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Research Article

Cancer Prevention Research

Phase II Trial of Chemopreventive Effects of Levonorgestrel on Ovarian and Fallopian Tube Epithelium in Women at High Risk for Ovarian Cancer: An NRG Oncology Group/GOG Study

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Abstract

A large body of epidemiologic evidence has shown that use of progestin-containing preparations lowers ovarian cancer risk. The purpose of the current study was to gather further preclinical evidence supporting progestins as cancer chemopreventives by demonstrating progestin-activation of surrogate endpoint biomarkers pertinent to cancer prevention in the genital tract of women at increased risk of ovarian cancer. There were 64 women enrolled in a multi-institutional randomized trial who chose to undergo risk-reducing bilateral salpingo-oophorectomy (BSO) and to receive the progestin levonorgestrel or placebo for 4 to 6 weeks prior to undergoing BSO. The ovarian and fallopian tube epithelia (FTE) were compared immunohistochemically for effects of levonorgestrel on apoptosis (primary endpoint). Secondary endpoints included TGFβ isoform expression, proliferation, and karyometric features of nuclear abnormality. In both the ovary and fallopian tube, levonorgestrel did not confer significant changes in apoptosis or expression of the TGFβ1, 2, or 3 isoforms. In the ovarian epithelium, treatment with levonorgestrel significantly decreased the proliferation index. The mean ovarian Ki-67 value in the placebo arm was 2.027 per 100 cells versus 0.775 per 100 cells in the levonorgestrel arm (two-sided P value via Mann–Whitney U test = 0.0114). The karyometric signature of nuclei in both the ovarian and FTE deviated significantly from normal controls (women at average risk of ovarian cancer), but was significantly less abnormal in women treated with levonorgestrel. These karyometric data further support the idea that progestins may clear genetically abnormal cells and act as chemopreventive agents against ovarian and fallopian tube cancer.

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Introduction

Along with parity, oral contraceptive use has consistently been associated with decreased ovarian cancer risk. Three or more years of oral contraceptive use reduces the risk of ovarian cancer by 30% to 50% (1). The association increases with the duration of use and is independent of inherent ovarian cancer risk, occurring in women at average as well as increased hereditary risk of ovarian cancer (2).

The mechanisms underlying the marked protective effect of oral contraceptive use against ovarian cancer have not been well defined. However, it is commonly believed that ovulation, with its associated disruption and subsequent repair of the ovarian epithelium, can lead to acquisition of genetic damage in ovarian epithelial cells and, in turn, to ovarian cancer (3). This hypothesis is supported by epidemiologic evidence linking ovulation with ovarian cancer risk (1–3) and by the finding that ovarian cancers arise frequently in hens, which ovulate daily (4). Historically, reproductive and hormonal factors such as oral

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contraceptive use and pregnancy have been presumed to lower ovarian cancer risk via inhibition of ovulation. However, both of these factors confer a degree of ovarian cancer protection much greater than would be expected on the basis of the number of ovulatory cycles that are inhibited, suggesting they may impact ovarian cancer risk through additional biologic mechanisms (5, 6).

Previously, a 3-year study in primates demonstrated that the progestin component of an oral contraceptive has a potent apoptotic effect on the ovarian epithelium, providing support for the hypothesis that oral contraceptives may lower ovarian cancer risk via induction of cancer preventive molecular pathways in the ovarian epithelium (7). Activation of apoptosis leads to clearance of cells that have undergone irreparable genetic damage and are prone to neoplastic transformation (8). It is a key molecular pathway for elimination of premalignant cells *in vivo* (9–11). In animal models of cancer as well as in humans, the efficacy of cancer preventive agents has been shown to correlate with the degree of apoptosis induced (12, 13). The finding that progestins activate this critical pathway in the ovarian epithelium raises the possibility that progestin-mediated apoptotic effects, and not solely ovulation inhibition, may underlie ovarian cancer risk reduction associated with oral contraceptive use.

Analysis of data from the Cancer and Steroid Hormone (CASH) study investigated the relationship between progestin and estrogen potency in oral contraceptives and risk of developing ovarian cancer. When comparing oral contraceptives categorized by estrogen and progestin potency in 400 ovarian cancer cases and 3,000 controls, oral contraceptive formulations with increased progestin potency conferred twice the reduction in ovarian cancer risk than those with lower progestin potency, irrespective of the estrogen content ($P < 0.001$). The analyses even demonstrated a 60% to 70% reduction of ovarian cancer risk in women who took high progestin potency oral contraceptives for relatively short duration (<18 months). The finding that the degree of protection afforded by oral contraceptives is related to progestin potency further supports the hypothesis that biologic effects related to the progestin component may be a key mechanism underlying the reduction in ovarian cancer risk associated with oral contraceptive use (14).

The results of these studies led us to hypothesize that progestins might be highly effective ovarian cancer preventives. We sought to gather further preclinical data in support of progestins as ovarian cancer preventives by demonstrating induction of surrogate endpoint biomarkers relevant to cancer prevention in the ovarian epithelium of women at high risk for ovarian cancer treated with the progestin, levonorgestrel. The primary endpoint of our study was demonstration of progestin-activation of apoptosis in the ovarian epithelium, similar to what had been shown in a primate model. Secondary endpoints included (i) activation of TGFβ signaling, given the relevance of this pathway to cancer prevention, and demonstration previously in primates that progestin differentially regulates TGFβ in ovarian epithelium, concomitant with activation of apoptosis (7), and (ii) the effect of progestin on cellular proliferation. As an exploratory objective, we sought to determine whether levonorgestrel conferred normalization in the karyometric signature, shown previously to be abnormal in the ovarian epithelium in women at high risk for ovarian cancer (15). In addition to investigation of the ovary, our study provided a unique opportunity to measure the impact of progestins on chemopreventive biomarkers in the fallopian tube, the organ thought increasingly to harbor the cell of origin for many high-grade ovarian cancers (16).

