

# Exogenous Testosterone Does Not Influence 11-Oxygenated C19 Steroid Concentrations in Healthy Postmenopausal Women

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**Context:** 11 $\beta$ -Hydroxyandrostenedione (11OHA4), 11 $\beta$ -hydroxytestosterone (11OHT), and their respective peripheral derivatives, 11-ketoandrostenedione (11KA4) and 11-ketotestosterone (11KT), have been implicated in androgen-related physiopathology. Little is known of these steroids in postmenopausal women or whether exogenous testosterone therapy influences their levels.

**Objective:** The impact of exogenous testosterone on serum levels of 11-oxygenated steroids was determined in healthy postmenopausal women.

**Participants and Methods:** Levels of 19-carbon (C19) steroids were measured by liquid chromatography–tandem mass spectrometry in serum obtained at baseline and at 12 and 26 weeks from 73 healthy postmenopausal women, aged 55 to 65 years, who participated in a randomized, double-blind, placebo-controlled clinical trial assessing the effects of transdermal testosterone on cognitive performance.

**Results:** Of the 11-oxygenated androgens, 11OHA4 was the most abundant (median, 6.46 nmol/L; range, 1.51 to 23.82 nmol/L), with concentrations several fold greater than its precursor androstenedione (median, 1.38 nmol/L; range, 0.52 to 2.92 nmol/L). Baseline median (range) testosterone and 11KT levels were similar [0.56 (0.23 to 1.48) nmol/L; 0.85 (0.25 to 2.86) nmol/L, respectively]. 11OHT was closely correlated with 11KT (Spearman rank correlation coefficient, 0.79;  $P < 0.001$ ) and 11OHA4 correlated with 11KA4 (Spearman rank correlation coefficient, 0.73;  $P < 0.001$ ). Testosterone therapy resulted in an increase in serum testosterone level, whereas all 11-oxygenated androgens remained unchanged throughout the 26 weeks of treatment.

**Conclusion:** After menopause, the adrenal production of 11-oxygenated derivatives of androstenedione and testosterone contributes importantly to the total circulating androgen pool. Exogenous testosterone does not influence the circulating levels 11-oxygenated C19 steroids.

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**Freeform/Key Words:** androgens in women, 11-oxygenated testosterone, postmenopause

In women, the adrenals are a critical source of androgen precursors. Dehydroepiandrosterone sulfate (DHEAS) is the most abundant adrenal preandrogen, whereas the unconjugated 19-carbon (C19) steroids dehydroepiandrosterone (DHEA), 11 $\beta$ -hydroxyandrostenedione (11OHA4), androstenedione, and 11 $\beta$ -hydroxytestosterone (11OHT) are produced in

Abbreviations: 11KA4, 11-ketoandrostenedione; 11KT, 11-ketotestosterone; 11OHT, 11 $\beta$ -hydroxytestosterone; 11OHA4, 11 $\beta$ -hydroxyandrostenedione; BMI, body mass index; C19, 19-carbon; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; DHT, dihydrotestosterone.

lesser amounts [1]. 11OHA4 and 11OHT are precursors for the peripheral production of 11-ketotestosterone (11KT). Traditionally, the biologically active androgens produced in non-adrenal tissues were considered to include testosterone and dihydrotestosterone (DHT). The 11-oxygenated androgen 11KT and its metabolite, 11-ketodihydrotestosterone, are androgen receptor agonists with similar binding affinities to the androgen receptor as testosterone and DHT, respectively [2]. Recent studies have implicated the 11-oxygenated androgens as having important roles in adrenarche [3], congenital adrenal hyperplasia [4, 5], and polycystic ovary syndrome [6].

Although circulating levels of DHEAS and androstenedione decline with age [7], the adrenals remain the major source of these steroids in postmenopausal women [8, 9], with potentially a small ovarian contribution [7, 10]. As yet, little is known of 11-oxygenated steroids in postmenopausal women. Testosterone therapy improves sexual desire, and may improve musculoskeletal health and enhance cognitive function in postmenopausal women [11]. We now report levels of these steroids in healthy postmenopausal women before and after 26 weeks of treatment with transdermal testosterone [12]. Because testosterone is a precursor for adrenal 11OHT biosynthesis, we investigated the effects of exogenous testosterone on the levels of 11OHT and 11KT.

