Journal of
Steroids & Hormonal Science

Research Article **Open Access**

Tobacco Smoke Exposure, C-reactive Protein and Steroid Hormones Measured by Tandem Mass Spectrometry in Healthy Women

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Abstract

Introduction: High sensitivity C-reactive protein (hsCRP) is a reliable biomarker of inflammation. Plasma CRP increases dramatically after severe trauma, bacterial infection and inflammation. The hsCRP accurately and sensitively measures basal levels of CRP, including in the areas of increased risk of developing myocardial infarction. Tobacco smoking has also been indicated to influence pregnancy outcomes and infertility.

Methods: We examined the associations between smoking, circulating hsCRP, and steroid hormone levels in healthy, non-pregnant women of reproductive age. Serum hsCRP concentrations were analyzed using immunoturbidimetry. Based on cotinine levels and self-questionnairres, the women were divided into three separate groups of smokers, non-smokers and passive smokers (secondhand exposure). Steroids and cotinine levels were measured by isotope dilution tandem mass spectrometry.

Results: A significant, positive correlation was observed between hsCRP and cotinine levels indicating an association between cigarette smoking and inflammation. However, no association was found between hsCRP and cotinine levels in both the non-smoker and passive smoker group. Also, hsCRP levels were significantly associated with increased BMI scores.

Conclusions: Combined factors of increased smoke exposure and obesity were significantly correlated with increased hsCRP levels, suggesting that multiple conditions confer additive risk to an inflammatory state. To determine the role of inflammation in women's health, further studies are essential to determine the interacting relationship between hsCRP and sex hormone levels in women with disease.

Introduction

Tobacco smoking and secondhand smoke exposure have been identified as agents that cause cardiovascular disease and precipitate cancer development. Smoke exposure, particularly for women, heavily influences pregnancy outcomes and is associated with infertility, preterm delivery, stillbirth, low birth weight, and sudden infant death syndrome.

The association between smoking and inflammation has been examined mainly in the context of cardiovascular disease. It was demonstrated that cigarette tobacco smoke causes direct vascular injury, subsequently leading to an immunological response [1]. These immunological responses involve the release of potent cytokines that stimulate hepatic synthesis of acute phase reactants, proteins that are thought to be anti-infective, anti-proliferative, and pro-coagulative.

In the mid-1990s, more sensitive methods for the measurement of CRP were introduced. These methods, referred to as hs-CRP, can accurately measure basal levels of CRP throughout the currently accepted cardiovascular risk assessment range (0.20-10.0 mg/L). The hs-CRP assays were used to assess outcomes in patients with unstable angina and showed that hs-CRP values in the upper tertile (>3.0 mg/L) were associated with increased risk of developing myocardial infarction [1,2].

Much attention has been given to CRP as a potential biomarker for pathologies involving inflammation. As one of the first reactants released into plasma, its measurement has been considered a classic indicator for the acute phase response in inflammation. CRP is synthesized in the liver in response to interleukin (IL)-6, IL-1, and tumor necrosis factor (TNF) released from the inflamed tissue. It plays a key role in activating the complement system, inducing adhesion molecule expression, enhancing phagocytosis and macrophage and leukocyte activation and opsonization [1]. CRP levels may directly reflect the expression of an IL-6 polymorphic genotype [1]. Further, studies in monozygotic twins demonstrate that CRP is related to total and central obesity, blood pressure, and lipid profile independent of genetics [3].

CRP is easily and inexpensively measured compared to other inflammatory markers, making it an attractive candidate as a prognostic marker for inflammation. The high sensitivity CRP assay (hsCRP) can detect serum CRP concentrations in the range of 0 to 2 mg/L. Normal CRP levels are defined as < 10 mg/L. Individuals with no disease exhibit CRP levels $\leq$ 1 mg/L [1]. CRP assays have been used to detect and monitor inflammation in conditions such as rheumatoid

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arthritis, polymyalgia rheumatica, inflammatory bowel disease, and temporal arthritis. Physiologically, CRP levels are elevated during the third trimester of pregnancy, and in patients taking oral contraceptives, hormone replacement therapies, and during menopause. Elevated levels are also seen in obesity [1]. In children of 3-16 years of age, CRP levels were demonstrated to be higher in those of low socioeconomic status, attributable to recent illness or increased adiposity in these children [4].

