Positive association of genetic variations in the phospholipase C-like 1 gene with dermatomyositis in Chinese Han

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Abstract  Idiopathic inflammatory myopathies (IIMs) are autoimmune diseases with an underlying yet undefined genetic component. Recently, phospholipase C-like 1 (PLCL1) has been identified as a potential genetic susceptibility locus for dermatomyositis (DM) in patients of European ancestry. Here, association between PLCL1 polymorphisms and IIMs was investigated in Chinese Han. Genomic DNA was isolated from blood samples (2 mL) collected from Chinese Han (≥18 years) with polymyositis (PM, n = 286) or dermatomyositis (DM, n = 535) and ethnically matched controls (n = 968). Patients and controls were genotyped for five SNPs (rs938929, rs1518364, rs6738825, rs2117339, and rs7572733) previously associated with DM, with the Sequenom MassARRAY system. SNPs rs6738825 and rs7572733 were found to be associated with the development of DM in Chinese Han (Pc = 0.015; Pc = 0.025, respectively) as well as the risk A allele of rs938929 and T allele of rs1518364 (Pc = 0.030; Pc = 0.029). None of the five SNPs were associated with PM (all Pc > 0.05). The frequency of the two haplotypes of these five SNPs was also significantly different between DM patients and healthy controls. In addition, conditional analysis with rs6738825 revealed that these SNPs were not independent factors contributing to DM. Finally, a novel association between rs6738825 and DM with complicating interstitial lung disease was observed (ILD; Pc = 0.040; Pc = 0.030, respectively). A positive association between PLCL1 polymorphisms and DM patients and DM patients with ILD was observed, indicating that PLCL1 might be the susceptibility gene for DM patients in Chinese Han.

Keywords  Dermatomyositis · Polymyositis · Phospholipase C-like 1 gene · Polymorphism · Association

Introduction  The idiopathic inflammatory myopathies (IIMs) are a heterogeneous group of rare autoimmune diseases affecting skeletal muscles. They are primarily characterized by symmetric, proximal muscle weakness, muscle enzyme elevations, inflammatory cell infiltrates in muscle biopsy specimens, and electromyographic abnormalities on neurophysiological testing [1–6]. On the basis of well-defined clinical and immunopathological criteria, IIMs are subdivided into four major and discrete subgroups, including polymyositis (PM), dermatomyositis (DM), inclusion body myositis (IBM), and myositis overlapping with another connective tissue disease (myositis–CTD overlap syndrome) [1]. Interstitial lung disease (ILD), also known as diffuse parenchymal lung disease (DPLD), is a group of lung disorders in which the deep lung tissues become inflamed and then damaged. ILD is a critical complication in PM/DM patients and associated with treatment effect.
and increased mortality [7, 8] and is the predominant reason for hospital admission in a majority of patients [9]. Although the pathogenesis of IIMs remains unclear, IIMs are thought to be complex diseases, triggered by immune activation following specific environmental factors in genetically susceptible individuals. However, IIMs are relatively rare, with a prevalence of 10–15 cases per 100,000, and this low incidence has obstructed progress in the identification of the underlying genetic susceptibilities [10].

Until now, the major histocompatibility complex (MHC) gene region has been determined to have the strongest genetic association with the development of IIMs, and candidate gene studies have designated IIMs share genetic susceptibility with other autoimmune diseases [11]. Recently, HLA-DRB1 alleles have been associated with anti-melanoma differentiation-associated gene 5-antibody (anti-MDA-5)-positive dermatomyositis in Japanese [12]. Furthermore, HLA-DRB1 alleles have been linked to smoking as a risk factor for the development of antibodies against histidyl-tRNA synthetase (anti-Jo-1 antibodies), which are considered to be a specific marker of IIMs [13].

Several genes outside of MHC regions, including the proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) [14–17], interleukin (IL)-1α, IL-1β [16], and interferon (IFN)-γ [18], interferon-induced helicase (IFIH1) [19], mannose-binding lectin 2 (MBL2) [20], NF-kB-related genes [21], and an immunoglobulin gene [22], were found to be associated with specific IIMs subgroups. Furthermore, other studies demonstrated that the protein tyrosine phosphatase N22 gene (PTPN22) [23] and the signal transducer and activator of transcription 4 (STAT4) [24] were associated with specific IIM phenotype, indicating that IIMs shared genes commonly associated with the risk of other autoimmune diseases.