## 1. Materials and Methods

### A. Study Design and Participants

Study participants were naturally postmenopausal women who, at randomization, had at least 12 months of amenorrhea, were aged 55 to 65 years, had participated in previous study of transdermal testosterone therapy [12]. Women were excluded if they had used any systemic estrogen, progestogen, or androgen therapy (including testosterone, DHEA, and tibolone) or hormonal contraceptive in the prior 12 months or medications known to affect cognitive performance, including antidepressants. Other criteria for exclusion included body mass index (BMI) <18 or >32 kg/m<sup>2</sup>, serious endocrine disorder (*e.g.*, Addison disease or Cushing disease), severe depressive symptoms (Center for Epidemiological Studies Depression Scale score  $\geq$ 16), a condition that might affect cognition, being a current smoker, or consuming more than three standard alcoholic drinks per day.

The study was a single-center, randomized, double-blind, placebo-controlled, parallel-group trial conducted between 24 April 2010 and 23 April 2012 [12]. It consisted of a 4-week screening period plus a 26-week treatment phase involving three morning study visits. Recruitment was from the community via advertisements in electronic and print media. Participants attended the Monash University Women's Health Research Program in Melbourne, Australia, for all study visits. Participants were randomly allocated to apply a transdermal testosterone gel (1.0% testosterone in a hydroalcoholic gel formulation providing 300  $\mu$ g of testosterone; LibiGel) or identical placebo gel (both supplied by BioSante Pharmaceuticals, Lincolnshire, IL) for 26 weeks, as previously described [12]. Blood samples were collected at baseline and study weeks 13 and 26, and samples were stored at  $-80^{\circ}\text{C}$  until used for steroid assays.

The study was approved by the Monash University Human Research and Ethics Committee, and all participants provided written informed consent. This trial was registered with the Australian New Zealand Clinical Trials Registry.

### B. Steroid Quantitation by Liquid Chromatography–Tandem Mass Spectrometry

Using mass spectrometry, 19 steroids, including the 11-oxygenated derivatives of testosterone and androstenedione, were measured in sera obtained at baseline and at weeks 12 and 26 of treatment with testosterone or placebo. Serum samples first were processed by supported liquid extraction. In brief, a 100- $\mu$ L aliquot of serum was mixed with 100  $\mu$ L of internal standard deuterated steroids (C/D/N Isotopes, Pointe-Claire, Quebec, Canada, and

Sigma-Aldrich, St. Louis, MO) at known concentrations (ratio of methanol to water, 4:6), and 280  $\mu\text{L}$  of deionized water and loaded on ISOLUTE supported liquid extraction columns (Biotage, Charlotte, NC). The columns were then washed twice with 0.7 mL of methyl-*tert*-butyl ether. The eluent was concentrated under nitrogen and the dried extract was reconstituted with 100  $\mu\text{L}$  of methanol and deionized water at a 4:6 ratio and transferred to a 0.25-mL vial insert.

Steroid levels were measured as previously described [3]. Briefly, 10- $\mu\text{L}$  samples were injected via autosampler and resolved with a two-dimensional chromatography method via a C4 column (10  $\times$  2.1 mm; Restek, Bellefonte, PA) on an Agilent 1260 binary pump HPLC, and a Kinetex biphenyl column (50  $\times$  2.1 mm, 2.5  $\mu\text{m}$  particle size; Phenomenex, Torrance, CA) on an Agilent 1290 binary pump, using gradient elution with 0.2 mmol/L ammonium fluoride and methanol. The column effluent was directed into the source of an Agilent 6495 triple quadrupole mass spectrometer using electrospray ionization in positive ionization mode and analyzed using multiple reaction monitoring mode. Quantitation was accomplished by comparing ion currents for the monitored ions with 13-point quadratic external calibration curves (minimum  $R^2$ , 0.995) and corrected for recovery of internal standards using ChemStation and MassHunter software (Agilent). The assay limits of detection and limits of quantification, respectively, for the key androgens were as follows: testosterone, 0.019 nmol/L and 0.049 nmol/L; androstenedione, 0.01 nmol/L and 0.024 nmol/L; 11OHA4, 0.238 nmol/L and 0.569 nmol/L; 11KA4, 0.054 nmol/L and 0.135 nmol/L; 11OHT, 0.073 nmol/L and 0.183 nmol/L; and 11KT, 0.034 nmol/L and 0.085 nmol/L.

