

# A preliminary study on the association between pro-inflammatory cytokine IL-1 $\beta$ polymorphisms and susceptibility to hepatitis C infection in Malay male Malay drug abusers

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## ABSTRACT

**Introduction:** Hepatitis C virus (HCV) infection frequently leads to liver complications, such as fibrosis, cirrhosis, and hepatocellular carcinoma. The incidence of HCV infection transmission among drug abusers is concerning. Interleukin-1 $\beta$  (IL-1 $\beta$ ) is a pro-inflammatory cytokine secreted during innate and adaptive immune responses and plays a pivotal role in chronic inflammatory diseases. Functional single nucleotide polymorphisms in IL-1 $\beta$  cause it to play different roles in disease susceptibility and progression. This study aimed to investigate the association between genetic polymorphisms of pro-inflammatory cytokines (IL-1 $\beta$ ) and HCV infection susceptibility in Malay male drug abusers. **Methods:** In total, 48 male Malay drug abusers were included in this retrospective case-control study. Genomic DNA was extracted from whole blood samples and analyzed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) for the IL-1 $\beta$  rs16944 and rs1143634 polymorphisms. **Results:** Analysis of IL-1 $\beta$  rs1143634 revealed that the C/C genotype was common in both the case and control groups; however, no statistical significance was observed ( $p = 0.068$ ,  $\chi^2 = 3.755$ ). Genotyping of IL-1 $\beta$  demonstrated that all samples were of the homozygous mutant type (T/T). **Conclusion:** There was no association between IL-1 $\beta$  polymorphism (rs1143634 and rs16944) and hepatitis C infection susceptibility among Malay male drug abusers.

**Key words:** hepatitis C, interleukin-1 $\beta$  (IL-1 $\beta$ ), polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), Malay male, single nucleotide polymorphism (SNP)

## INTRODUCTION

Hepatitis C virus (HCV) infection has been recognized as a major global public health issue since 2015. It is estimated that 71 million people have been diagnosed with HCV; currently, there are 58 million chronically infected individuals<sup>1</sup>. Although the decrease in the number of chronic infections is cause for optimism, it should not detract from the severity of this infection. According to the World Health Organization (2022), approximately 290,000 HCV-related deaths were in 2019. These deaths were primarily due to chronic infections progressing to cirrhosis and hepatocellular carcinoma (HCC), as 20% of persistent HCV infection cases develop these conditions<sup>2,3</sup>. Up to 80% of acute HCV infections are asymptomatic, making it difficult to diagnose, which, in turn, increases the risk of latent infection transmission and progression to chronic infection<sup>4,5</sup>.

HCV is a bloodborne liver disease. Injecting hazardous parenteral drugs, engaging in high-risk sexual activity, and receiving blood transfusions are

the three most common modes of HCV transmission<sup>3,6-9</sup>. Therefore, healthcare patients, healthcare workers, and drug abusers are at the highest risk of contracting HCV. Due to this, drug abusers have been the focus of recent studies<sup>10</sup>. Hazardous activities, such as illicit drug use and the sharing of contaminated syringes, escalate HCV transmission<sup>11,12</sup>.

HCV infection is endemic globally; however, the prevalence of the disease varies globally<sup>6,13</sup>. Studies on the global distribution and prevalence of HCV genotypes revealed the diverse prevalence of genotypes 1, 2, 3, and 6 across Southeast Asian countries<sup>13,14</sup>. Southeast Asia has a particularly high prevalence of genotype 6, with 94.6 million infected individuals<sup>15</sup>. Studies on HCV genotype prevalence in Malaysia reported that genotype 3 is the most common, followed by genotype 1<sup>16,17</sup>. In 2017, HCV infection had an incidence of 9.54 in 100,000 people, while the mortality rate was 0.29 per 100,000 people<sup>18</sup>.