Materials and Methods

Study design

GOG 0214 was a blinded, two-arm phase II study of the impact of levonorgestrel on chemopreventive surrogate endpoint biomarkers in the epithelium of the ovary and fallopian tube. Women at increased ovarian cancer risk intending to undergo prophylactic salpingo-oophorectomy were randomized by the GOG Data Center to receive levonorgestrel (0.150 mg/day) or placebo for 4 to 6 weeks prior to surgery. Eligibility criteria were generally similar to that of GOG protocol 199 (17). Eligible subjects needed to be ≥30 years of age and have at least one intact ovary. Exclusion criteria included (i) history of myocardial infarction, thromboembolic, liver and coagulation disorders; (ii) current malignancy, other than nonmelanoma skin cancer, and history of ER^+ and/or PR^+ breast cancer; (iii) current/recent use of any hormone, including contraceptives or hormone replacement therapy (oral or intrauterine <3 months, injectable Depo-Provera <12 months); (iv) breastfeeding; (v) being within one year of systemic cancer therapy or pelvic radiation therapy (systemic therapy must have been for <12 months); and (vi) history of ovarian cancer. Although the study design was based on data from premenopausal primates, postmenopausal women were not excluded. Published epidemiologic evidence suggests that menopausal estrogen replacement therapy (ERT) significantly increases ovarian cancer risk, but that addition of progestins abrogates this, consistent with a chemopreventive effect (18). The protective effect of progestins is likely underestimated in these studies, in that ERT users (as compared with EPRT users) typically have had hysterectomy, which lowers ovarian cancer risk (19). Thus, we hypothesized that the ovarian epithelium would be responsive to progestins in postmenopausal females.

Subjects were to take one capsule containing either 0.150 mg of levonorgestrel or placebo daily orally until the day (including morning) of surgery. A missed dose could be taken up to 12 hours later; the drug/placebo

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regimen would resume the following day. Surgery was to be completed 4 to 6 weeks after beginning drug/placebo; for surgical delays, continuation of the drug/placebo was allowed for no more than 5 months, after which the subject would be removed from the study. Compliance was assessed using a Pill Count Form and further confirmed with preoperative blood collection the day of surgery for determination of serum levonorgestrel (inVentiv Health).

Capsules contained 0.150 mg levonorgestrel (IND #79,610) or matching lactose placebo (Flourish Integrative Pharmacy). This dose of levonorgestrel was comparable with that commonly used in oral contraceptives without the estrogen component and to that shown to effectively impact apoptosis and TGFβ expression in the ovarian epithelium in a prior primate study, and thus, presumed adequate to induce surrogate biomarker effects in the gynecologic tract in women (7). Four weeks of intervention was presumed to be adequate in that the monkeys had undergone oophorectomy after only 2 to 3 weeks of progestin treatment. Notably, the significant effects of levonorgestrel shown in the primate study occurred despite the fact that half of control monkeys were in the luteal phase of the menstrual cycle, and thus, were producing endogenous progesterone. With cynomolgus macaques (*Macaca fascicularis*) having a reproductive tract anatomically and hormonally similar to that of humans, including a 28-day menstrual cycle (20), it was anticipated that levonorgestrel would have an effect in women similar to what was observed in primates. Thus, in the current study, neither the timing of start of the study drug nor the surgeries were timed to the menstrual cycle in premenopausal subjects.

Tissue acquisition

To minimize ovarian epithelium loss at surgery, surgeons were given detailed instructions to minimize contact with the ovarian surface. For each case, 25 5-μm serial sections from each formalin-fixed, paraffin-embedded ovary and fallopian tube were prepared for IHC and karyometry. In all cases, step-sectioning and histologic evaluation of fallopian tubes and ovaries were performed according to standard of care to rule out occult cancers.

Proliferation

IHC detection of Ki-67 was performed by NorthShore University HealthSystem Clinical Pathology Laboratory using standard protocol and a nuclear Ki-67-specific antibody (EMD Millipore) diluted 1:100 using small intestine as positive control. Ovarian and fallopian sections were evaluated by microscopy using a 40× objective. For ovary, all surface epithelial cells in an ovarian section were counted and the number of Ki-67–positive cells recorded. For fallopian tube, 500 epithelial cells from a randomly chosen field of the fimbria (or isthmus, when fimbria not available) were counted and the number of Ki-67–positive cells was recorded. The percentage of positively stained cells in each section was determined and compared statistically between treatment groups.

Apoptosis

Ovarian surface epithelium (OSE) and fallopian tube epithelium (FTE) were evaluated for prevalence of epithelial cell apoptosis after terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) using the Apoptag Plus Kit (EMD Millipore; ref. 7). Dark brown nuclear staining identified apoptotic cells. Tonsillar tissue, exhibiting high apoptosis, served as positive control. Tissue sections treated with label only, without the terminal transferase, served as negative controls. Positively stained cells in the OSE and FTE were counted as described for Ki-67. Two different investigators counted TUNEL-positive cells, both blinded to treatment group identity. The proportion of TUNEL-positive cells was compared statistically between treatment groups.

Expression of TGFβ isoforms

TGFβ expression was determined by IHC, as described previously (7) using primary antibodies specific for individual isoforms, TGFβ1, -β2, and -β3 (R&D Systems, EMD Millipore, and Thermo Fisher Scientific, respectively, each diluted 1:100). Relative staining intensity in OSE and FTE was graded on a 0 to 4 point scale, by three observers blinded to treatment, with 0 being completely negative, and 4 being intense staining. The average intensity of immunostaining for each TGFβ isoform was determined and compared statistically between treatment groups.

Karyometry

Sample collection. Analytical samples consisted of deidentified 5-μm unstained paired ovarian and fallopian tube tissue sections from 58 patients, (6 cases not evaluable) received at the University of Arizona Cancer Center Quantitative Histopathology lab. Among the 58 cases, 29 received placebo. The other 29 paired ovarian and fallopian tube samples came from levonorgestrel-treated patients. In addition, six ovarian and seven fallopian tube samples came from women at average risk for ovarian cancer, from NorthShore University HealthSystem's tissue bank.

