Podocalyxin variants and risk of prostate cancer and tumor aggressiveness

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We previously reported linkage of a prostate cancer tumor aggressiveness locus to chromosome 7q32–q33, a region also associated with a high frequency of allelic imbalance in prostate tumors. The smallest region of allelic imbalance contains the podocalyxin-like (PODXL) gene, which we evaluate here as a candidate prostate cancer aggressiveness gene mapping to 7q32–q33. DNA from probands of linked families was examined for germ-line mutations in PODXL. A variable in-frame deletion, four missense variants and two nonsense variants were identified in linked men. Variants that affected amino acid sequence were further evaluated for association with risk of prostate cancer and tumor aggressiveness in a family-based case–control population (439 cases and 479 sibling controls). The presence of any single in-frame deletion was positively associated with prostate cancer [odds ratio (OR) = 2.14, 95% confidence interval (95%CI) = 1.09–4.20, P = 0.03] and the presence of two copies of any deletion further increased risk (OR = 2.58, 95%CI = 1.23–5.45, P = 0.01). This finding was strengthened when stratifying among men with more aggressive disease (high grade or stage): OR = 3.04 for one deletion (95%CI = 1.01–9.15) and OR = 4.42 for two deletions (95%CI = 1.32–14.85, P = 0.02). A weak positive association was also observed between prostate cancer risk and PODXL variant 340A (in linkage disequilibrium with another variant, 587T) (OR = 1.48, 95%CI = 1.02–2.14, P = 0.04). These results implicate PODXL as a candidate prostate cancer tumor aggressiveness gene mapping to chromosome 7q32–q33.

INTRODUCTION

The aggressiveness of prostate cancer varies widely among patients, with some cancers rapidly progressing to invasive, life-threatening disease, whereas others remain indolent for many years. The mechanisms underlying this variability in disease presentation and course are not known, but there is evidence that genetics may play a role, as prostate cancer tumor aggressiveness is influenced by a family history of this disease (1–4).

To identify genes that affect the aggressiveness of prostate cancer, we previously performed a genome-wide scan of 594 affected siblings, using Gleason score as a surrogate for tumor aggressiveness, and reported strong linkage to chromosome 7q32–q33 (5,6). Linkage to this region has been replicated in two independent studies (7,8) and a third report has linked a neighboring region on chromosome 7q to prostate cancer susceptibility among 36 Jewish families (9). Chromosome 7q32–q33 has also been shown to exhibit a high frequency of allelic imbalance in prostate tumors, with the smallest region of allelic imbalance spanning approximately 1.1 Mb and corresponding to the peak linkage marker from our affected sibling pair study (5.6,8).

The 1.1 Mb region on chromosome 7q32–q33 contains the podocalyxin-like (PODXL) gene, which encodes a 528 amino acid protein. PODXL, also known as podocalyxin,
podocalyxin-like protein 1 (PCLP-1), Myb-Ets-transformed progenitor 21 (MEP21) and thrombomucin, is a transmembrane sialomucin structurally related to the vascular endothelium marker CD34 (10). Studies reveal two diverse roles for the podocalyxin protein. In podocytes of the renal glomeru- }lae, podocalyxin functions as an anti-adhesion molecule that maintains an open filtration pathway between neighboring podocyte foot processes by charge repulsion (11). In the vasculature, podocalyxin acts as an adhesion molecule on high endothelial venules, supporting the tethering and rolling of lymphocytes through binding to L-selec tin (12).

Podocalyxin has been implicated in the development of cancer and, in particular, more aggressive cancer. Podocalyxin is a downstream target gene of the WT1 tumor suppressor (13,14) and has been implicated in beta catenin signaling (15). Studies have also shown that podocalyxin expression is negatively regulated by TP53 in kidney cells (16). Recent data have shown a correlation between high levels of podocalyxin protein expression and a more aggressive breast cancer (17). Furthermore, gene expression studies have implicated podocalyxin in angiogenesis, where a positive correlation was seen between expression of PODXL and ETS1, a regulator of angiogenesis, in human vascular endothelial cells (18). Additionally, podocalyxin was recently reported commonly expressed by blasts in both acute myeloid and acute lymphoblastic leukemias (19).

