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ORIGINAL ARTICLE

Assessment of microRNA-related SNP effects in the 3′ untranslated region of the *IL22RA2* risk locus in multiple sclerosis

Christina M. Lill · Marcel Schilling · Sara Ansaloni · Julia Schröder · Marian Jaedicke · Felix Luessi · Brit-Maren M. Schjeide · Andriy Mashychev · Christiane Graetz · Denis A. Akkad · Lisa-Ann Gerdes · Antje Kroner · Paul Blaschke · Sabine Hoffjan · Alexander Winkelmann · Thomas Dörner · Peter Rieckmann · Elisabeth Steinhagen-Thiessen · Ulman Lindenberger · Andrew Chan · Hans-Peter Hartung · Orhan Aktas · Peter Lohse · Mathias Buttmann · Tania Kümpfel · Christian Kubisch · Uwe K. Zettl · Joerg T. Epplen · Frauke Zipp · Lars Bertram

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Abstract Recent large-scale association studies have identified over 100 MS risk loci. One of these MS risk variants is single-nucleotide polymorphism (SNP) rs17066096, located ~14 kb downstream of *IL22RA2. IL22RA2* represents a compelling MS candidate gene due to the role of IL-22 in autoimmunity; however, rs17066096 does not map into any known functional element. We assessed whether rs17066096 or a nearby proxy SNP may exert pathogenic effects by affecting microRNA-to-mRNA binding and thus *IL22RA2* expression using comprehensive *in silico* predictions, *in vitro* reporter assays, and genotyping experiments in 6,722 individuals. *In silico* screening identified two predicted microRNA binding sites in the 3′UTR of *IL22RA2* (for hsa-miR-2278 and hsa-miR-411-5p) encompassing a SNP (rs28366) in moderate linkage disequilibrium with rs17066096 (r^2=0.4). The binding of both microRNAs to the *IL22RA2* 3′UTR was confirmed in vitro, but their binding affinities were not significantly affected by rs28366. Association analyses revealed significant

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C. M. Lill (✉) · M. Schilling · S. Ansaloni · J. Schröder · M. Jaedicke · B.-M. M. Schjeide · A. Mashychev · L. Bertram
Neuropsychiatric Genetics Group, Department of Vertebrate Genomics, Max Planck Institute for Molecular Genetics, Ihnestr. 63-73, Berlin, Germany
e-mail: lill@molgen.mpg.de

C. M. Lill · F. Luessi · C. Graetz · F. Zipp
Department of Neurology, Focus Program Translational Neuroscience, University Medical Center of the Johannes Gutenberg University Mainz, Mainz, Germany

D. A. Akkad · S. Hoffjan · J. T. Epplen
Department of Human Genetics, Ruhr University, Bochum, Germany

L.-A. Gerdes · T. Kümpfel
Institute for Clinical Neuroimmunology, Ludwig Maximilian University, Munich, Germany

A. Kroner · P. Rieckmann · M. Buttmann
Department of Neurology, University of Würzburg, Würzburg, Germany

P. Blaschke · A. Winkelmann · U. K. Zettl
Department of Neurology, University of Rostock, Rostock, Germany

T. Dörner
Department of Medicine, Rheumatology, and Clinical Immunology, Charité University Medicine, Berlin, Germany

E. Steinhagen-Thiessen
Interdisciplinary Metabolic Center, Lipids Clinic, Charité University Medicine, Berlin, Germany

U. Lindenberger
Max Planck Institute for Human Development, Berlin, Germany

A. Chan
Department of Neurology, St. Josef-Hospital, Ruhr-University, Bochum, Germany

H.-P. Hartung · O. Aktas
Department of Neurology, Medical Faculty, Heinrich Heine University, Düsseldorf, Germany

association of rs17066096 and MS risk in our independent German dataset (odds ratio =1.15, $P=3.48\times10^{-4}$), but did not indicate rs28366 to be the cause of this signal. While our study provides independent validation of the association between rs17066096 and MS risk, this signal does not appear to be caused by sequence variants affecting microRNA function.