For ovarian epithelium, 3,264 nuclei were recorded from levonorgestrel-treated patients, and 2,889 nuclei were recorded from placebo-treated cases. For FTE, 3,264 nuclei were recorded from levonorgestrel-treated patients and 3,122 nuclei from placebo-treated patients. In addition, there were 1,171 nuclei analyzed from normal (nonhigh risk) ovarian tissue and 1,205 from normal fallopian tubes.

Tissue preparation. Sections were stained using synthetic hematoxylin and eosin, with human tonsil tissue utilized for quality control (21). Images of ovarian and FTE were

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captured at 100× and consisted of a random accumulation of well-defined nonoverlapping nuclei.

Karyometric imaging protocol. Investigators conducting the karyometric analysis were blinded to group identity of the specimens. Images were analyzed using a semiautomated segmentation program with manual correction, thereby isolating individual nuclei for further investigation (22). Following segmentation, 93 karyometric features were used to analyze the nuclear chromatin pattern (23–25). These features are related to nuclear area, total optical density, and chromatin distribution (26, 27).

The 93 karyometric features collectively created the "nuclear signature." Our linear discriminant analysis (LDA) used a two-step procedure to identify important nuclear features for discrimination: at step 1 (initial screening), a Kruskal–Wallis test and the ambiguity function after Genchi and Mori (23) were utilized to detect submicroscopic differences not detectable by visual analysis alone. For each case (individual), the average feature values were computed over nuclei separately for each of the 93 karyometric features. The test was conducted at the case level, with observations from different cases assumed to be independent. More than 20 nuclear features exhibited statistical differences at $P < 0.0001$ between two treatments, and they were used as inputs to perform LDA. The discriminant function (DF) was derived from the LDA to discriminate between two classes of tissues (28). At step 2 (refined selection), we further identified the most statistically significant features by using Wilks' lambda test (a variance–covariance matrix function varying from 0 to 1, with lower values indicating greater discrimination), and these comprised the final DF that determined differences between normal nuclei and nuclei deviating from normal.

Statistical analysis

Sample size calculation ($n = 64$) for the study was designed for alpha = 0.05 and power of 80% for the primary endpoint of activation of apoptosis in the ovarian epithelium. Both proliferation and percent apoptotic cells in the two treatment groups were compared using the nonparametric Mann–Whitney *U* test, for fallopian tube and ovary separately. Differences were deemed significant at $P < 0.05$ (two-tailed). There was no control mechanism put in place for multiple testing, either across the sample types or across the two separate evaluations of apoptosis. TGFβ1, TGFβ2, and TGFβ3 were treated as dichotomous variables, with differences in expression estimated using Fisher exact test. TGFβ expressions were also analyzed as continuous variables. Karyometric statistical methods are described above within the karyometry protocol. Tissue sections were considered inevaluable, for IHC (i) when no epithelium was present, (ii) where staining characteristics were inconsistent across sections, (iii) in cases where there was significant nonspecific staining across sections, and (iv) in cases lacking stretches of epithelium spanning two 40× fields; and for karyometry when sections lacked 100 evaluable epithelial nuclei.

Table 1. Patient characteristics for all subjects in GOG 214

	Treatment		
Characteristic	Levonorgestrel *N* (%)	Placebo *N* (%)	Total *N* (%)
Age group			
30–39	4 (12.1)	6 (19.4)	10 (15.6)
40–49	14 (42.4)	13 (41.9)	27 (42.2)
50–59	14 (42.4)	9 (29.0)	23 (35.9)
60–69	1 (3.0)	3 (9.7)	4 (6.3)
Ethnicity			
Hispanic or Latino	2 (6.1)	0 (0)	2 (3.1)
Non-Hispanic	29 (87.9)	27 (87.1)	56 (87.5)
Unknown/not specified	2 (6.1)	4 (12.9)	6 (9.4)
Race			
Race unknown	0 (0)	1 (3.2)	1 (1.6)
American Indian/Alaskan Native	2 (6.1)	0 (0)	2 (3.1)
White	31 (93.9)	30 (96.8)	61 (95.3)
Menopausal status			
Premenopausal	20 (60.6)	19 (61.3)	39 (60.9)
Postmenopausal	13 (39.4)	12 (38.7)	25 (39.1)
Total	33 (51.6)	31 (48.4)	64 (100.0)

Results

Study subjects were accrued between April 9, 2008 and June 11, 2012. Characteristics of enrolled subjects are summarized in Table 1. Average patient age was 48 years. A total of 95.3% subjects were white, 87.5% nonHispanic descent, and 60.9% were premenopausal. Levonorgestrel was tolerated well, with no significant adverse effects, and no significant difference in recorded side effects between control and treated subjects according to CTC version 3 criteria. Preoperative levonorgestrel blood levels were available for 30 of 33 treated subjects. Six did not have detectable levonorgestrel levels (<10 pg/mL), indicative of the subjects not having taken study drug for at least

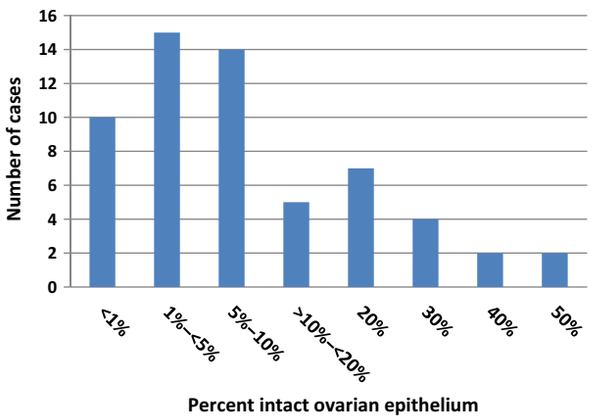

Figure 1.
Percent intact OSE in specimens analyzed in this study. Each bar represents the number of cases for which the indicated relative percent of the surface epithelium was present.

