

Keywords: metastatic colorectal cancer; irinotecan; KRAS; plasma; prediction; prognosis

KRAS-mutated plasma DNA as predictor of outcome from irinotecan monotherapy in metastatic colorectal cancer

K G Spindler^{1,2,4}, A L Appelt^{1,2}, N Pallisgaard^{2,3}, R F Andersen^{2,3} and A Jakobsen^{1,2}

¹Department of Oncology, Vejle Hospital, Vejle, Denmark; ²Danish Colorectal Cancer Group South, Vejle Hospital, Kabbeltoft 25, 7100, Vejle, Denmark and ³Department of Biochemistry, Vejle Hospital, Vejle, Denmark

Background: We investigated the clinical implications of KRAS and BRAF mutations detected in both archival tumor tissue and plasma cell-free DNA in metastatic colorectal cancer patients treated with irinotecan monotherapy.

Methods: Two hundred and eleven patients receiving second-line irinotecan (350 mg m⁻² q3w) were included in two independent cohorts. Plasma was obtained from pretreatment EDTA blood-samples. Mutations were detected in archival tumour and plasma with qPCR methods.

Results: Mutation status in tumor did not correlate to efficacy in either cohort, whereas none of the patients with mutations detectable in plasma responded to therapy. Response rate and disease control rate in plasma KRAS wt patients were 19 and 66% compared with 0 and 37%, in patients with pKRAS mutations, ($P=0.04$ and 0.01). Tumor KRAS status was not associated with PFS but with OS in the validation cohort. Plasma BRAF and KRAS demonstrated a strong influence on both PFS and OS. The median OS was 13.0 mo in pKRAS wt patients and 7.8 in pKRAS-mutated, ($HR=2.26$, $P<0.0001$). PFS was 4.6 and 2.7 mo, respectively ($HR=1.69$, $P=0.01$). Multivariate analysis confirmed the independent prognostic value of pKRAS status but not KRAS tumor status.

Conclusion: Tumor KRAS has minor clinical impact, whereas plasma KRAS status seems to hold important predictive and prognostic information.

Worldwide, colorectal cancer (CRC) remains a significant cause of cancer morbidity and mortality, with an overall incidence of >16 000 new cases per year in the Nordic countries (Ferlay *et al*, 2010). Unfortunately, the majority of the patients will eventually develop metastatic disease, and despite the availability of several effective cytotoxic drugs and new biological agents, the prognosis remains poor and the curative options for metastatic disease limited.

The cornerstone in the treatment of mCRC has been fluoropyrimidines and related pro-drugs for more than four decades. 5-Fluorouracil alone has increased the OS beyond 6 months (Prescrire, no authors listed, Chemotherapy of metastatic colorectal cancer, 2010; Jemal *et al*, 2011). The introduction of

oxaliplatin- and irinotecan-based combination therapies has improved OS even further, whereas the addition of biological agents targeting the EGFR system and the anti-angiogenetic drug avastin eventually has led to median OS results beyond 2 years (Cunningham *et al*, 2004; Prescrire, no authors listed, Chemotherapy of metastatic colorectal cancer, 2010; Jemal *et al*, 2011). Consequently, the majority of patients will be offered several lines of palliative chemotherapy, with an accompanying risk of substantial side effects. Careful selection of patients and monitoring during therapy are therefore essential.

Irinotecan is a widely used semisynthetic analogue of camptothecin, a naturally occurring quinolone alkaloid targeting the topoisomerase I, which is responsible for maintaining the

*Correspondence: Dr KG Spindler; E-mail: k.g.spindler@rm.dk or Spindler@hafnet.dk

⁴Current address: Department of Oncology, Aarhus University Hospital, Aarhus, Denmark.

Received 15 July 2013; revised 17 September 2013; accepted 20 September 2013; published online 21 November 2013

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functional compact and supercoiled DNA double-helix structure. Thus, Irinotecan generates single-stranded DNA breaks, which can lead to overall DNA damage and thereby cell death. Efficacy of irinotecan in mCRC was demonstrated more than a decade ago; response rates from monotherapy have been recorded to ~14%, with an overall benefit of PFS and OS in the second-line setting. The major dose-limiting toxicities include diarrhoea and myelosuppression, rendering a strong need for predictive and prognostic markers to optimise patient treatment.

