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Original Research Article

Potential relative quantities of miR-122 and miR-150 to differentiate hepatocellular carcinoma from liver cirrhosis

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ABSTRACT

Cirrhosis and hepatocellular carcinoma (HCC) are related to chronic liver diseases. Diagnostic algorithms are needed to discriminate HCC from cirrhosis for better patient management. This study aimed to determine the potential of miR-122 and miR-150 to differentiate HCC from liver cirrhosis. This study used a cross-sectional method involving 66 patients with liver cirrhosis, 27 subjects with HCC, and 29 healthy controls. Examination of miR-122 and miR-150 levels from blood plasma used real-time quantitative polymerase chain reaction and their relative expressions were calculated. Clinical and laboratory data were collected and graphed for the Area Under the Curve (AUC) and also for comparison using unpaired T-tests, Kruskal-Wallis, Mann-Whitney, and Chi-square tests with significance set as $p < 0.05$. The relative expressions of miR-122 and miR-150 could differentiate HCC from cirrhosis, with cut-off 9.11, AUC 53.84%, $p = 0.2120$, and cut-off 1.47, AUC 67.65%, $p = 0.0001$, respectively. Meanwhile, the combined relative expressions of miR-122 and miR-150 can distinguish HCC from cirrhosis, with AUC 71.94%, $p = 0.0006$. The combination of miR-122 and miR-150 has the potential as a biomarker to differentiate HCC from liver cirrhosis.

1. Introduction

Liver cirrhosis is the final stage of chronic liver disease, characterized by the formation of regenerative nodules of the liver parenchyma surrounded by fibrotic connective tissue, leading to the loss of liver function and disruption of the portal system [1]. Cirrhosis is a risk factor and associated with the development of hepatocellular carcinoma (HCC). Chronic cirrhosis can develop into HCC through a carcinogenesis mechanism that induces malignant cirrhotic nodules to become HCC [2]. HCC arising from liver cirrhosis accounts for 90% of cases [3]. Damage and chronic inflammation of the liver cause fibrosis, which then proceeds to the pathway of carcinogenesis. The composition of the extracellular matrix changes, and non-parenchymal cell activation occurs, resulting in an anti-apoptotic environment that can promote uncontrolled hepatocyte growth [3].

Liver cirrhosis and HCC are diseases with a high mortality rate. Globally in 2017, deaths from liver cirrhosis were 1.32 million, compared to 899,000 in 1990. These deaths from cirrhosis accounted for 2.4% of total global deaths in 2017 [4]. Meanwhile, HCC was the fourth leading cause of cancer death in 2018 [5]. In the Asia Pacific in 2015, liver cirrhosis was the leading cause of liver-related death and represented 54.3% of 1,161,914 cirrhosis-related deaths globally [6]. Meanwhile, in Asia, especially East Asia, the mortality rate has decreased, although the rate still ranges from 10 to 24/100,000 in men [7].

The most common etiologies of liver cirrhosis and HCC are hepatitis B (HBV), hepatitis C (HCV), and alcoholism [6,8]. Integration of the HBV genome into the host chromosome causes a *cis*-effect, abolishes the function of tumor suppressor genes, and activates tumor-promoting genes. The role of the HBx HBV protein also affects intracellular signal transduction and alters host gene expression [9]. HCV plays a direct and indirect role in the HCC mechanism. The indirect process is through activation of hepatic stellate cells (HSCs) followed by activation of fibrosis and cirrhosis, which then initiates HCC. The direct process occurs through the role of HCV proteins that activate cellular signaling pathways, thus providing a preconditioning effect for HCC induction [10]. Meanwhile, alcohol is associated with the development of HCC through a genotoxic process and the development of cirrhosis.

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Abbreviation

HCC	hepatocellular carcinoma
AUC	area under the curve
miR	microRNA
HBV	hepatitis B virus
HCV	hepatitis C virus
HBx HBV	hepatitis B virus X protein
HSCs	hepatic stellate cells
CYP2E1	Cytochrome P450 2E1
ROS	reactive oxygen species
CT	computerized tomography
AFP	alpha-fetoprotein
HBsAg	hepatitis B surface antigen
anti-HCV	hepatitis C antibody
PCR	polymerase chain reaction
cDNA	complementary DNA
RT-qPCR	real-time quantitative PCR
RQ	relative quantities/relative expressions
SD	standard deviation
ROC	receiver operating characteristic
AST	aspartate aminotransferase
ALT	alanine aminotransferase
CTP	Child Turcotte Pugh
BCLC	Barcelona-Clinic Liver Cancer staging

Long-term alcohol intake causes the induction of the CYP2E1 enzyme, increases hepatic acetaldehyde production, while drastically increases reactive oxygen species (ROS) production and liver oxidative stress [11].

