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Alternative splicing in *CEP290* mutant cats results in a milder phenotype than LCA^{CEP290} patients

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Abstract

Purpose: The *rdAc* cat has an intronic mutation in the centrosomal 290 kDa (*CEP290*) gene resulting in a frameshift and a premature stop codon (c.6960 + 9 T > G, p.Ile2321AlafsTer3) predicted to truncate the protein by 157 amino acids. *CEP290* mutations in human patients cause a range or phenotypes including syndromic conditions and severe childhood loss of vision while the *rdAc* cat has a milder phenotype. We sought to further characterize the effect of *rdAc* mutation on *CEP290* expression.

Methods: TaqMan quantitative real-time polymerase chain reaction assays were used to compare wildtype and truncated transcript levels. Relative protein abundance was analyzed by Western blot. Immunohistochemistry (IHC) was performed to detect CEP290 protein.

Results: *CEP290* mutant cats show low-level (17.4% of wildtype cats) use of the wildtype splice site and usage of the mutant splice site. Western analysis shows retina from cats homozygous for the mutation has CEP290 protein that likely comprises a combination of both wildtype and truncated protein. IHC detects CEP290 in affected and control retina labeling the region of the interconnecting cilium.

Conclusions: The comparably milder phenotype of *CEP290* mutant cats is likely due to the retained production of some full-length CEP290 protein with possible functional contributions from presence of truncated protein.

KEYWORDS
CEP290, Leber congenital amaurosis, progressive retinal atrophy, splice site mutation

1 | INTRODUCTION

Leber Congenital Amaurosis (LCA) is a group of debilitating inherited retinal diseases that cause retinal degeneration and eventual blindness in children.[1] About 20% of LCA cases are caused by mutations in the centrosomal 290 kDa (*CEP290*) gene.[1] Mutations in this gene have been associated with a broad spectrum of diseases, ranging from neonatal lethal syndromes to retina-only phenotypes.[2–9] The most common retinal phenotype caused by mutations in this gene is LCA, with the commonest mutation being a mutation in intron 26 (c.2991 + 1655A > G) that creates a strong splice donor site, leading to the introduction of a cryptic exon in the *CEP290* transcript.[10] The additional

intron results in a frameshift and a premature stop codon (p.Cys998Ter). There are currently no treatments outside of clinical trials for *CEP290*-LCA; however, experimental procedures aimed at replacing or repairing the causative genetic defect show great promise, including therapies aimed at correcting splicing alterations.[11–16]

A naturally occurring genetic variant in the *CEP290* gene in the cat resulting in an autosomal recessive retinal degeneration has provided a naturally occurring feline model for LCA caused by *CEP290* mutations known as the *rdAc* (retinal degeneration, Abyssinian cat) cat.[17–36] The feline defect arises from an intronic T to G substitution 9 base-pairs downstream from the wildtype splice donor site (c.6960 + 9 T > G, p.Ile2321AlafsTer3).[10] This is predicted to result in a canonical GT splice donor site 4 base-pairs downstream of the wildtype GC donor site that is preferentially used. Use of the alternative splice site in mutant cats results in a frameshift and a premature termination codon following two altered codons.[10] The predicted effect on the protein, if translated, would be introduction of 2 altered amino acids followed by a truncation of 157 amino acids out of the total 2479. This feline mutation offers an opportunity to study splice-altering mutations, how they affect transcript levels relative to the wildtype state, and what types of molecular intervention could reverse the dysfunction. This model therefore provides a valuable tool for better understanding this commonly mutated gene, as well as for the development and testing of treatments.

Cats homozygous for the *rdAc* genetic variant show a significantly milder phenotype than most human *CEP290* retinopathy patients, with a later-onset and slowly-progressing photoreceptor degeneration.[17,18] The underlying mechanism for this milder phenotype may shed light on *CEP290*-associated *retinopathies* and for the development of treatments. This mutation in felines occurs further downstream than the similar mutation in people, suggesting that a possible mechanism could be the translation of a longer, more functional, protein due to a less severe truncation. Furthermore, the wildtype splice site remains, raising the question of whether some amount of full-length protein is still produced in homozygous mutant cats.