The largest study investigating the genetic component of myositis to date was a genome-wide association study (GWAS) undertaken on DM patients with European ancestry [25]. Three genes with DM were revealed in this study: phospholipase C-like 1 (PLCL1; rs6738825, rs7572733, rs1518364, rs938929), B lymphoid tyrosine kinase (BLK; rs2736340), and chemokine (C-C motif) ligand 21 (CCL21; rs951005, rs2492358). None of these genes had been previously reported to be associated with DM. However, variants of the PLCL1 gene had been associated with allergic disease (rs10497813) [26] as well as Crohn’s disease (CD) [27] and systemic lupus erythematosus (SLE) (rs6738825, $r^2 = 0.97$ with rs10497813) [27, 28].

The emphasis on the molecular characterization of PM/DM will hopefully lead to improvements in the clinical management of the disease. However, the association between SNPs in the PLCL1 gene region with PM/DM patients has not yet been investigated in Asians. Here, five PLCL1 SNPs were selected to examine the association with PM/DM in a cohort of Chinese Han: four common SNPs (rs7572733, rs1518364, rs2117339, and rs938929) and a PLCL1 SNP (rs6738825) that was previously associated with CD [27] and SLE [28]. In addition, the status of PLCL1 SNPs was also examined in order to predict whether a subgroup of PM/DM patients was predisposed to accompanying ILD.

Materials and methods

Ethics statement

Approval for the study was obtained from the Institutional Review Board of the Peking Union Medical College Hospital (Beijing, China). Informed consent was obtained from all individuals participating in the study.

Subjects

PM ($n = 286$) and DM patients ($n = 535$) ≥18 years at the onset of disease were predetermined to be probable or definite myositis by at least two rheumatologists according to the criteria of Bohan and Peter [29]. Patients were enrolled from the Peking Union Medical College Hospital from February 2013 to May 2014 ($n = 443$; PM, $n = 131$; DM, $n = 312$) and through the cooperation of three additional centers in China on a grant from the Research Special Fund for Public Welfare Industry of Health ($n = 378$; PM, $n = 155$; DM, $n = 223$). ILD in patients was identified with high-resolution chest computed tomography (HRCT) [30]. Patients with myositis–CTD overlap syndrome were excluded who fulfilled any one of the following published criteria: American College of Rheumatology (ACR) criteria for systemic sclerosis (SSc) [31], SLE [32], and rheumatoid arthritis (RA) [33], the American and European consensus criteria for Sjögren’s syndrome (SS) [34], or the criteria for mixed CTD defined by Sharp et al. [35]. Amyopathic dermatomyositis (ADM) patients were excluded who were difficult to diagnose according to the traditional criteria of Sontheimer [36]. According to the patient’s medical history, laboratory findings, imaging data, and so on, and the comprehensive judgments by two rheumatologists, every patient recruited in this study was excluded malignancy. IBM patients were also excluded, as the disease is much less prevalent among Chinese than Caucasian populations.

Ethnically matched healthy controls ($n = 968$) were recruited from the Peking Union Medical College Hospital based on the following criteria: (1) no significant history of disease; (2) no family history of rheumatologic diseases;
(3) normal biochemical and immunological profiles; and
(4) negative serology for anti-Jo-1 or anti-Mi-2 antibodies.

DNA extraction and genotyping

Peripheral blood (2 mL) was collected from each participant, and genomic DNA was extracted with a genomic DNA kit (Tiangen, Beijing, China), according to the manufacturer’s instructions. The five SNPs of the PLCL1 gene were genotyped with the MassARRAY iPLEX system (Sequenom; San Diego, USA), according to the manufacturer’s instructions. Briefly, after multiplex PCR amplifications, the products were used for locus-specific single-base extension reactions. The final products were desalted and transferred to 384-element SpectroCHIP arrays. Allele detection was carried out by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The resultant mass spectrograms and genotype data were analyzed with MassARRAY Typer 4.0 software. The primers for polymerase chain reaction (PCR) and single-base extension were designed with MassARRAY Assay Design 4.0 (Sequenom) and synthesized by Life Technologies (Grand Island, NY, USA).

