Association of Toll-Like Receptor-9 Polymorphism with Host Susceptibility to Chronic Hepatitis B Virus Infection Among Iraqi Patients

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Abstract
TLR9 is essential for the innate immune response by detecting intracellular viral dsDNA, which activates the immune response against HBV. In this research, we aimed to see whether the TLR9 rs187084, rs352140, and rs5743842 polymorphisms were associated with HBV persistence in Iraqi chronic HBV patients. These SNPs were genotyped by the RFLP-PCR technique in 100 HBV patients and 120 healthy controls. The frequencies of the rs187084 G allele were highly significant in HBV patients compared to controls (133 vs. 84 %; p 4.681e-11). with an OR of 3.687. (95% CI: 2.482-5.476) The GG genotype was significantly more prevalent in patients than in controls. (55 vs 10%; p 0.0001), with an OR of 10.00 (95% confidence interval: 4.48-22.32). Also, the frequency of the rs352140 C allele was higher in HBV patients than control (104 vs. 72 %; p= 2.72e-6), with an OR of 2.527 (95% CI: 1.709-3.74). The frequency of rs352140 SNP CC genotypes in patients was also significantly increased. (38 vs 8.3%; p= 0.0001) with an OR of 6.741 (95% CI: 3.14-14.45). Haplotype analysis revealed that the A-T haplotype of TLR-9 SNPs (rs187084 and rs352140) was associated with a considerably lower HBV risk. The other haplotypes of TLR-9 SNPs (rs187084 and rs352140) didn't show any significant. In conclusion, these SNPs are associated with the susceptible persistence of HBV infection, and this is the first study to look for an association between polymorphisms in genes implicated in the innate immune response and HBV infection in the Iraqi population.

Keywords: HBV persistence, haplotype, innate immune response, polymorphism, Iraq.

1. Introduction

The hepatitis B virus causes a possibly fatal liver infection (HBV). It's a worldwide health issue. Chronic infection may induce cirrhosis and liver cancer (WHO, 2021). Tokunaga and colleagues identified it in 1984 as a bacterial DNA recognizer in human immune cells (Tokunaga et al., 1984). Then, (Krieg et al., 1995), linked activation B cell to a highly conserved cytidine-phosphate-guanosine (CpG) DNA pattern seen in bacterial and viral genomes. TLR9 regulates the cellular response to CpG DNA, as demonstrated in mice for the first time by (Hemmi et al., 2000). TLR9 gene is found on the 3p21.3 chromosome, as well as the protein produced by it is highly preserved that plays a critical role in pathogen identification and immune response activation. TLRs are important for immunological responses, particularly innate immunity, versus viral illnesses like hepatitis B. TLR9 identifies viral dsDNA within cells and triggers an immune response towards HBV. A flaw within this system, on the other hand, might result in a weaker response, leading to long-term HBV infections. HBV's replication cycle makes its eradication challenging. Hepatitis B virus (HBV), unlike HCV, creates a covalently closed circular DNA (cccDNA) minichromosome in the nucleus that is untargeted by conventional therapies. It functions as the transcriptional template for HBV-encoding pre-genomic RNA and messenger RNAs (mRNAs) which are required for replication, secretion, and reinfection of virus. (Revill et al., 2019).

TLR9 is only found in the endoplasmic reticulum of dormant cells (ER) (Leifer et al., 2004). TLR9 is activated by CpG DNA recognition and passes through the Golgi apparatus from the Endoplasmic Reticulum (ER) to the endolysosomes (Latz et al., 2016). TLR9 is driven to endosomes by the endoplasmic reticulum protein UNC93B1, and once there, it is cleaved by the resident protease, allowing the shortened version of the receptor to bind MyD88 and begin downstream signaling (Yasuda et al., 2005). TLR9 and MYD88 interact with each other through TIR after TLR9 and CpG-ODNs interact. When MYD88 is activated, it interacts with IRAK1 and IRAK4, activating TRAF3 and TRAF6 via the death domain. Mitogen-activated protein kinase (MAPK), transforming growth factor β-activated kinase 1 (TAK1), Activator protein 1(AP-1), and NF-B will be activated as a result of this events (Chang and Toledo-Pereyra, 2012; Sajadi et al., 2013; Wu et al., 2012).