Keywords Genetic association · Immunogenetics · Interleukin-22 · MiRNA · Luciferase reporter assay

Introduction

Multiple sclerosis (MS) is the most common auto-inflammatory disease of the central nervous system and is caused by an interplay of genetic and environmental risk factors. Recent genome-wide association studies (GWAS) and follow-up assessments have established more than 100 risk loci in MS [1–4], the majority of which point to a predominant role of T cell-mediated immune mechanisms in MS pathogenesis [1]. By design, single-nucleotide polymorphisms (SNPs) assessed in GWAS can be informative markers tagging disease-associated regions, but often do not represent the variants exerting a functional role, some may even be located between genes. The identification of functional variants and the elucidation of underlying pathomechanisms will thus be a major focus in MS genetics research in the coming years.

One typical example is the MS risk locus on chromosome 6q23.3, in which rs17066096, the SNP most significantly associated with MS risk ($P=6.0\times10^{-13}$)[1], maps into an intergenic region. The region surrounding this SNP contains several potentially relevant immunological candidate genes: rs17066096 is located ~14 kb downstream of *IL22RA2* as well as ~66 kb downstream of *IFNGR1* and ~115 kb upstream of *IL20RA*. A previous genetic association study [5] assessed 29 SNPs across *IL22RA2* and *IFNGR1* in Swedish subjects partially overlapping with the original GWAS[1] and concluded that the association in this region was mostly driven by the 3′ end of *IL22RA2*. Supporting this conclusion, the same study found no convincing evidence for an association between SNPs in *INFGR1* and MS risk [5].

Interleukin 22 (IL-22) is produced by proinflammatory T helper cells and acts synergistically with IL-17 [6]. While the exact role of the IL-22 pathway is not fully unraveled, it appears to be highly relevant for the pathophysiology of autoimmune diseases including MS [6, 7]. Given the emerging evidence for a pathogenic role of IL-22 in autoimmune processes as well as the importance of small non-coding microRNAs (miRNAs) for the regulation of gene expression in MS (e.g. [8–10]), we set out to explore whether the MS association signal at the 3′ end of *IL22RA2* may be owing to one or more functional sequence variants in or near miRNA binding sites located in the *IL22RA2* 3′ untranslated region (UTR). Such variants may alter the binding of certain miRNA(s) to the mRNA 3′UTR and, as a result, the expression levels of *IL22RA2*. To assess this hypothesis, we performed a comprehensive in silico assessment of the potential role of DNA sequence variants on miRNA-to-mRNA binding. This led to the identification of SNP rs28366 (which is in linkage disequilibrium (LD) with the original MS GWAS SNP rs17066096[1]) located in the seed region of a site predicted to be bound by two miRNAs (hsa-miR-2278 and hsa-miR-411-5p). Both SNPs were genotyped in a large German case-control dataset (independent of the original GWAS[1]) comprising 6,722 subjects. In parallel, we investigated the potential role of rs28366 on the binding of both hsa-miR-2278 and hsa-miR-411-5p to the *IL22RA2* 3′UTR in vitro using customized luciferase reporter assays.

Methods

In silico assessments of IL22RA2 3'UTR SNPs predicted to affect miRNA binding First, miRNA binding sites were predicted for 3′UTRs of all currently known protein-coding *IL22RA2* transcripts (downloaded from Ensembl Genes 67, http://www.ensembl.org/biomart/martview) using miRanda v. 3.3a [11] (using the no-energy option), TargetScan 5.0 [12] and PITA [13] and v18 of the mirBASE database (http://www.mirbase.org). These predictions were performed with both the reference and alternative alleles of known SNPs in this region (based on build 137 of NCBI's dbSNP; http://www.ncbi.nlm.

P. Lohse
Department of Clinical Chemistry, Ludwig Maximilian University, Munich, Germany

C. Kubisch
Institute of Human Genetics, University of Ulm, Ulm, Germany

L. Bertram
School of Public Health, Department of Medicine, Imperial College London, London, UK

Present Address:
A. Kroner
Centre for Research in Neuroscience at McGill University, Montreal, Canada

Present Address:
P. Rieckmann
Department of Neurology, Sozialstiftung Bamberg Hospital, Bamberg, Germany

Present Address:
P. Lohse
Institute of Laboratory Medicine and Human Genetics, Singen, Germany

nih.gov/SNP/). To ensure high sensitivity in identifying miRNA binding sites, all potential miRNA binding sites predicted by at least one of the three prediction programs listed above [11–13] were included. Only SNPs displaying at least moderate LD (i.e. r^2 of 0.3; estimated from whole genome sequence data of the 1000 Genomes phase 1 CEU reference panel [14]) with rs17066096 were considered further. Finally, we estimated the potential effect of each eligible SNP on miRNA-to-mRNA binding using a modified version [15] of the support vector regression (SVR) method developed by Betel et al. [16].

