A novel intergenic variant linked to IFIH1 rs1990760 polymorphism, rs2111485, shows an association with susceptibility to coronavirus disease 2019 and influences IFIH1 protein levels.

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Abstract

Interferon-induced helicase C domain-containing protein 1 (IFIH1) is one of the main pattern recognition receptors that sense viral RNA and activate host cells to mount an effective antiviral immunity. Therefore, a case-control study (90 patients with mild/moderate COVID-19 and 90 matched controls) was performed to explore the association of two variants of the *IFIH1* gene with COVID-19 risk using the tetra-primer amplification refractory mutation system-polymerase-chain-reaction method. The first is a missense variant, rs1990760 C/T, and the second is an intergenic variant, rs2111485 A/G. In addition, serum IFIH1 levels were assessed using an ELISA kit. Results revealed that mutant alleles (*T* and *G*, respectively) and corresponding homozygous genotypes (TT and GG, respectively) of both variants were significantly associated with increased risk of COVID-19. IFIH1 levels were significantly higher in patients compared to controls and were favorably affected by the rs1990760 and rs2111485 mutant-type genotypes. In conclusion, IFIH1 protein showed up-regulated levels in the serum of patients with mild/moderate COVID-19. In addition, the *IFIH1* gene variants rs1990760 C/T and rs2111485 A/G were associated with susceptibility to COVID-19, and the study suggests that their mutant-type genotypes are not only associated with increased risk of COVID-19 but also contributed to higher serum IFIH1 levels.

1. Introduction

Globally, coronavirus disease 2019 (COVID-19) remains one of the newest and most widespread respiratory viral infections affecting humanity, with millions of cases and deaths recorded. The disease is associated with a wide range of clinical manifestations, from asymptomatic to mild to moderate and may progress to severe pneumonia requiring intensive care (Lamers and Haagmans, 2022). The causative agent of COVID-19 is a novel coronavirus (severe acute respiratory syndrome coronavirus-2; SARS-CoV-2), a single-strand positive RNA virus belonging to the Coronaviridae family (Li et al., 2021). The virus infects lung alveolar cells by attaching its spikes to a receptor on host cell, angiotensin-converting enzyme 2 (ACE2). This leads to hyperactivity of the angiotensin II receptor axis and facilitates virus entry into the cell (Beyerstedt et al., 2021). As an outcome, pro-fibrotic, pro-apoptotic and pro-inflammatory signal pathways are activated to mediate the pathogenesis of SARS-CoV-2 infection, which is characterized by an exacerbated host inflammatory response known as a cytokine storm that may lead to the development of acute respiratory distress syndrome (Lamers and Haagmans, 2022).

Pattern recognition receptors (PRRs) are the first line of host defense system response against viral attack, and interferon-induced helicase C domain-containing protein 1 (IFIH1, also known as MDA5; melanoma differentiation associated gene 5 protein) is one of the main PRRs that first sense viral RNA and activate host cells to produce interferon (IFN) in order to mount an effective antiviral immunity (Brisse and Ly, 2019).

In vitro analysis showed that IFIH1 protein was effective in restricting replication of human respiratory syncytial virus and rhinoviruses, and in children with *IFIH1* deficiency increased susceptibility to common respiratory RNA viruses was indicated (Asgari et al., 2017).

The IFIH1 protein is encoded by *IFIH1* , a gene located in the long arm of human chromosome 2 at position 2q24.2. Naturally occurring single nucleotide polymorphisms (SNPs) of the *IFIH1* gene have been studied worldwide, and some have shown an association with susceptibility to a number of autoimmune diseases (type 1 diabetes, systemic lupus erythematosus, rheumatoid arthritis, and others) as well as infectious diseases such as COVID-19 (Muñiz-Banciella et al., 2023; Xiao et al., 2023). Among the *IFIH1* SNPs that have attracted attention in the topic of COVID-19 susceptibility is rs1990760. Although the evidence is not conclusive, studies have linked rs1990760 to the risk of developing this respiratory infection, especially in cases where the disease is severe (Dieter et al., 2023; Feizollahi et al., 2023; Maiti, 2020). The rs1990760 SNP is a missense variant and there is evidence to suggest that this SNP is in strong linkage disequilibrium (LD) with an intergenic variant located between the *FAP*(fibroblast activation protein alpha) and *IFIH1* genes; it is rs2111485. Studies have revealed that rs2111485 is associated with susceptibility to type 1 diabetes, vitiligo, and hepatitis C virus infection (Gootjes et al., 2022; Jiang et al., 2019; Onan et al., 2019). In addition, a protective role of rs2111485 in spontaneous hepatitis B virus clearance has also been suggested (Yao et al., 2021). In the context of COVID-19 susceptibility and to the researchers' best knowledge, rs2111485 has not been investigated.