There is an association between liver cirrhosis and HCC. Cirrhosis can accompany HCC, or cirrhosis can occur which later develops into HCC [12]. The diagnosis of HCC is often delayed because signs and symptoms are not noticeable when the tumorogenesis is small. Finally, detection and therapy are also often delayed, causing the patient's life expectancy to be poor [13]. Therefore, it is necessary to develop a diagnostic algorithm with better methods to detect and differentiate cirrhosis and HCC. The correct diagnosis will determine the appropriate therapy.

MicroRNA is a small non-coding RNA molecule, which plays a role in regulating human biological functions [14]. MiR-122 is a microRNA abundant in the liver. MiR-122 expression is driven by liver-enriched transcription factors (LEFTs), and miR-122 expression plays a role in balancing hepatocyte proliferation and differentiation [15]. It functions as a tumor suppressor and plays an essential role in regulating liver function [16]. Decreased miR-122 expression is associated with liver disease, and it has been suggested that reduced miR-122 levels are associated with poorer prognosis and metastasis in liver cancer [15].

Meanwhile, miR-150 is antifibrotic agent in chronic liver disease [17] and tumor suppressor [18]. Studies show that miR-150 can reduce fibrosis levels and inhibit stellate cell activation by inhibiting c-Myb expression [19]. In HCC, miR expression is decreased in metastatic cancer tissue compared to primary tissue. Inhibition of miR-150 promotes migration and invasion of liver cancer cells, whereas overexpression of miR-150 suppresses migration and invasion of cancer cells in vitro [18].

In hepatitis B virus-associated HCC, miR-122 can differentiate HCC from healthy (AUC = 0.726) [20]. MiR-122 also differentiates HCC from chronic hepatitis C (cut-off value < 0.21, AUC = 0.98) [21]. Meanwhile, research on miR-150 for diagnostic tests is still limited. Studies on hepatitis B virus-associated HCC indicate that miR-150 can differentiate HCC from healthy (AUC = 0.931) and HCC from chronic hepatitis B (AUC = 0.881) [22]. In hepatitis C virus-associated HCC, miR-150 can differentiate HCC from healthy at a cut-off value of 0.67453 (AUC 0.638) [23].

There have been only limited studies of miR-122 and miR-150 as biomarkers of liver cirrhosis and HCC. In addition, previous studies have focused on hepatitis B virus- or hepatitis C virus-associated HCC, and there have been no studies on the application of microRNA as a diagnostic tool to differentiate cirrhosis and HCC regardless of etiology. This study aimed to determine the potential of miR-122 and miR-150 to differentiate HCC from liver cirrhosis.

2. Materials and methods

2.1. Study population and data collection

This study used a cross-sectional method. Research respondents consisted of 66 patients with liver cirrhosis, 26 subjects with HCC, and 29 healthy controls. Patients with liver cirrhosis and HCC were recruited from Dr. Sardjito General Hospital Yogyakarta, while healthy controls were from blood donors at the Indonesian Red Cross Blood Transfusion Unit, Banyumas.

Gastroenterolo hepatologists diagnosed liver cirrhosis based on clinical, laboratory examinations, and abdominal computerized tomography (CT). Meanwhile, the diagnosis of HCC was confirmed with alpha-fetoprotein (AFP) and/or histopathology. Healthy controls were healthy adults, without hepatitis B, no hepatitis C with HBsAg and anti-HCV negative, no liver cirrhosis, and no cancer [24].

Once the patients had agreed to be research respondents then they had their venous blood taken from their cubital vein. The samples were stored in the Research Laboratory of the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada.

2.2. MicroRNA analysis

Plasma was isolated from peripheral venous blood and then stored at −80 °C. Hematology and blood biochemistry samples were examined at the Clinical Pathology Laboratory of Dr. Sardjito General Hospital.