Viral proteins and double-stranded RNA (dsRNA) from HCV induce the production of pro-

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inflammatory cytokines<sup>19</sup>. During the early stages of infection, the intricate cytokine network enables the formation of coordinated and efficient innate and adaptive immune responses<sup>20-23</sup>. A viral infection outcome is determined by the interaction between the host's capacity to generate a powerful antiviral response and the viral mechanisms that neutralize such responses<sup>24</sup>. It is impossible to adequately emphasize the significance of pro-inflammatory cytokines, such as interleukin-1 (IL-1), in immune response regulation. This is because many factors influence immune system function, particularly when it is affected by diseases such as HCV. Addressing the genetic diversity of a host during a latent infection is one way to ascertain the significant associations between the behaviors of different cytokines. Functional single nucleotide polymorphisms (SNPs) of IL-1 have diverse distributions among major populations in America, Europe, South Asia, East Asia, and Africa. Studies have reported that different IL-1 expression levels and polymorphisms may impart different effects on disease conditions<sup>25-28</sup>. Therefore, there is a growing need to understand the role that SNPs play in the progression of chronic HCV infection due to the growing body of evidence suggesting a relationship between SNPs and disease progression, particularly in diseases involving inflammation. Genotyping and SNP analysis provide information that can be used to build genome-based knowledge regarding individual susceptibility to a variety of common diseases, produce safer and more effective personalized diets and treatments for patients, and improve the understanding of the evolutionary processes of diseases<sup>29</sup>. This study aimed to investigate the association between the genetic polymorphisms rs16944 and rs1143634 of pro-inflammatory cytokine IL-1 and HCV infection susceptibility in male Malay drug abusers.

## MATERIALS AND METHODS

### Subject recruitment and DNA extraction

A retrospective case-control study design was used involving male Malay drug abusers. The sample size was calculated using a web calculator and in reference to a previous study<sup>30,31</sup>. The sample size consisted of 29 male Malay drug abusers with chronic HCV infection (case group) and 19 male Malay drug abusers without HCV (control group). The participants' ages ranged from 35–60 years old. Medical histories were obtained, and physical examinations were performed for each participant. Subject recruitment received ethical review and approval from the Medical Research

and Ethics Committee, Ministry of Health Malaysia (NMRR-19-399-45866), and the USM Human Research Ethics Committee (USM/JEPeM/18010012). Several inclusion criteria were used: 1) participants had to be third-generation Malay, 2) be at least 18 years old and have a history of drug abuse, and 3) for the control group, have tested positive for HCV for six months based on a structured clinical review using the DSM-V. Participants who met any of the exclusion criteria were not included in the analysis: 1) had non-HCV-related liver disease, 2) had a mental disorder, and 3) refused or were unable to provide informed consent.

A 10 mL blood sample was collected from each patient in a sterile tube containing heparin K<sub>2</sub>EDTA. Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Spectrophotometry was used to determine the purity and concentration of the extracted DNA. DNA quality was reflected by a consistent ratio of 1.8 – 2.0. The coded genomic DNA solution was stored at 4°C.

### Genotyping with PCR-RFLP

The extracted genomic DNA extracted was genotyped using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The amplification was performed in 25  $\mu$ L of reaction solution containing a mixture of PCR buffer (1 $\times$ ), dNTPs (2 mM), MgCl<sub>2</sub> (1.5 mM), forward and reverse primers (20 pmol) (Table 1), Taq polymerase (2.5 U), and sufficient nuclease-free water. PCR amplification targeting IL-1 $\beta$  rs16944 was performed following several steps: an initial denaturation at 95°C for 5 minutes followed by 35 cycles at 95°C for 30 seconds, 57°C for 30 seconds, 72°C for 30 seconds, and a final extension at 95°C for 5 minutes. The PCR product was then mixed with 2  $\mu$ L of AvaI (5 U) and incubated at 37°C for 2 hours. Genotypes of rs16944 were identified using their base pair size, where the wildtype (C/C) corresponded to two bands of 190 and 116 base pairs, heterozygous (C/T) corresponded to three bands of 190, 116, and 306 base pairs, and mutant (T/T) corresponded to a single band of 306 base pairs. For rs1143634, different PCR steps were followed: an initial denaturation at 94°C for 5 minutes followed by 35 cycles at 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds, and a final extension at 94°C for 5 minutes. The PCR product was then mixed with 2  $\mu$ L of TaqI (5 U) and incubated at 65°C for 2 hours. Genotypes were identified according to their base pair size, where wildtype (C/C) corresponded to two bands of 126 and 104 base pairs, heterozygous

(C/T) corresponded to three bands of 126, 104, and 230 base pairs, and mutant (T/T) corresponded to a single band of 230 base pairs. Digestion fragments were separated by electrophoresis on 2% agarose with gel stain (Invitrogen SYBR Safe DNA, USA) using the available size ladder for comparison.