Although the wave of COVID-19 has subsided worldwide, there are surged in reported cases among few countries in January 2024 as infection of mutational variances becoming more common, there is still a need to understand the factors that may influence the development of the viral respiratory infection. In this study, two SNPs of the *IFIH1* gene, rs1990760 and rs2111485, were genotyped in patients with mild/moderate COVID-19 with the aim of understanding their role in disease susceptibility. In addition, serum IFIH1 levels were also determined. The impact of rs1990760 and rs2111485 genotypes on IFIH1 levels was also evaluated.

2. Materials and methods

2.1 Patients and controls

The current study was approved by the Institutional Ethics Committee of the Iraqi Ministry of Health. All participants were informed of the study objectives and agreed to participate in it through written consent. In this case-control study, 90 patients diagnosed with mild/moderate COVID-19 with a median age of 40 years and an interquartile range (IQR: 25-75%) of 33-46 years (60% males and 40% females) were enrolled. A control group (HC) of 90 blood donors matched patients for age (39 [IQR: 34-43] years) and gender (64.4% males and 35.6% females) was also enrolled in the study. The HC group included apparently healthy individuals, and serum tests for the blood bank antibody panel, including SARS-CoV-2 IgM and IgG antibodies, were negative. The study was conducted on patients admitted to healthcare units in Anbar Governorate due to signs and symptoms of COVID-19. Molecular examination of nasopharyngeal swabs confirmed infection with SARS-CoV-2 (RealLine SARS-CoV-2 kit, Bioron Diagnostics GmbH). In addition, sera of patients were tested for SARS-CoV-2 IgM and IgG antibodies (VIDAS SARS-CoV-2 IgM and IgG assay kits; bioMerieux, France). Inclusion criteria were positive molecular test, positive serum IgM antibody test, age [?] 18 years, and mild/moderate disease as defined in WHO interim guidelines (WHO, 2020). Exclusion criteria were pregnancy and severe and/or critical COVID-19.

2.2 Baseline laboratory tests

Patients and HC were tested for erythrocyte sedimentation rate (ESR) following the conventional Westergren method. In addition, a complete blood count (total white blood cells [WBC], granulocytes, lymphocytes, and monocytes was conducted using an automated hematology analyzer (CELL-DYN Automated Emerald, Abbott, USA). Granulocyte-to-lymphocyte ratio (GLR) was obtained by dividing the absolute count of granulocytes with the absolute count of lymphocytes. Lymphocyte-to-monocyte ratio (LMR) was obtained by dividing the absolute count of lymphocytes with the absolute count of monocytes.

2.3 SNP selection and detection

According to the literature, two SNPs of the *IFIH1* gene were selected; rs1990760 and rs2111485 (Feizollahi et al., 2023; Jiang et al., 2019; Maiti, 2020; Muniz-Banciella et al., 2023). SNPs were genotyped using the tetra-primer amplification refractory mutation system-polymerase-chain-reaction (T-ARMS-PCR) method as previously described (Medrano and De Oliveira, 2014). A web-based service for T-ARMS-PCR was used to design primers, which were validated using the PrimerBLAST-NCBI database. Details of primers and T-ARMS-PCR conditions are given in Table 1.

2.4 Serum IFIH1 levels

Serum IFIH1 levels were measured using an ELISA kit, and the protocol provided by the manufacturer was followed (ELK biotechnology, USA). The detection range of the kit was 0.32-20 ng/mL with a sensitivity of 0.115 ng/mL.

2.5 Bioinformatic analysis

The protein-protein interaction network of the IFIH1 protein was determined using the Search Tool for the Retrieval of Interacting Genes (STRING) database (von Mering et al., 2003). Only proteins with an interaction score > 0.9 were shown.