2.2.1. - RNA extraction and cDNA synthesis

RNA was isolated using the miRNeasy Serum/Plasma Kit from Qiagen, and the concentration was measured. Synthesis of cDNA was using miRCURY LNA RT from Qiagen, with a mix composition of 5x reaction buffer 2 μl, nuclease-free water 4.5 μl, enzyme mix 1 μl, spike in 0.5 μl, and RNA 2 μl (1 μl = 5 ng). Then cDNA was synthesized using polymerase chain reaction (PCR) with the following program: incubation for 60 min at 42 °C, inactivation of the reverse transcriptase enzyme at 95 °C for 5 min, and cooling at 4 °C.

2.2.2. - Real-time quantitative PCR (RT-qPCR)

RT-qPCR of miR-122 and miR-150 used Qiagen's miRCURY LNA SYBR Green. The miR-122 primer was 5′UGGAGUGUGACAAUGGUGUUUG and the miR-150 primer was 5′UCUCCCAACCCUUGUACCAGUG. The results of the cDNA synthesis were diluted with nuclease-free water, with the ratio of 1:60. The reagent composition for RT-qPCR was SYBR Green 5 μl, primer 1 μl, cDNA 3 μl, up to a total mix of 10 μl. The PCR program involved initial denaturation 95 °C for 2 min, 95 °C for 10 s, annealing 56 °C for 60 s, for a total of 40 cycles.

2.2.3. - Relative quantities calculation

The relative quantities were calculated using miR-16 as an endogenous control. The miR-16 primer was 5′UAGCAGCACGUAAAUAUUGGCG. MiR-16 levels were confirmed to be stable from previous studies. The relative expressions (RQ) of miR-122 and miR-150 were calculated using $2^{-\Delta\Delta C}$ [25].

2.3. Statistical analysis

Data were analyzed using STATA 15. Patient characteristics were presented in mean and standard deviation (SD), percentage, median, and maximum-minimum values. The statistical analyses used unpaired T-test, Kruskal-Wallis, Mann-Whitney, and Chi-square tests. The results were significant if $p < 0.05$.

The Receiver Operating Characteristic (ROC) curve analysis was used to determine the sensitivity, specificity, and cut-off values, based on the Youden index. Tiered logistic regression analysis was used to measure the combination of miR-122 and miR-150 to differentiate between HCC and cirrhosis. The *p*-values included in the logistic regression analyses were any results with <0.25. The results were significant if $p < 0.05$.

3. Results

3.1. Characteristics and clinicopathological of the study group

This study involved 122 respondents, including 66 patients with liver cirrhosis, 27 with HCC, and 29 healthy control subjects. Table 1 shows the subjects' characteristics.

There were significant differences in platelet counts and AST levels between the cirrhosis and HCC groups. Relative quantities (RQ) of miR-122 were significantly different between cirrhosis to healthy and HCC to healthy. Meanwhile, the RQ of miR-150 was significantly different between cirrhosis against healthy and between cirrhosis against HCC (Table 1).

3.2. Receiver Operating Characteristic (ROC) curve analysis

The ROC test to analyze the diagnostic ability of RQ miR-122 and RQ miR-150 to discriminate HCC from cirrhosis. The diagnostic performance of RQ miR-122 and RQ miR-150 to discriminate between cirrhosis and HCC is shown in Figs. 1 and 2. The combined diagnostic performance of RQ miR-122 and RQ miR-150 to predict HCC from cirrhosis was 71.94% (Table 2) (Fig. 3).

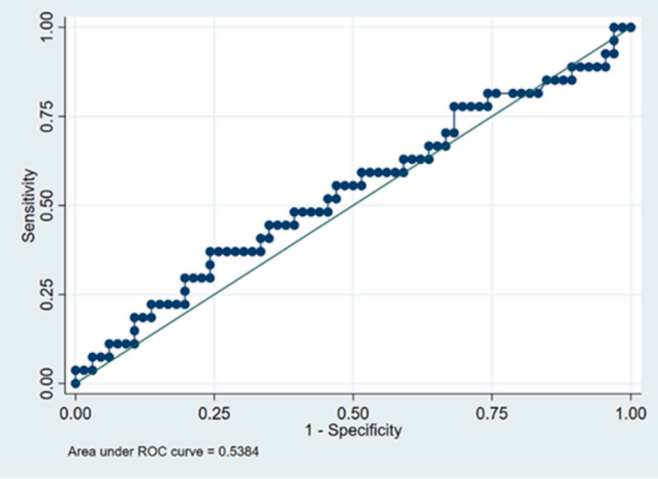

Fig. 1. ROC RQ miR-122 to discriminate HCC from cirrhosis.