The linkage and allelic imbalance studies noted previously, along with the known functions of podocalyxin, make it an attractive candidate gene for the involvement in tumor aggressiveness. To assess the potential role of podocalyxin in prostate cancer risk, we first performed PODXL germ-line mutation analyses of probands from families linked to chromosome 7q32–q33 (5). We then evaluated the relationship between these PODXL variants and prostate cancer and tumor aggressiveness in a family-based association study. A variable in-frame deletion and a missense variant of PODXL were associated with risk of prostate cancer and tumor aggressiveness.

## RESULTS

### PODXL variants in families linked to chromosome 7q32–q33

Mutation analyses of PODXL were performed by DNA sequencing, using genomic DNA from white blood cells of probands of the 17 families that showed linkage to chromosome 7q32–q33. Primers were designed to amplify all the eight exons of PODXL including the intron splice donor and acceptor regions (Table 1). A number of coding sequence variants in PODXL were identified (Table 2). Specifically, we identified a common variable in-frame deletion, four common missense variants, two silent variants and two intronic substitutions (g.1759+1 T>C and g.1759+5 T>G). The variable in-frame deletion in exon 1 was 6 bp (TCGCCG) and 12 bp (TCGCCGTCGCCG) nucleotides in length (herein termed del6 and del12) and resulted in the loss of one or two serine–proline repeats (two or four amino acid residues, respectively). The in-frame deletion variant and missense variants 340G>A, 587C>T and 802C>G were genotyped in the case–control population. A reliable assay for the 982G>A variant could not be developed and was not investigated further.

### Allele frequencies for PODXL variants in a family-based case–control population

Allele frequencies of the PODXL variants in the case–control population are shown in Table 3. Three cases were not successfully genotyped in the case–control population and were not included in the results. Note that a third form of the in-frame deletion, a deletion of 18 bp (TCGCCGTCGCCG) nucleotides in length (herein termed del18), was identified in our case–control population but was not observed in our original 17 linked families. The del18 deletion occurs at the same locus as the del6 and del12 deletions and results in the loss of an additional serine–proline repeats (for a total of six amino acid residues). This deletion was almost exclusively seen in African American men, with 20/76 (26%) African American men carrying at least one copy of the del18 deletion. In contrast, 1/831 (<0.1%) Caucasian men in the study carried this deletion.

Note that as the 340G and 587C alleles and the 340A and 587T alleles were in complete linkage disequilibrium, we present findings only for the former. Alleles for G340A/587T and C802G were in Hardy–Weinberg equilibrium among controls within ethnic groups (P > 0.3).

### Associations between the PODXL variants and prostate cancer

No specific model was assumed in our initial regression analysis, and odds ratios (ORs) comparing each observed variant genotype to the wild-type homozygote were calculated. For

## Table 1. PODXL primers used for PCR amplification and DNA sequencing

<table>
<thead>
<tr>
<th>Exon</th>
<th>Forward primer sequence</th>
<th>Reverse primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5’-CTCCTCTGCCACTGGCTCTG-3’</td>
<td>5’-GTGGATGTGCCAGGTCAG-3’</td>
</tr>
<tr>
<td>2A</td>
<td>5’-GGGACGGGATTACAGGAGA-3’</td>
<td>5’-GTGGATGTGCCAGGTCAG-3’</td>
</tr>
<tr>
<td>3</td>
<td>5’-ACGCGATGACCCCTTGGATT-3’</td>
<td>5’-ATGGGAAAGGACCACCTTCT-3’</td>
</tr>
<tr>
<td>4</td>
<td>5’-GCGTTGGAGGAAAGAACAGA-3’</td>
<td>5’-ATCATTGGGAAGAAGACAGA-3’</td>
</tr>
<tr>
<td>5</td>
<td>5’-TCCACCTCTGGCTTCGAC-3’</td>
<td>5’-TCTTCGCAAATCGGGAATC-3’</td>
</tr>
<tr>
<td>6</td>
<td>5’-GCTGGGATTACAGGAGA-3’</td>
<td>5’-ACTTGGGGGTGGTTGGTCTG-3’</td>
</tr>
</tbody>
</table>

*Exon 2 was sequenced using two sets of overlapping primers.*
Table 2. Sequence alterations identified in PODXL.