Subjects The genetic association analyses of this study comprised 3,737 unrelated MS cases (71 % females) and 2,985 healthy control subjects (60 % females) of self-reported European descent from Germany diagnosed according to standard diagnostic criteria (for further details see 2). All samples were collected with informed written consent and appropriate ethical approval at the respective sites.

Genotyping Rs17066096 and rs28366 were genotyped using allelic discrimination assays based on TaqMan chemistry on a 7900HT Fast Real-Time PCR System (Life Technologies) following the manufacturer's instructions. Each 384-well plate contained approximately 5 % CEU HapMap samples to assess genotyping accuracy. Genotyping and genotype calling were performed blind to phenotypic status.

Genetic association analysis Hardy–Weinberg equilibrium (HWE) in controls was assessed using Pearson's $\chi 2$ as implemented in PLINK v1.07 (http://pngu.mgh.harvard.edu/purcell/plink/) [17]. All association analyses were based on an additive transmission model and adjusted for age and sex via logistic regression. Conditional logistic regression analysis was performed for rs28366 while conditioning on rs17066096. All reported *P* values are two tailed.

In vitro assessments of potential effects on miRNA-to-mRNA binding via luciferase reporter assays A luciferase reporter vector that contained the 3′UTR sequence of *IL22RA2* (ENST00000296980) subcloned into the pLightSwitch_3UTR vector 3′ of the *Renilla* luciferase gene along with miRNA mimics for hsa-miR-2278 and hsa-miR-411-5p as well as non-binding scrambled miRNA molecules were purchased from SwitchGear Genomics (Menlo Park, CA, USA). The correct orientation and the sequence of the reporter construct were verified by Sanger sequencing. The construct containing the alternative G instead of the reference A allele of rs28366 was generated by site-directed mutagenesis, and the base change was confirmed by Sanger sequencing. Two hundred ninety three human embryonic kidney (HEK293) cells were cultured at 37 °C and 5 % CO_2 in DMEM GlutaMax medium (Invitrogen) with 10 % fetal bovine serum (Biochrom). Cells were split in 96-well plates and after 24 h at 50 % confluency, transfected with 50 ng plasmid and 50 nM miRNA using DharmaFECT (ThermoScientific) according to the manufacturer's instructions. The following transfection conditions were used: (1) the reporter construct with the A (reference) allele of rs28366 and scrambled miRNA, (2) the reporter construct with the G (alternative) allele of rs28366 and scrambled miRNA, (3) the reporter construct with the A allele and miRNA hsa-miR-2278, (4) the reporter construct with the G allele and miRNA hsa-miR-2278, (5) the reporter construct with the A allele and miRNA hsa-miR-411-5p, 6) the reporter construct with the G allele and miRNA hsa-miR-411-5p. Twenty four hours after transfection, luciferase assays were conducted using the LightSwitch assay reagents (SwitchGear Genomics) according to the manufacturer's instructions. *Renilla* luciferase intensity values were recorded by endpoint reading with 3 s integration time and 3,500 gain per well using a POLARStar Omega plate reader (BMG Labtech). Ten independent experiments were performed, using different cell batches and independent transfection mixes. In each experiment, six wells were transfected per experimental condition. The in vitro assay conditions had been optimized previously based on an established miRNA-mRNA binding effect [18, 19], i.e. using co-transfections of the pLightSwitch_3UTR vector containing the 3′UTR of *APP* (ENST00000348990) with hsa-miR-101 in HEK293 cells, by testing miRNA concentrations of 12.5 to 100 nM, 25 to 50 ng vector, and endpoint readings 24 and 48 h post transfection. The most stable miRNA binding effects without cytotoxicity were observed using 50 nM miRNA, 50 ng vector, and endpoint readings after 24 h and were subsequently used for the current study as described above.