### Statistical analysis

The independent samples t-test and Mann–Whitney test were used to compare continuous data. The Hardy–Weinberg equilibrium (HWE) was used to ascertain the quality of the genotyping method for genotype and allelic analysis. The HWE p-value was determined by comparing the control group with a reference population in a  $2 \times 2$  contingency table (CC vs. CT+TT). Differences in allele and genotype frequencies between the study groups were compared using chi-squared tests. Statistical significance was defined as  $p < 0.05$ . Fischer's exact test was employed in place of the chi-squared test when more than 20% of the expected counts were less than five in the  $2 \times 2$  contingency table.

## RESULTS

**Table 2** shows the demographic and clinical characteristics of the study participants. **Table 3** shows the association between HCV infection and human immunodeficiency virus (HIV) co-infection in male Malay drug abusers. **Figure 1** and **Figure 2** present the genotyped samples targeting IL-1 $\beta$  at positions –511 (rs16944) and +3954 (rs1143634). The genotype and allele distributions of rs16944 and rs1143634 between the case and control groups are presented in **Table 4**.

No significant differences were seen in age, weight, height, body mass index (BMI), systolic blood pressure, and diastolic blood pressure between the case and control groups. In addition, liver test results (bilirubin level, AST, ALP, ALT, and GGT) did not significantly differ between the case and control groups. However, there was a significant correlation between HCV infection and HIV co-infection; 58.62% of case group participants were co-infected with HIV, while the control subjects had a co-infection rate of 21.05% ( $p = 0.01$ ,  $\chi^2 = 6.583$ ).

For rs16944, the HWE value deviated from the reference population ( $p < 0.05$ ). Allelic analysis revealed similar findings. Genotyping of IL-1 $\beta$  rs16944 showed that all samples from the case and control groups were of the homozygous mutant type (T/T). Allelic frequency analysis indicated that the T allele was dominant. Due to the non-varied frequencies of alleles and genotypes, no analysis could be performed.

The chi-squared test results and p-values of the genotypic and allelic frequency for this gene were undefined.

The HWE result for the genotyping of rs1143634 was reliable ( $p = 0.581$ ). The frequency of this genotype in the control group was comparable to the reference population. Allelic analysis demonstrated similar results ( $p = 0.593$ ). Genotyping of IL-1 $\beta$  rs1143634 revealed that 21 (72.41%) HCV-infected patients had the homozygous wildtype (C/C), 7 (24.14%) were heterozygous (C/T), and 1 (3.45%) was mutant (T/T). The frequencies between the reference population and the control group demonstrated similar distributions. The genotypic frequency of the homozygous wildtype was much higher in the control group than in the case group; the observed frequency of the C/C genotype in the control group was 94.74%. However, the heterozygous genotype in the control group was much lower than in the case group, with only one (5.26%) group participant demonstrating this genotype. There were no homozygous mutant (T/T) genotypes reported in the control group. The frequency of the C allele in the control group (37; 97.37%) was higher than in the case group (49; 84.48%), while the frequency of the T allele was higher in the case group (9; 15.52%) compared to the control group (1; 2.63%). Statistical evaluation of IL-1 $\beta$  rs1143634 revealed no significant association between HCV infection and genotype distribution ( $p = 0.068$ ,  $\chi^2 = 3.755$ ). Furthermore, there was no significant association between the allelic distribution and HCV infection ( $p = 0.083$ ).

## DISCUSSION

Since its discovery, HCV infection has been a continuous public health concern. The acute stage of the infection is typically followed by the progression to chronic disease if the infection is allowed to persist. There are several factors that affect HCV progression and an individual's susceptibility to the transformation of an acute HCV infection into a chronic condition. For instance, the illicit use of controlled substances disrupts liver metabolism. This disruption occurs proportionally to the role of specific substances as immunological mediators. Drugs are well-known immune modulators, and excessive use of these substances can lead to elevated levels of inflammatory cytokines and an increased risk of infection<sup>34</sup>.