2.6 Statistical analysis

SNP alleles and genotypes were described in terms of absolute number and percentage. Hardy-Weinberg equilibrium (HWE) analysis was performed using Pearson's chi-square test. A web-based platform, SHEsis, was used to perform LD and haplotype analyses (http://analysis.bio-x.cn/myAnalysis.php). Risk assessment, odds ratio (OR) and its 95% confidence interval (CI), was estimated using age- and gender-adjusted multinomial logistic regression analysis, which was performed under five genetic models; allele, recessive, dominant, over-dominant, and co-dominant. Median and IQR were used to express continuous variables because they did not follow a normal distribution (Shapiro-Wilk and Kolmogorov–Smirnov tests), and Mann-Whitney U test was used to assess significant differences. The discriminatory performance of serum IFIH1 levels between patients and HC were evaluated using receiver-operating characteristic (ROC) curve analysis, which was presented in terms of area under the curve (AUC), 95% confidence interval, cut-off point (adjusted with Youden index; YI), sensitivity, and specificity. Correlation coefficient (r_s) was determined using Spearman's rank-order correlation analysis. The level of significance was set at a probability (p) of less than 0.05, which was corrected (pc) for multiple comparisons with the Bonferroni correction method or the false discovery rate method. Statistical analysis was performed using IBM SPSS Statistics 25.0 (Armonk, NY: IBM Corp.) and graphs were generated using GraphPad Prism version 9.2.0 (San Diego, CA, USA).

3. Results

3.1 Baseline laboratory data

Baseline laboratory data included ESR and total and absolute counts of WBC (granulocytes, lymphocytes, and monocytes). COVID-19 patients showed a significantly increased median (IQR) ESR compared to HC (45 [37-55] *vs* . 13 [9-16] mm/h; $p < 0.001$). Likewise, the total count of WBC was significantly higher in patients than in HC (7.8 [6.2-9.8] *vs* . 7 [6.0-8.1] x 10^9/L; $p = 0.001$), but the count in both groups was within the reference range. Granulocytes were significantly elevated in patients compared to HC (5.2 [4.0-6.9] *vs* . 3.8 [3.1-4.8] x 10^9/L; $p < 0.001$), while lymphocytes were significantly lower in patients (1.8 [1.4-2.4] *vs* . 2.5 [2.2-3.1] x 10^9/L;$p < 0.001$). Monocyte counts showed no significant difference between patients and HC ($p = 0.567$). GLR was significantly higher in patients than in HC (2.3 [1.8-4.5]*vs* . 1.6 [1.3-1.8]; $p < 0.001$), while LMR decreased significantly in patients (3.5 [3.0-4.3] *vs* . 4.8 [3.7-5.6]; $p < 0.001$) (Table 2).

3.2 *IFIH1* SNPs

HWE analysis demonstrated that genotype frequencies of both SNPs (rs1990760 and rs2111485) did not deviate significantly from HWE in the HC group ($p = 0.523$ and 0.253, respectively). Association analysis

was conducted under five genetic models; allele, recessive, dominant, over-dominant, and co-dominant. For SNP rs1990760, the genotype frequencies of CC, CT, and TT were 4.4, 37.8, and 57.8%, respectively, in COVID-19 patients. The corresponding frequencies in HC were significantly different (24.4, 53.3, and 22.2%, respectively;$p < 0.001$). Under the allele model (T *vs* .C), the mutant T allele was associated with a significantly increased risk of COVID-19 (OR = 3.44; 95% CI = 2.19-5.39; $p < 0.001$; $pc < 0.001$). A higher risk of infection with COVID-19 was associated with the mutant TT genotype under recessive (TT *vs* . CC+CT: OR = 4.86; 95% CI = 2.52-9.38; $p < 0.001$; $pc < 0.001$) and co-dominant (OR = 14.59; 95% CI = 4.42-48.19; $p < 0.001$; $pc < 0.001$) models. Under dominant model (CT+TT*vs* . CC), the CT+TT genotypes were also associated with an increased risk of COVID-19 (OR = 6.95; 95 CI = 2.27-21.28; $p = 0.001$; pc = 0.005). Under over-dominant model (CT *vs* . CC+TT), there was no significant risk of COVID-19 associated with SNP rs1990760 ($p = 0.035$; $pc = 0.175$) (Table 3).