Statistical analysis of acquired in vitro data The statistical analyses of the luciferase intensity data were performed in R language (http://www.r-project.org) and based on a framework for the analyses of luciferase experiments introduced by Jacobs and Dinmann [20]: The luciferase activity of the 3′UTR reporter construct containing the A or G allele and co-transfected with a functional miRNA was divided by the baseline luciferase activity of the reporter construct containing the A or G allele, respectively, and co-transfected with the scrambled, non targeting miRNA as negative control. Outliers were defined as quotients that deviated more than 1.5 times the fold spread (fold spread = third quartile - first quartile) from the median as described in Jacobs and Dinmann [20] and were subsequently excluded from further analysis (applicable to one out of 236 data points in this study). Next, the expression change of the *Renilla* gene caused by substituting the A (reference) allele with the G allele in the 3'UTR of *IL22RA2* was assessed based on the *t* test statistic for two independent samples (Welch test).

Results

Our in silico assessments identified two highly correlated SNPs (rs28366 and rs202566, r^2=0.92) in moderate LD with rs17066096 (r^2=0.40) that mapped within 80 bp of predicted binding sites of 693 miRNAs in the 3′UTR of all three protein-coding *IL22RA2* transcripts (i.e. transcripts ENST00000296980 and ENST00000349184 [the 3′UTR sequences of which are 100 % identical], as well as ENST00000339602; Suppl. Table 1). The modified SVR approach used to calculate the difference in binding strength between 3′UTR sequences containing the reference vs. alternative allele suggested that the bindings of miRNAs hsa-miR-2278 and hsa-miR-411-5p to transcript ENST00000296980 were most likely to be affected by SNP rs28366 (a similar but slightly weaker effect on binding was predicted for ENST00000339602). This SNP is located in the seed regions of both predicted miRNA binding sites (Fig. 1). The alternative G allele allows for an additional Watson-Crick binding in the seed region of the hsa-miR-2278 binding site, which may increase the strength of the miRNA-to-mRNA binding. For hsa-miR-411-5p, the predicted allele-specific effects on miRNA-to-mRNA binding were opposite to that of hsa-miR-2278: here, the alternative G allele results in the disruption of a Watson-Crick pairing and the formation of a weaker wobble base pair instead. As a result, this may decrease the binding strength of hsa-miR-411-5p to its target mRNA. The predicted effects on miRNA-to-mRNA binding of the second SNP, rs202566, were overall much less compelling than for rs28366; therefore, this SNP was not considered further.

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miRNA: hsa-miR-2278
mRNA: ENSG00000164485|ENST00000296980:rs28366

miRNA 5'    GAGAGCAGUGUGUGUUGCCUGG    3'
             |||| ||   | : |||  ||
mRNA  3' ...GUCUCAUCUGAAGGAACA-ACA... 5'

miRNA 5'    GAGAGCAGUGUGUGUUGCCUGG    3'
             |||||||   | : |||  ||
mRNA  3' ...GUCUCGUCUGAAGGAAC-AACA... 5'

miRNA: hsa-miR-411-5p
mRNA: ENSG00000164485|ENST00000296980:rs28366

miRNA 5'    UAGUAGACCGUAUAG-CGUACG    3'
             |||||||  : | |  ||  |
mRNA  3' ...CUCAUCUGAAGGAACAACAA-C... 5'