Table 1
Characteristics of the study group.

Variable	Cirrhosis (n = 66)		HCC (n = 27)		Healthy (n = 29)		*p*-Value*
	Number/Level/Mean/Median	%/SD/Min-Max	Number/Level/Mean/Median	%/SD/Min-Max	Number/Level/Mean/Median	%/SD/Min-Max	
Gender							
* Male	48	39	19	16	26	21	0.146[#]
* Female	18	15	8	7	3	2	
Age	57.50[a]	23–74	58[b]	21–80	48[a,b]	40–68	0.0079^
Etiology							
* Hepatitis B	58	62	18	19			
* Hepatitis C	4	4	2	2			
* Unspecific	4	4	7	8			
Platelet (x10^3/µL)	95.5	28–242	283	91–556			0.000[‡]
Albumin (g/dL)	3.53	0.78	3.33	0.73			0.2778[∞]
AST (U/L)	43	18–250	97	18–903			0.0010[‡]
ALT (U/L)	32	13–148	39	10–350			0.4115[‡]
CTP							
* A	34	37	14	15			0.976[#]
* B and C	32	34	13	14			
BCLC							
A			2	7			
B			13	48			
C			11	41			
D			1	4			
RQ miR-122	2.70[a]	0.06–72	2.98[b]	0.23–162.53	0.98[a,b]	0.14–8.95	0.0108^
RQ miR-150	0.63[a,c]	0.06–35.22	2.79[c]	0.09–185.18	2.43[a]	0.05–44.88	0.0039^

AST: aspartate transaminase, ALT: alanine transaminase, CTP: Child Turcotte Pugh, BCLC: Barcelona-Clinic Liver Cancer staging, SD: standard deviation, RQ: relative quantities.
*: Significant, $p < 0.05$, [#] Chi-square test, ^: Kruskal-Wallis test, [‡]: Mann-Whitney test, ∞: Unpaired T-test.
[a] Significantly different between cirrhosis from healthy.
[b] Significant difference between HCC from healthy.
[c] Significant difference between cirrhosis from HCC.

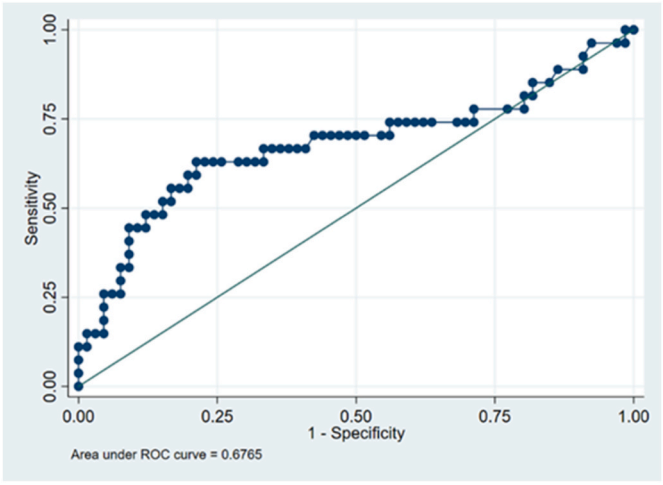

Fig. 2. ROC RQ miR-150 to discriminate HCC from cirrhosis.

Table 2
Diagnostic performance of RQ miR-122 and RQ miR-150 to discriminate HCC from cirrhosis.

	Cut off	Sensitivity	Specificity	AUC (95% CI)	*p*-Value
Cirrhosis vs HCC					
RQ miR-122	9.11	37.04%	75.76%	53.84% (40.35%–67.34%)	0.2120
RQ miR-150	1.47	62.96%	78.79%	67.65% (53.69%–81.61%)	0.0001*
RQ miR-122 + miR-150	–	–	–	71.94% (60.98%–82.91%)	0.0006*

AUC: area under the curve.
RQ: relative quantities.