The *CEP290* gene encodes a ciliary protein that localizes to the interconnecting cilium of rod and cone photoreceptors and is integral for protein transport between the inner and outer segments.[10] The large size of this gene precludes packaging of the full-length cDNA into the most effective retinal vector system; adeno-associated virus (AAV) vectors and other vector types have shown poor retinal transduction, making gene therapy based treatments challenging. However, a possible method to circumvent this obstacle to gene therapy could be to deliver a partial *CEP290* transcript if this provides some protein function. Studies in zebrafish and mice support this approach with rescue of vision noted in each model after injection of a partial transcript.[10,37] However, the effect of a truncated protein has not been studied in a large animal model, leaving an information gap before this form of treatment could reach human clinical trials. The *CEP290* mutant feline may provide information on the retinal phenotype associated with a truncated protein. However, detailed characterization of the *rdAc* mutation on retinal *CEP290* transcripts in affected cats has not been reported. We therefore analyzed *CEP290* transcript levels and the protein profile in *rdAc* cats versus wildtype and heterozygous carrier cats aiming to investigate whether the canonical splice site resulting from the intronic mutation is used exclusively and whether the mutant mRNA avoids nonsense-mediated decay to result in a truncated protein.

2 | METHODS

2.1 | Animals and tissue processing

All cats were purpose bred as part of a *CEP290* retinopathy breeding colony maintained at Michigan State University. All animal practices were approved by the Michigan State University Institutional Animal Care and Use Committee and conducted according to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Colony cats were genotyped utilizing a standard assay developed by our laboratory. A 240 base-pair genomic region spanning the mutation site was amplified by polymerase chain reaction. The amplicons were then differentially cut by restriction enzyme digestion with the TasI/Tsp509I enzyme (NEB, Beverly) (Figure S1).

2.2 | Retinal RNA extraction and cDNA synthesis

Following euthanasia by intravenous pentobarbital overdose (Fatal-Plus, Vortech Pharmaceuticals), retinas were collected from nine 16-week-old cats, three from each genotype; homozygous wildtype, *CEP290* mutation heterozygous, and *CEP290* mutation homozygous, flash frozen and stored at −80°C until use. RNA was extracted using the 5 Prime PerfectPure RNA Tissue Kit (Fisher Scientific Co.) following the standard kit protocol. RNA quality and quantity were analyzed using the Agilent BioAnalyzer 2100 (Agilent Technologies, Palo Alto) with a minimum RNA Integrity Number (RIN) set at 6 (Table S1). cDNA was synthesized using the Transcriptor First Strand cDNA synthesis kit (Roche Diagnostics) following standard kit protocol using the provided random primers and including a DNase step to prevent DNA contamination.

2.3 | Transcript analysis

For quantitative real-time polymerase chain reactions (qRT-PCRs), custom TaqMan assays were designed (Applied Biosystems, Foster City, CA, USA) to detect the wildtype *CEP290* transcript, the mutation-created alternative *CEP290* transcript, and *Tuba1b* transcript as an endogenous control (Table 1). qRT-PCR was carried out on an ABI 7500 Fast qRT-PCR system (Applied Biosystems) under standard conditions which included a 2-minute UNG incubation at 50°C, a 10-minute polymerase activation step at 95°C, and then 40 cycles of a denature and anneal protocol of 95°C for 15 seconds and 60°C for 1 minute, respectively. All samples were run in triplicate and technical replicates were analyzed and only used if within 0.3 Ct of each other. Samples were re-run if outside of this level of consistency. *CEP290* data were normalized to the *Tuba1b* data and analyzed using the comparative delta delta Ct method with the wildtype samples set as the reference. Data were analyzed for normality using the Shapiro–Wilk test for normality. Since the datasets did not pass normality testing, non-parametric tests were used to compare genotypes. For wildtype transcript level data, in which three genotypes were compared, the Kruskal–Wallis one-way analysis of variance on ranks was used. To compare the two genotypes in which the truncated transcript was analyzed, the Mann–Whitney rank sum test was used. Statistical significance was set at $p < .05$ for all testing. All statistical analyses and graphing were completed in SigmaPlot12 statistical software (Systat Software Inc.).