Whereas high sensitivity CRP (hsCRP) assays have been used to detect and monitor inflammatory conditions such as rheumatoid arthritis, polymyalgia rheumatica, and inflammatory bowel disease, there still remains ambiguity regarding its use as a prognostic marker for other diseases that exhibit downstream inflammatory effects, such as atherosclerosis or obesity. Many contemporary studies suggest that increased adipose tissue induces a chronic and sub-acute inflammatory state. Whether or not these individuals exhibit higher hsCRP levels has not yet been shown. Additionally, hsCRP levels in the context of abnormal serum hormone levels have not been adequately explored. Data elucidating the role of inflammation in reproductive health can contribute to our understanding of how inflammation might be indicative of stress, infertility, and complications in pregnancy related to abnormal hormone levels.

While an inflammatory state has been shown to be associated with atherosclerosis and cardiovascular disease, studies examining the relationship between CRP and cardiovascular risk factors, such as obesity and smoking habits, remain uncertain. Our objective was to examine the association between serum hsCRP concentrations and serum cotinine, a long-lived nicotine metabolite and classic indicator of smoking status. Additionally, we examined the associations between serum hsCRP levels, body mass index, and serum hormone levels, exploring a mechanism for the harmful effects of smoking on infertility, and complications in pregnancy through inflammation and certain steroid hormones [5].

Methods

Subjects

This is a cross-sectional, three-arm study on adult women. The study was approved by the Georgetown University Medical Center Institutional Review Board (IRB). Blood samples were collected from a total of 325 healthy, non-pregnant women. 293 women of reproductive age (18-45 years) completed all study requirements and are included. Exclusion criteria included illness, infection, immunological or endocrine disorders diagnosed within six months prior to blood draw, as well as surgery with general anesthesia within the prior three months. Women having used birth control pills, steroids, or immunosuppressive medications within the previous two months were excluded. Participants that were male, post-menopausal, or did not reside in Virginia (Arlington, Alexandria, Fairfax, Loudoun), Maryland, or the District of Columbia were also excluded from the study. A summary of subject demographics is presented in Table 1.

Study participants were identified as non-smokers, passive smokers (secondhand smoke (SHS) exposure [6]) and as active smokers. The smoking status of these three groups was defined using: (1) serum cotinine levels (2) self-reported identification and (3) a combination of serum cotinine and self-reported identification. Cotinine, a stable metabolite of nicotine and with a half-life of 18-24 hours, was used as the biomarker for cigarette smoke exposure. Using these definitions the women were divided to the 3 smoking groups. First, subjects defined themselves as active smokers if they had smoked at least one cigarette in the previous five days. Passive smokers were defined as subjects who reported to have been regularly exposed to SHS (lived or worked with a smoker on a daily basis) within the previous six months. Non-smokers were subjects who had not smoked or been exposed to SHS within the previous six months. Smoking history was also collected using a validated smoking questionnaire described below. Serum cotinine analysis of the samples was conducted at the laboratory medicine Clinical Chemistry section at Children's Hospital Boston.

Questionnaire

All eligible study participants completed a structured, in-person interview and questionnaire conducted by a trained clinical research assistant dedicated to this study. The smoking questionnaire is based on the U.S. National Institutes of Health's National Cancer Institute Transdisciplinary Tobacco use Research centers (TTURC) questionnaire. Information collected included detailed medical history, reproductive health, tobacco smoke exposure history and use, dietary habits, and lifestyle. Each of the study participants also provided blood samples at the time corresponding to the follicular stage of her cycle. Subjects were instructed to make their appointments two to three days after the beginning of their menstrual flow. To minimize diurnal variation, blood samples were drawn in the morning hours between 7:00-11:30am. Blood was collected in two 10-mL red top vaccutainer tubes without serum separators (for LC/MS/MS and immunoassay hormone analysis), processed, serum separated into multiple labeled (ID number only) aliquots and frozen at -80ºC until analysis.