miRNA 5'    UAGUAGACCGUAUAG-CGUACG    3'
             ||:||||  : | |  ||  |
mRNA  3' ...CUCGUCUGAAGGAACAACAA-C... 5'
```

Fig. 1 Predicted miRNA binding sites in the *IL22RA2* 3′UTR possibly affected by rs28366. SNP rs28366 is located in the seed region (*highlighted in blue*) of two predicted miRNA target sites (for miRNAs hsa-miR-2278 and hsa-miR-499b-5p) for all three protein-coding transcripts of *IL22RA2 (*shown here: ENST00000296980). The alternative G allele of rs28366 (*highlighted in red*) may alter binding affinity of hsa-miR-2278/hsa-miR-499b-5p to the *IL22RA2* 3′UTR and subsequently alter *IL22RA2* mRNA stability and/or protein levels.

Next, we genotyped SNPs rs17066096 (originally identified by GWAS[1]) and rs28366 (potentially affecting miRNA-to-mRNA binding) in a large German case-control dataset that was independent of the original study [1]. The aims of these analyses were to (1) independently validate the association between *IL22RA2* and MS risk in this dataset and (2) to assess whether or not the association at rs17066096 is entirely or in part due to rs28366. Importantly, rs28366 was not assessed in the previous GWAS analysis [1] (data available to the authors); thus, this signal may have been missed before. In our dataset, genotyping efficiencies for rs17066096 and rs28366 were 99.1 and 98.7 %, respectively, and genotyping accuracy was 100 % for both SNPs. Genotypes for both SNPs were distributed according to HWE in control subjects (P=0.155 and 0.141, respectively). Logistic regression analyses confirmed the association of rs17066096 and MS risk (odds ratio (OR) [95 % confidence interval (CI)]=1.15 [1.07-1.25], P= 3.48×10^{-4}) showing the same directionality as reported in the original study [1] (i.e., risk increase conferred by the alternative G allele). In contrast, rs28366 demonstrated a smaller effect size estimate (OR [95 % CI]=1.10 [1.02-1.20]) and statistically weaker evidence for association (P=0.0209) with MS susceptibility (Table 1). To assess whether rs28366 may exert an effect on MS risk independently of rs17066096, we repeated analyses for rs28366, this time conditioning on rs17066096. However, these analyses did not reveal evidence of genetic association of rs28366 independent of rs17066069 (OR [95 %CI]=0.99 [0.88-1.11], P=0.813; Table 1).

In parallel to the genetic association analyses, we assessed whether our in silico predictions that (1) hsa-miR-2278 and hsa-miR-411-5p bind to the 3′UTR of the *IL22RA2* mRNA molecule, and that (2) the presence of rs28366 affects the binding of both miRNAs to their target sites, could be observed in vitro using customized luciferase reporter assays. While these experiments clearly show that both miRNAs bind to reporter constructs containing the full-length 3′UTR of *IL22RA2* (as evidenced by significant reduction of luciferase luminescence in experiments co-transfecting the reporter constructs with either miRNA compared to the co-transfection with a non-binding negative miRNA control); this binding was not affected by the allele status at rs28366 in the reporter constructs (P=0.144 and P=0.473 for hsa-miR-2278 and hsa-miR-411-5p, respectively, based on 10 experiments with 6 replicates each; Fig. 2). Overall, these results are in line with our genetic association data indicating that rs28366 is not the causal variant underlying the association signal between MS

Table 1 Association results for *IL22RA2* rs17066096 and rs28366 across 3,737 German MS cases and 2,985 controls

SNP (min/maj)	Genotype cases	Genotype controls	MAF (%) cases	MAF (%) controls	OR (95 % CI)	P	OR_{cond} (95 % CI)	P_{cond}
rs17066096 (C/T)	301/1488/1953	200/1078/1669	27.9	25.1	1.15 (1.07-1.25)	3.48×10^{-4}	-	-
rs28366 (G/A)	208/1306/2207	150/967/1825	23.1	21.5	1.10 (1.02-1.20)	0.0209	0.99 (0.88-1.11)	0.813

Association analysis of rs17066096 and rs28366 and MS risk was performed using logistic regression adjusted for age at examination, and sex and was based on an additive model. Conditional logistic regression analysis of rs28366 and MS additionally included rs17066096 genotype status in the model.