2.4 | Protein analysis

Protein was extracted from retinal tissue as follows: Retinal tissue was immersed in a Tris-Triton cell lysis buffer and homogenized with a pestle. Samples were then vigorously mixed on a vortex machine and centrifuged, saving the supernatant sample. Protein was quantified by spectrophotometry using a ND-1000 NanoDrop machine (NanoDrop Technologies) and stored at −20°C.

Relative quantification of CEP290 protein across genotypes was determined by automated capillary Western blot utilizing the WES system (ProteinSimple) following standard kit protocols. All samples were run in duplicate and were run against a calnexin control. Relative protein concentration was compared across genotypes. To do this, the area under the curve of the CEP290 peaks for each sample was generated by the WES Compass software (ProteinSimple). Genotypes were then statistically compared using the Kruskal–Wallis one-way analysis of variance on ranks using SigmaPlot12 software (Systat Software Inc.) (Table 2 shows antibodies used).

2.5 | Immunohistochemistry

One eye from each of four mid-stage (4 year-old) *CEP290* mutant cats and one eye from an adult wildtype cat were processed for IHC as previously described.[38] Briefly, eyes were promptly enucleated, fixed in 4% paraformaldehyde, and dissected along the limbus to separate the posterior eye cup which was then embedded in optimal cutting temperature gel (OCT. Sakura Finetek USA, Inc.) and flash frozen in liquid nitrogen.

For visualization of CEP290 protein in retinal sections, serial 14-micron vertical retinal sections were taken from the above eyes and sections from the area centralis region were labeled with a CEP290 specific antibody (Table 2). IHC was performed as previously reported by our laboratory to label CEP290 protein against a nuclear counterstain and images from the region of the area centralis were collected on a Nikon Eclipse 80i microscope (Nikon instruments Inc.) equipped with a CoolSnap ESv camera (Photometrics, Tuscon).[10]

3 | RESULTS

qRT-PCR revealed that *CEP290* homozygous mutant felines produce both full-length wildtype and truncated mutant transcript. Mutant cats produce 17.4% as much

TABLE 1 TaqMan Primers and Probes. Primers and probes designed by Applied Biosystems for Taqman assays, designed to differentially target the wildtype and mutant alleles as well as Tuba1b as an endogenous control.

Forward primer	Reverse primer	Reporter
Full length wildtype transcript assay		
AGAGAGGGAGCAAAAAGCTAAGAAA	GCACCTTCAGGAACATGTTTGAGA	CCTTGAGCAACAAATTGA
Truncated mutant transcript assay		
AGAGAGGGAGCAAAAAGCTAAGAAA	GCACCTTCAGGAACATGTTTGAGA	TTGAGCAACAAGCAAATTG
Tuba1b endogenous control assay		
GCCCCAACCTACACTAACCTAAATAG	ACATTCAGGGCTCCATCAAATCTG	TCCATCACTGCTTCCC

TABLE 2 Antibodies used for Protein Analysis. The same CEP290 antibody was used for both WES Western blotting and IHC. Different secondary antibodies appropriate to the assays were used, with the WES-provided secondary antibody used for the WES and an IHC verified antibody used for the IHC. A nuclear counterstain was used on all IHC slides.