In the general population, the rs16944 SNP was found to exhibit a heterogenous genotype (C/T). The frequency of the T allele was found to be slightly higher than the C allele. In the East Asian (EAS) population, the most common genotype was the heterogenous (C/T) genotype. Furthermore, the C allele was

**Table 1: Primers and restriction enzymes used for PCR-RFLP**

	RE	Forward Primers (5'-3')	Reverse Primers (5'-3')	Ref.
rs16944	<i>AvaI</i>	TGGCATTGATCTGGTTCATC	GTTTAGGAATCTTCCCCTT	32
rs1143634	<i>TaqI</i>	GACTTTGACCGTATATGCTCAG	ATGGACCAGACATCACCAAGC	33

**Table 2: Demographic and clinical characteristics of all subjects**

Characteristics	Drug abuser		P-value
	Case Group (n = 29)	Control Group (n = 19)	
Age (year)	44.59 (6.23)	43.05 (7.48)	0.445a
Weight (kg)	164.76 (4.76)	164.37 (7.85)	0.847a
Height (m)	58.18 (7.33)	59.58 (9.08)	0.560a
BMI (kg/m <sup>2</sup> )	21.46 (2.67)	22.02 (2.86)	0.491a
Systolic BP (mm/Hg)	120.55 (16.94)	120.68 (10.68)	0.378b
Diastolic BP (mm/Hg)	82.37 (8.87)	79.26 (7.73)	0.221b
Bilirubin (μmol/L)	9.89 (9.84)	6.45 (2.8)	0.176b
ALT (U/L)	33.43 (18.91)	42.31 (38.8)	0.987b
AST (U/L)	49.48 (24.64)	48.15 (34.31)	0.429b
ALP (U/L)	103.39 (29.13)	96.23 (31.61)	0.497a
GGT (U/L)	65.35 (42.03)	79.85 (75.63)	0.908b

<sup>a</sup>Independent-sample t-test, <sup>b</sup>Mann-Whitney test; Data were presented in Mean (SD).

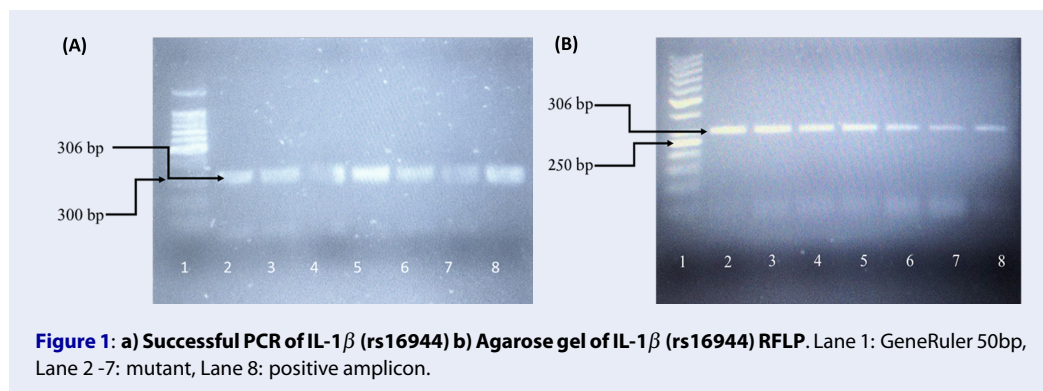
\*Statistically significant at P < 0.05.

**Abbreviations:** ALT: Alanine aminotransferase, AST: Aspartate transaminase, ALP: Alkaline phosphatase, GGT: Gamma-glutamyl transpeptidase

**Table 3: Association of HCV infection and HIV coinfection in Malay male drug abusers**

Characteristics	Drug abuser		P value
	Case Group (n=29)	Control Group (n=19) N (%)	
HIV co-infection	17 (58.62)	4 (21.05)	0.01c*

<sup>c</sup>Pearson Chi-square test; \*Statistically significant at P < 0.05.

