
(1) **Latency**: the time from tumor injection until first palpable tumor; (2) **Multiplicity**: The appearance of multiple independent tumors at the site of injection; (3) **Tumor burden over time**: the total tumor volume calculated at each palpation (4) **Tumor growth rate**: The change in palpable tumor volume from palpation until sacrifice, divided by time. (4) **Tumor burden at sacrifice**: The most accurate measurement of volume and tumor weight at necropsy.

**Statistical Analysis**  
Statistical analyses were performed using SigmaStat (Jandel Scientific, San Jose, CA). Analysis of effects of dietary group on dietary consumption, mouse weights at sacrifice, and lung weights, counting of surface metastases, and histologic analysis of lung metastases were performed using a one way ANOVA.

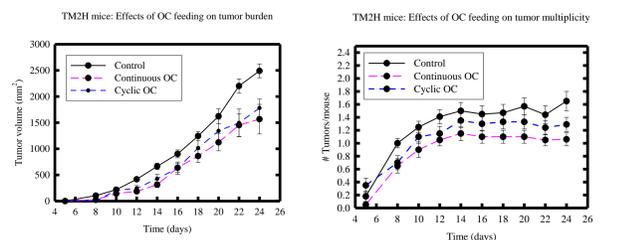
## Results



## Results

	Control	Continuous OC	P value vs. Control	Cyclic OC	P value vs. Control
Latency (d)	7.6 ± 0.3	<b>10.2 ± 1.4</b>	0.021	7.75 ± 0.6	0.900
Survival (d)	27.5 ± 1.3	32.1 ± 2.24	0.159	28.6 ± 1.4	0.578
Tumor volume (mm <sup>3</sup> ):					
Day 7	91 ± 22	<b>24 ± 10</b>	0.001	<b>25 ± 17</b>	<0.001
Day 14	630 ± 65	<b>313 ± 53</b>	<0.001	<b>427 ± 87</b>	0.015
Day 21	2133 ± 139	<b>1452 ± 214</b>	0.036	<b>1496 ± 264</b>	0.002
Tumor growth rate	269 ± 32	342 ± 76	0.796	512 ± 260	0.888

**Table 1: Effects of OC on TM2H mammary tumor latency, survival and multiplicity.** Continuous OC significantly increased tumor latency (time from injection to first palpation), compared to control. No effect on survival (time from injection to sacrifice when tumor diameter greater than 1 cm) was seen. Palpable tumor volume was significantly decreased by both OC regimens at day 7, 14 and 21. Growth rate was not significantly altered by OC; cyclic OC showed a nonsignificant trend to increase growth rate. Data represent means +/- SEM, n=22 (control), 20 (continuous OC) or 20 (cyclic OC). Bold face indicates significant differences (p<0.05).



**Figure 5: Effects of OC regimen on palpable tumor burden and multiplicity.** Left: Both continuous and cyclic OC decreased palpable tumor burden. Right: Despite injection into a single site in the mammary fat pad, multiple lesions arose at the site of injection. Both continuous and cyclic OC decreased multiplicity. Data represent means +/- SEM, n=22 (control), 20 (continuous OC) or 20 (cyclic OC). Asterisks indicate significant differences (p<0.05).

## Summary

- Both continuous and cyclic OC can alter mouse mammary gland structure (decreased TEBs, increased alveolar buds, and increased white adipose cellular infiltration) when administered for seven estrous cycles post-puberty.
- Continuous OC increased epithelial density.
- Cyclic OC significantly increased epithelial/stromal proliferation
- Only continuous OC increased tumor latency.
- Both OC regimens reduced tumor burden and multiplicity.
- Once tumors were established, OC did not affect tumor growth rate.

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