<table>
<thead>
<tr>
<th>Location</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
<th>Allele frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 1</td>
<td>67–72 del6 (−6 bp)</td>
<td></td>
<td>9/34 (27)</td>
</tr>
<tr>
<td></td>
<td>67–78 del12 (−12 bp)</td>
<td></td>
<td>10/34 (29)</td>
</tr>
<tr>
<td>Exon 2</td>
<td>237G&gt;A</td>
<td></td>
<td>5/34 (15)</td>
</tr>
<tr>
<td>Exon 2</td>
<td>587C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exon 2</td>
<td>919G</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exon 2</td>
<td>802G&gt;C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exon 2</td>
<td>1320G&gt;A</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#Nucleotides are numbered from the ATG-translation initiation codon. Note that the numbering of variants was based on the 'no deletion' (full length) variant, whereas the UCSC browser numbers according to the −6 bp deletion variant and differs by two amino acids.

#Amino acids are numbered from the translation initiation methionine (+1). Also see footnote ‘a’.

#Allele frequency reflects the frequency of the variant allele in the total number of chromosomes tested (percentage).

#Deleted amino acids were SerPro.

#Deleted amino acids were SerProSerPro.

individual in-frame deletions, positive associations with prostate cancer were observed for almost all of the comparisons (including deletion heterozygotes, homozygotes and compound heterozygotes), with ORs ranging from 2.19 to 3.71 (corresponding P-values ≤ 0.05). The exceptions were no deletion/del12 heterozygotes where only a weak positive association (OR = 1.67) was seen and del18 carriers where no association was found (not shown).

Combining the in-frame deletion genotypes in our family-matched analysis, the presence of any single deletion gave an OR = 2.14 [95% confidence interval (CI) = 1.09–4.20, P = 0.03], whereas the presence of two PODXL deletions further increased this risk, giving an OR = 2.58 (95%CI = 1.23–5.45, P = 0.01) (Table 4, upper panels).

These findings for the in-frame deletion were strengthened among men with more advanced disease at diagnosis. Specifically, for men with more aggressive prostate cancer, positive associations were found for individual in-frame deletions for almost all of the comparisons, with ORs ranging from 4.00 to 7.39 (corresponding to P ≤ 0.03). Once again, the exceptions were a weak positive association for the no deletion/del12 heterozygotes (OR = 2.15) and no association for carriers of the del18 (not shown).

Combining the deletion genotypes, the presence of any single deletion gave an OR = 3.04 (95%CI = 1.01–9.15, P = 0.05); carrying two copies of any deletion gave an OR = 4.42 (95%CI = 1.32–14.85, P = 0.02) (Table 4, lower panels). No association was found between any deletion and prostate cancer among men with less aggressive disease (not shown).

Analysis of the G340A variant suggested that genotypic one or two of the G340A variants increased prostate cancer risk by approximately 50% (Table 5). Comparing individuals carrying either one or two G340A variants versus none gave an OR = 1.48 (95%CI = 1.02–2.14, P = 0.04). Restricting this analysis to men with more aggressive disease did not strengthen these results. Restricting our analyses to Caucasians only did not materially change any of the results (not shown). No associations were found for other variants.

Table 3. Allele frequencies for PODXL variants in the family-based case–control study.

<table>
<thead>
<tr>
<th>Alleles</th>
<th>Controls (total = 479)</th>
<th>Cases (total = 439)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low aggressive</td>
<td>High aggressive</td>
</tr>
<tr>
<td>Exon1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No del</td>
<td>221</td>
<td>82</td>
</tr>
<tr>
<td>del6</td>
<td>452</td>
<td>187</td>
</tr>
<tr>
<td>del12</td>
<td>272</td>
<td>100</td>
</tr>
<tr>
<td>del18</td>
<td>13</td>
<td>5</td>
</tr>
<tr>
<td>Exon2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>340G</td>
<td>551</td>
<td>206</td>
</tr>
<tr>
<td>340A</td>
<td>407</td>
<td>170</td>
</tr>
<tr>
<td>Exon3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>802C</td>
<td>919</td>
<td>344</td>
</tr>
<tr>
<td>802G</td>
<td>69</td>
<td>32</td>
</tr>
</tbody>
</table>

#Some cases did not have complete clinical data and therefore were not included in the analyses stratified by disease aggressiveness.