In mCRC, a major proportion of tumors harbour KRAS or BRAF mutations, which are negative predictors of outcome from EGFR-targeted therapies, predominantly in third-line settings of combination therapy with irinotecan (Qui *et al*, 2010; Adelstein *et al*, 2011). Tumour mutation status does not seem to affect outcome from chemotherapy alone; however, its role has been only sparsely investigated. However, archival tumour tissue obtained several years earlier and prior to multiple lines of therapy previously may not sufficiently reflect disease biology at the time of therapy. Addressing of this by repeated biopsies is not applicable because of both ethical and practical reasons.

Recently, we and others have shown that small fragments of free DNA measured in the plasma from cancer patients is a feasible source for mutation detection and quantification in the peripheral blood (Spindler *et al*, 2012; Murtaza *et al*, 2013). We have used a feasible in-house qPCR method to detect KRAS and BRAF mutations in the plasma from patients with mCRC treated with irinotecan and cetuximab and shown a clear correlation with outcome from plasma analysis (Spindler *et al*, 2012).

The present study aimed at investigating the predictive and prognostic value of KRAS and BRAF mutations in tumour and plasma from patients treated with irinotecan monotherapy.

MATERIALS AND METHODS

The study included a retrospective test cohort and a cohort of patients included in a prospective biomarker study for validation, in total comprising 211 mCRC patients treated with irinotecan at the Department of Oncology, Vejle Hospital, Denmark.

Retrospective cohort. Irinotecan monotherapy is the standard second-line therapy for mCRC in our department, and KRAS and BRAF mutation analysis is routinely performed prior to potential third-line therapy with irinotecan- and EGFR-targeted therapy. Mutational status in archival tumour tissue and clinical data was collected for the retrospective analysis from 111 consecutively treated patients receiving at least one cycle of irinotecan monotherapy. The majority of patients had also participated in a prospective third-line biomarker study. The purpose of this study was to investigate the predictive and prognostic value of tumour mutation status in regard to irinotecan monotherapy. Patients were treated with intravenous irinotecan monotherapy 350 mg m⁻² q3w and supportive care according to the local guidelines. Computed tomography scans of the chest and abdomen were performed every 9 weeks.

Prospective biomarker study. Patients who were candidates for irinotecan monotherapy were included in a prospective non-randomised phase II and biomarker study (Protocol ID S-20090114). Inclusion criteria were as follows: histopathologically verified metastatic colorectal cancer, measurable disease according to RECIST version 1.1, indication for irinotecan monotherapy according to local guidelines, informed consent to therapy and biobank collection and age ≥18 years. Patients with other concurrent cancer diseases (within 5 years of inclusion, apart from squamous cell carcinoma of the skin), having received experimental therapy <30 days prior to inclusion, or with planned radiotherapy to target lesions were not eligible. Patients were

treated with intravenous irinotecan monotherapy 350 mg m⁻² q3w and supportive care according to the local guidelines. Response evaluations with CT scans of the chest and abdomen were performed every 9 cycles according to RECIST v 1.1.

All patients provided signed informed consent before study entry and the ethics committee (The Regional Scientific Ethical Committee for Southern Denmark) approved the studies prior to commencement.

Specimen characteristics. Archival formalin-fixed, paraffin-embedded tumour tissue was used for tumour mutation detection. After informed consent, pretreatment blood samples were drawn prior to the first cycle of therapy and at each visit until the time of progression. Plasma was obtained from blood samples collected in EDTA tubes and centrifuged at 2000 g for 10 min within 2 h of collection, before being stored at -80 °C until use. All samples were analysed, blinded to the study end points.

DNA purification. DNA was extracted from formalin-fixed paraffin-embedded tissue using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) after histological confirmation of viable tumour cells on HE-stained slides. DNA was purified from 1 ml of plasma using a QIASymphony virus/bacteria midi-kit on a QIASymphony robot (Qiagen), according to the manufacturer's instructions. DNA was eluted in 110 µl.