min minor allele, *maj* major allele, *MAF* minor allele frequency, *OR (95%CI)* odds ratio and 95 % confidence interval (the reference allele with an OR of 1 corresponds to the major allele in all settings), *cond* conditional on rs17066096

risk and the *IL22RA2* containing region on chromosome 6q23.3.

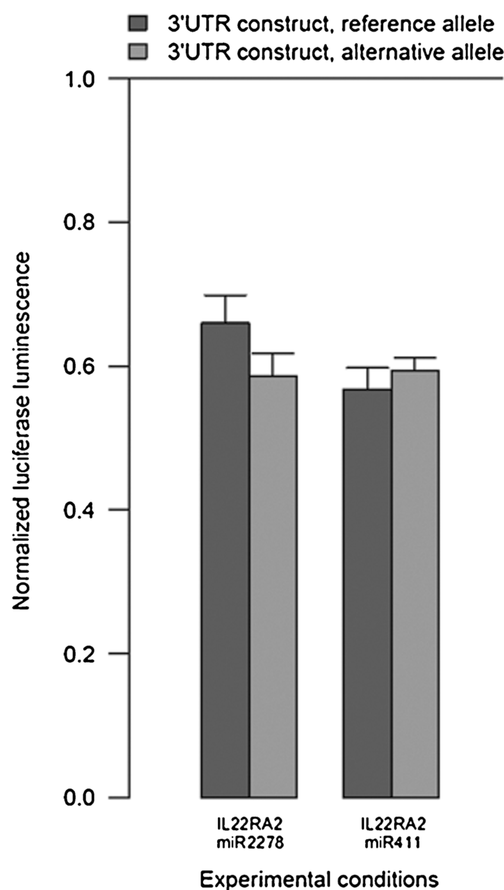

Fig. 2 Influence of rs28366 (A/G allele) in the 3′UTR of *IL22RA2* on gene expression. This bar chart displays *Renilla* luciferase expression under the control of the *IL22RA2* 3′UTR construct with the A (reference) or G (alternative) alleles of rs28366 that was co-transfected with hsa-miR-2278 and hsa-miR-411-5p, respectively, in HEK293 cells. Depicted are the mean luciferase luminescence and standard errors relative to the control luciferase luminescence of the *IL22RA2* 3′UTR construct co-transfected with non-binding negative miRNA control (corresponding to the *horizontal line*). The relative mean luciferase luminescence of the construct containing the A and the G allele was 0.660 (±0.0393) and 0.585 (±0.0321), respectively, for hsa-miR-2278 (P=0.144) and 0.568 (±0.0296) and 0.593 (±0.0183), respectively, for hsa-miR-411-5p (P=0.473).

Discussion

In this study, we performed a "functional mapping" of the previously identified association signal near *IL22RA2* with MS risk. Specifically, we tested the hypothesis that the *IL22RA2* association—which was previously suggested to originate from near the 3′ end of *IL22RA2* [1, 5]—may exert its pathogenetic effects by affecting miRNA-to-mRNA binding and, therefore, expression of *IL22RA2*. While our comprehensive in silico and in vitro assessments successfully identified two novel miRNA binding sites in the 3′UTR of *IL22RA2*, the binding of either miRNA was not affected by the presence of rs28366 in vitro. Thus, this SNP does not appear to represent a functional variant underlying the MS association signal in this region. This conclusion was further supported by the observation that the nominally significant MS association signal exerted by rs28366 was much weaker and dependent of that observed for rs17066096 in our German case-control dataset. Thus, in light of this comprehensive in silico and in vitro assessments, it currently appears unlikely that the functional basis of this association is due to major effects of DNA sequence variants on miRNA-regulated expressional regulation of *IL22RA2*.

One possible limitation of our study relates to the fact that all functional experiments were based on reporter assays applied to HEK293 cells. This model system may not sufficiently mirror physiologic conditions in MS effector cells such as Th17. Thus, our overall negative findings do not entirely exclude the presence of subtle miRNA-mediated SNP effects in *IL22RA2* in MS relevant cells. However, our negative functional data are corroborated by our genetic association results with rs28366, making it unlikely that this SNP exerts a strong and clinically relevant effect on miRNA-to-mRNA binding.

The results of this study, however, do not exclude the possibility that *IL22RA2* is, indeed, the functional gene at this locus. While a recent international collaborative fine-mapping effort of this region (using the Immunochip design [4]) did not provide any additional information on the nature of the putative underlying functional variant, the possibility remains that DNA sequence changes in the coding region of *IL22RA2* may

play a role in this setting. Alternatively, the association could be explained by SNPs in the 5′UTR or more distant upstream regulatory regions potentially affecting *IL22RA2* expression via effects on mRNA transcription.

In summary, our study provides independent genetic evidence supporting that *IL22RA2* (or a gene nearby) is significantly associated with MS risk. Functionally, this association is unlikely caused by impaired miRNA-related expressional regulation of *IL22RA2*. Further studies are needed to elucidate potential alternative pathophysiological mechanisms underlying this association.

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Conflicts of interest None of the authors report any disclosures.

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