Two additional variants within the PODXL gene region were genotyped to determine the specificity of the observed associations. The variants are located on either side of the PODXL transcript. One variant (rs15956) lies approximately 8 kb 5’ to the PODXL deletion variant and the other (rs1733877) approximately 54 kb 3’ to the G340A variant. Neither variant exhibited any association with the risk of prostate cancer (OR = 1.31, 95%CI = 0.85–2.02, P = 0.23 for rs15956; OR = 1.16, 95%CI = 0.74–1.81, P = 0.52 for rs1733877) or advanced tumor aggressiveness (OR = 0.99, 95%CI = 0.54–1.83, P = 0.98 for rs15956; OR = 1.21, 95%CI = 0.67–2.17, P = 0.53 for rs1733877) in this population.

**DISCUSSION**

Both linkage and allelic imbalance studies support the mapping of a gene influencing prostate cancer and tumor aggressiveness on (or near) chromosome 7q32–q33 (5–9). The sialomucin podocalyxin maps within this region, and here we provide evidence that two novel PODXL variants (a variable in-frame deletion and G340A) are associated with the risk of both prostate cancer and tumor aggressiveness in a moderately large-sized family-based prostate cancer case–control population.

We observed a positive association between men carrying one or two deletion copies and prostate cancer, which was strengthened among men with more advanced disease. The corresponding ORs suggest that carrying two copies of the variable in-frame deletion doubles a man’s risk of developing more aggressive prostate cancer. The impact of each individual deletion varied slightly, with the strongest association seen with the 6 bp deletion. This may simply reflect the imprecision in the estimates [i.e., because the CIs for the different deletions overlapped] or suggest that different deletion variants have different biological consequences on the podocalyxin protein. In addition, we observed positive associations between men carrying at least one copy of the G340A variant (which was in linkage disequilibrium with the PODXL variant C587T) and prostate cancer risk. Two
This essential role of podocalyxin in the kidney was through its highly negatively charged extracellular domain. The maintenance of foot processes and filtration slits mechanism may come from studies of podocalyxin in the progression is not known. However, insight into a possible lylxin function and, subsequently, the risk of prostate cancer African American men are warranted. Additional studies focused on clarifying the relationship too small to determine any associations with this variant, unfortunately, our African American population was of prostate cancer when compared with other ethnic groups they are at higher risk of developing more aggressive forms African American men are of particular interest because which was found predominantly in African American men and appears to be a founder mutation in this population. We also identified an 18 bp deletion (del18) in PODXL, Na+/H+-exchanger regulatory factor 1/2 (NHERF1/2), the metastasis-associated protein EZrin and F-actin (21,22).

Ezrin plays a critical role in a number of signaling pathways implicated in metastasis (23–29). Furthermore, increased ezrin expression correlated with the metastatic potential of both rhabdiosarcomas and osteosarcomas (28,30) and with a metastatic phenotype or poor outcome in a number of other cancers (17,26,30–33). Ezrin overexpression has been reported in prostatic intraepithelial neoplasia and prostate tumors (34). However, it should be noted that a role in prostate cancer for podocalyxin and other members of this complex has not yet been reported.

PODXL gene variants may influence prostate cancer and tumor aggressiveness through neutralization of the negative charge of the extracellular domain of podocalyxin. Charge neutralization has previously been shown to disrupt the association between PODXL, Na+/H+-exchanger regulatory factor 1/2 (NHERF1/2), the metastasis-associated protein EZrin and F-actin (21,22).

