

## Research Article

Smoking, Gender, and Ethnicity Predict Somatic *BRAF* Mutations in Colorectal Cancer

Laura S. Rozek<sup>1,2</sup>, Casey M. Herron<sup>3,4</sup>, Joel K. Greenson<sup>6</sup>, Victor Moreno<sup>7</sup>, Gabriel Capella<sup>8</sup>, Gad Rennert<sup>9</sup>, and Stephen B. Gruber<sup>3,4,5</sup>

## Abstract

Approximately 5% to 15% of all colorectal cancers (CRC) have an activating *BRAF* somatic mutation, which may be associated with a distinct risk profile compared with tumors without *BRAF* mutations. Here, we measured the prevalence and epidemiologic correlates of the *BRAF* V600E somatic mutation in cases collected as a part of a population-based case-control study of CRC in northern Israel. The prevalence of *BRAF* V600E was 5.0% in this population, and the mutation was more likely to be found in tumors from cases who were of Ashkenazi Jewish descent [odds ratio (OR), 1.87; 95% confidence interval (95% CI), 1.01-3.47], female (OR, 1.97;  $P = 1.17$ -3.31), and older (73.8 years versus 70.3 years;  $P < 0.001$ ). These results were similar when restricting to only tumors with microsatellite instability. Whether smoking was associated with a *BRAF* somatic mutation depended on gender. Although men were less likely to have a tumor with a *BRAF* somatic mutation, men who smoked were much more likely to have a tumor with a somatic *BRAF* mutation (OR<sub>interaction</sub>, 4.95; 95% CI, 1.18-20.83) than women who never smoked. We note the strong heterogeneity in the reported prevalence of the *BRAF* V600E mutation in studies of different ethnicities, with a lower prevalence in Israel than other Western populations but a higher prevalence among Jewish than non-Jewish Israeli cases. Epidemiologic studies of CRC should incorporate somatic characteristics to fully appreciate risk factors for this disease. *Cancer Epidemiol Biomarkers Prev*; 19(3); 838-43. ©2010 AACR.

## Introduction

The heterogeneity of colorectal carcinogenesis gives rise to distinct tumor phenotypes with corresponding differences in disease prognosis and potentially even treatment (1-6). Examination of these phenotypes in population-based studies has led to an appreciation of distinct epidemiologic risk factors corresponding to the tumor phenotypes. For example, the concordant expansion or contraction of nucleotide repeats in microsatellite markers is indicative of a microsatellite instable (MSI-high) tumor with defective mismatch repair. The MSI-high phenotype, corresponding to 10% to 20% of all colorectal cancers (CRC), is associated with epidemiologic risk factors not appreciated in studies that do

not consider MSI status of the tumor. These risk factors include female gender, estrogen withdrawal, smoking, and nonsteroidal anti-inflammatory drug (NSAID) use (6-14).

Approximately 5% to 15% of all CRCs have an activating *BRAF* somatic mutation, the vast majority of which are a substitution of glutamic acid for valine at codon 600, or V600E. These *BRAF*-mutated CRCs are composed predominantly of sporadic rather than familial tumors that often arise from a methylator pathway (15-18). *BRAF* mutations are clinically useful in distinguishing MSI-high tumors resulting from Lynch syndrome from sporadic MSI-high tumors (19-21). Studies of *BRAF* mutations in CRC find that *BRAF*-positive tumors may be associated with age, family history of CRC, female gender, and ethnicity (17, 22). Multiple studies have noted that CRC patients with *BRAF* mutations have a shorter survival than CRC patients with *BRAF* wild-type (WT) tumors (23-25), further indicating the importance of this subgroup of CRC.

To try to better understand the epidemiologic risk factors for clinically relevant subtypes of CRC, we genotyped the *BRAF* V600E somatic mutation in tumors collected as a part of the Molecular Epidemiology of Colorectal Cancer (MECC) study, a population-based study in northern Israel. We found that there is a distinct risk profile of gender, smoking, and ethnicity beyond that which is found when only considering MSI status of the tumor.