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72 hours (29). For the rest of the cases, the mean levonorgestrel level ± SD was 1289 ± 891 pg/mL with a range of approximately 300 to 3893 pg/mL. These measurements were within the anticipated range for subjects who took study drug within the past 24 hours, with the majority having taken drug within hours of undergoing surgery (29). Drug levels were not impacted by patient age or menopausal status.

Overall, the quality of the ovarian tissue sections was poor. There were no cases in which the ovarian epithelium was greater than 50% intact. In over 60% of cases, more than 80% of the epithelium was missing or denuded. In 39% of cases, less than 5% of the epithelium remained intact (Fig. 1). The quality of epithelium was significantly better in fallopian tube sections, but there was heterogeneity in the types of tube sections submitted, with only 32/64 (50%) of sections containing fimbriated end.

Apoptosis

On initial examination and scoring, the ovarian epithelium in 50 of 64 cases was considered evaluable (24 in the placebo arm and 26 in the levonorgestrel arm). Representative fields are shown in Fig. 2. Examination of ovarian sections for epithelial apoptosis revealed marked variability, both in percent of TUNEL-positive nuclei, as well as in amount of nonspecific staining. Median percent apoptosis was 9.3% [interquartile range (IQR) 22.6] in the levonorgestrel group and 11.5% (IQR 29.6) in the placebo group. The overall range of apoptotic cells was 0% to 89.7%. There was no difference in mean percent of apoptotic cells between the two treatment groups, with a mean ± SD of 19.8% ± 21.9% in controls and 18.0% ± 20.8% in levonorgestrel-treated subjects ($P = 0.749$). Results were not affected by menopausal status.

For fallopian tube, 62 of 64 cases were considered evaluable (30 placebo and 32 in the levonorgestrel arm). Median percent apoptosis was 20.5% (IQR 32.5) in the levonorgestrel group and 7.9% (IQR 24) in the placebo group. The overall range of apoptotic cells was 0% to 95.7%. There was no difference in mean percent of cells staining positive for TUNEL, with a mean ± SD of 16.8% ± 19.7% in the placebo group and 26.7% ± 25.3% in the levonorgestrel group ($P = 0.082$). Again, results were not impacted by menopausal status.

TGFβ

Expression of the TGFβ1, TGFβ2, and TGFβ3 isoforms in both ovarian and FTE did not differ between the two groups. Representative fields are shown in Fig. 3. Mean expression of TGFβ isoforms was similar (Table 2A). The data were also analyzed with TGFβ expression dichotomized (low expression <3 and high expression ≥3), with similar results (Table 2B).

For both apoptosis and TGFβ expression, results were not impacted by the exclusion of women who had

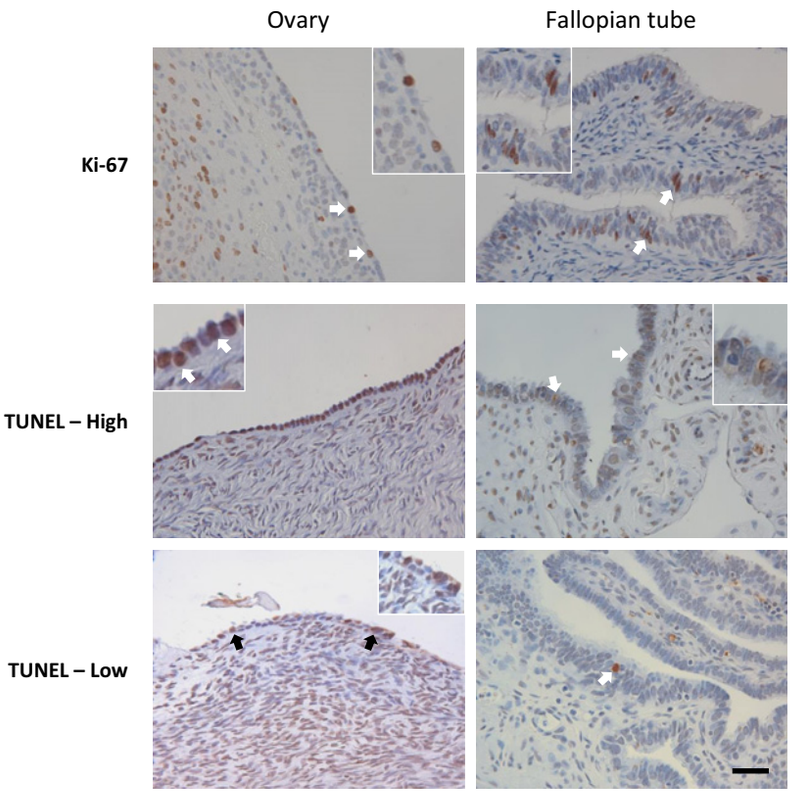

Figure 2.
Proliferation and apoptosis in ovarian and FTE. Each image represents an example of the Ki-67 IHC or TUNEL label (arrows) present in OSE or FTE, including both high and low TUNEL label. Insets show 2-fold increased magnification for detail. Magnification bar, 50 μm.