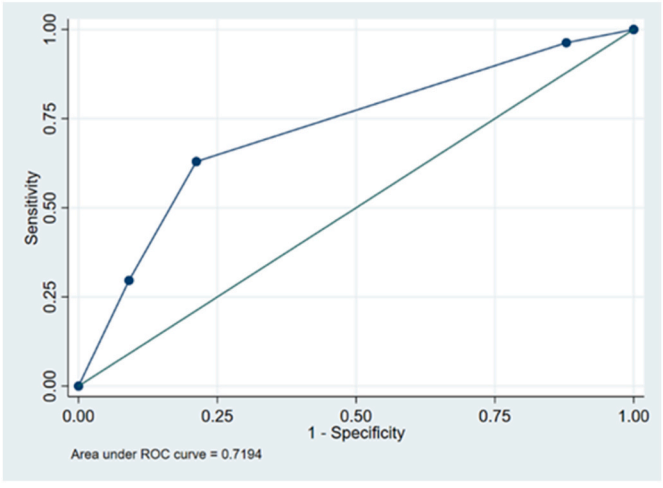

Fig. 3. ROC of combined RQ miR-122 and RQ miR-150 to discriminate HCC from cirrhosis.

4. Discussion

The results showed that there were significant differences in platelet count and AST levels between the cirrhosis and HCC groups. Cirrhosis tends to have thrombocytopenia, whereas, in HCC, some patients have a platelet count of more than 450 × 10^3/mL (Table 1). The increase in platelets in HCC is associated with facilitating an ideal environment for cancer growth [26,27]. Meanwhile, decreased platelet count in cirrhosis is associated with decreased production, sequestration in the spleen, and increased platelet destruction [28].

The AST levels tend to be elevated in both patients with cirrhosis and those with HCC. Results showed that there were significant differences in AST levels between cirrhosis and HCC (Table 1). AST is less specific because it is also found in organs other than the liver [29]. High AST levels in HCC may be due to damage to organs other than the liver. Meanwhile, albumin and ALT levels of cirrhosis and HCC tend to be as expected (average range values of albumin in the laboratory: 3.40–5.00 g/dL, ALT: 12–78 U/L). There was no difference in albumin and ALT levels between cirrhosis and HCC (Table 1). In this study, the difference in platelets and AST and no difference in albumin and AST between cirrhosis and HCC were probably due to the clinical condition of the patients (Table 1). The CTP levels that did not differ between cirrhosis and HCC could possibly have influenced these results (Table 1). In addition, it can also be caused by albumin levels that can still be compensated, and the exchange in capillaries and interstitials is still good [30].

Meanwhile, the characteristics of patients in both the cirrhosis and HCC groups were primarily male, and the most common etiology was hepatitis B. Although there was an age difference between cirrhosis with healthy and HCC with healthy (Table 1), there was no correlation between gender and age with RQ miR-122 and miR-150 (data not shown). These results indicate that the RQ of miR-122 and miR-150 was not influenced by gender and age in this study. There was no difference in CTP levels between cirrhosis and HCC, showing similar clinical characteristics in both groups. Furthermore, in patients with HCC, more than 80% fell into the classifications B and C BCLC (Table 1).

The RQ of miR-122 tended to be higher in HCC and cirrhosis than in healthy controls but did not differ between HCC and cirrhosis (Table 1). Studies in hepatitis C virus-associated HCC have shown a decrease in the relative quantity of miR-122 in HCC compared to patients with chronic hepatitis C [21]. Meanwhile, another study on hepatitis B virus-associated HCC showed a significant increase in the relative expression of miR-122 in HCC compared to hepatitis B virus-associated liver cirrhosis [31]. Another study on HCC and hepatitis B virus-associated cirrhosis showed increased miR-122 expression in HCC but were not significantly different [30]. Race [32] and etiologic factors seem to influence the increase and decrease in the relative expression of miR-122. In this study, most of the etiology was hepatitis B. MiR-122 is a tumor suppressor and plays a role in hepatic homeostasis [16]. However, miR-122 also regulates oncogenic genes such as ADAM17, Bcl-w, Wnt1, and cyclin G1 [33]. Previous studies have shown inconsistent regulation. Some studies show an increase in HCC, but other studies show a decrease. However, it was stated that miR-122 is a biomarker used for cirrhosis and HCC caused by HBV or HCV, where levels are elevated in both subjects with cirrhosis and HCC [34].