NF-B plays an important function since it is a transcription factor for numerous genes, including those encoding cytokines, chemokines, addressing and directing molecules, and cost molecules like CD80 and CD86. IRAK1 and TRAF3 also activate IRF7, which is involved in type I interferon transcriptional activation, both directly and indirectly (Hu and Shu, 2020). Interferons type I are the early immune responses to virus infection such as HBV (Alsharifi et al., 2005). TLR9 polymorphisms can influence gene function or protein structure, mRNA subcellular

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localization and conformation, in addition to promoter activity (Zhang et al., 2013). Previous clinical studies indicated the anti-inflammatory action of TLR9 agonist CpG ODN in vitro prevention of hepatitis B virus multiplication (Cooper et al., 2004).

TLR9 mRNA expression is reduced in the peripheral blood mononuclear cells of patients with chronic hepatitis B, according to Xu et al, (2008). As a result, TLR9 may represent a potential gene involved in HBV progression or disease development. This led researchers to investigate if variations in this gene have a role in the development of hepatitis B symptoms.

To begin with, (Jia et al., 2009) found a significant association between that SNP (rs187084) T-1486C allele inside the TLR9 gene and HBV-related liver cirrhosis. Then (Wu et al., 2012) showed the SNP (rs5743836) C allele at the TLR9 promoter region were significantly linked to earlier spontaneous HBeAg seroconversion. SNP (rs352140) It plays a role in the neonatal susceptibility to Transmission of HBV intrauterine (Gao et al., 2015). He et al. found the SNP (rs352140) has a substantial impact in the susceptibility to chronic hepatitis B in Chinese patients (He et al., 2015a).

Two studies on SNP (rs5743842) found no link between it and susceptibility to lung disease (Lee et al., 2006; Torres-García et al., 2013). There does not seem to be any research linking this SNP with hepatitis B, to the best to our knowledge. so, it's has been studied.

2. Methods and Patients

subjects

A case-control study was carried out. 100 patients were HBsAg-positive (67 males and 33 females). The samples were collected from March to September 2021 in Al-Anbar province, west of Iraq, in order to determine SNPs of the TLR-9 gene. The samples were obtained at the Central Public Health Laboratory's Viral Hepatitis Reference, by patients' reviewers at hepatology clinics, and by government and private hospitals. It was observed that the mean age of the patients was 38.54 ± 12.7 years.

The diagnosis of HBV was made using the EASL (European Association for Liver Research) criteria.(Lampertico et al., 2017a), Anti-HbsAg antibodies, anti-HBc IgM, and anti-HBc IgG, were used in the serological diagnostic tests. Detection of HBV through S genes was part of the molecular evaluation. There were no participants in the study who had Infection with HCV or another chronic liver disease disorders. The study also included a group of 120 healthy control patients who were matched for age (40.47 ± 11.03 years), gender (81 men and 39 women), and ethnicity. Iraqi authorities, including the Biology Department's Ethics Committee and the Directory of Health for Anbar in the Iraq Ministry of Health (Reference: 4972-8/2/2021), authorized the study's protocol.

serological tests

A total of three anti-HBV ELISA kits were utilized to examine samples from patients (HbsAg antibodies,Anti-HBc(IgM, & IgG). The kits had supplied by CTKBIotech and MyBioSource (USA) and were utilized in accordance with the instructions.

HBV molecular detection

DNA was extracted from the blood sample with EDTA.following the procedure of the ABIOpure Extraction kit, USA. As shown in (Table 1), Using sense and antisense primers, a 1063 bp area of the pre-S1 through S genes was amplified by PCR.(Naito et al., 2001). The universal primers (P1: sense primer and S1-2: antisense primer) made the mixture of PCR reactions, 2 µl primer (forward and reverse) (10 pmol each), 8 µl DNA, 2.5 µl of nuclease- free water, The combination was put into a tube with a lyophilized master mix (Promega, USA) to make up the initial PCR reaction mixture (25 µl). The PCR program was established. At the thermocycling settings, at 94°C for 5 minutes was initial denaturation followed by denaturation for 40 cycles at 94°C (1 minute), annealing at 58°C (1 minute), and extension at 72°C for 2 minutes. The step is then followed by a 7-minute final extension at 72°C. The products of PCR were electrophoresed on a 2 % agarose-gel at 100 volts/50 mAmp for 60 min. The Gel imaging system was used to see the ethidium bromide-stained bands in the gel. Based on the migration of PCR product size along a DNA ladder pattern.