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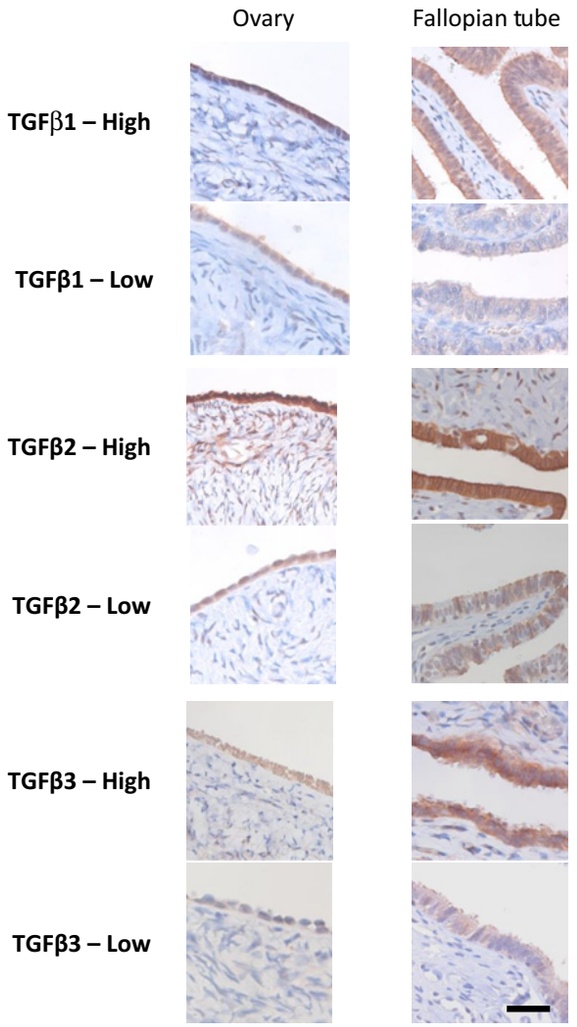

Figure 3.
TGFβ expression in the ovarian and FTE. Each image represents an example of the TGFβ1, -β2, or -β3 immunolabeling in OSE or FTE, including high and low intensity stain for each. Magnification bar, 50 μm.

nondetectable serum levels of levonorgestrel at the time of surgery.

Proliferation

In the OSE, treatment with levonorgestrel significantly decreased the proliferation index (Fig. 2). The mean Ki-67 value in the placebo arm was 2.027/100 cells versus 0.775/100 cells in the levonorgestrel arm ($P = 0.0114$). Proliferation as measured by Ki-67 was rare in FTE, and thus, not statistically evaluable.

Karyometry

The following DFs (DF1–DF4) were derived by karyometric analysis:

DF1, placebo-treated ovarian tissue versus normal controls.

Table 2A. TGFβ isoform expression in ovarian and FTE in subjects with and without levonorgestrel treatment

Tissue	TGFβ Isoform	Treatment	Mean stain intensity ± SD (95% CI)	*P*
Ovary	TGFβ1	Placebo	0.95 ± 1.11 (0.43–1.47)	
		Levonorgestrel	1.46 ± 1.22 (0.99–1.92)	0.139
	TGFβ2	Placebo	2.29 ± 1.01 (1.89–2.68)	
		Levonorgestrel	2.32 ± 1.09 (1.90–2.74)	0.899
	TGFβ3	Placebo	1.50 ± 1.31 (1.01–1.99)	
		Levonorgestrel	1.52 ± 1.26 (1.05–1.98)	0.961
Fallopian tube	TGFβ1	Placebo	1.67 ± 1.15 (1.21–2.14)	
		Levonorgestrel	2.12 ± 1.33 (1.63–2.63)	0.185
	TGFβ2	Placebo	2.45 ± 1.05 (2.06–2.84)	
		Levonorgestrel	2.67 ± 0.90 (2.35–2.99)	0.374
	TGFβ3	Placebo	2.25 ± 1.02 (1.87–2.63)	
		Levonorgestrel	2.16 ± 1.26 (1.70–2.61)	0.748

Abbreviation: CI, confidence interval.

Table 2B. TGFβ results dichotomized by high (intensity ≥3) and low (intensity <3) expression

Tissue	TGFβ Isoform	*P*
Ovary	TGFβ1	0.720
	TGFβ2	1.000
	TGFβ3	0.384
Fallopian tube	TGFβ1	0.395
	TGFβ2	0.302
	TGFβ3	0.793

DF2, levonorgestrel-treated ovarian tissue versus normal controls.

DF3, placebo-treated fallopian tube tissue versus normal controls.

DF4, levonorgestrel-treated fallopian tube tissue versus normal controls.

Karyometric feature values are given in arbitrary, relative units.

Ovarian nuclei

Twenty-nine cases of placebo-treated ovarian samples from women at risk for ovarian cancer, with 2,889 nuclei recorded, and six cases of ovarian tissue from women with no such known risk, as controls, with 1,171 nuclei recorded, were analyzed. At the initial screening step, each of more than 20 significant nuclear features in the initial DF reflected first and second order spatial/statistical differences in nuclear chromatin distribution. There were no features indicative of a progression to abnormality, such as atypical hyperplasia, or premalignancy. At the refined selection step, Wilks' lambda test was used to identify a set of four features that were most statistically significant for discrimination. The four karyometric variables that specifically determined functions DF1 (run length nonuniformity, cooccurrence matrix, run length matrix, and cooccurrence matrix change from lowest entry, all related to texture analysis) reduced Wilks' lambda to 0.625, which suggests a distinct difference from normal. The nuclear classification matrix, which summarizes the degree of correspondence between true and predicted classes for

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Table 3A. Ovarian nuclear classification matrix for the LDA to distinguish placebo-treated and levonorgestrel-treated versus normal controls, showing percent correct matching of true versus karyometry-predicted classification[a]

	Karyometry-predicted classification	
True classification	**Placebo-treated**	**Normal controls**
Placebo-treated	84.5%	15.5%
Normal controls	25.3%	74.7%
	Levonorgestrel-treated	**Normal controls**
Levonorgestrel-treated	76.4%	23.6%
Normal controls	25.3%	74.7%

Table 3B. Fallopian tube nuclear classification matrix for the LDA to distinguish placebo-treated and levonorgestrel-treated versus normal controls, showing percent correct matching of true versus karyometry-predicted classification[a]

	Karyometry-predicted classification	
True classification	**Placebo-treated**	**Normal controls**
Placebo-treated	60.9%	39.1%
Normal controls	34.0%	66.0%
	Levonorgestrel-treated	**Normal controls**
Levonorgestrel-treated	48.1%	51.9%
Normal controls	34.0%	66.0%