KRAS and BRAF mutation detection. The KRAS analysis of archival tumour tissue was performed with the DxS kit (Garm Spindler *et al*, 2009) and validated in-house assays as also previously described (Garm Spindler *et al*, 2009; Spindler *et al*, 2012). Analyses of plasma DNA were performed with the in-house assays that are based on the Amplification Refractory Mutation System-Quantitative PCR (ARMS-qPCR) methodology. The assays detect six mutations in KRAS codon 12 (Gly12Ala, Gly12Arg, Gly12Asp, Gly12Cys, Gly12Ser and Gly12Val), one mutation in KRAS codon 13 (Gly13Asp) and one BRAF codon 600 mutation (Val600Glu).

Statistical analysis. Data are presented according to the REMARK guidelines. Correlation between variables and mutation status were analysed with cross tabulations. The Kaplan–Meier method was applied to estimate PFS and OS, and differences in outcome between subgroups were compared using the log-rank test. A multivariate Cox regression analysis was performed to examine the association of tumour and plasma mutation status with overall and disease-free survival, whereas controlling for effects of age and PS. *P*-values referred to two-tailed tests and were considered significant when *P* ≤ 0.05. Statistical analyses were carried out using the NCSS statistical software 2007 v.07.1.5 (NCSS Statistical Software, Kaysville, UT, USA, www.ncss.com).

RESULTS

Patients. Baseline characteristics from the two cohorts are presented in Table 1, which also shows the number of samples available for the biomarker analyses. The baseline characteristics were similar in the two cohorts.

Retrospective data. One hundred and eleven patients were evaluated; the analysis included 48 female and 68 male patients, with a median age of 62 years. The median number of cycles received was six (range 2–15). The median progression-free and overall survivals were 4.9 months (95% CI 4.3–6.1 months) and 16.1 months (95% 13.7–18.2 months), respectively. Response evaluation according to RECIST revealed a partial response rate of 14% and SD in 50% of patients, with a subsequent disease control rate of 64%, in agreement with the literature. Thirty-six (32%) patients progressed after the first evaluation.

Prospective study. All but one patient commenced therapy as planned and the median number of cycles received was four (range 1–15). One patient withdrew consent for data analysis and blood sampling and is included in the ITT population; however, baseline characteristics and outcome parameters were consequently not recorded. The overall toxicity was comparable to the literature, with 41% who experienced diarrhoea and 21% neutropaenia. Response evaluation was not available for 13 patients but the overall response rate was 13%, whereas 57% obtained disease control and 43% progressed before or at first response evaluation. At the time of analysis, the median observation time was 9.5 month, with 90 patients dead and 9 still alive. The median PFS was 4.6 mo (95% CI 3.7–5.8) and the median OS 9.5 mo (95% CI 8.4–11.8) in the ITT cohort.

Mutations and relation to demographic characteristics. In the retrospective cohort, tumour DNA was available for the KRAS

analysis in 109 (99%) of the patients and included 42 (39%) patients with mutations and 67 (60%) wild type (wt) (that is, absence of mutations). Eight patients had a BRAF V600 mutation (7%). Mutational status was not associated with baseline patient characteristics (data not shown). Data from the prospective biomarker study showed similarly that KRAS mutations were detected in 44 (45%) tumours and BRAF mutations in 8 (8%), with no association to baseline characteristics.

KRAS and BRAF mutation detection in plasma and tumour. Ninety-seven patients had available tumour and plasma samples for mutation analysis, respectively; however, only 95 patients had matching tumour and blood samples available for comparison of mutation status. In these, KRAS mutations were detected in 44 tumours and BRAF mutations in 7 tumours. The overall concordance between tumour and plasma KRAS status was high. Fifty patients had wt tumour and corresponding plasma KRAS status, whereas 28 had detectable mutations in both. In 16 patients with wt plasma sample, a previous mutations in the tumour had been detected, whereas only one patient had a detectable plasma KRAS mutation not previously found in the tumour. For BRAF status, the agreement was complete = 100% (but one patient with a BRAF-positive tumour sample did not have a matching plasma sample).

The predictive value of KRAS mutations in tumour. Retrospective analysis revealed no correlation between mutation status in tumour and outcome in terms of tumour response, disease control rates or progression rates according to the KRAS tumour status as demonstrated in Table 2. This was confirmed in the prospective study.