**Authors' Affiliations:** <sup>1</sup>Environmental Health Sciences, <sup>2</sup>Department of Otorhinolaryngology, <sup>3</sup>Department of Internal Medicine, <sup>4</sup>Department of Human Genetics, and <sup>5</sup>Department of Epidemiology, School of Public Health and <sup>6</sup>Department of Pathology, University of Michigan Medical School, Ann Arbor, Michigan; <sup>7</sup>Biostatistics and Bioinformatics Unit and <sup>8</sup>Translational Research Laboratory, Catalan Institute of Oncology-IDIBELL, Barcelona, Spain; and <sup>9</sup>Department of Community Medicine, Carmel Medical Center, Technion Institute of Technology, National Israeli Cancer Control Center, Haifa, Israel

**Corresponding Author:** Stephen B. Gruber, University of Michigan Medical School, 1524 BSRB, 109 Zina Pitcher, Ann Arbor, MI 48109-2200. Phone: 734-615-9712; Fax: 734-763-7672. E-mail: sgruber@umich.edu

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**Table 1.** Characteristics of samples with MSI status and BRAF genotyping

	<b>BRAF WT (n = 1,232)</b>	<b>BRAF V600E (n = 65)</b>	<b>OR (95% CI)</b>
Age (y)	70.3 (SD = 11.5)	73.8 (SD = 11.1)	<i>P</i> = 0.02
Range	21-99	45-93	
Pack-years	33.7 (SD = 28.4)	36.4 (SD = 25.7)	<i>P</i> = 0.68
Ever smoked	514 (95.5)	24 (4.5)	0.85 (0.50-1.44)
Never smoked	693 (94.8)	38 (5.2)	1.00
Ashkenazi Jewish	840 (94.2)	52 (5.8)	1.87 (1.01-3.47)
Non-Ashkenazi Jewish	392 (96.8)	13 (3.2)	1.00
Female	593 (93.4)	42 (6.6)	1.97 (1.17-3.31)
Male	639 (96.5)	23 (3.5)	1.00
HRT use	22 (93.1)	2 (6.9)	1.15 (0.26-5.10)
No HRT use	417 (92.7)	33 (7.3)	1.00
Aspirin/NSAID use	326 (95.0)	17 (5.0)	1.00 (0.56-1.77)
No aspirin/NSAID use	863 (95.0)	45 (5.0)	1.00
First-degree family history of CRC	123 (94.6)	7 (5.4)	1.08 (0.48-2.42)
No family history of CRC	1,103 (95.0)	58 (5.0)	1.00
MSI-high	106 (72.1)	41 (27.9)	18.1 (10.5-31.2)
MSS/MSI-low	1,126 (97.9)	24 (2.1)	1.00

## Materials and Methods

### Study Population

The MECC study is a population-based, matched case-control study that includes 2,126 incident CRC cases and corresponding matched controls. The MECC study participants have previously been described (26). Eligible cases include any person newly diagnosed with CRC between March 31, 1998 and April 1, 2004 in northern Israel. Eligible cases were invited to participate and interviewed. Potential controls were matched for exact year of birth, sex, and primary residence. Individuals previously diagnosed with cancer of the colorectum were not

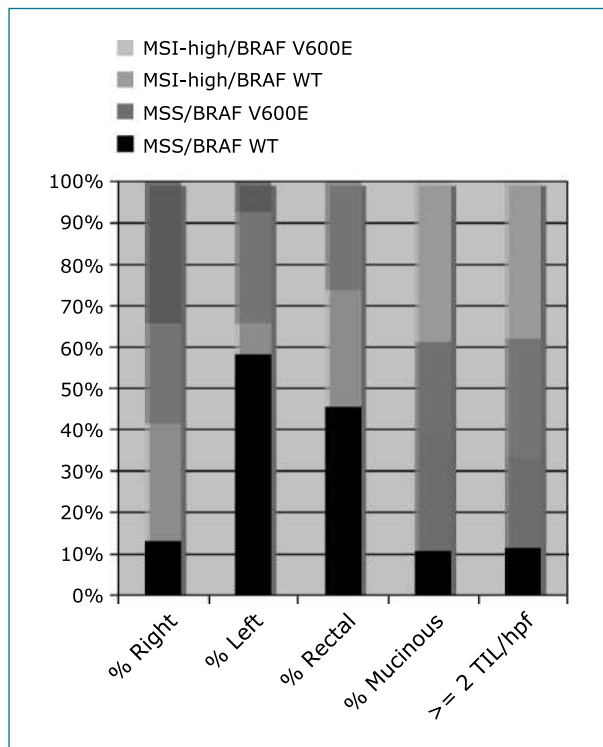
eligible to participate. The study was approved by the Institutional Review Boards at the University of Michigan and Carmel Medical Center in Haifa. Written informed consent was required for eligibility.