[a]There are four 2-by-2 classification matrices in Tables 3A and 3B, which are used to evaluate the classification accuracy of the proposed karyometric analysis under four scenarios. Following is an explanation of how the four entries of the first 2-by-2 matrix in Table 3A are interpreted, and then those of the remaining three matrices are interpreted in a similar way. In the first classification matrix, four entries are respectively: the top row 84.5%, 15.5%, the bottom row 25.3%, and 74.7%, which are used to show the accuracy of the karyometric method in discriminating two types of ovarian tissues, placebo-treated tissues (type 1) versus normal controls (type 0). In the top row, the first entry, 84.5%, is called the true positive rate, which is calculated as the percentage of the tissues belonging to type 1, which are correctly predicted as type 1 by the karyometry method; the second entry, 15.5%, is called the false negative rate, which is calculated as the percentage of the cases in type 1, which are incorrectly predicted as type 0 by the karyometry classifier. For the placebo-treated tissues, the karyometric analysis can correctly predict 84.5% of them (as placebo-treated tissue) and incorrectly predict 15.5% of them (as normal controls). In the second row, the first entry 25.3% is called the false positive rate, which is calculated as the percentage of the tissues belonging to type 0, which are incorrectly predicted as type 1 by the karyometric method; the second entry 74.7% is called the true negative rate, which is calculated as the percentage of the cases in type 0 that are correctly predicted as type 0 by the karyometry method. For the normal control tissues, the karyometric analysis predicts 25.3% of them incorrectly (as placebo-treated tissue) and predicts the remaining 74.7% of them correctly (as normal controls). The same analysis is done for the other three classification matrices, with the only differences being the definitions of type 1 and type 0 tissues. For example, in the second matrix, type 1 refers to levonorgestrel-treated tissues and type 0 refers to normal controls.

ovary, is shown in Table 3A (top). The average classification accuracy was 81.7%.

The distribution of DF scores is shown in Fig. 4A. Approximately 60% of nuclei in placebo-treated ovarian tissue had karyometric features with values deviating from normal. For levonorgestrel-treated ovarian tissues there were 29 cases, with 3,264 nuclei recorded. When function DF2 (based on the same four features as DF1; Fig. 4B) was applied, the average correct classification rate was 75.9% (Table 3A, bottom). Levonorgestrel treatment reduced the percentage of nuclei with karyometric deviations from normal by 8.1%. This reduction from about 85% to 75% was statistically significant at $P < 0.01$, given the sample size of more than 3,000 nuclei, consistent with the chemopreventive intervention effectively shifting the karyometric nuclear signature toward normal in the ovarian epithelium. Case classification, using the convention of the 50% rule (if more than half the cells showed change), classified 26/29 or 89.6% of the treated cases correctly.

Fallopian tube nuclei

There were 29 cases, with 3,122 nuclei recorded from fallopian tube tissue, and 1,205 nuclei from seven fallopian tube control cases, totaling 4,327 nuclei. The algorithm identified more than 20 karyometric features with statistically significant differences at $P < 0.0001$. Seven features descriptive of the statistical and spatial distribution of the nuclear chromatin (nuclear area, optical density SD, optical density histogram, run length matrix, long run length emphasis, short run length emphasis, and optical density mean in grey level) determined DF3 for fallopian tube nuclei (Fig. 4C). Table 3B shows the placebo-treated fallopian tube tissue classification matrix. Average correct classification rate was 62.3%.

Figure 4D shows the distribution of DF4 scores. About 25% of fallopian tube nuclei had karyometric values deviating from normal controls, compared with approximately 60% doing so in ovarian tissues. The levonorgestrel-treated fallopian tube classification matrix is in Table 3B. The percentage of fallopian tube nuclei with karyometric variables in the "placebo-treated state" was reduced from 26.9% to 12.8%, that is, treatment returned fallopian tube tissue almost to the condition of normal controls. The reduction from 60% to 48% was statistically significant at $P < 0.01$, that is, the chemopreventive intervention effectively shifting the karyometric nuclear signature toward normal in the fallopian tube.

Discussion

When the current study was conceived, the objective was to gather further preclinical evidence for progestins as chemopreventive agents for ovarian cancer. We sought to demonstrate activation of surrogate endpoint biomarkers relevant to chemoprevention in the OSE, long presumed to be the site of origin for ovarian cancers. The hypothesis that the fallopian tube harbors the cell of origin for many high-grade ovarian cancers had not yet been validated. In light of the finding that progestins had been shown in a relevant primate model to markedly activate apoptosis and differentially regulate TGFβ in OSE (7), we sought to demonstrate similar effects in the OSE in women at high risk for ovarian cancer.

Via conventional IHC, our study failed to confirm in humans the results shown previously in primates. Progestin treatment did not significantly alter apoptosis or TGFβ signaling in the OSE in women at high risk for ovarian cancer. There are a number of possible reasons for our