The predictive value of KRAS mutations in plasma. Interestingly, in the prospective study, none of the patients with KRAS mutations detectable in plasma responded to therapy (RR = 0), whereas the RR in pKRAS wt patients was 19%, ($P = 0.014$). The disease control rate in pKRAS wt patients was 66% compared with 37% in the patients with pKRAS mutations ($P = 0.01$). These differences were highly significant, indicating a predictive value of KRAS when detectable in the plasma (Table 2).

The prognostic value of KRAS detection in tumour in test and validation cohorts. The KRAS tumour mutation status did not significantly influence PFS or overall survival after second-line irinotecan therapy in the retrospective evaluation (Table 2). In the prospectively investigated cohort, KRAS tumour mutation status was significantly associated with OS but not with PFS, as shown from Table 2 and Figure 1.

Table 1. Patient characteristics

Colorectal cancer cohorts		
Characteristics	Retrospective n = 111	Prospective n = 100
Age (median/range)	62 (26-80)	66 (37-83)
Gender		
Female	48 (43)	34 (34)
Male	63 (57)	65 (65)
PS		
0	61 (55)	45 (45)
1	47 (42)	46 (46)
2	3 (3)	8 (8)
Tumour KRAS	109	97
Mutation	42 (39)	44 (45)
Wild type	67 (60)	53 (55)
Plasma KRAS	NA	97
Mutations		30 (31)
Wild type		67 (69)
Tumour BRAF	109	97
Mutation	8 (7)	8 (8)
Wild type	101 (93)	89 (92)
Plasma BRAF	NA	97
Mutation		7 (7)
Wild type		90 (93)

Table 2. Univariate analysis of outcome parameters according to KRAS and BRAF mutations

Marker	Cohort	Response				PFS			OS		
		RR	P	DCR	P	HR	95% CI	P	HR	95% CI	P
KRAS tumour wt/m	R	15/12	>0.05	73/60	>0.05	1.08	0.68–1.71	>0.05	1.33	0.89–1.97	>0.05
BRAF tumour wt/m	R	15/0	>0.05	68/63	>0.05	0.56	0.22–1.42	>0.05	0.95	0.40–2.23	>0.05
KRAS tumour wt/m	P	17/8	>0.05	62/53	>0.05	0.84	0.56–1.26	>0.05	0.61	0.40–0.95	0.02
BRAF tumour wt/m	P	14/0	>0.05	62/14	0.02	0.28	0.08–1.01	0.0001	0.30	0.09–1.04	0.0005
KRAS plasma wt/m	P	19/0	0.01	66/37	0.01	0.59	0.36–0.97	0.01	0.44	0.26–0.76	0.0002
BRAF plasma wt/m	P	14/0	>0.05	60/17	0.04	0.29	0.08–1.13	0.0006	0.34	0.09–1.19	0.003

Abbreviations: CI = confidence interval; DCR = disease control rate in per cent; HR = hazard ratio; m = mutation; OS = overall survival; P = prospective cohort; p = P-value; PFS = progression-free survival; RR = response rate in per cent; R = retrospective cohort; wt = wild type.