There is a precedent for the association of a common in-frame deletion in a protein and the risk of developing cancer: transforming growth factor-beta receptor, Type I (TGFBR1) (36). A common variant (TGFBR1*6A) resulting in the loss of three alanine amino acids within a nine alanine repeat at the end of exon 1 of TGFBR1 results in a reduction in the anti-proliferative effects of TGF beta (37,38). This variant (TGFBR1*6A) has been associated with increased risk of a number of malignancies, including breast, colon and other cancers (37–44).
In summary, we report an association between two PODXL variants and increased risk of developing prostate cancer and increased tumor aggressiveness. Our data suggest that individually or in concert, PODXL variants or variants on their haplotypes may alter the expression or function of the podocalyxin protein possibly through its interactions with the metastasis-related gene EZRIN, leading to an increase in prostate cancer risk. Further studies will be needed to clarify the relationship between podocalyxin and ezrin in the prostate and the role of PODXL variants in prostate cancer aggressiveness.

MATERIALS AND METHODS

Study subjects used in mutation studies

Seventeen probands from families that showed linkage with D7S1804, the peak linkage marker in our original sibling-pair study (5), were chosen for mutation analysis. Marker D7S1804 was chosen because it also mapped to the distal boundary of the smallest common region of allelic imbalance found in prostate tumors (6). The 17 families showed mean allelic sharing between parents of greater than 70% (with 11 of the 17 families sharing at >95%) at marker D7S1804 with respect to Gleason score. Mutation analyses were performed on DNA isolated from blood leukocytes. Details about recruitment of the subjects that were included in the original linkage analysis are reported in Suarez et al. (45).

Study subjects used in association studies

The family-based case-control study population was recruited through the major medical institutions in the Greater Cleveland, Ohio area and from the Henry Ford Health System in Detroit, Michigan between 1998 and 2001. Men with histologically confirmed prostate cancer were invited to join the study if they fulfilled specific criteria, that is, they were diagnosed at 73 years or younger and had a living, unaffected brother who was either older or at most eight years younger than their age at diagnosis. These criteria were selected in an attempt to increase the likelihood of detecting genetic factors influencing prostate cancer and to minimize potential for misclassification of prostate cancer status among controls. We chose to use this sibling-based study design to ensure that our control group was selected from the same genetic source population as the cases, and thus not to be subject to population stratification from factors such as ethnicity.

The clinical characteristics (e.g. Gleason score, tumor stage) of each case were obtained from medical records. Prostate specific antigen (PSA) levels were measured for all potential controls to screen for possible asymptomatic disease. If a control had PSA levels above 4 ng/ml, they were informed and advised by our collaborating urologists that they should schedule an appointment with their primary care physicians to further evaluate this finding. Such individuals were retained in the study as controls unless a subsequent diagnosis of prostate cancer was made. Their inclusion in the study is important because automatically excluding men with elevated PSA levels regardless of their ultimate prostate cancer status can lead to biased estimates of association (46,47).

A total of 918 men (439 cases and 479 controls) were included in the present study. Each proband had at least one unaffected brother, with some families having multiple affected and unaffected brothers. Ninety-one percent of the subjects were Caucasian, 8% were African American and 1% were Asian or Latino. The mean age at diagnosis of the cases was 61 years and the mean age of controls at enrollment was 62.7 years. This study was approved by the institutional review boards of all participating institutions and all study participants provided written informed consent.

Mutation analysis of PODXL

Genomic DNA samples from the probands of 17 families that showed linkage to chromosome 7q32–q33 were subjected to mutation analyses of the PODXL gene by DNA sequencing. Primers used to amplify all eight exons including the intron splice donor and acceptor regions are shown (Table 1). PCR amplification was performed separately for each exon. Each 15 μl PCR reaction contained 20 ng of DNA, 1.25 mM of each dNTP, 67 mM Tris–HCL (pH 8.8), 6.7 mM magnesium chloride, 16.6 mM ammonium sulfate, 10 mM β-mercaptoethanol, 10% DMSO, 0.5 units Platinum Taq Polymerase (Invitrogen, Carlsbad, CA, USA) and 0.5 μM of forward and reverse primers. The PCR amplification reaction was performed using a 5 min denaturing step at 94°C, 35 cycles of 94°C for 45 s, optimal annealing temperature for 1 min, 72°C for 1 min, followed by a final 5 min extension at 72°C. PCR products were purified using the QIAquick PCR purification kit from QIAGEN (Valencia, CA, USA) according to the manufacturer’s instructions. Sequencing was performed using an ABI 377 (Applied Biosystems, Foster City, CA, USA) automated sequencer or a MegaBACE 1000 (Amersham Biosciences, Piscataway, NJ, USA) automated DNA analysis system. Sequences were aligned with and compared with reference PODXL sequence (NM_005397) and analyzed using Sequencher 4.1 (Gene Codes Corp., Ann Arbor, MI, USA).