In the case of SNP rs2111485, genotype frequencies (AA, AG, and GG) showed significant differences between COVID-19 patients and HC (6.7, 64.4 and 28.9% *vs* . 42.2, 41.1 and 16.7%, respectively; $p< 0.001$). The mutant G allele (allele model; Gvs . A) and GG genotype (co-dominant model; GG *vs* . AA) were associated with a higher risk of COVID-19 (OR = 2.65, 95% CI =1.73-4.05, $p < 0.001$, $pc < 0.001$; OR = 10.72, 95% CI = 3.66-31.40, $p < 0.001$, $pc< 0.001$, respectively). Under dominant (AG+GG *vs* . AA: OR = 10.15; 95% CI = 4.01-25.69; $p < 0.001$; $pc< 0.001$) and over-dominant (AG *vs* . AA+GG: OR: 2.62; 95% CI = 1.43-4.80); $p = 0.002$; $pc = 0.01$) models, a significant increased risk of COVID-19 was also associated with SNP rs2111485. Under recessive model (GG *vs* . AA+AG), there was no significant risk of COVID-19 associated with SNP rs2111485 ($p = 0.063$; $pc = 0.315$) (Table 3).

To further explore the association of *IFIH1* SNPs with COVID-19, haplotype analysis of the two SNPs was conducted (haplotype: rs1990760-rs2111485). Four haplotypes were encountered (T-A, T-G, C-A, and C-G) with frequencies of 22.2, 54.4, 16.7 and 6.7%, respectively, in COVID-19 patients, and 20.6, 28.3, 42.2 and 8.9%, respectively, in HC. The T-G haplotype was significantly associated with an increased risk of the disease (OR = 3.02; 95% CI = 1.95-4.68; $p< 0.001$; $pc < 0.001$), while the C-A haplotype was associated with a significantly decreased risk of contracting COVID-19 (OR = 0.27; 95% CI = 0.18-0.45; $p < 0.001$;$pc < 0.001$). The T-A and C-G and haplotypes showed no association with COVID-19 risk (Table 3). LD analysis revealed that the rs1990760 and rs2111485 polymorphisms were moderately linked as indicated by the LD coefficient (D') of 0.57 and correlation coefficient (R^2) of 0.18 (Figure 1).

3.3 Serum IFIH1 levels

IFIH1 levels (median and IQR) were significantly higher in the serum of COVID-19 patients than in HC (15.35 [13.28-17.30] *vs* . 4.60 [3.17-6.50] ng/mL; $p < 0.001$) (Figure 2A). ROC curve analysis demonstrated the potential of IFIH1 in distinguishing between COVID-19 patients and HC as indicated by an AUC of 0.999 (95% CI = 0.999-1.0; $p < 0.001$). At a cut-off point of 10 ng/mL, the sensitivity and specificity of IFIH1 were 100.0 and 97.8%, respectively (Figure 2B).

To evaluate the impact of *IFIH1* SNPs on IFIH1 serum levels, these levels were stratified by genotypes of SNPs rs1990760 and rs2111485 in all participating individuals (COVID-19 plus HC patients; n=180). Individuals with the mutant-type genotypes rs1990760 TT (14.2 [8.2-16.9] *vs* . 3.4 [2.5-4.8] ng/mL; $p <$ 0.001) and rs2111485 GG (13.4 [7.4-16.4] *vs* . 6.4 [3.4-8.1] ng/mL; $p < 0.001$) showed significantly elevated levels of IFIH1 compared to the wild-type genotypes (rs1990760 CC and rs2111485 AA, respectively) (Figure 3). Of note, the mutated genotypes rs1990760 TT and rs2111485 GG were significantly associated with an increased risk of infection with COVID-19 (Table 3).

3.4 Correlation analysis

Spearman's rank-order correlation analysis indicated that *IFIH1*was positively correlated with ESR (r_s = 0.708; $p< 0.001$; Figure 4A), WBC (r_s = 0.183; $p= 0.014$; Figure 4B), and GLR (r_s = 0.463; $p< 0.001$; Figure 4C), while it showed a negative correlation with LMR (r_s = -0.415; $p < 0.001$; Figure 4D).