### DNA Extraction from Tumor Slides

DNA was extracted from tumor slides as previously described (27). Briefly, tumor DNA was microdissected from unstained, recut slides of paraffin-embedded tumors. Areas for microdissection were circled by one pathologist (J.K.G.), and the H&E-stained slide was used as a template. Following dissection from slides, xylene was added to remove paraffin and the DNA was precipitated with

**Table 2.** Characteristics of MSI-high tumors by BRAF mutation status

	<b>BRAF WT (n = 106)</b>	<b>BRAF V600E (n = 41)</b>	<b>OR (95% CI)</b>
Age (y)	68.0 (SD = 12.1)	77.7 (SD = 9.2)	<i>P</i> < 0.0001
Female	53 (65.4)	28 (34.6)	2.15 (1.01-4.61)
Male	53 (80.3)	13 (19.7)	1.00
Ashkenazi Jewish	70 (66.7)	35 (33.3)	3.00 (1.15-7.79)
Non-Ashkenazi Jewish	36 (85.7)	6 (14.2)	1.00
Ever smoked	51 (76.1)	16 (23.9)	0.71 (0.33-1.50)
Never smoked	52 (69.3)	23 (30.7)	1.00
Pack-years	38.8 (SD = 26.3)	36.1 (SD = 26.1)	<i>P</i> = 0.75
HRT use	2 (66.6)	1 (33.3)	0.74 (0.06-8.63)
No HRT use	34 (59.7)	23 (40.4)	1.00
Aspirin/NSAID use	25 (69.4)	11 (30.6)	1.24 (0.54-2.85)
No aspirin/NSAID Use	79 (73.8)	28 (26.2)	1.00
First-degree family history of CRC	9 (69.2)	4 (30.8)	1.15 (0.33-3.97)
No family history of CRC	96 (72.2)	37 (27.8)	1.00
MSI-high	106 (72.1)	41 (27.9)	—



**Figure 1.** Relationship between clinicopathologic characteristics of MECC tumors, MSI, and *BRAF* somatic mutation. TIL/hpf, tumor-infiltrating lymphocytes per high-powered field.

ethanol. Following centrifugation, the supernatant was discarded and the pellet was lyophilized. The pellet was resuspended in 100  $\mu$ L of proteinase K buffer (50 mmol/L Tris and 200 ng/ $\mu$ L proteinase K). The samples were incubated overnight at 37°C and then denatured at 95°C.

### MSI Analyses

MSI analyses were done as previously described (27). Normal and tumor DNA were extracted from microdissected DNA and analyzed for the consensus panel of seven markers (28). Briefly, forward primers for BAT25, BAT26, BAT40, TGF $\beta$ II, D2S125, and D5S346 and reverse primers for D17S250 were labeled with [ $\gamma$ - $^{32}$ P]ATP and included in a 20  $\mu$ L PCR that included 1  $\mu$ L of microdissected DNA. PCR products were run on 6% polyacrylamide gels for  $\sim$ 3 h at 65 W and exposed to film at  $-80^{\circ}$ C for 12 to 20 h. Films were double scored and en-

tered as stable, unstable, or loss of heterozygosity; markers with loss of heterozygosity were not counted in MSI calculations. The threshold for MSI-high for less than seven markers was  $\geq 30\%$ . Where data for all seven markers were available, tumors were designated as MSI-high if there was instability at three or more markers, MSI-low if there was instability at one or two markers, and microsatellite stable (MSS) if stable at all markers. Where data were available for less than all seven markers, tumors had to have data for at least three markers to be scored, one of which had to be a mononucleotide marker (BAT25, BAT26, BAT40).