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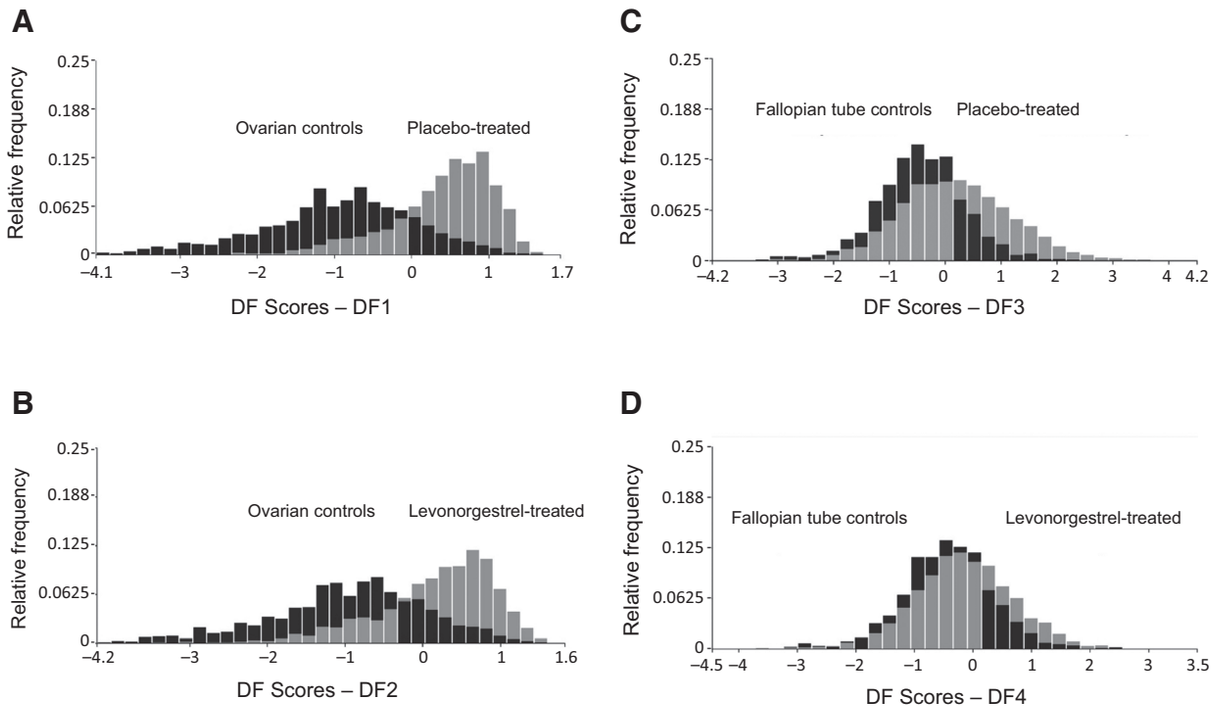

Figure 4.

Distribution of DF scores of ovary and fallopian tube. The dark bars indicate the relative frequency of occurrence of nuclei of controls with a discriminant score in each interval of the DF score axis, and the grey bars indicate those for the nuclei of placebo- or levonorgestrel-treated tissues. The score differences shown in **A**/**B** (and **C**/**D**) are due to the different cases used in training the discriminating function. For **A**, the classifier aims to discriminate placebo-treated tissues versus normal controls, whereas in **B**, the goal is to discriminate levonorgestrel-treated tissues versus normal controls. Thus, we have two different classification questions and, because the scores are not directly comparable, the graphs are shown separately. **A,** The distribution of DF scores of ovarian controls versus placebo-treated ovarian epithelium (based on DF1). A shift to higher scores indicates higher deviation from normal. **B,** The distribution of DF scores of ovarian controls versus levonorgestrel-treated ovarian epithelium (based on DF2). A shift to lower scores indicates less deviation from normal. **C,** The distribution of DF scores of fallopian tube controls versus placebo-treated fallopian tube cells (based on DF3). A shift to higher scores indicates higher deviation from normal. **D,** The distribution of DF scores of fallopian tube controls versus levonorgestrel-treated fallopian tube cells (based on DF4). A shift to lower scores indicates less deviation from normal.

findings: (i) it is possible that dysfunction in progestin-mediated molecular pathways in women with hereditary risk of ovarian cancer may render progestins ineffective as chemopreventives. However, this is inconsistent with published evidence that use of progestin-containing regimens lowers ovarian cancer risk in women with known alterations in BRCA 1 and 2 (2). (ii) It is possible that OSE in women at hereditary risk of ovarian cancer is resistant to progestin-mediated changes in apoptosis or TGFβ signaling. It has been shown, for example, that expression of Bcl-2, an antiapoptotic protein, is increased in ovaries and fallopian tubes of women at increased risk of ovarian cancer, thereby potentially rendering these organs more resistant to apoptosis (30). In addition, mutation or loss of p53 expression, as commonly occurs in high-risk fallopian tubes, may blunt responsiveness to TGFβ (31). (iii) The chemopreventive effect of progestins may be due to effects unrelated to apoptosis or TGFβ signaling. A recent study, for example, demonstrated *in vivo* and *in vitro* that progestins clear p53-deficient fallopian tube cells via necroptosis and not apoptosis (32). (iv) For fallopian tube, our cases were half fimbrial and half isthmus in location. This lack of homogeneity among types of tubal specimens could have impacted our findings in that the sensitivity of FTE to the biologic effect of progestins may vary by location in the tube. It has been shown, for example, that a number of cytokines, including members of the TGFβ superfamily, are differentially expressed depending on the location in the fallopian tube, as well as the hormonal milieu as influenced by stage in the menstrual cycle, menopausal status, or exposure to reproductive hormones (33–37). (v) It is possible that progestin activation of surrogate endpoint biomarkers related to apoptotic and TGFβ pathways may depend on the presence of estrogen priming, thus potentially impacting the results of our study. Our study design was based on results in premenopausal primates, whereas 40% of women enrolled in our study were postmenopausal. (vi) Our results may have been adversely impacted by limitations related to either the quality of tissues collected or the IHC technique itself. The majority of OSE

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was missing in most of our cases. This rendered visual interpretation of staining intensity difficult, especially in cases that lacked long stretches of continuous epithelium. (vii) Because half the tubal cases lacked fimbriated end, which is the portion of the tube at greatest cancer risk and the target for chemoprevention, our study may have lacked sufficient statistical power to demonstrate a difference in fallopian tube apoptosis results, even though mean values were increased by >60% in the levonorgestrel group. In addition, marked, nonspecific TUNEL staining in some specimens made scoring/interpretation of apoptosis difficult in both ovarian and FTE. In contrast to IHC, our application of karyometry for computer analysis of cellular nuclear features is not subject to the variability that can occur with staining techniques, nor dependent on activation of any single biomarker. In addition, the karyometric results are standardized, reproducible, and allow for identification of changes not visible to the human eye.