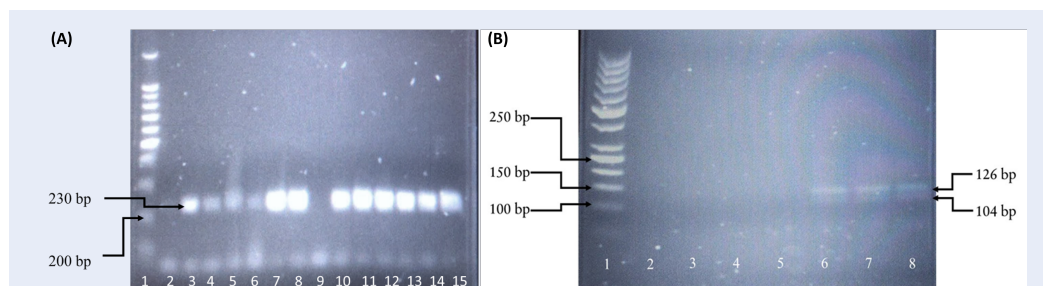


**Table 4: Statistical analysis of allelic and genotypic frequencies of IL-1β (rs16944 and rs1143634)**

IL-1β	Genotype and allele	Control group (n=19), N (%)	Case group (n=29), N (%)	EAS HWE (n=504), N (%)	HWE P-valued (<0.05)	χ <sup>2</sup> (df)	p-value
-511 (C>T) rs16944	CC	0	0	145 (28.80)	0.006*	-	-
	CT	0	0	245 (48.61)			
	TT	19 (100)	29	114 (22.62)	0.000*	-	-
	C	0	0	535 (53.10)			
	T	38 (100)	58 (100)	473 (46.92)			
+3954 (C>T) rs1143634	CC	18 (94.74)	21 (72.41)	482 (95.63)	0.581	3.755 (1)	0.068
	CT	1 (5.26)	7 (24.14)	21 (4.17)			
	TT	0	1 (3.45)	1 (0.20)			
	C	37 (97.37)	49 (84.48)	985 (97.72)	0.593	4.085 (1)	0.083
	T	1 (2.63)	9 (15.52)	23 (2.28)			

**Abbreviations:** EAS: East Asian, HWE: Hardy-Weinberg Equilibrium, Fisher exact, \*Statistically significant at p < 0.05.

**Note:** The HWE p-value was determined by comparing the frequencies (CC vs. CT+TT) from HN (control group) to the reference population, East Asia (EAS). The normal p-value is evaluated by comparing the frequencies (CC vs. CT+TT) from control group to the case group.



**Figure 2: a) Successful PCR of IL-1β (rs1143634) b) Agarose gel of IL-1β (rs1143634) RFLP.** Lane 1: GeneRuler 50bp, Lane 2-5: Not applicable, Lane 6 – 8: Wild type

slightly more prevalent in the EAS population. Comparing the allelic distribution in the control group and the EAS population, a significant deviation in genotypic and allelic distribution was found. Regarding the rs1143634 SNP, the C/C genotype was predominant in the general population. Additionally, the C allele frequency was increased in the general population. The C/C genotype was the most common within the EAS population, and the C allele had a higher frequency than the A allele. The rs1143634 genotype in the control group exhibited a similar trend to the ref-

erence population; the HWE values were in equilibrium and, therefore, did not deviate from the reference population.

In the absence of disrupting events, the HWE states that genetic variation in a population will remain constant from generation to generation. This principle is based on several assumptions, including random mating, the absence of natural selection, a large population size, no gene flow or migration, no mutation, and the autosomal nature of the locus<sup>35</sup>. The deviation from the HWE observed in the control group may be

indicative of significant issues, such as selection bias, population stratification, and genotyping errors<sup>36,37</sup>. The results of the current study indicated no relationship between the genotypic frequency of IL-1 $\beta$  rs1143634 and HCV infection. Furthermore, the frequency of homozygous C/C—which corresponds to the G/G genotype—was higher in the control group. The frequency of the C allele was not significantly correlated with chronic HCV illness. In a previous study, no genotypes of IL-1 $\beta$  rs1143634 demonstrated a significant association with the progression to chronic HCV infection among Tunisian patients<sup>38</sup>. However, the C/C genotype frequency in the G2 group (62.5%) was higher than the frequency of heterozygous C/T and homozygous mutant (T/T) genotypes. The G2 group was comprised of 24 participants who were thought to have recovered from HCV infection without medical intervention, as evidenced by two HCV-PCR tests taken one year apart that were both negative<sup>39</sup>.