Statistical analysis

The five SNPs were examined for departure from Hardy–Weinberg equilibrium (HWE) in healthy controls by using a Chi-square ($\chi^2$) test. Any SNPs that deviated significantly from HWE ($P < 0.05$ in the control groups) were excluded from subsequent analyses. The genetic power for this case–control study was calculated using the statistical program, Genetic Power Calculator (http://pngu.mgh.harvard.edu/~purcell/gpc/). For the association analysis between PLCL1 polymorphisms and the two clinical subsets (PM patients and DM patients vs. control subjects), statistical analyses were performed using PLINK v1.07 software (Shaun Purcell; Boston, MA, USA) [37]. Differences in genotype and allele frequencies between cases and controls were analyzed using a $\chi^2$ test. The odds ratio (OR) with a 95 % confidence interval (95 % CI) was calculated, and $P$ values, corrected for multiple testing by Bonferroni adjustment, <0.05 were considered to be statistically significant. Genotype frequencies were further analyzed by three logistic regression genetic models: additive, dominant, and recessive. Haploview analysis was performed with Haploview software v4.2 [38]. Subphenotype stratification analysis regarding the association study for PLCL1 polymorphisms and the presence of ILD was performed based on the results of the following three comparisons: patients (PM and DM patients) with ILD vs. all controls, patients without ILD vs. all controls, and patients with ILD vs. those without ILD.

Results

Clinical characteristics of patients and healthy controls

The clinical characteristics of the patients ($n = 821$) and healthy controls ($n = 968$) are summarized in Table 1. A total of 821 adult-onset PM/DM patients (74.9 % female) were enrolled, including 286 PM patients (73.4 % female) and 535 DM patients (76.4 % female). The mean ages for PM and DM patients were 45.2 ± 14.9 and 47.0 ± 15.5 years, respectively. The ethnically matched healthy controls included 968 subjects (83.7 % female; mean age 42.9 ± 12.5 years).

There was no deviation of the five PLCL1 SNPs from HWE in the healthy controls. The genotyping success rate for rs938929, rs1518364, rs2117339, rs6738825, and rs7572733 was 97.7, 97.1, 97.2, 98.1, and 97.3 %, respectively. The size of the cohort had >80 % power ($\alpha = 0.05$) for detecting an association with an OR of 1.10–1.60 for both heterozygotes and homozygotes [39].

Association of the SNPs with PM/DM patients in the Han population

The risk allele and genotype frequencies among PM and DM patients were compared to healthy controls, and the $P_c$ were calculated (Table 2; Fig. 1a). The rs6738825 and rs7572733 alleles and genotypes were found to be associated with the development of DM ($P_c = 0.015$ and $P_c = 0.020; P_c = 0.025$ and $P_c = 0.020$, respectively). The percentage of DM patients with the risk A allele of rs938929 or T allele of rs1518364 was also significantly greater than that in the healthy controls ($P_c = 0.030$ or $P_c = 0.029$). However, there was no statistically significant difference in the allele or genotype frequencies of rs2117339 between DM patients and healthy controls (all $P_c > 0.05$; Table 2). Finally, allele and genotype frequencies of the five SNPs were not significantly different between PM patients and healthy controls (all $P_c > 0.05$; Table 2).

Table 1 Clinical data for PM/DM patients and controls

<table>
<thead>
<tr>
<th>Clinical parameter</th>
<th>Patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects (DM/PM)</td>
<td>821 (535/286)</td>
<td>968 (–)</td>
</tr>
<tr>
<td>Female (%)</td>
<td>74.9</td>
<td>83.7</td>
</tr>
<tr>
<td>Average age (years)</td>
<td>46.1 ± 15.2</td>
<td>42.9 ± 12.5</td>
</tr>
<tr>
<td>DM with ILD [n/total (%)]</td>
<td>303/535 (56.6)</td>
<td>–</td>
</tr>
<tr>
<td>PM with ILD [n/total (%)]</td>
<td>157/286 (54.8)</td>
<td>–</td>
</tr>
</tbody>
</table>