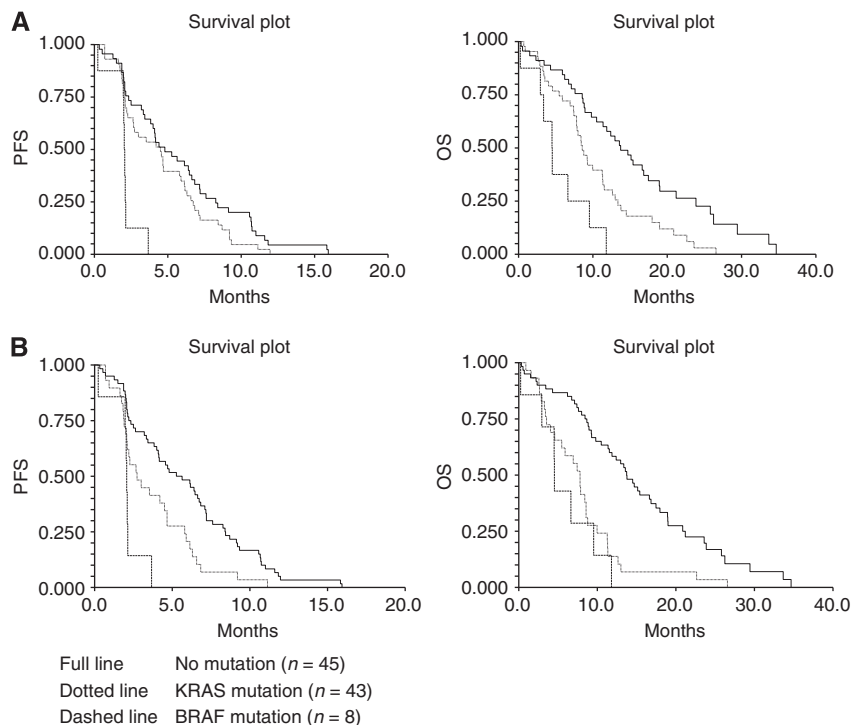


Figure 1. Kaplan–Meier estimates of survival among patients with KRAS or BRAF mutations detected in archival tumour tissue (A) and in plasma (B) in the prospective cohort.

The prognostic value of KRAS detection in plasma. A clearly enhanced prognostic value of plasma mutations compared with tumour mutations was revealed when analysing the relationships between OR and DFS and the plasma KRAS mutation status (Table 2 and Figure 1). The median OS was 13.0 months (95% CI 9.5–15.1) in plasma KRAS wt patients and 7.8 months (4.6–8.4) in patients with plasma KRAS mutations, HR 2.26 (95% CI 1.31–3.90), $P < 0.0001$. The median PFS were 4.6 months (95% CI 3.3–6.4) and 2.7 months (95% CI 2.1–4.5), respectively, HR 1.69 (95% CI 1.03–2.77), $P = 0.01$.

BRAF mutations in tumour and plasma. The number of BRAF mutations was small ($n = 7(8)$), and conclusions drawn from this sample size should therefore be regarded with caution. Data revealed – similarly to the results for KRAS – that none of the patients with detectable BRAF mutations in the plasma responded to therapy and, although these differences did not reach significance, they translated into differences in DCR, PFS and OS (Table 2 and Figure 1).

Multivariate survival analysis. The Cox multivariate regression analysis is presented in Table 3. The model included age ($> / < 66$ years), PS and mutation status in tumour and plasma. The model confirmed an independent prognostic value of plasma KRAS status, whereas the effect of tumour KRAS status seemed to diminish.

Table 3. Multivariate Cox regression analysis				
	PFS		OS	
Variables	Hazard ratio (95% CI)	P-value	Hazard Ratio (95% CI)	P-value
PS ^{a,b}	1.2 (0.8–1.8)	0.35	1.9 (1.3–2.8)	0.001
Age				
< Median ^c	0.6 (0.4–1.0)	0.03	0.8 (0.5–1.2)	0.22
> Median				
Tumour KRAS status				
Wild type ^c	1.0	0.92	1.2	0.52
Mutation	(0.6–1.7)		(0.7–2.3)	
Plasma KRAS status				
Wild type ^c	1.9	0.03	2.7	0.004
Mutation	(1.1–3.5)		(1.4–5.2)	
Plasma (and tumour) BRAF status				
Wild type ^c	5.3	0.0002	4.3	0.002
Mutation	(2.1–13.0)		(1.7–10.6)	

The Hazard ratio refers to moving from the reference group to the other group or changing one step in parameters entered as continuous variables.

^aPS = Performance Status (ECOG).

^bEntered as a continuous variable.

^cReference group.

DISCUSSION

KRAS is the most commonly mutated gene in colorectal cancer and is regarded as an early event in carcinogenesis (Andreyev *et al*, 2001). The constitutive activation of KRAS as well as BRAF mutations leads to EGFR-independent downstream signalling and hereby tumorigenicity in metastatic colorectal cancer, which drives tumour growth and progression and impairs response to EGFR inhibition. The prognostic relevance of these downstream

mutations in CRC has been investigated for decades with inconsistent results but has attracted increasing focus lately as a consequence of the collection of prospective clinical data and availability of mutation status in larger cohorts.