### C. Statistical Analysis

Descriptive statistics were used to characterize the women whose data were included in this analysis and to describe the steroid hormone levels at baseline. Spearman rank coefficients were generated to assess the association between the different steroid hormone levels at baseline ( $n = 73$ ). Box-and-whisker plots were generated to show the distribution of each hormone in women in the treatment and placebo groups separately, at baseline, week 12, and week 26. Mixed ANOVA was used to investigate statistically significant ( $P < 0.05$ ) differences in each hormone level between groups, between times, and for a group  $\times$  time interaction. For this analysis, the hormone results were log transformed to normalize the distribution of data. Visually, it appeared that women maintained consistent levels of each hormone throughout the study, such that the same women were ranked in the upper part of the range at each time point (and the same women were ranked in the lower part of the range of results at each time point). This was tested by conducting a rank correlation of values for each hormone at baseline and at week 26, performed separately for women in the placebo- and testosterone-treated groups. Analysis was performed using SPSS Statistics for Windows, version 25.0 (IBM, Armonk, NY) and Stata Statistical Software, release 12 (StataCorp. College Station, TX).

## 2. Results

Of the 92 women who were randomly assigned and received study medication, stored serum was available for analysis for 73 women ( $n = 32$  randomly assigned to testosterone gel treatment;  $n = 41$  randomly assigned to receive placebo). Their median age was 60 (range, 55 to 65) years, and mean BMI was 25.9 (SD 4.1)  $\text{kg}/\text{m}^2$  (Table 1). Baseline concentrations of the androgens of interest are listed in Table 2. Of the androgens measured, the most abundant was 11OHA4. The median levels (range) of androstenedione and 11OHA4 were 1.38 (0.52 to 2.92) nmol/L and 6.46 (1.51 to 23.82) nmol/L, respectively, and for testosterone and 11KT were 0.56 (0.23 to 1.48) nmol/L and 0.85 (0.25 to 2.86) nmol/L, respectively. 11OHT was most closely correlated with 11KT (Spearman rank correlation coefficient, 0.789;  $P < 0.001$ ) and 11OHA4 was most closely correlated with 11KA4 (Spearman rank correlation coefficient, 0.733;  $P < 0.001$ ). Correlations between BMI and each of 11OHA4 and testosterone approached significance ( $P = 0.06$ ).

**Table 1. Characteristics of Study Participants**

Variable	Baseline Data (N = 73)
Age, median (IQR); range, y	60.0 (57–63); 55–65
Time since last menstrual period, median (IQR); range, y <sup>a</sup>	10.0 (5–12); 1–30
Weight, kg	68.7 (11.3)
BMI, kg/m <sup>2</sup>	25.9 (4.1)
Systolic blood pressure, mm Hg	129 (13)
Diastolic blood pressure, mm Hg	80 (10)

Data given as mean (SD) unless otherwise indicated.

Abbreviation: IQR, interquartile range.

<sup>a</sup>Data missing for two women.

### A. Impact of Exogenous Testosterone on Circulating 11-Oxygenated C19 Steroid Levels

Treatment with exogenous testosterone resulted in a significant increase in serum testosterone level in the treated group vs placebo (values at 26 weeks [SD]: 1.89 [0.99] nmol/L [54.5 ng/dL (28.5)] vs 0.60 [0.24] nmol/L [17.41 (6.9) ng/dL], respectively;  $P < 0.001$ ; Fig. 1). With testosterone therapy, statistically significant differences were seen for time ( $P < 0.001$ ), treatment group ( $P < 0.001$ ), and the treatment group  $\times$  time interaction variable ( $P < 0.001$ ) in the mixed ANOVA analysis. Treatment with exogenous testosterone was not associated with a statistically significant change in any other measured steroid. In the rank correlation analysis for each of the six hormones (*i.e.*, testosterone, androstenedione, 11OHT, 11KT, 11OHA4, and 11KA4) at weeks 0 and 26, the correlations were all statistically significant at the 5% level except for testosterone in the active treatment group and androstenedione in the placebo group (Table 3).