Previous studies have found that IL-1 $\beta$  rs1143634 genotypes have no significant influence on liver cirrhosis predisposition in patients with chronic HCV infection<sup>40</sup>. The genotypic distribution of IL-1 $\beta$  rs1143634 has also been shown to have no influence on spontaneous HCV clearance among the European population<sup>41</sup>. Another study on the genotypic frequencies of the IL-1 $\beta$  rs1143634 gene and HCV treatment inferred that the C/C genotype was strongly associated with interferon therapy<sup>38</sup>. In addition, the examination of allelic distribution revealed that the T allele was associated with treatment non-response, increased fibrosis, and increased hepatic activity. In contrast, the C allele was significantly associated with decreased fibrosis, lower hepatic activity, and persistent viral response.

According to existing research<sup>32,33,39,42–45</sup>, polymorphisms of IL-1 $\beta$  at locus –511 are associated with progression to chronic HCV infection. However, the genotypes associated with HCV infection susceptibility are diverse. While one study found that the C/C genotype was predominant in HCV-positive patients<sup>42</sup>, another reported that the C/T genotype was more prevalent in Wang *et al.*'s study (2003)<sup>43</sup>. Moreover, other research has concluded that the T/T genotype was a significant risk factor for the progression of chronic HCV infection to HCC<sup>44</sup>. In addition, the mutant T/T allele has been frequently reported in patients with chronic HCV and HCV-induced liver cirrhosis<sup>42</sup>. An extensive inflammasome gene polymorphism study reported that the C allele of IL-1 $\beta$  rs16944 was less likely to advance hepatitis C development<sup>45</sup>. These differences in IL-1 $\beta$  rs16944 genotypes are primarily caused by the ethnic background

of the population observed in each respective study, namely, American, Asian, and European

In Malaysia, there is currently a lack of research regarding the effects of IL-1 $\beta$  gene polymorphisms (IL-1 $\beta$  rs16944 and rs1143634) in drug abusers with chronic HCV infection.

## CONCLUSIONS

There was no association between IL-1 $\beta$  polymorphisms (rs1143634 and rs16944) and HCV infection susceptibility among Malay male drug abusers. The partial results of IL-1 $\beta$  genotype and allele frequency analysis are in line with the findings of several previous studies; however, some previous studies contradict the current findings.

## ABBREVIATIONS

**ALP:** Alkaline phosphatase, **ALT:** Alanine aminotransferase, **AST:** Aspartate transaminase, **BMI:** Body Mass Index, **DNA:** Deoxyribonucleic acid, **DSM-V:** Diagnostic and Statistical Manual of Mental Disorders, **dsRNA:** Double strand ribonucleic acid, **EAS:** East Asian, **GGT:** Gamma-glutamyl transpeptidase, **HCC:** Hepatocellular carcinoma, **HCV:** Hepatitis C virus, **HIV:** Human Immunodeficiency Virus, **HWE:** Hardy-Weinberg Equilibrium, **IL-1 $\beta$ :** Interleukin 1-beta, **PCR-RFLP:** Polymerase Chain Reaction Restriction Fragment Length Polymorphism, **RNA:** Ribonucleic acid, **SNP:** Single nucleotide polymorphism, **WHO:** World Health Organization

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## AUTHOR'S CONTRIBUTIONS

Conceptualization, R.A.B, I.C.N and I.A.; methodology, R.A.B., I.C.N., J.L. and A.K.C.M.N.; software, J.L.; validation, J.L., N.S.B. and R.A.B.; formal analysis, J.L. and N.S.B.; investigation, J.L.; resources, R.A.B., I.C.N. and A.K.C.M.N.; data curation, J.L., N.S.B. and R.A.B.; writing—original draft preparation, J.L. and N.S.B., writing—review and editing, J.L., N.S.B. and R.A.B.; visualization, J.L.; supervision, N.S.B, A.K.C.M.N. and R.A.B.; project administration, R.A.B.; funding acquisition, R.A.B. All authors have read and agreed to the published version of the manuscript.

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## AVAILABILITY OF DATA AND MATERIALS

The data used and analyzed in this study are available from the corresponding author on request. This will require an ethical permit.

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was conducted in accordance with the Declaration of Helsinki and approved by the ethical review and the Medical Research & Ethics Committee, Ministry of Health Malaysia (NMRR-19-399-45866) and USM Human Research Ethics Committee (USM/JEPeM/18010012).

## CONSENT FOR PUBLICATION

Informed consent was obtained from all subjects involved in the study.

## COMPETING INTERESTS

The authors declare that they have no competing interests.

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