Table 1 : sense and antisense primers

Primer	Sequence	Genotype	Annealing Temp. (°C)	Size (bp)
P1	5'-TCACCATAT TCT TGGGAACAA GA-3'	Universal	58	1063
S2-1	5'-CGAACCACTGAACAAATGGC-3'	Universal		

TLR-9 gene SNPs

Three genetic variations of TLR9 SNPs were examined: rs178084 in the promoter region (generated by changing the A allele to G on the reverse DNA strand), rs352140 in the second exon region (changing the C allele to T allele on the forward DNA strand), and rs5743842, also in the second exon region (changing the G allele to A allele on the reverse DNA strand). The polymerase chain reaction with restriction fragment length polymorphism (PCR-RFLP) technique was used to detect the SNPs using specific primers (Table 2). PCR primers are designed in accordance with the methodology of (Hashim et al., 2015). with the aid of the NCBI-primer BLAST online software(http://www.ncbi.nlm.nih.gov/tools/primerblast/index.cgi?LINK_LOC=BlastHome). Using the BLAST algorithm on the human genome, the specificity of primers for their target sequences was examined. Then the mutations were interred according to the design demands.The ability of the

primer to generate secondary structure was evaluated using oligo calc onlinesoftware.(http://www.basic.northwestern.edu/biotools/oligocalc.html).

Table 2. Profiles and primers utilized for the RFLP-PCR analysis of TLR-9 gene SNP.

SNP	Primer sequence	Tm°C	Size of product (bp)	Restriction enzyme	Cut product size
rs187084 A>G	F:5′-GCTAGCACACCGGATCATT-3′ R:5′AGCCTTCACTCAGAAATACCCTC -3′	57.61 59.80	216	Tru9I	A=75+141
rs352140 C>T	F:5′-ATGCGGTTGGAGGACAAGGA-3′ R:5′- GAGACTGAGTTCAGGTTCCCC-3′	61.49 59.72	167	Bst FNI	C= 141+26
rs5743842 G>A	F:5′- ATGCGGTTGGAGGACAAGGA-3′ R:5′- GAGACTGAGTTCAGGTTCCCC-3′	55.00 57.14	404	PstI	A=212+123+69 G=335+69

The (F: forward; R: reverse) sequence of primers; Tm: melting temperature and bp: Bas pair

Using the online software "Multiple Primer Analyzer" from Thermo Fisher Scientific Inc., each pair of primers was analyzed for dimer formation. The appropriate restriction enzyme was chosen with the assistance of the online software WatCut (http://watcut.uwaterloo.ca/template), (Table 2) shows the primers, cycling conditions for PCR, and RFLP.Every amplification was performed in a final volume of 20 µL including contains 8µl of Master Mix (Promega Taq PCR Master mix), 1µl of one of the forward primers (10 pmol/ml), 1µl reverse primer (10 pmol/ml), 2 µl of genomic DNA, and 7.5µl of nuclease-free water). The DNA fragments were electrophoresed on 1.5 % agarose gels (Promega Company, USA) after PCR amplification or RFLP analysis. The PCR products were electrophoresed for 60 minutes at 100 volts/50mAmp and stained with ethidium bromide. The migration of PCR products was then seen utilizing a gel-documentation technique in conjunction with a pattern of 100bp DNA ladders.

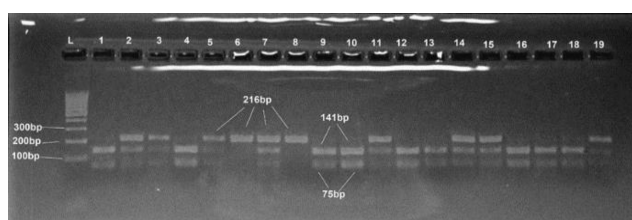

Figure 1: genotyping of rs187084 polymorphism by PCR-RFLP technique, lane L 100bp DNA ladder; lanes 6 and 8 GG genotype; Lanes 2,3,5,7,11,14,15 and19 AG genotype; lanes 1, 4,9, 10,12,13,16,17and 18 AA genotype.