## 3. Discussion

The results of this study demonstrate that 11-oxygenated C19 steroids comprise a significant proportion of the circulating pool of androgens in the postreproductive years. As in premenopausal women, 11OHA4 was the most abundant of the 11-oxygenated C19 steroids, with levels four- to five-fold greater than those of androstenedione. An important finding is that exogenous testosterone does not provide a source for additional 11OHT or 11KT production, nor does it suppress their synthesis.

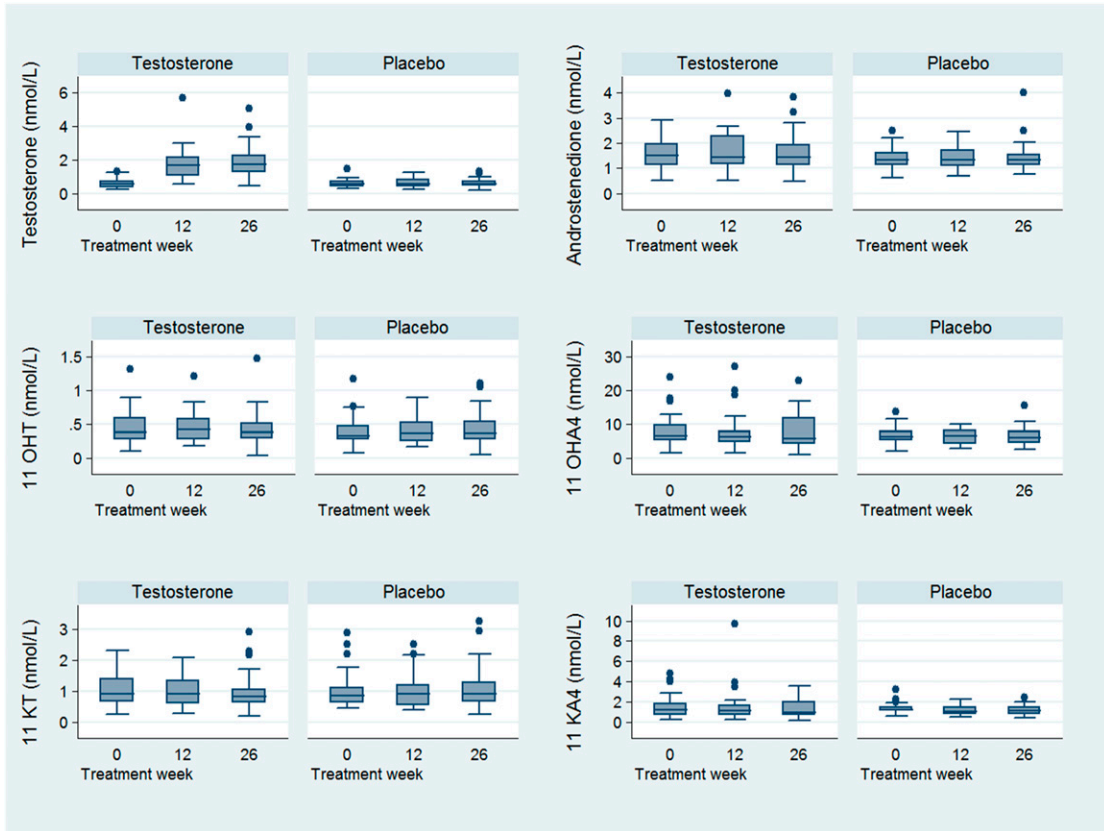
Consistent with 11OHA4 being the precursor for 11KA4, circulating concentrations of these steroids were closely correlated. Both 11KA4 and 11OHT are precursors for peripheral

**Table 2. Serum Concentrations of Unconjugated 19-Carbon Steroid Levels in 73 Postmenopausal Women in Baseline Samples**

Steroid	Mean	SD	Median	Percentile			
				10th	90th	Minimum	Maximum
Testosterone	0.61 (17.51)	0.26 (7.50)	0.56 (16.24)	0.35 (10.16)	0.95 (27.29)	0.23 (6.64)	1.48 (42.58)
A4	1.48 (42.33)	0.50 (14.20)	1.38 (39.41)	1.00 (28.53)	2.21 (63.15)	0.52 (14.95)	2.92 (83.53)
11OHA4	7.20 (218.05)	3.69 (111.87)	6.46 (195.86)	3.74 (113.39)	12.01 (364.05)	1.51 (45.67)	23.82 (721.67)
11KA4	1.41 (42.43)	0.90 (26.90)	1.17 (35.18)	0.66 (19.77)	2.28 (68.47)	0.26 (7.91)	4.78 (143.67)
11OHT	0.42 (12.79)	0.23 (6.97)	0.35 (10.68)	0.21 (6.33)	0.75 (22.72)	0.08 (2.40)	1.31 (39.96)
11KT	1.02 (30.85)	0.52 (15.75)	0.85 (25.74)	0.54 (16.30)	1.66 (50.23)	0.25 (7.63)	2.86 (86.43)