### Identification of *BRAF* Mutations

Mutations in *BRAF* codon 600 were identified by direct sequencing of exon 15 of *BRAF* following PCR amplification of DNA extracted from paraffin-embedded samples. PCRs included 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L MgCl $_2$ , 200  $\mu$ mol/L deoxynucleotide triphosphates, 100 ng of both forward and reverse primer, 1.5 units of AmpliTaq Gold (Applied Biosystems), and 2  $\mu$ L of microdissected tumor DNA in a total volume of 50  $\mu$ L. Samples were denatured for 5 min at 95°C and passed through 40 cycles of amplification, which consisted of 60 s of denaturation at 95°C, 1 min of primer annealing at 56°C, and 1 min of elongation at 72°C. The DNA sequences of the primers are 5'-TCATAATGCT-TGCTCTGATAGGA (forward) and 5'-GGCCAAAATTAATCAGTGGA (reverse; ref. 21). A primer sequence with a smaller product (forward, 5'-TTCTTCATGAA-GACCTCAC; reverse, 5'-CCATCCACAAAATGGATCC) was used on a small subset of samples that failed to amplify with the original primer set. All sequencing for *BRAF* was done on an ABI 3700 sequencer in the University of Michigan Sequencing Core Facility. Mutations were detected by using Mutation Surveyor software (Softgenetics, Inc.). All chromatograms were also manually reviewed to confirm the presence or absence of mutations.

### Statistical Analyses

We used a case-only study design to evaluate the association between somatic *BRAF* mutations and classic risk factors for CRC.  $\chi^2$  Analyses, Fisher's exact test, and unconditional logistic regression were used for analyzing factors associated with the presence or absence of *BRAF* V600E somatic mutations. Factors evaluated included Ashkenazi versus non-Ashkenazi ethnicity, family history

**Table 3.** Gender, smoking, and *BRAF* mutation status

	Male: MSS		Female: MSS		Male: MSI-high		Female: MSI-high	
	<i>BRAF</i> WT	<i>BRAF</i> V600E	<i>BRAF</i> WT	<i>BRAF</i> V600E	<i>BRAF</i> WT	<i>BRAF</i> V600E	<i>BRAF</i> WT	<i>BRAF</i> V600E
Never smoked	208	3	433	12	20	2	32	21
Ever smoked	369	7	94	1	31	11	20	5
OR (95% CI)	1.32 (0.34-5.14)		0.38 (0.05-2.99)		3.55 (0.71-17.7)		0.38 (0.12-1.17)	

**Table 4. Case-only multivariate model: risk of BRAF V600E CRC**

	OR (95% CI)
MSI-high	19.24 (10.88-34.02)
Age (per year)	1.02 (1.00-1.05)
Ethnicity (Ashkenazi Jewish ethnicity vs non-Ashkenazi Jewish ethnicity)	1.65 (0.81-3.34)
Gender (male)	0.30 (0.11-0.82)
Smoking (ever vs never)	0.44 (0.17-1.17)
Smoking*gender	4.95 (1.18-20.83)

of CRC in a first-degree relative, weekly use of NSAIDs/ aspirin for at least 1 y, use of hormone replacement therapy (HRT), smoking status, and pack-years of smoking. Stepwise forward regression was used for multivariate model building for prediction of the presence of a BRAF V600E mutation using a threshold *P* value of 0.10. Polychotomous logistic regression was used to model the risk of CRC cases with and without a BRAF mutation compared with controls using the proc catmod command. All analyses used SAS version 9.1 (SAS Institute).

## Results

Paraffin-embedded tumors were available for 1,737 MECC cases, MSI analyses were available for 1,592 MECC cases, and both BRAF and MSI analyses were available for 1,297 MECC tumors. Cases with and without BRAF status available did not differ on any baseline characteristics (age, gender, ethnicity) or tumor characteristics (stage, grade, location). Of these tumors, 65 (5.0%) carried the BRAF V600E mutation and 147 (11.3%) were MSI-high (Table 1). As expected, tumors with the BRAF V600E mutation were 18.1 times more likely to be MSI-high than those tumors with WT BRAF [95% confidence interval (95% CI), 10.5-31.2]. Cases with BRAF-mutated tumors were more likely to be female, of Ashkenazi Jewish ethnicity, and older than subjects with BRAF WT tumors (Table 1). There was no difference in smoking history or duration, use of NSAIDs/aspirin or HRT, or family history of CRC between subjects with and without BRAF-mutated tumors (Table 1). These characteristics were not simply due to the strong association between BRAF mutation and the MSI-high phenotype. When restricting the sample to only MSI-high CRCs, age, ethnicity, and gender continue to be associated with presence of a BRAF V600E somatic mutation with a similar or stronger magnitude of association (Table 2). Despite the differences in epidemiologic characteristics, pathologically the BRAF V600E tumors have very similar characteristics to MSI-high tumors overall (Fig. 1). The majority of BRAF V600E tumors were right sided, with only four tumors located in the rectum (one MSI-high, three MSS). A high proportion of BRAF V600E tumors, regardless of instability status, showed a