Study Demographic Data					
	Total	**Active smokers**	**Passive smokers**	**Non-smokers**	
Characteristic	(n=293)	(n=107)	(n=86)	(n=100)	P*
Age (years)					
< 28	29.1	29.7	27.4	29.9	
28-37	23.4	27.7	17.8	23.7	0.69
38-47	23.4	21.8	25	23.7	
> 47	24.1	20.8	29.8	22.7	
Race/Ethnicity					
White	46.5	51.5	34.1	51.5	
Black	40.8	37.9	47.6	38.1	<0.01
Asian	5.7	3.9	3.7	9.3	
Mixed/Other	7	6.7	14.6	1.1	
Education level					
< high school	3.7	2.8	5.9	3	
High School	19.1	16.8	20	20.2	
Some college	25.2	30.8	28.2	17.2	0.32
College/technical	32.2	31.8	24.7	39.4	
Professional	19.8	17.8	21.2	20.2	
Household income					
< 10,000	30.7	32	31	29	
10,000-30,000	17.8	15.5	19	19	
30,000-60,000	33.2	35	35.7	29	0.54
60,000-90,000	7	9.7	3.6	7	
> 90,000	11.5	7.8	10.7	16	
Marital status					
Single	73.7	81.3	69.8	72	
Married	13	5.6	15.1	17	
Has partner	4.3	7.5	3.5	0	<0.01
Divorced/ separated/ widowed	9	5.6	11.6	11	
*p values from chi-square test					

Table 1: Distribution of Data on study characteristics by smoking status on the day the serum was obtained.

Steroid hormone analysis

Steroid hormones analyses were conducted at the Bioanalytical Core Laboratory at Georgetown University Medical Center using isotope dilution LC/MS/MS API-5000 (Applied Biosystems, CA) equipped with an atmospheric pressure photoionization source and deuterium-labeled internal standards as described previously [7,8]. The detection limit was defined as the concentration two standard deviations above the response at zero doses. The sensitivity of each assay, within run (intra-assay) and between run (inter-assay) imprecision, as well as the coefficients of variation (% CV) for replicate quality control samples averaged for different concentrations of steroids were low and dependent on analyte concentrations (4-12%). These are now standard and are conducted routinely at the Bioanalytical Core Laboratory at Georgetown University Medical Center. The measurement of estradiol

was precise and accurate down to 1 pg/mL. Eleven steroid hormones were measured in one profile: four androgens: androstenedione (AE), dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS) and testosterone (T, testosterone), the corticosteroids (aldosterone (A), 11-deoxycortisol (DOC), and cortisol (F), progestin 17-β-hydroxyprogesterone (17OHP), and estrogens 17-β-estradiol (E_2) and estriol (E_3). Luteal hormone (LH) and follicle-stimulating hormone (FSH) concentrations were measured using immunoassays to confirm that each subject was in the follicular phase of her menstrual cycle at the time of the blood draw (follicular phase: LH <20 μIU/mL and FSH 2.5-10.2 μIU/mL).

CRP analysis

CRP concentrations were determined using an immunoturbidimetric high sensitivity CRP (hsCRP) assay on the Roche P Modular system (Roche Diagnostics, Indianapolis, IN), using reagents and calibrators from DiaSorin (Stillwater, MN). Analysis was conducted at the clinical and epidemiological research laboratory of Children's Hospital Boston Department of Laboratory Medicine. Serum CRP concentrations in healthy adults are usually lower than 10 mg/L, slightly increasing with age. CRP levels are slightly higher in late pregnancy, viral infections
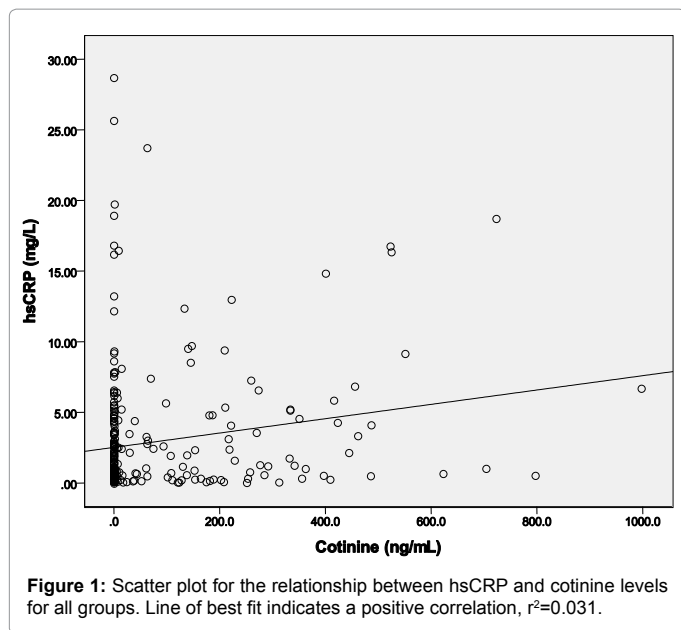
Cotinine		N=	Mean	Std. Deviation
Cotinine (ng/mL)	Nonsmokers	100	0.5	0
	Passive smokers	86	1.9	3.3
	Smokers	107	207.5	193.1