PODXL genotyping

Several coding sequence variants were identified in PODXL and these are shown in Table 2. Genotyping was performed on those variants that affected amino acid sequence. The PODXL variable in-frame deletion in exon 1 was genotyped following PCR amplification, using the primers 5’-CTCCCTCTGGCCTGCTTG-3’ and 5’-CTGTTGATGGTGAAGGTCAG. The forward primer included a 5’ fluorophore for analysis using a MegaBACE 1000 DNA analysis system. Allele sizes were assessed using Genetic Profiler v1.5 software (Amersham Biosciences).

The PODXL 340G>A variant was detected following PCR amplification, using the primers 5’-AAGAGCTGGGAGAAGCTGGG-3’ and 5’-GGCTGTTGTGGTCTAGGTTA-3’. The 414 bp PCR product was digested with AciI (New England Biolabs, Beverly, MA, USA) for 1 h at 37°C. Digested products were resolved on a 6% polyacrylamide gel. PCR products containing the 340A variant are not cleaved by restriction enzyme AciI, whereas 340G fragments are cleaved into 301 and 113 bp fragments. The PODXL 587C>T variant was detected following PCR amplification,
using the primers 5'-AGGCAACCTACTACCACCA-3' and 5'-GCTGGATTACAGGCTAGA-3'. The 459 bp product was digested with TaqI (New England Biolabs) at 65°C for 1 h and fragments were separated on a 6% polyacrylamide gel. The 587 PCR product is cleaved into two fragments of 438 and 21 bp, whereas the 587 PCR product is cleaved into three fragments of 221, 217 and 21 bp. The PODXL 802C> G variant was detected by PCR amplification, using the primers 5'-GACCAAAGAAGCTGGGAAA-3' and 5'-GGTTTGGAGAAGACAGA-3'. The 412 bp product was digested with HaeIII (New England Biolabs) for 1 h at 37°C, resulting in two fragments of 248 and 164 bp for the 802C genotype and three fragments of 221, 164 and 27 bp for the 802G genotype. A reliable assay for the 982G>A variant could not be developed and no further analyses were performed on this polymorphism.

Two additional variants (rs15956 and rs1733877) within the PODXL gene region were genotyped using pre-made TaqMan assays (Applied Biosystems). Reactions were performed according to the manufacturer’s instructions, using the RealMasterMix Probe (+ROX) kit (Eppendorf), and genotypes determined using a Prism 7900HT instrument (Applied Biosystems).

Genotyping calls were made independently by two investigators. A randomly selected 10% of samples were re-genotyped and an additional 1% of samples were sequenced using an ABI 377 automated sequencer (Applied Biosystems). No discrepancies in the genotype calls were identified.

Statistical analysis
Genotype frequencies were calculated for each of the alterations being studied, and tested for Hardy–Weinberg equilibrium. Conditional logistic regression (using family as the matching variable, and a robust variance estimator that incorporates familial correlations) was used to estimate ORs and corresponding 95%CIs for the relationship between genotypes and prostate cancer. Sibling sets are treated appropriately by conditional logistic regression in which each family is analyzed as a matched set, so all men are counted only once in the analysis. The regression model was adjusted for potential confounding by age. To investigate the potential effect of geno-
type on disease aggressiveness, we stratified the analyses by the case’s clinical characteristics at diagnosis. Aggressiveness was defined as ‘low’ if a case’s Gleason score was <7 and their tumor category was <T2c, and ‘high’ if their Gleason score was ≥7 or their tumor category was ≥T2c. The tumor category reflects the TNM System. All P-values are from two-sided tests, and all analyses were undertaken with SAS software (version 8.2, SAS Institute, Cary, NC, USA).

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Conflict of Interest statement. The authors declare no conflicts of interest.

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