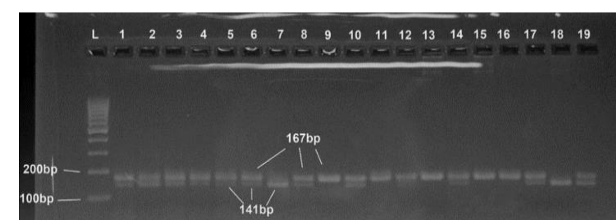

Figure 2: genotyping of rs352140 polymorphism by PCR-RFLP technique, lane L 100bp DNA ladder; lanes 9,11,12,13,15, and 16 TT genotype; Lanes 1,2,3,4,5,6,8,10,14,17 and19 CT genotype; lanes 7and 18and CC genotype.

3. Statistical Methods

The size of sample determined by Open Epi software (Dean, 2010), the power was 80% with 95% confidence level. The SPSS program was employed for all statistical calculations (IBM Corp., 2020). IBM SPSS Statistics for Windows, Version 27.0 (Armonk, NY: IBM Corp. (2019, Microsoft Corp. USA). Odds ratios (OR) and confidence intervals (CIs 95%) were computed. Hardy-Weinberg Equilibrium (HWE) determined by comparing the observed numbers of different genotypes to those predicted based on the estimated frequency of genotypes. A p-value of 0.05 or less was considered as statistically significant. Chi-square test for categorically correlated variables.

4. Results

Diagnosis of Hepatitis B Virus

HBsAg (hepatitis B surface antigen), anti-HBc (hepatitis B core antigen) IgM, and anti-HBc IgG were evaluated qualitatively in the serum of HBV patients and healthy controls. According to the data, all of the patients tested positive for anti-HBc IgG and -HBsAg antibodies but negative for anti-HBc IgM antibodies. As a result, the current sample of HBV patients was classified as having a chronic infection. This profile is compatible with the interpretation of HBV serology used to diagnose chronic HBV infection (Lampertico et al., 2017; Tan et al., 2021). Molecular analysis revealed that following PCR amplification and gel electrophoresis, the CHB patients had a band of 1063 bp. On the other hand, Anti-HBV antibodies were not detected in any of the subjects serving as controls, and the gel electrophoresis results verified the absence of viral DNA.

TLR-9 polymorphism

HBV-infected (n = 100) and healthy control (n = 120) were fully tested for the tlr-9 SNPs using PCR-RFLP methods. A comparison of the two studied groups was made to see if there was a significant difference between the allele and genotype frequency for each SNP. (Table 2). All of the SNPs examined were found at varying frequencies in the populations studied. There were statistically significant differences in the allele and genotype frequencies of the rs187084SNP between patients and the control group. the G allele

frequency was significantly increased in patients (133 vs. 84 %; p 4.681e-11). with an OR of 3.687. (95% CI: 2.482-5.476). The A allele had a significantly lower frequency in patients than in controls (67 vs. 156 %; p< 0.001), with an OR of 0.271. (95 % CI: 0.183-0.403). While genotype frequency, the GG genotype was more frequent in patients with HBV than in controls. (55 vs 10%; p<0.0001) with an OR of 10.00. (95 % CI: 4.48-22.32). and the AA genotype was less prevalent in the HBV patients as compared to the control group (22 vs 48%; p0.00048) Although the difference was a significant (p0.00048), in contrast, While the AG genotype in the patients' HBV patients was less frequent than control (23vs50%; p0.0001), these results showed that the TLR-9 SNP A>G was a significant difference between HBV patients and control (p values<0.05) There was a significant relationship observed under both the dominant a recessive model. The genotypes and allele frequency distributions of the A/G SNP in the TLR-9 gene are shown in (Table2).

Patients and controls had statistically significant differences in allele and genotype frequencies for the rs352140SNP. The frequency of the C allele was significantly higher among patients than control (104 vs. 72 %; p 2.72e-6). with an OR of 2.527 (95% CI: 1.709-3.74) Patients, on the other hand had lower frequency of the T allele than controls (96 vs. 168%; p 0.0001), with an OR of 0.55 (95% CI: 0.196-0.450).

Table 2: The frequency of the TLR9 single nucleotide polymorphism (rs187084), (rs352140) allele and genotype, were determined in total HBV patients and controls.