mucinous histology (52.2% MSS/BRAF V600E, 73.2% MSI-high/BRAF V600E versus 44.6% MSI-high/BRAF WT, 19.4% MSS/BRAF WT; *P* < 0.001 for both comparisons within MSI status). However, the number of tumor-infiltrating lymphocytes per high-powered field was similar within MSI grouping regardless of BRAF mutation status, shown to be a powerful pathologic predictor of MSI-high in this study population (29).

It is necessary to be attentive to the role of gender in understanding the relationship between smoking history and the somatic profile of the tumor. Table 3 shows the relationship between smoking and BRAF V600E somatic mutation stratified by MSI status of the tumor and gender. Women who smoke are less likely to have a BRAF V600E somatic mutation, despite the fact they are twice as likely overall to have a BRAF somatic mutation. Men are more likely to have a somatic BRAF mutation if they smoke, and this effect is stronger in MSI-high tumors. A case-only analysis shows that female CRC cases are approximately twice as likely to have a BRAF mutation than male cases. A multivariate logistic model evaluating only cases shows that the interaction between smoking and gender is highly significant (Table 4) after adjusting for age, MSI status of the tumor, ethnicity, gender, and smoking.

Recognizing that the overall case-control study is a design matched on gender and that the risk of cancer by gender cannot be estimated directly, it is interesting to note that gender is associated with BRAF V600E CRC compared with controls (Table 5). Next, we used polychotomous logistic regression to estimate the risk of BRAF-related CRC compared with controls (Table 6). As a note, pack-years was modeled as 0 (a nonsmoker), <27 pack-years (the median), and ≥27 pack-years of smoking. Female gender was associated with BRAF-positive cancer [odds ratio (OR), 3.66; 95% CI, 2.35-5.69] but not BRAF-negative cancer (OR, 0.97; 95% CI, 0.88-1.07). Ashkenazi Jewish ethnicity and history of CRC in a first-degree relative were associated with an increase in risk of CRC regardless of BRAF status. Pack-years of smoking were associated with risk of BRAF-positive cancer, and an interaction term modeling pack-years and gender was highly significant. As indicated by the

**Table 5. Gender and BRAF in case-control comparisons and case-only analyses**

	Female	Male	OR (95% CI)
BRAF-positive cases	42 (64.6)	23 (35.4)	
Controls	998 (49.4)	1,021 (50.6)	1.87 (1.12-3.13)
BRAF-positive cases	42 (64.6)	23 (35.4)	
BRAF-negative cases	591 (48.2)	635 (51.8)	1.96 (1.17-3.30)

**Table 6.** Case-control multivariable model: risk of *BRAF* V600E CRC

	<i>BRAF</i> -negative CRC vs controls	<i>BRAF</i> -positive CRC vs controls
Gender: female vs male	0.97 (0.88-1.07)	3.66 (2.35-5.69)
Age	0.99 (0.99-1.00)	1.02 (1.00-1.03)
Ashkenazi Jewish ethnicity	1.30 (1.20-1.41)	2.22 (1.59-3.09)
Family history of CRC	1.35 (1.19-1.53)	1.55 (1.03-2.34)
Pack-years	0.99 (0.92-1.05)	1.78 (1.35-2.35)
Pack-years*gender	0.82 (0.73-0.93)	0.36 (0.23-0.57)

case-only analyses, CRC female cases were much less likely to have a history of smoking compared with controls, and this effect was stronger in *BRAF*-positive cases.

## Discussion

Defining the somatic fingerprint of a tumor may be a powerful way to precisely define subgroups of cancer that correspond to distinct risk factors. Here, we evaluated risk factors associated with one mutation, *BRAF* V600E, in incident cases of CRC from northern Israel. This has provided insight into how to interpret epidemiologic studies of CRC, particularly with regard to heterogeneity in reported risk factors for CRC between populations.