PM polymyositis, DM dermatomyositis, ILD interstitial lung disease
Logistic regression analysis was performed to investigate associations of the SNPs with PM/DM under three genetic models: additive, dominant, and recessive (Table 3). Significant associations were observed for rs6738825 and rs7572733 with DM in additive and recessive models ($P_c = 0.017$ and $P_c = 0.030$; $P_c = 0.012$ and $P_c = 0.008$, respectively). The SNPs rs938929 and rs1518364 were associated with DM only in the additive model ($P_c = 0.035$; $P_c = 0.033$, respectively), while rs2117339 was associated with DM only in the recessive
model ($P_c = 0.026$). However, the five SNPs under these three genetic models were not associated with PM (all $P_c > 0.05$; Table 3). Conditional analysis of the most significant SNP, rs6738825, revealed no evidence of additional independent associations at the locus (data not shown).

**Association between PLCL1 polymorphisms and the ILD phenotype of PM/DM patients**

PLCL1 polymorphisms were further investigated for associations with the ILD phenotype of PM and DM patients. Over 50% of cases were PM or DM complicated with ILD, 54.8% (157/286) and 56.6% (303/535), respectively (Table 1). The association of two SNPs, rs6738825 and rs7572733, surprisingly, reached statistical significance in DM complicated with ILD ($P_c = 0.040$ and $P_c = 0.030$, respectively, Table 4). However, the other three SNPs, rs938929, rs1518364, and rs2117339, were not found to be associated with PM or DM complicated with ILD in this cohort.

**Haplotype analysis of PLCL1 SNPs and DM patients**

Strong pairwise LD between rs938929, rs1518364, rs2117339, rs6738825, and rs7572733 was found (Table S1; data in File S1). Linkage data from the HapMap CHB database corroborated the LD analysis performed, indicating that a strong LD association existed between these five SNPs in Chinese Han (Figure S1; data in File S1). Two haplotypes formed by rs938929, rs1518364, rs2117339, rs6738825, and rs7572733 were revealed: GCACC and ATGTT. The percentages of DM patients with the haplotypes differed significantly from healthy controls. Moreover, the GCACC haplotype occurred less often in DM patients than in the healthy controls ($P_c = 0.019$; Table S1; data in File S1). In order to identify candidate causal SNPs for PLCL1, the genotyping data of the HapMap CHB population were downloaded and viewed in Haplovlew. An LD block of 409 kb extended from chr2: 198545462 (rs7340470) to chr2: 198954774 (rs11684176) and contained the 5' region of PLCL1 as well as the MARS2 and BOLL genes (Fig. 1b).

**A novel SNPs in second exon of PLCL1 associated with DM**

The second and third exon of the PLCL1 gene was contained within the LD block of 409 kb. A common SNP, rs1064213, located in the second exon was found to be strongly linked to rs6738825, the index SNP, as well as rs7572733 (Fig. 1c). These results indicated that in this cohort, rs1064213 was likely to also be associated with DM. Rs1064213 changes codon 667 from Val (GTA) to Ile (ATA). PolyPhen-2 was applied to predict the possible impact of the change of Val to Ile on the structure and function of PLCL1. The mutation was predicted to be “probably damaging” with a score of 1.00, indicating this mutation be functionally impairing.

**Discussion**

A more complete understanding of the genetic component of complex, rare diseases such as PM/DM is necessary to improve diagnosis and treatment in populations of diverse ancestries. Here, the large case–control study was undertaken in Chinese individuals with PM/DM in order to examine a
potential association with \textit{PLCL1} polymorphisms. Five SNPs (rs938929, rs1518364, rs2117339, rs6738825, and rs7572733) in the \textit{PLCL1} gene were confirmed to be associated with DM in Chinese Han. Importantly, our results were consistent with previous findings from a population of European ancestry [25–28], indicating that \textit{PLCL1} might play a role in the pathogenesis of DM regardless of ethnic origin.