SNP	Allele/ genotype	N (%)		OR (95%CI)	*P value*
		Patients(N=100)	Control(N=120)		
rs187084	A	67 (34%)	156(65%)	0.271(0.183-0.403)	----
	G	133(66%)	84(35%)	3.687(2.482-5.476)	4.681e-11(S)
	AA	22(22%)	48(40%)	1.00	----
	AG	23(23%)	60(50%)	0.84(0.42-1.68)	0.0001(S)
	GG	55(55%)	12(10%)	10.00(4.48-22.32)	<0.0001(S)
HWE *p*			0.32(NS)		
Recessive model					
AA &AG		45(45%)	108(90%)	1.00	----
GG		55(55%)	12(10%)	11.00(5.38-22.48)	<0.0001(s)
Dominant model					
AA		22(22%)	48(40%)	1.00	
AG&GG		78(78%)	72(60%)	2.36(1.30-4.30)	0.0039
rs352140	T	96(48)	168(70)	0.396(0.267-0.585)	----
	C	104(52)	72(30)	2.527(1.709-3.74)	2.72e-6(S)
	TT	34(34)	58(48.3)	1.00	----
	CT	28(28)	52(43.3)	0.92(0.49-1.72)	0.018(S)
	CC	38(38)	10(8.3)	6.48(2.87-14.65)	<0.0001(S)
HWE *p*			0.83(NS)		
Recessive model					
TT&CC		62(62%)	110(91.7%)	1.00	----
CC		38(38%)	10(8.3)	6.74(3.14-14.46)	<0.0001(S)
Dominant model					
TT		34(34%)	58(48.3%)	1.00	----
CT&CC		66(66%)	62(51.7%)	1.82(1.05-3.14)	0.031

In aspects of genotype frequency, The CC genotype was much more prevalent among HBV patients than of controls. (38 vs 8.3%; p0.0001) with an OR of 6.741 (95% CI: 3.14-14.45). whereas the TT genotype was less frequent in HBV patients than in the control group (34 vs 48.3%; p0.032), While the CT genotype was less frequent in HBV patients than in controls (28 vs 52 %; p0.019), with an OR of 0.51. (95 % CI:0.29-0.90). The TLR-9 SNP C>T was a significant difference between HBV patients and controls (p values 0.05) in both dominant and recessive models. The genotypes and allele frequency distributions for the C/T SNP in the TLR-9 gene are shown in (Table2) For the G/A SNP rs5743842 There was no restriction digestion Although the internal control was digested, only a 404 bp band was found after running on gel electrophoresis. There are no possible genotypes that can be found among Iraqi people, whether they are patients or healthy controls.

Linkage disequilibrium (LD) and haplotype frequencies

The calculated D' value, which had a maximum value of 0.486 for TLR-9 SNPs (rs187084 and rs352140), revealed a moderate LD between TLR-9 SNPs in patients (Figure 3). The calculated two-locus haplotype frequencies, on the other hand, showed some significant differences between patients and controls. The haplotypes A-T of TLR-9 SNPs (rs187084 and rs352140) had a considerably higher frequency in the control group than in hepatitis B virus infection patients and it was associated with a protective role against HBV infection (Table 3).

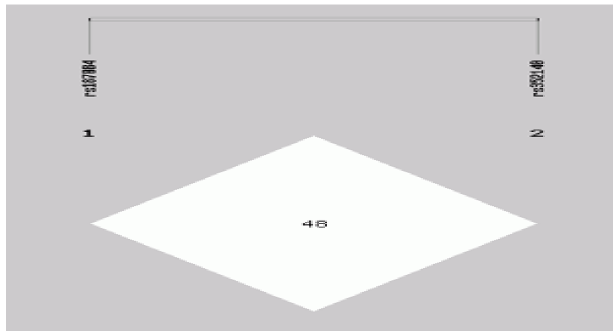

Figure 3The (D') coefficient of linkage disequilibrium between TLR-9 SNPs (rs187084 and rs352140) in patients infected with hepatitis B virus.

Table 3: Haplotype frequencies of TLR-9 (rs187084 and rs352140) in hepatitis B patients and controls.