Antibody	Host	Target	Concentration and use	Source
Primary antibodies				
CEP290	Rabbit	CEP290 in interconnecting cilium	1:50, WES 1:500, IHC	Thermo fisher scientific
Calnexin	Rabbit	Calnexin protein	1:500, WES	Novus biologicals
Secondary antibodies				
Goat anti-rabbit IgG (H + L) Cross-Adsorbed secondary antibody, Alexa Fluor 488	Goat	Rabbit primary antibody	1:500, IHC	Thermo fisher scientific
Goat anti-rabbit HRP conjugate	Goat	Rabbit primary antibody	1:50, WES	Protein simple
Nuclear counterstain				
DAPI (4′,6-Diamidino-2-Phenylindole, Dihydrochloride)	NA	Nuclei	1:10 000, IHC	Thermo fisher scientific

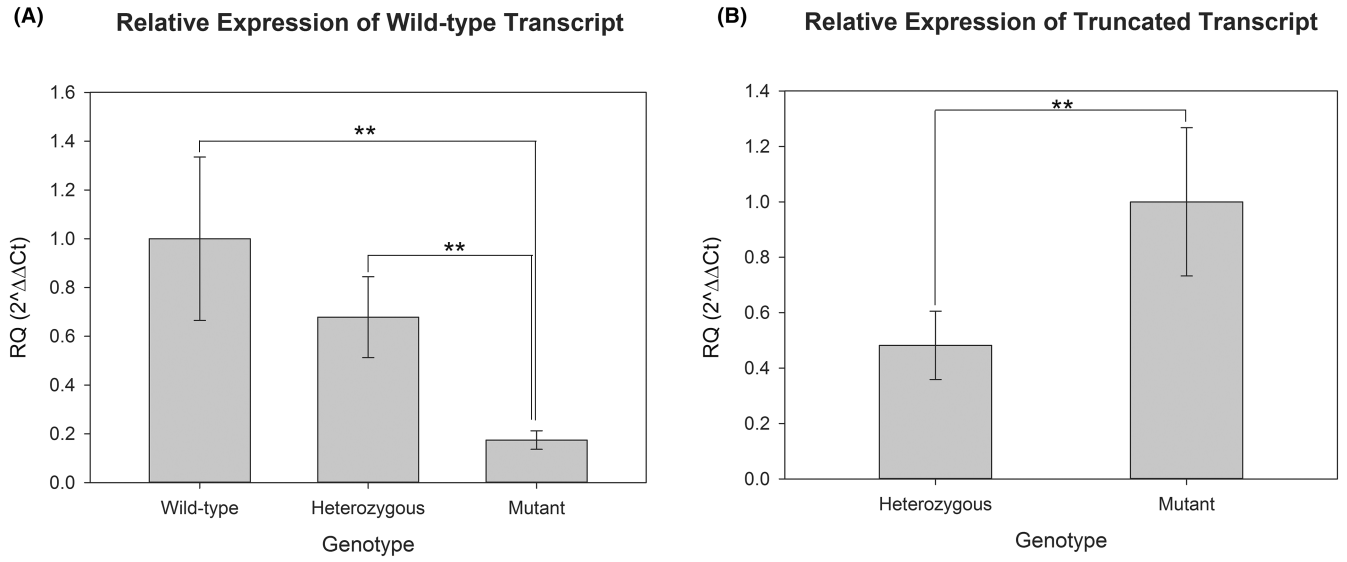

FIGURE 1 Relative expression of full-length wild-type and truncated mutant transcripts. (A) Mutant cats produce 17.4% as much full-length transcript as wild-type cats. (B) Mutant cats show production of truncated transcript and heterozygous cats show 48% as much truncated transcript as mutant cats. ** = $p < .005$.

full-length transcript as wildtype cats ($p < .001$), a statistically significant difference that shows that mutant cat cells do utilize the wildtype splice site at a low level. Heterozygous mutant (carrier) cats produce 67.8% as much full-length transcript as wildtype cats, a difference which was not statistically significant ($p = .125$). Mutant cats also showed usage of the mutant splice site, with heterozygous cats showing 48% as much mutant transcript as mutant cats ($p = .026$). Taken together, these data suggest that homozygous mutant cats produce a combination of shortened mutant transcript and full-length wildtype transcript (Figure 1).