Notably, the frequency of the *BRAF* V600E mutation varies widely between population groups, both within this study (5.8% in the Ashkenazim versus 3.2% in the non-Ashkenazim) and reported by other groups (Samowitz et al., 9.5%; English et al., 16.3%; here, 5.0% overall). Heterogeneity of rates between studies may reflect an underlying genetic predisposition to *BRAF*-mutated tumors that differ by ethnic group, the sensitivity of the assay, specific environmental influences present at different levels in different populations, a combination of both, or chance. Sanger sequencing may have lower analytic sensitivity for somatic mutations than Pyrosequencing (30), although another study of *BRAF* mutations reported a higher-frequency mutations while using Sanger sequencing (12% in CRC cases; ref. 25). There is no reason to think that the detection method would be associated with epidemiologic risk factors within our study. It is especially intriguing to consider the hypothesis that specific somatic alterations are associated with distinct environmental exposures. There are several examples of this in cancers, most notably in the distinct *KRAS* mutational profile in never smokers with lung adenocarcinoma (31). This is further underscored by the distinct histology of *BRAF*-mutated tumors, with a high proportion of the tumors showing a mucinous histology and overwhelmingly right sided regardless of MSI status, possibly indicating a specific etiologic pathway. Combining comprehensive epidemiologic data with high-throughput sequencing of somatic tissue could elucidate previously unappreciated environmental and genetic risk factors for cancers.

There is a 2-fold difference in the odds of a *BRAF* CRC between those of Ashkenazi Jewish ethnicity and those

who are not Ashkenazi Jewish. Incidence rates of CRC vary widely in Israel, with the highest rates found in the Ashkenazim and the lowest in the Arab population; Israeli Jews are at intermediate risk (32). English et al. describe a higher incidence of *BRAF*-mutated tumors in the Anglo-Saxon population compared with the southern European population of Australia, with similar incidence rates of CRC without *BRAF*. Given that the risk profile likely differs by somatic profile of the tumor, it is essential to consider the somatic profile to appreciate differences in CRC rates between different populations.

The picture becomes complicated when evaluating MSI-high/*BRAF*-mutated tumors with respect to smoking. Women are twice as likely to have a tumor with a *BRAF* mutation, but this is not strongly associated with smoking. On closer inspection, men who smoke have a significantly higher risk of CRC with a *BRAF* mutation (OR, 4.95; 95% CI, 1.18-20.83; *P* interaction = 0.03, after adjusting for age, ethnicity, and MSI status of tumor). When evaluating risk factors in a multinomial model that includes controls, gender is strongly associated with risk of *BRAF* -mutated CRC but much less likely to have a history of smoking regardless of mutational status. This suggests that tumors with *BRAF* somatic mutations arise from a different pathway in women. Slattery et al. (12) hypothesize that estrogen HRT may reduce the risk of MSI-high CRC in women by reducing the likelihood of estrogen receptor methylation, as loss of ER expression, possibly due to gene silencing by methylation, was shown to be ubiquitous in colon cells that give rise to cancer (33). The low prevalence of HRT use in the MECC study does not allow an adequate comparison in this population. The exact pathways of how smoking and estrogen may affect colorectal carcinogenesis are unknown. Large studies such as the MECC study are needed to continue to address these questions in a meaningful way.

There are several limitations to this study. First, despite the large sample size, *BRAF* mutations are rare and the number in this population is relatively small (5.0%). This limits the study to detect only large effects. We do find significant associations with many epidemiologic risk factors, including a significant interaction between male gender and smoking and *BRAF* tumor status. The smoking rate in Israeli women is low, and thus, the study is less generalizable to other female populations where

smoking is more prevalent. However, our findings in this case-only study are consistent with case-control studies showing that smoking in females is not strongly associated with risk of colon cancer.

Here, we show that the prevalence of *BRAF* V600E mutations, although relatively rare (~5% of all CRCs in the Israeli population), is associated with distinct risk factors in the Israeli population. In contrast to other populations, we note striking differences in the prevalence of this somatic mutation. Epidemiologic studies should consider somatic alterations to best understand the risk factors for this common complex disease.

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No potential conflicts of interest were disclosed.

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