Hormone levels reported as nmol/L (ng/dL).

Abbreviation: A4, androstenedione.



**Figure 1.** Box-and-whisker plots for testosterone, 11OHT, 11KT, androstenedione, 11OHA4, and 11KA4 levels at 0, 12, and 26 weeks of treatment with transdermal testosterone or placebo. The box represents the interquartile range (IQR), the line in the box is the median. The whiskers extend to the upper and lower adjacent values. The upper adjacent value is defined as the largest data point not greater than the 75th percentile + 1.5 × IQR. The lower adjacent value is defined as the smallest data point no less than the 25th percentile - 1.5 × IQR. Outliers are any values beyond the whiskers.

11KT biosynthesis [13]. The closer correlation between 11OHT and 11KT suggests that 11OHT, not 11KA4, is the primary precursor for 11KT in postmenopausal women. Because testosterone is the precursor for 11OHT biosynthesis, we were interested in whether the

**Table 3. Spearman Rank Correlation Coefficients for the Steroid Hormone Levels and BMI (N = 73)**

	Testosterone	11OHT	11KT	A4	11OHA4	11KA4	BMI
Testosterone	1	0.095	0.083	0.376	0.139	0.108	0.219
<i>P</i> value		0.424	0.484	<0.001	0.241	0.362	0.063
11OHT	0.095	1	0.789	0.588	0.554	0.261	0.133
<i>P</i> value	0.424		0.001	0.001	0.001	0.026	0.263
11KT	0.083	0.789	1	0.545	0.529	0.414	0.105
<i>P</i> value	0.484	0.001		0.001	0.001	0.001	0.376
A4	0.376	0.588	0.545	1	0.399	0.125	0.135
<i>P</i> value	0.001	0.001	0.001		0.001	0.293	0.253
11OHA4	0.139	0.554	0.529	0.399	1	0.733	0.221
<i>P</i> value	0.241	0.001	0.001	0.001		0.001	0.060
11KA4	0.108	0.261	0.414	0.125	0.733	1	0.061
<i>P</i> value	0.362	0.026	0.001	0.293	0.001		0.605
BMI	0.219	0.133	0.105	0.135	0.221	0.061	1
<i>P</i> value	0.063	0.263	0.376	0.253	0.060	0.605	

Abbreviation: A4, androstenedione.



administration of exogenous testosterone influences 11OHT or 11KT levels. This was not the case, because the only steroid that increased with transdermal testosterone therapy was testosterone. Thus, in postmenopausal women, there does not appear to be significant peripheral biosynthesis of 11OHT from extra-adrenal testosterone. This finding is consistent with previous data from adult men and women showing that despite large sex differences in testosterone levels, peripheral 11OHT and 11KT levels were similar between sexes before and after cosyntropin stimulation [4]. This study also provides safety information pertaining to the use of testosterone therapy for women, in that exogenous testosterone does not change endogenous androgen synthesis and metabolism.

The correlations between hormone levels at baseline and week 26 indicate that women tended to have consistent levels of each of these hormones, except for testosterone in the active treatment group and androstenedione in the placebo group. For women treated with testosterone, it was expected that testosterone levels at 26 weeks would not be predicted by, and hence correlated with, baseline levels, because of variable absorption and timing of dosing related to collection of blood samples. The lack of correlation between androstenedione levels at baseline and week 26 in the placebo group is most likely a chance finding.