Via karyometry, digital microscopy provides a quantitative approach that discriminates malignancy-associated changes in nuclear chromatin from benign, and targets features reflecting an estimate of the percentage of nuclei affected by the intervention. Utilizing this approach, a previous study demonstrated that both histologically normal-appearing ovarian epithelium in women at high risk for ovarian cancer, as well as normal ovarian epithelium adjacent to ovarian cancer harbored abnormal submicroscopic changes in nuclear chromatin, thereby suggesting potential for malignancy (15). This has been similarly shown in nuclei of normal, preinvasive, and invasive lesions in other tissues including breast, rectal, urinary tract, prostate, and skin (15, 38–42). Taken together, these data suggest that karyometry may allow identification of changes in nuclear signature typical of early transformative events occurring during carcinogenesis, as well as serve as a surrogate endpoint for assessment of chemopreventive agents.

Our karyometry results confirm prior findings of Brewer and colleagues (15) showing a deviation from normal in the karyometric nuclear signature in OSE in women at high risk for ovarian cancer. In addition, we further found a deviation from normal in the karyometric signature in the nuclei of the FTE in women at high risk. Recent studies suggest that the cell of origin for many high-grade ovarian cancers may reside in the fallopian tube (16). Several compelling lines of evidence support this hypothesis. These include frequent molecular/genetic abnormalities noted in the fallopian tube in women at high risk for ovarian cancer who have undergone prophylactic salpingo-oophorectomy, as well as the discovery of identical genetic/molecular abnormalities in both intraabdominal metastatic deposits of putative ovarian cancer and early fallopian tube lesions identified in the same patient, suggesting that abnormal cells from the fallopian tube are likely to shed into the abdominal cavity and transform into clinical ovarian cancer (43). In light of the high frequency of abnormal molecular signatures in the fallopian tube, it is not surprising that we found that the high-risk fallopian tube has an abnormal karyometric nuclear signature.

Demonstration of an abnormal karyometric signature in both the ovary and fallopian tube in women at hereditary ovarian cancer risk supports the notion that both the ovary and fallopian tube are at risk of neoplastic transformation in these women. This is consistent with the surgicopathologic study of gynecologic cancers identified in >1,000 women who underwent risk reducing salpingo-oophorectomy, in which approximately equal proportions of ovarian and fallopian tube cancers were identified (44). It is further supported by the finding in genome-wide expression studies of numerous differentially expressed genes in both ovaries and fallopian tubes in women at high ovarian cancer risk (45). In addition, a recent series using detailed genomic analysis of serous tubal intraepithelial carcinomas (STICs), invasive fallopian tube lesions, invasive ovarian lesions, and omental metastases, demonstrated that although at least half of cases had a STIC as a precursor lesion, some STICs represented intraepithelial metastases to the fallopian tube (46).

The finding that, in response to a progestin, the karyometric nuclear signature in high-risk OSE and FTE is more like that in normal controls provides supportive evidence for progestins as chemopreventive agents for ovarian cancer. The observation of these effects in both ovary and fallopian tube further strengthens these results. Remarkably, progestins conferred a karyometric signature in the fallopian tube similar to that of women at average risk of ovarian cancer. These data are consistent with the notion that progestins clear genetically damaged cells, as has been proposed by Wu and colleagues, in cellular and animal models of fallopian tube cancer (32). In addition, the data support a chemopreventive effect regardless of whether abnormal cells at risk of transforming to ovarian cancer reside in the ovary or in the fallopian tube.

Compelling human and animal data are supportive of the notion that a biologic effect of progestins may be a major mechanism underlying the ovarian cancer preventive effect for both the oral contraceptive as well as pregnancy, which is associated with high serum progesterone levels. These data include (i) the CASH study, which demonstrated that progestin-potent oral contraceptives confer greater protection against ovarian cancer than oral contraceptives containing weak progestin formulations (14); (ii) data from the World Health Organization, demonstrating a 60% reduced risk of nonmucinous ovarian cancer in women who have ever used depo-medroxyprogesterone acetate, a progestin-only contraceptive (47). Because progestin-only oral contraceptives do not reliably inhibit ovulation, this suggests that progestins have a direct chemopreventive biologic

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effect; (iii) epidemiologic evidence showing that twin pregnancy, which is associated with higher progesterone levels, is more protective against subsequent ovarian cancer than singleton pregnancy and that pregnancy at a later age is more protective than pregnancy early in life. It has been proposed that this suggests a protective effect of pregnancy unrelated to effects on ovulation, and supporting the notion that pregnancy-induced progesterone may clear premalignant or damaged cells from the ovary (48); (iv) *in vivo* evidence of a chemopreventive effect, unrelated to ovulation, of progestins against reproductive tract (ovarian and oviductal) tumors in egg laying hens (49); (v) evidence that use of the levonorgestrel intrauterine device lowers the risk of subsequent ovarian cancer (50); and (vi) *in vivo* and *in vitro* evidence revealing that progestins clear cells containing abnormal molecular signatures in the fallopian tube (32).

Our karyometric results add support to the idea that progestins may clear genetically abnormal cells, adding preclinical evidence in support of progestins as chemopreventive agents against ovarian and fallopian tube cancer. If the mechanism underlying the protective effects of oral contraceptives involves a biologic effect of progestins as we hypothesize, and not simply ovulation inhibition, it may be possible via a potent progestin-based chemopreventive strategy to achieve protective effects even greater than the 30% to 50% reduction associated with routine use of oral contraceptives, with the potential to prevent most ovarian cancers. Finally, our results support karyometry as a valid surrogate biomarker that may have utility in future ovarian cancer chemoprevention trials.

Disclosure of Potential Conflicts of Interest

G.C. Rodriguez has ownership interest in patents for the use of progestins for ovarian cancer prevention (of no financial value). No potential conflicts of interest were disclosed by the other authors.

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Cancer Prevention Research

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