Although the majority of studies have indicated a worse prognosis in patients with KRAS-mutated CRC, a large number of investigations have failed to demonstrate an association between the mutations and outcome, as discussed recently by Yokota (2012) and Phipps *et al.* (2013). The Rascal study demonstrated a clear prognostic impact of KRAS (Andreyev *et al.*, 2001); however, translational research data from the PETCAC-3, EORTC 40993 and SAKK 60-00 trials failed to demonstrate a relevant prognostic impact (Roth *et al.*, 2010). A very recent retrospective study of two major Scandinavian cohorts have also shown inconsistent results, BRAF being the only prognostic factor in the first study, whereas KRAS had a strong prognostic impact in the second (Eklöf *et al.*, 2013). Such inconsistencies between studies have been attributed to the differences in patient selection, sample sizes, methods used and lack of control for other relevant prognostic markers such as BRAF, MSI and PTEN expression. Furthermore, a recent population-based study from the Western Washington State of 1989 patients diagnosed with CRC revealed an overall association with disease-specific survival but not in patients who presented with distant-stage disease (Phipps *et al.*, 2013). It has consequently also been suggested that the prognostic role of KRAS may differ by stage of the disease.

Regarding the predictive value, testing for KRAS mutations in late-stage disease prior to anti-EGFR-targeted treatment has been implemented in clinical practice as a consequence of the overall consistent results in this setting; however, the role of KRAS mutations as biomarker for outcome of similar combination regimens in the first-line settings is less clear. Two large phase III studies failed to demonstrate a PFS or OS improvement from the addition of cetuximab to oxaliplatin-based first-line combination therapy (Maughan *et al.*, 2011; Tveit *et al.*, 2012). Interestingly, these trials even seemed to suggest a detrimental effect of the EGFR inhibition in patients with KRAS mutations; however, no clear explanation for this potential negative interaction has been revealed. Similar data were found in the randomised PRIME study, investigating the addition of panitumumab to oxaliplatin also in the first-line setting (Douillard *et al.*, 2010). Curiously, a single recent evaluation has shown a possible predictive value of KRAS mutations for outcoming of oxaliplatin-based first- and second-line therapies, with a significantly higher RR and longer PFS in patients with KRAS-mutant disease compared with KRAS wt patients, primarily in the first-line setting (Basso *et al.*, 2013); however, the sample size was small and results should be validated.

The present study supplements the current knowledge in two major aspects; firstly, it addresses the possible predictive and prognostic value of KRAS and BRAF in the second-line setting of irinotecan monotherapy as opposed to combination with anti-EGFR antibodies; secondly, it examines the value of measuring the mutations in the more timely pretreatment plasma sample. The role of mutation status in the archival tumour tissue was first investigated in a test cohort and validated in a prospectively collected study. Results showed that KRAS mutations detected in the archival tumour tissue were not predictive for response and we were unable to reveal a prognostic value of KRAS in archival tissue. In contrast, we found a strong independent prognostic value of the plasma analysis. Furthermore, patients with detectable KRAS mutations in the plasma had a poor chance of tumour response to therapy. In line with the data from third-line combinations of irinotecan with anti-EGFR antibodies, none of the patients with KRAS-mutated disease achieved an objective response, resulting in statistically significant differences.

The frequency of BRAF in both cohorts was low, resulting in broad confidence intervals, which indicate that results should be interpreted with caution. However, data suggested a confirmation of the poor prognosis in patients with BRAF-mutant disease (Ogino *et al.*, 2012).

This study presents new evidence, which at least partially may explain the diverging and contradictive results with respect to the

clinical importance of KRAS mutations. Intratumoral heterogeneity as well as heterogeneity among tumour and metastases exist and could have clinical implications. Another problem is 'heterogeneity' over time. Clonal selection – especially provoked by treatment – may well result in major quantitative differences in the ratio between mutated and wt cells. This aspect has never been addressed. Taken together, it may well be hypothesised that tumour tissue is not the relevant starting material and the liquid biopsy represented by a blood sample should be the material of choice.

The obvious limitations of a small sample size and non-randomised design should be acknowledged and any conclusions on the predictive value of mutations detected in the plasma should therefore be regarded with caution. However, this study indicates that timely molecular characterisation of the disease is important and that plasma KRAS mutation status holds important predictive and prognostic information regarding the outcome of irinotecan monotherapy in mCRC, whereas the use of archival tumour tissue seems insufficient. These findings are hypothesis-generating but novel and call for validation in a randomised trial.

ACKNOWLEDGEMENTS

We sincerely thank Tryg Fonden and the Research Council Hospital Lillebaelt for financial support for laboratory analysis in this study.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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