The clinical importance of the 11-oxygenated androgens in postmenopausal women is yet to be elucidated. The androgenic activity of each of testosterone and DHT is influenced by the extent to which testosterone is aromatized to estradiol in target tissues and potentially by its high binding affinity to SHBG, such that in women, only 1% to 2% of the circulating fraction is not SHBG or albumin bound [14]. It remains unclear whether 11KT is aromatizable and thus a substrate for estrogen biosynthesis. The binding affinities of 11KT and 11DHT to SHBG have not been determined; thus, the tissue bioavailability of these hormones remains to be established. In addition, the target tissue effects of these steroids are modulated at the cellular level by the relative levels of the enzymes 11 $\beta$ -hydroxysteroid dehydrogenase types 1 and 2. This is because within cells, 11 $\beta$ -hydroxysteroid dehydrogenase type 2 catalyzes the pathway to 11KT production, whereas 11 $\beta$ -hydroxysteroid dehydrogenase type 1 reduces (*i.e.*, inactivates) 11KA4 and 11KT [13].

The adrenals appear to be the primary source of 11OHT and 11OHA4 [13]. 11KT is believed to be produced primarily in peripheral tissues from both 11OHT and 11KA4 [15–17]. It has been suggested that the ovaries may also be a source of 11KT during the reproductive years, but this needs confirmation [18]. Peripheral concentrations of 11KT are much greater than those of testosterone during adrenarche, in premature adrenarche, and in polycystic ovary syndrome [3, 6, 19]. The serum concentrations of 11KT in postmenopausal women in our study were similar to those of testosterone. Studies of the impact of natural and surgical menopause on peripheral levels of 11OHT, 11KA4, and 11KT are needed to determine whether the ovaries are an important source of these steroids. In addition, documentation of levels by decade of age will inform whether there is a decline in these steroids with age, as seen for DHEA, androstenedione, and testosterone [7].

The associations between ACTH and the steroids we have measured have been reported by Rege *et al.* [1] and Turcu *et al.* [4], who found that acute ACTH stimulation increases adrenal preandrogen production. Peripheral 11KT and DHT did not increase with ACTH stimulation. Overall, however, available data show that adrenal androgens are not simply produced in response to ACTH. Circulating DHEA and DHEAS levels may be normal or suppressed in acute stress [20], severe systemic illness [21], anorexia nervosa [22], and Cushing syndrome [23], which are otherwise characterized by elevated cortisol levels. Consequently, the feedback mechanism that controls adrenal preandrogen and androgen production remains elusive.

Strengths of this study include the measurement of the steroids of interest by liquid chromatography–tandem mass spectrometry in healthy, postmenopausal women not using hormone therapy, and the opportunity to investigate the impact of transdermal testosterone therapy on the steroid levels. The ability to explore associations between the steroids measured and BMI was limited by having excluded from the study underweight women and women with a BMI >32 kg/m<sup>2</sup>. Thus, our findings apply to normal to overweight, healthy,

postmenopausal women. We also did not measure levels of the potent androgens DHT or 11-ketodihydrotestosterone.

In summary, 11-oxygenated derivatives of androstenedione and testosterone make an important contribution to the total circulating androgen pool in postmenopausal women, and their biosynthesis does not appear to be modulated by exogenous testosterone therapy. Additional research to elucidate the roles of these little-studied steroids offers the possibility to broaden and deepen our understanding of androgen action and conditions of androgen depletion and excess in postmenopausal women.

## Acknowledgments

**Financial Support:** This study was supported by a research grant from the Judith Jane Mason & Harold Stannett Williams Memorial Foundation to S.R.D. and an unrestricted grant to S.R.D. from BioSante Pharmaceuticals USA, which also provided study medication. S.R.D. is an Australian National Health and Medical Research Council Senior Principal Research Fellow (Grant 1135843). The study sponsor was Monash University. A.F.T. was supported by Grant 1K08DK109116 from the National Institutes of Health (NIH).

Neither BioSante Pharmaceuticals nor the Judith Jane Mason & Harold Stannett Williams Memorial Foundation had any role in study design; data collection, analysis, or interpretation; writing of the report; or in the decision to submit the paper for publication.

**Clinical Trial Information:** Australian New Zealand Clinical Trials Registry no. ACTRN12609000981224 (registered 13 November 2009).

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**Disclosure Summary:** S.R.D. has received honoraria from Besins Healthcare and Pfizer Australia, and has been a consultant to Mayne Pharmaceuticals, Lawley Pharmaceuticals, and Que Oncology. She is an Investigator for Que Oncology. The remaining authors have nothing to disclose.

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