Table 2: Serum Cotinine Concentrations for the three smokers groups.

Steroid Hormone		N	Mean	Std. Deviation	Normal* range
DHEAS (ug/dL)	Nonsmokers	100	144.7	52.5	10.0-237.0
	Passive smokers	86	145.5	75.4	
	Smokers	107	137.7	62.7	
Aldosterone (ng/dL)	Nonsmokers	100	43.5	60.8	1.0-32.0
	Passive smokers	86	19.8	49.1	
	Smokers	107	24.6	43.3	
Cortisol (ug/dL)	Nonsmokers	100	13.6	7.8	2.4-28.6
	Passive smokers	86	12	6.9	
	Smokers	107	11	6.1	
11-Deoxycortisol (ug/dL)	Nonsmokers	100	0.0198	0.0345	0.012-0.050
	Passive smokers	86	0.0129	0.0122	
	Smokers	107	0.0141	0.0127	
Androstenedione (ng/dL)	Nonsmokers	100	79.3	47.1	50.0-250.0
	Passive smokers	86	69.4	44.3	
	Smokers	107	63.7	35.9	
Testosterone (ng/dL)	Nonsmokers	100	29.6	17.7	16.0-50.0
	Passive smokers	86	37.7	64.3	
	Smokers	107	32	54.3	
17-OH progesterone (ng/dL)	Nonsmokers	100	55	65	3.0-91.0
	Passive smokers	86	54.1	53.4	
	Smokers	107	47.8	49.3	
DHEA (ng/dL)	Nonsmokers	100	337.3	239.6	130.0-980.0
	Passive smokers	86	311	267.3	
	Smokers	107	301.5	249.3	
Progesterone (ng/mL)	Nonsmokers	100	150.2	413.4	10.0-950.0
	Passive smokers	86	42.9	198.1	
	Smokers	107	90.9	281.6	
Estrone (pg/mL)	Nonsmokers	100	63.9	59.8	29.0-105.0
	Passive smokers	86	63	55	
	Smokers	107	53	35.6	
Estradiol (pg/mL)	Nonsmokers	100	80.4	92.1	34.4-170.2
	Passive smokers	86	79.8	152.3	
	Smokers	107	72.6	94.4	
SHBG (nmol/L)	Nonsmokers	100	64.3	40.2	9.3-75.2
	Passive smokers	86	62	40.3	
	Smokers	107	68.6	46.8	

* Normal reference intervals for females older than 18 y

Table 3: Serum Hormone Concentrations in the three smokers groups.

Figure 1: Scatter plot for the relationship between hsCRP and cotinine levels for all groups. Line of best fit indicates a positive correlation, $r^2=0.031$.

and mild inflammation (10–40 mg/L), active inflammation, bacterial infection (40–200 mg/L), and in severe burns and bacterial infections (>200 mg/L) [9].

Statistical analysis

Sample characteristics were presented as frequencies and percentages for categorical data. Scatter plots were constructed to obtain a visual impression of the association between two continuous variables. Chi-square tests and correlation coefficients were computed to assess the association between categorical variables and continuous variables, respectively. Analysis was conducted in SPSS version 19 for Windows. All statistical tests were two-sided and significance was judged at 0.05 levels.

Results

Serum cotinine and hormone concentrations are detailed in Tables 2 and 3.