The \textit{PLCL1} SNPs were found to be associated with DM, but, interestingly, not PM. Clinical presentation of the two subtypes is in fact distinguishable on the basis of a number of parameters. First, the immunopathology differs between PM and DM. In DM, muscle biopsies display a mononuclear, inflammatory cell exudate arranged predominantly in perivascular regions or in the interfascicular septae rather than within the fascicles [1, 2]. In muscle biopsies from PM patients, the lymphocyte (CD8-positive cells) is the primary inflammatory cell type invading histologically healthy muscle fibers expressing MHC class I antigens [1–3]. Second, the clinical features and age of onset of DM and PM are distinctive. DM is typically diagnosed by a characteristic rash accompanying muscle weakness, which affects both children and adults, and women more than men. PM is best defined as a subacute myopathy that evolves over weeks to months, affects adults but rarely children, and presents with weakness of the proximal muscles. Unlike DM in which the rash assures early recognition, the actual onset of PM cannot be easily determined. Taken together, our findings possibly indicate that DM and PM are genetically distinct diseases. However, the relatively small sample size of PM patients in our cohort might also have reduced power to detect marginal associations between PM and the \textit{PLCL1} polymorphisms examined in this study.

Nevertheless, rs6738825, which was previously associated with other autoimmune disorders, CD [27] and SLE [28], was found to be strongly associated with DM in our cohort. Moreover, the risk allele and genotype frequencies between DM patients and controls indicated weak associations with rs2117339. The trend was not statistically significant, however, after Bonferroni correction, which was conducted in order to avoid false-positive loci [40]. In recessive models, significant associations were observed for rs2117339 as well as rs6738825 and rs7572733, indicating that susceptibility to DM may be due to homozygosity in these three SNP risk genotypes (TT, GG, and TT, respectively). Although our study confirmed the \textit{PLCL1} polymorphisms were associated with DM in Chinese Han, the \textit{P} value was within 0.01–0.05. This may be due to a relatively weaker genetic influence and stronger environmental influence on PM/DM susceptibility compared to other rheumatic diseases, or it could be a manifestation of disease heterogeneity [41]. Miller et al. [25] demonstrated the study was first and largest GWAS of any form of myositis, and the sample size was 1178 DM patients and

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|c|}
\hline
\textbf{Disease} & \textbf{Groups} & \textbf{rs938929} & \textbf{rs1518364} & \textbf{rs2117339} & \textbf{rs6738825} & \textbf{rs7572733} \\
\hline
\multirow{2}{*}{DM} & P versus N & 3.95 & 1.29 (0.79–1.37) & 1.30 (1.05–1.59) & 1.25 (0.99–1.57) & 1.25 (0.99–1.57) \\
& P versus C & 3.89 & 1.30 (1.05–1.59) & 1.31 (1.07–1.62) & 1.33 (1.08–1.63) & 1.33 (1.08–1.63) \\
& N versus C & 0.07 & 0.97 (0.79–1.19) & 0.97 (0.79–1.19) & 0.97 (0.79–1.19) & 0.97 (0.79–1.19) \\
\hline
\multirow{2}{*}{PM} & P versus N & 2.44 & 1.12 (0.76–1.54) & 1.16 (0.88–1.53) & 1.16 (0.88–1.53) & 1.16 (0.88–1.53) \\
& P versus C & 2.54 & 1.04 (0.77–1.41) & 1.04 (0.77–1.41) & 1.04 (0.77–1.41) & 1.04 (0.77–1.41) \\
& N versus C & 0.90 & 0.78 (0.59–1.02) & 0.78 (0.59–1.02) & 0.78 (0.59–1.02) & 0.78 (0.59–1.02) \\
\hline
\end{tabular}
\caption{Association of the five \textit{PLCL1} SNPs and PM/DM patients with ILD.}
\end{table
4724 healthy controls. However, no genetic signals with a genome-wide level of significance were observed outside of the MHC (the details of the characteristics and results are provided in Table S3 in File S1). Jani et al. [42] suggested the research had larger sample size, yet the results of this study were not within the genome-wide level of significance (the details of the characteristics and results are provided in Table S3 in File S1). Thus, the results of GWAS conducted on PM/DM indicated that IIMs are complex and heterogeneous autoimmune diseases, and genetic heterogeneity was existed between PM and DM.