WES analysis shows production of CEP290 protein in cats of all genotypes. The homozygous mutant *CEP290* cats showed a protein blot that appeared as a smear with a wider band that extends from 290 kDa to slightly lower. Because the truncated protein is only shortened by 159 of 2479 amino acids (approximately 6%), it is difficult to separate the truncated and full-length proteins on Western blot. The area under the CEP290 peaks shows a stronger CEP290 signal for wildtype cats (average area = 24917.5 chemiluminescence) than the other genotypes, with heterozygous cats having an intermediate protein signal (average area = 16140.8 chemiluminescence) and

homozygous mutant cats having the weakest signal (average area = 13466.25 chemiluminescence). Statistical comparison of the genotypes shows a significant difference between the protein signals of mutant cats and wildtype cats ($p = .005$) and between wildtype cats and heterozygous cats ($p = .01$) (Figure 2).

IHC in wildtype cats showed strong labeling of the photoreceptors at the inner segment/outer segment junction. IHC on sections from mid-stage (4-year-old) *CEP290* mutant cats also shows immunolabeling for CEP290 in the region of the connecting cilium (Figure 3). There was no detected off-target expression of CEP290 in the sections examined suggesting that the mutant transcript, as well as the wildtype transcript was trafficked appropriately.

4 | DISCUSSION

CEP290 mutant cats have a later-onset slower retinal degenerative condition than is typical for children with *CEP290*-LCA, who have an early and severe loss of vision. Furthermore, some *CEP290* mutations in humans result in a range of syndromic conditions affecting, in addition to the retina, the kidney, and brain. A precise phenotype:genotype correlation for these disorders is not apparent.[39] However, some studies have suggested that the severe syndromic effects associated with *CEP290* mutations are avoided with hypomorph mutations where either some wildtype CEP290 is produced or when an in-frame deletion results in a protein that may still have some function, despite missing a series of amino acids.[1,40,41] For example, it was reported than using lymphoblastoid cell lines from human subjects homozygous for the common c.2991+1655A>G; p.Cys988Ter variant, which results in non-syndromic LCA, a small amount of wildtype product was produced, making this a hypomorph mutation.[1] Studies with mouse models also support this hypothesis. *Cep290* knockout mice have severe vision loss, a fatal hydrocephalus with the longer surviving animals also developing cystic kidneys.[42] The *rd16* mouse has a naturally occurring, in-frame deletion of *Cep290*, resulting in a protein lacking amino acids 1606–1904.[43] The homozygous mice ($Cep290^{rd16/rd16}$) have a milder phenotype than *Cep290* knockout mice showing just non-syndromic, but early (LCA-like), retinal degeneration. The truncated protein in *rd16* mice is present at reduced levels, but presumably with sufficient residual function to avoid the syndromic changes associated with complete absence of Cep290, but not enough to prevent an LCA-like phenotype.[43]

Interestingly, despite the *rdAc* mutation introducing an alternative stronger splice site we showed that some transcripts were still produced using the original splice site. This resulted in wildtype mRNA being present at 17.4% of normal levels in the homozygous cats. The total amount of CEP290 protein detected on Western blot was, however, 54% the level of that in wildtype cats, suggesting a significant portion of the product was the truncated protein. This indicates that the truncated mRNA resulting from the introduced splice site at least partly escapes nonsense-mediated decay and that the truncated protein is not degraded. The CEP290 protein produced by homozygous *rdAc* cats is visualized in the correct retinal location on IHC of retinal sections. However, as the antibody used for IHC cannot distinguish between full-length and truncated protein, we cannot ascertain how much of the immunolabeling is due to wildtype versus truncated product. The predicted truncated protein resulting from use of