The relative quantity of miR-150 increased in HCC and was significantly different between cirrhosis and HCC but could not be used to differentiate between HCC and healthy controls (Table 1). Other studies on hepatitis B virus- and hepatitis C virus-associated HCC have shown a pattern of decreased relative expression of miR-150 [22,23]. MiR-150 has an antifibrotic role and works to inhibit the activation of liver stellate cells [19]. In addition, miR-150 also acts as a tumor suppressor [18]. According to the theory, miR-150 has decreased expression in cirrhosis and HCC. However, the results of this study differ from this theory. Wang et al. (2014) showed increased levels of miR-150 in patients with intrahepatic cholangiocarcinoma [35]. Although in vitro studies generally show the role of miR-150 as an antifibrotic and tumor suppressor, it seems that there is still a need for more in-depth studies regarding the role of miR-150 in HCC patients, considering that studies of miR-150 in HCC are still very few, especially regarding miR-150 as a diagnostic tool. Shen et al. (2016) explained that miR-150 could be used

as a diagnostic and prognostic biomarker for hepatitis B virus- and hepatitis C virus-associated HCC [34].

This study indicates that miR-122 can differentiate patients with cirrhosis and HCC from healthy controls, whereas miR-150 can distinguish healthy subjects and patients with HCC from those with cirrhosis (Table 1). The combined ROC results of RQ miR-122 and miR-150 to discriminate HCC from cirrhosis were AUC 71.94% (CI: 60.98%–82.91%), with p-value = 0.0006 (Table 2). If the diagnostic algorithm uses only the RQ of miR-122, then there is less potential to differentiate HCC from cirrhosis. In hepatitis B virus- and hepatitis C virus-associated HCC, meta-analyses have shown that miR-122 is less potent at differentiating HCC from cirrhosis [36,37]. Meanwhile, we found miR-150 to be better at differentiating HCC from cirrhosis. Studies of miR-150 as a diagnostic tool for cirrhosis against HCC are still minimal. The results of Yu et al. (2015) showed that the AUC of HCC vs. healthy and HCC vs. chronic hepatitis B were 0.931 and 0.881, respectively [22]. Meanwhile, the study by Shaheen et al., in 2018 found the cut-off of 1.0005 can detect malignant transformation of HCC in non-cirrhotic HCV (AUC 0.704), and the cut off value of 0.67453 can be used as a diagnostic marker of HCC from healthy controls (AUC 0.638) [23].

Our results found that the combination of miR-122 and miR-150 has the potential to serve as a biomarker of HCC in patients with liver cirrhosis. Our study examined the potential application of miR-122 and miR-150 as biomarkers of HCC pathogenesis and disease progression regardless of the underlying etiology. One of the weaknesses of this study is that we did not classify patients with combined cirrhosis with HCC (cirrhosis and HCC), and there has been no attempt to classify them. Additional research is needed for the identification, explanation, and exploration of possible comorbid etiologies.

5. Conclusions

There are differences in the relative quantity of miR-122 between cirrhosis and healthy individuals and between HCC and healthy individuals. Our results show that there is a difference in the relative quantity of miR-150 between patients with cirrhosis and healthy controls and between subjects with cirrhosis and those with HCC. The combination of miR-122 and miR-150 has the potential as a biomarker to differentiate HCC from liver cirrhosis.

Ethics approval

The Medical and Health Research Ethics Committee of the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada - Dr. Sardjito General Hospital approved this research, with the number KE/FK/0631/EC/2020.

CRediT authorship contribution statement

Nur Signa Aini Gumilas: Investigation, Formal analysis, Writing – original draft. **Irianiwati Widodo:** Conceptualization, Writing – review & editing, Supervision, Project administration. **Neneng Ratnasari:** Resources, Writing – review & editing. **Didik Setyo Heriyanto:** Investigation, Writing – review & editing.

Declaration of competing interest

The authors have no conflicts of interest to declare.

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