Figure 1 illustrates a positive correlation between hsCRP levels and cotinine levels. There was a significant, positive correlation between hsCRP and cotinine levels in the active smokers group. Cotinine explained about 6% of the variance seen in hsCRP levels in this group. Moreover, in all groups, serum cotinine levels explain approximately 3% of the variance seen in hsCRP levels and indicated a significant predictor of hsCRP concentrations. When examining hsCRP levels within each of the three reclassified groups (non-, passive, and active smokers), however, no association was found between hsCRP and cotinine levels in both the non-smoker and passive smoker group.

hsCRP levels were significantly associated with increased BMI scores. BMI appeared to be a significant predictor of hsCRP after adjusting for age, explaining 15% of the variance seen in hsCRP levels. Stratified analysis by BMI indicated differences in mean hsCRP levels among normal (≤25), overweight (25-29.99), and obese (≥30) BMI categories. Significant differences were seen between the normal and obese groups and between the overweight and obese groups.

When classifying subjects into one of three BMI groups—normal (BMI < 25), overweight (25-30), and obese (>30)—there were no

significant associations between hsCRP levels and a combination of the reclassified smoking status groups and the BMI groups. In addition, among individuals who were non-smokers there was a negative correlation between hsCRP levels and obesity (BMI > 30). A negative correlation was also observed between hsCRP levels and overweight status (BMI 25-30) in the reclassified passive smoker group. All other combinations yielded positive correlations.

One-way ANOVA indicates significant differences among the three smoking groups with combined risk factors of smoke exposure and obesity. Of note, mean hsCRP levels were significantly elevated in obese smokers compared to both non-obese non-smoker and non-obese smoker groups.

Correlation studies between hsCRP levels and cortisol, aldosterone, estrone, progesterone, and SHBG showed no significant associations between these serum hormones and hsCRP levels.

Discussion

Cigarette tobacco smoking is the second largest preventable public health cause of mortality and morbidity worldwide. Exposure to tobacco smoke is known to cause coronary heart disease, atherosclerosis and ischemic disease. The degree of this risk is proportional to cigarette tobacco smoke exposure, and it varies from individual to individual because of between-individual differences in genetic differences and other environmental exposures and behaviors. While the chemical properties of the tobacco smoke are relatively well characterized, the mechanisms by which smoking leads to disease and the genetic factors that determine susceptibility to these diseases are not well understood. Since smoking induces an inflammatory state, elevated CRP levels have been associated with smoking-related diseases. Levels of serum cotinine, a metabolite of nicotine, have been positively associated with CRP among smokers and non-smokers [10]. In adult smokers receiving 3.0-6.9 mg tar delivery per cigarette, hsCRP levels were greater than in non-smoking adults over a six-week period and proved to discriminate between the smoker and non-smoker groups with high sensitivity and specificity [6]. Individuals merely exposed to smoke also exhibit higher levels of CRP and increased risk for CVD-related death [11]. Lifetime secondhand smoke exposure appears to continually impact CRP levels.

Our results align well with recent studies exploring the relationship between elevated CRP levels and smoking-related diseases. We confirm the finding that tobacco smoke exposure is associated with increased levels of hsCRP. Cotinine, a nicotine metabolite with a half-life of 20 hours, has consistently been used as a valid indicator of cigarette smoke exposure among the tobacco researchers. A recent study by Benowitz et al. utilized cotinine to screen hospitalized patients and found that of individuals who denied smoking, 32% were found to have had significant smoke exposure [12]. Thus, cotinine is particularly useful in assessing secondhand smoke exposure and together with self-reported data, was used to represent smoking status in this study.

Blood concentrations of CRP are determined only by its rate of production since CRP clearance rate is constant. Following tissue insult, serum CRP levels will rise within 4-6 hours, increase exponentially within 24-48 hours and fall to normal levels only when tissue structure and function have been fully re-established [4]. In theory, this suggests that while CRP may be an adequate indicator of current injury, its presence in serum, when measured at a single point in time, cannot convey much regarding the chronicity of inflammation. However, recent studies have demonstrated that even after complete cessation of smoking, CRP levels remain high and do not fall to normal immediately, indicating that the tissue damage continues to provide an inflammatory

stimulus [13]. In 152 smokers with CVD, hsCRP levels did not change with reduced smoking, nicotine-replacement therapy, and counseling six months later, further highlighting the prolonged effects smoking has on inflammatory response [14].