Haplotype	Patients(N=100)	Control(N=120)	OR (95% CI)	P value
A-C	35.08	45.00	0.906(0.545-1.508)	0.705(NS)
A-T	31.92	62.00	0.506(0.307-0.834)	0.007(S)
G-C	14.92	9.00	2.089(0.885-4.928)	0.087(NS)
G-T	64.08	58.00	1.564(0.993-2.463)	0.052(NS)

CI: confidence interval; p: fisher's exact two-tailed; p;S: Significant (≤ .05); NS: Non- significant (p > .05).

5. Discussion

The results of this study showed that the TLR-9 SNP A>G rs187089 and T/C rs352140 were a significant difference between CHB patients and control group (p value <0.05). There was an association between these SNPs within TLR-9 and chronic hepatitis B infection. There were significant differences in TLR9 haplotype frequencies between HBV infected patients and controls, suggesting that the A-T haplotype may be a protective haplotype did. Multiple studies have shown that the outcome of an HBV infection is influenced by the host's genetic background. This suggests that better understanding of the host-virus interaction could lead to better management of the disease. (Hirsch et al., 2010; Shi et al., 2012). Activating the body's innate immunity is essential for the treatment of CHB patients and the eventual development of a functional cure for the disease. Anti-cancer and anti-infective therapies have used TLR9 agonists because of their capacity to increase both innate and adaptive immune responses. (Krieg, 2006; Shahrakyvahed et al., 2014) Recent research has examined the association between single-nucleotide polymorphisms (SNPs) and genes for innate immune receptors, like (TLRs). Some research indicates that TLR-9 SNPs are associated with HBV infection, (Chihab et al., 2019) showed that the rs187084 G allele protects against HBV infection progression, and that the AA genotype is associated with HBV infection progression to advanced liver disease in chronic HBV patients in Morocco, these finding come on the contrary of our study results. According to (He et al., 2015b), in Chinese chronic HBV infection, the rs352140 variation was associated with chronic infection susceptibility, with CT genotype carriers having a lower risk than CC and TT genotype carriers. This complies with finding of this study that the CC genotype has a relative risk of 6.48(2.87-14.65). (Gao et al., 2015) It was also found in China that The A allele of the rs352140 polymorphism was associated with HBV prenatal transmitting susceptibility in China, whereas the GA genotype indicated protection in neonates. (Zhu et al., 2017) investigated the roles of TLR9 gene variant (rs187084 and rs352140) in a male population on HBV clearance and HCC development in Chinese individuals, SNPs revealed no significant associations with HBV having HCC and HBV clearance spontaneously. There are also other SNPs of TLR-9 like rs5743836 investigated for a significant role in HBeAg seroconversion in CHB in Taiwan (Wu et al., 2012a) and didn't show any association in Turkish population (Katrinli et al., 2018). The third SNP in this study was rs5743842.Two studies (Lee et al., 2006; Torres-García et al., 2013) looked for a link between SNP (rs5743842) and susceptibility to lung disease. This SNP didn't show any results, and there are no possible genotypes that can be found among Iraqi people, whether they are HBV patients or healthy controls.

One of the limitations of this study is that it doesn't include any advanced cases of HBV infection with hepatocellular carcinoma and liver cirrhosis. Serum TLR-9 levels were not measured in the present HBV chronic patient sample., hence no association with TLR-9 gene SNPs was established to realize the SNP's influence on TLR-9 level was made. Furthermore, it is important to measure TLR-9 levels and correlate them with the SNPs of the TLR-9gene in future investigations.

6. Conclusions

This study focused on assessing the potential association polymorphisms of the rs187084, rs352140, and rs5743842 located in the TLR9 promoter, exon, and intron with HBV effect. These SNPs are associated with the susceptibility to HBV infection persistence. TLR9 rs187084 GG genotype and rs352140 CC genotype are risky factors, whereas rs187084 AA genotype and rs352140 TT genotype are more likely to be protective for patients. The haplotypes A-T of TLR-9 SNPs (rs187084 and rs352140) had a considerably higher frequency in the control group and were associated with a protective role against HBV infection. This is the first study to try to find out if there is a link between the polymorphisms of gene within innate immune response and HBV infection in the Iraqi population.

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Author contributions

The three authors worked together on the project design, lab work, analysis of data and manuscript writing.

Declaration of competing interest

None

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