3.5 Bioinformatic analysis

STRING analysis demonstrated that IFIH1 protein interacts with five proteins, *DHX58* (DExH-box helicase 58), *MAVS*(Mitochondrial antiviral signaling protein), *NLRC5* (NLR family CARD domain containing 5), *ISG15* (ISG15 ubiquitin like modifier), and *ATG12* (Autophagy related 12) (Figure 5).

4. Discussion

In this study, genetic evidence is provided that two variants of the*IFIH1* gene, rs1990760 and rs2111485, are associated with susceptibility to COVID-19. The mutant alleles and the corresponding homozygous genotypes of SNPs rs1990760 (*T* and TT, respectively) and rs2111485 (*G* and GG, respectively) were found to be significantly associated with increased risk of COVID-19. In addition, the results revealed that haplotype containing mutant alleles, rs1990760 *T* -rs2111485 *G* haplotype, confers a 3.02-fold increased susceptibility to COVID-19. These data suggest that substituting C with T (rs1990760) and A with G (rs2111485) may frame a role for these two SNPs in the risk of COVID-19 infection. In addition, serum IFIH1 levels were significantly elevated in COVID-19 patients, and these levels were positively affected by the mutant-type genotypes of rs1990760 and rs2111485 (TT and GG, respectively).

For rs1990760, a number of studies have investigated its association with COVID-19 risk in populations of different ethnicities with inconsistent findings. Maiti and colleagues showed that African American and Chinese populations with a low frequency of the mutant *T*allele were more predisposed to contracting COVID-19. Furthermore, this allele was associated with decreased expression of IFN-β, a known cytokine that plays a role in protection against fatal viral infections (Maiti et al, 2020). Amado-Rodriguez and colleagues reported that COVID-19 patients with the rs1990760 TT genotype showed an attenuated inflammatory response and survived their intensive care unit (ICU) stay (Amado-Rodríguez et al., 2022). Conversely, the rs1990760 TT genotype was associated with worse COVID-19 outcomes and risk of ICU admission, especially in females and non-Caucasian populations (Dieter et al., 2023). Minashkin and colleagues reported a different observation, where the rs1990760 CC wild-type genotype was associated with an increased risk of COVID-19 (Minashkin et al., 2022). Consistent with this observation, the wild-type rs1990760 *C* allele showed significantly increased frequency in patients with early onset of disease and was associated with COVID-19 severity (Muñiz-Banciella et al., 2023). However, another study reported that rs1990760 was not associated with susceptibility to COVID-19 risk (Feizollahi et al., 2023). In the current study, the rs1990760 *T* allele and TT genotype were associated with an increased risk of COVID-19 and contributed to higher serum IFIH1 levels. Regardless of these conflicting results, the *IFIH1* rs1990760 variant appears to be associated with COVID-19 risk and the observed differences between studies may be related to ethnic diversity. The rs1990760 SNP is a missense variant resulting from a nitrogenous base substitution, C with T, and as a result an amino acid change, alanine to threonine, occurs at codon 946 of the *IFIH1* gene. This change may provide a potential molecular link between rs1990760 and viral infections such as COVID-19. In this context, it has been pointed out that the *IFIH1* gene encodes the interferon-inducible RNA helicase, which plays an important role in antiviral innate immune responses (Xiao et al., 2023).

This study also revealed that rs2111485 is another SNP of the*IFIH1* gene that shows a significant association with susceptibility to COVID-19. This SNP has not been investigated to determine its role in risk of COVID-19, but in other inflammatory diseases, such as type 1 diabetes and vitiligo, rs2111485 has shown an association with risk of developing these diseases (Gootjes et al., 2022; Onan et al., 2019). Regarding viral infections, such as hepatitis B and C virus infections, rs2111485 has either shown an association with their progression or may have a role in viral clearance (Jiang et al., 2019; Yao et al., 2021). SNP rs2111485 is an intergenic variant in LD with rs1990760 and maps to a non-coding region of the *IFIH1* gene. Non-coding SNPs in the human genome have been disclosed to be important in conferring susceptibility to complex diseases and determining human traits. In fact, most genome-wide association studies (GWAS) and meta-analysis studies have shown that the majority of disease-associated loci are located in non-coding regions and suggest a potential role for non-coding SNPs in human disease susceptibility, despite their functional interpretation remains to be discovered (Zhang and Lupski, 2015). Due to the accumulation of non-coding SNPs in DNA regulatory elements, it is hypothesized that these SNPs may disrupt the binding sites of transcription factors and thus indirectly participate in regulating the expression levels of genes (Cano-Gamez