Previous studies have demonstrated that the number of cigarettes might be unrelated to the elevated levels of CRP seen in smokers (Summarized in Table 3). Ohsawa et al. found that CRP levels were elevated in all smokers, regardless of the number of cigarettes smoked per day, and that a longer smoking cessation period was associated with lower levels of CRP in past smokers [15]. Similarly, Asthana et al. studied leukocyte counts and CRP levels in active smokers and found positive correlations between smoking intensity and leukocyte counts but not with CRP levels [16]. This may suggest that smoking causes a substantial inflammatory response independent of nicotine dose. Further studies should focus on cigarette dosage to better elucidate these findings.

While a poor indicator of fat mass and distribution, BMI appeared to be positively associated with hsCRP levels in our study. This was true when examining each of the reclassified smoking groups individually. Previous studies have not only shown elevated levels of hsCRP in obesity but have also demonstrated sex differences in hsCRP levels and body fat [17]. The quantity and distribution of fat (truncal versus lower body) appear to influence CRP levels more strongly in women than in men. Levels of SHBG have been negatively associated with levels of CRP in postmenopausal women [18], and this relationship between SHBG and CRP has been demonstrated to be stronger in women with leaner body mass [19]. It is possible that various hormones may mediate the effects of obesity and inflammation in men and women. In an epidemiological study using fractional polynomials to identify the dose/response relationship between hsCRP levels and indicators of adiposity, it was found that the sex difference seen in hsCRP levels and adiposity disappears when adiposity is adjusted for leptin [20].

Importantly, combined factors of smoke exposure and obesity were shown in this study to be significantly correlated with increased hsCRP levels, suggesting that multiple conditions might confer additive risk to an inflammatory state. Matthews et al. found that women with sleep disordered breathing exhibited elevated levels of inflammatory and coagulation biomarkers (CRP, PAI, fibrinogen, VIIc) [21]. Increased adiposity, increased immunological responses to environmental toxins, i.e. tobacco smoke, and decreased sleep all may impose physiological stress to women. While the non-specific nature of CRP makes it difficult to identify the particular cause of its release into plasma, hsCRP levels may prove useful in obtaining a general picture of inflammation in women. It was unexpected to observe that age was not a significant predictor of hsCRP levels. The high incidence and development of chronic inflammatory conditions and atherosclerotic changes in older age was thought to potentially contribute to a positive correlation between age and hsCRP levels.

While this study found no significant correlations between hsCRP levels and serum hormone levels, there was a dose-dependent effect of smoke exposure associated with levels of serum estrone, estriol, estradiol, and 16-OHE1. Smoking appears to decrease levels of aromatase in granulosa cells, resulting in an anti-estrogenic effect in women. The lack of correlation between serum hormone levels and hsCRP may have been simply from the fact that the subjects examined were healthy women without illness, infection, immunological, or endocrine disorders. Future studies should examine hsCRP levels in women with abnormal hormone levels, or in a population of women having had a prior miscarriage, infertility, or adverse outcome in prior pregnancy, to further identify the inter-relationships between hsCRP and sex hormone levels.

Currently, there remains some debate regarding the use of hsCRP levels as a prognostic marker for disease. It is argued that hsCRP assays may prove less helpful for patients with different chronic co-morbidities due to the broad range of diseases that possess an inflammatory component [22].

Others argue that the use of hsCRP assays can greatly improve the identification of individuals at risk for CVD, particularly if they have unremarkable low-density lipoprotein (LDL) levels yet would still benefit from statin therapy [23]. The AFCAPS/TexCAPS study demonstrated that individuals with low LDL levels yet elevated hsCRP levels still exhibited high risk for CVD, and that lovastatin therapy proved more cost-effective in patients with high CRP levels [24,25]. Similarly, the Jupiter study exhibited that lovastatin therapy in individuals with normal lipid profiles and abnormal CRP levels (> 2mg/L) lowered their risk for CVD [24].

Conclusions

Inflammation as indicated by hsCRP levels is strongly associated with increased smoke exposure and increased BMI in healthy women of reproductive age. No significant correlations were found between hsCRP levels and serum hormone levels. These studies suggest that multiple factors, endogenous and environmental, are additive in promoting an inflammatory state. Further studies examining the inter-relationships between hsCRP and sex hormone levels in women with disease are essential in determining the role of inflammation in women's health.

Declaration of Interests

The authors declare no conflict of interests.

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