One of the novel findings of the study was the association of rs6738825 and rs7572733 with susceptibility to DM complicated with ILD. The remaining three SNPs (rs938929, rs1518364, and rs2117339) demonstrated weak associations to DM with complicating ILD when they were directly analyzed. The trend, however, was not statistically significant after Bonferroni correction. Because the data of the PM/DM patients with anti-Jo-1 antibody from other three additional centers in China were missing, we could not analyze these patients. Thus, we only analyzed the PM/DM patients with anti-Jo-1 antibody from the Peking Union Medical College Hospital. Finally, the results of the analysis were not statistically significant (all \( P > 0.05 \)) (the details of the characteristics and results are provided in Table S2 in File S1). Our sample size of anti-Jo-1 antibody-positive patients was small, the results existed bias. In the subsequent research, we will further study the relationship between anti-Jo-1 antibody and PLCL1 gene polymorphisms. Conditional analysis of rs6738825 revealed no evidence of additional independent associations at the locus.

How PLCL1 functionally contributes to the pathogenesis of IIMs remains unclear. PLCL1 encodes an inositol 1,4,5-trisphosphate (IP3)-binding protein that inhibits IP3-mediated calcium signaling [43], and the gene is expressed in a variety of fetal and adult organs including brain, lung, and kidney. PLCL1 is not only involved in the inositol phospholipid-based intracellular signaling cascade, but the protein also regulates the turnover of receptors. PLCL1 participates in the phospho-dependent endocytosis process of \( \gamma \)-aminobutyric acid (GABA) A receptor, and thus, it contributes to the maintenance of muscle tone and of GABA-mediated synapatic inhibition [44, 45].

In order to locate potentially causal SNPs for PLCL1, an LD block of 409 kb extending from Chr2: 198545462 (rs7340470) to Chr2: 198954774 (rs11684176) was identified based on HapMap CHB. The block contains MARS2, BOLL, and the first three exons of PLCL1. MARS2 produces a mitochondrial methionyl-tRNA synthetase protein that is encoded by the nuclear genome and imported into mitochondria. BOLL encodes an RNA-binding protein, and loss of this gene function results in the absence of sperm in semen (azoosperma). Deficiencies in any one of these genes may contribute to the pathogenesis of the disease. However, an additional SNP leading to a nonsynonymous amino acid change in the second exon of PLCL1 may directly influence PLCL1 function and, thus, disease development. SNP rs1064213 was found to be strongly linked to the index SNP rs6738825 as well as rs7572733 (Fig. 1c) and changes codon 667 from Val (GTA) to Ile (ATA). A score of 1.00 determined with PolyPhen-2 predicted the change to be “probably damaging.”

The design of our study overall addressed some limitations of earlier studies. First, in general, genetic association studies for IIMs have always been hampered by factors such as the rarity and heterogeneity of these complex diseases. Importantly, our study was performed with a larger sample size than earlier candidate gene studies [14–24] and demonstrated the power (>80%) to examine prominent or even moderate associations. Second, because IIMs are rare diseases, early candidate gene studies with small numbers of patients often grouped clinical IIM subgroups together (including PM, DM, and myositis–CTD overlap syndrome) in an effort to increase statistical power. Third, patients of different ethnic populations have been combined in earlier studies. Our study was the first comprehensive report of PM/DM in Chinese Han, presenting clinical features in accordance with the international guidelines [29]. And our investigation had larger sample size than early candidate gene studies [14–24]. Finally, many prior studies have focused on the MHC gene region as the principal genetic risk of IIMs. This study in combination with a few other candidate gene studies broadens the potential basis for genetic susceptibility to genes outside of MHC region. However, one major limitation in the interpretation of the results is that the function of these PLCL1 genetic variants in the development of PM/DM was not investigated.

In summary, our results confirmed associations of PLCL1 polymorphisms rs938929, rs1518364, rs2117339, rs6738825, and rs7572733 with the risk of DM. Importantly, the research was performed exclusively on Chinese Han, and therefore, highlights a potential common genetic susceptibility shared by different ethnic populations. Whether the pathogenesis of DM is actually due to variants of the PLCL1 gene requires more investigation. Further large-scale mapping and sequencing of genomic DNAs from affected individuals are necessary to confirm these results and/or to identify novel candidate genetic susceptibility loci for DM in Asians, as well as other ethnic populations.

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Compliance with ethical standards The study was approved by the Ethics Committee of the Peking Union Medical College Hospital.

Conflict of interest The authors declare that they have no conflict of interest.

Informed consent Informed consent was obtained from all individual participants included in the study.

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