and Trynka, 2020). In the current study, the expression level of IFIH1 protein was stratified by rs2111485 genotypes in the serum of COVID-19 patients plus HC. It was found that the mutant-type GG genotype was associated with significantly elevated IFIH1 levels in serum compared with the wild-type AA genotype. These findings suggest that rs2111485 is a novel SNP involved in conferring susceptibility to COVID-19 and has a functional role in determining the serum IFIH1 levels. However, further studies are certainly warranted to confirm or refute these findings.

Serum IFIH1 levels were significantly elevated in COVID-19 patients compared to HC, and this elevation was found to be excellent in distinguishing between COVID-19 patients and HC patients as shown by the AUC of 0.999. Functionally, IFIH1 is involved in mediating antiviral responses by acting as a sensor of the early response to cytoplasmic RNA in viral infections by activating host cells to produce IFN (Brisse and Ly, 2019). Thus, IFIH1 is expected to have a role in the control of viral infections. Bioinformatics analysis using STRING database (https://string-db.org/) confirmed this role and demonstrated that IFIH1 interacts with five proteins involved in enhancing the antiviral immune response, including DHX58, MAVS, NLRC5, ISG15, and ATG12. DHX58 is probable ATP-dependent RNA helicase that acts as a regulator of IFIH1-mediated antiviral signaling (Xu et al., 2021). MAVS is a protein required for innate immune defense against viruses through recognition of viral RNA (Ren et al., 2020). NLRC5 is a protein involved in regulating the IFN signaling pathway and plays a role in homeostatic control of innate immunity and in antiviral defense mechanisms (Kienes et al., 2021). ISG15 is ubiquitin-like protein that plays a key role in the innate immune response to control viral infection (Freitas et al., 2020). ATG12 is another ubiquitin-like protein involved in the formation of autophagy vesicles and its role in antiviral innate immune responses has been recognized (Chawla et al., 2022). These data suggest that IFIH1 and its interacting proteins may have a pathophysiological role during the initiation and persistence of COVID-19 infection. Serum IFIH1 levels have not been evaluated in patients with COVID-19 and the current study is probably the first to conduct this evaluation. However, antibodies to MDA5, the alternative name for IFIH1, have been shown in some patients infected with SARS-CoV-2. Anti-MDA5 syndrome is a rare autoimmune disease that shows striking similarities to COVID-19, particularly elevated levels of pro-inflammatory cytokines (including type I IFNs) and the associated acute respiratory distress syndrome. Therefore, a common immunopathological mechanism between COVID-19 and anti-MDA5 syndrome has been proposed where MDA5 can be considered a cornerstone in this context (Giannini et al., 2020; Tonutti et al., 2022). Accordingly, understanding the mechanistic role of IFIH1 protein in the course of SARS-CoV-2 infection may have therapeutic potential and requires further studies.

IFIH1 levels showed a strong positive correlation with ESR and GLR, both non-specific inflammatory markers associated with COVID-19 pathogenesis (Al-Humairi et al., 2022). This may reflect the inflammatory state in COVID-19 patients and IFIH1 could be considered an important inflammatory marker interacting with other inflammatory markers during SARS-CoV-2 infection.

It is necessary to simultaneously evaluate the mRNA expression and genetic polymorphisms of *IFIH1* , *DHX58* , *MAVS* ,*NLRC5* , *ISG15* , and *ATG12* genes in patients with COVID-19 and this may represent a notable limitation of the current study.

In conclusion, IFIH1 protein showed up-regulated levels in the serum of patients with mild/moderate COVID-19. In addition, the *IFIH1* gene variants rs1990760 C/T and rs2111485 A/G were associated with susceptibility to SARS-CoV-2 infection, and the study suggests that their mutant-type genotypes are not only associated with increased risk of COVID-19 infection but also contributed to higher IFIH1 levels.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS

All authors have contributed equally to conceptualization, visualization, methodology, investigation, validation, and writing-reviewing and editing.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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