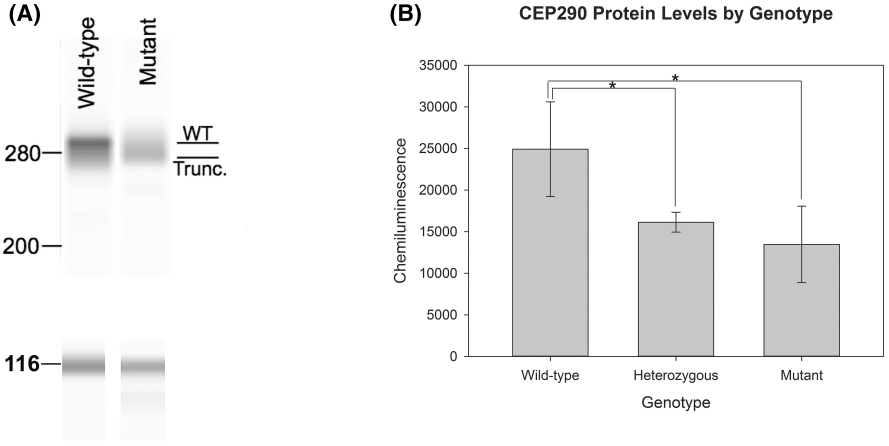

FIGURE 2 Automated capillary Western blot shows detectable CEP290 signal in mutant cats. (A) Representative blots generated by the WES software showing the shift toward a lower band in a *CEP290* homozygous mutant cat. The band at 116 shows the calnexin loading control. (B). Relative quantitation shows a significant difference between CEP290 protein levels in wildtype cats versus heterozygous or homozygous mutant cats. * = $p < .05$

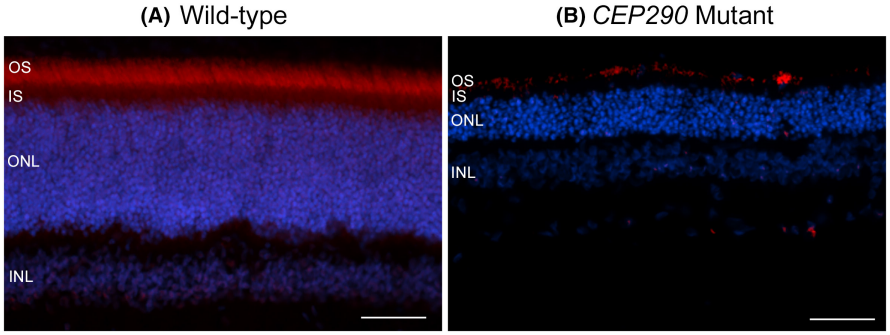

FIGURE 3 Immunohistochemistry shows detectable CEP290 protein in CEP290 mutant cats. (A) Wildtype cats show significant CEP290 protein signal in the inner/outer segment junction extending into the outer segments. (B) CEP290 mutant cats show a low but detectable signal for CEP290, which appropriately localizes to the region of the connecting cilium. Images from the *area centralis* region.

the introduced splice site would be shortened by 157 out of the 2479 amino acids.[10] The truncation would remove two kinase inducible domains but leave the rest of the protein, including other functional domains and localization signals, intact.

It is not possible from our studies to prove whether the presence of the low level of wildtype protein alone provides sufficient activity to result in the relatively mild phenotype or that the truncated protein also provides some activity, thus also contributing to the mild phenotype. Some human subjects have been reported with truncating mutations predicted to result in a similar shortening of CEP290 to that in the *rdAc* cats. Although studies have not been performed to show if nonsense-mediated decay is also avoided, and a truncated protein produced. However, all such patients were reported to have severe syndromic phenotypes. Two frameshift truncating mutations have been reported in exon 50, both associated with severe syndromic conditions. The first c.6869delA; p.Asn2290Ilefsx11 has only been identified in conjunction with a second different *CEP290* variant and the affected patient had cerebello-ocular-renal syndrome.[44] The second truncating mutation in exon 50, c.6870delT; p.Gln2290Ilefsx10, was identified in the homozygous state in a patient with the oculo-renal form of Joubert syndrome.[4] An exon 46 truncating nonsense mutation (C.6331C > T; p.Gln2111X) has also been described in the homozygous state with the affected patient having cerebello-ocular-renal syndrome.[45] Unlike the *rdAc* cat, these were not splice site variants so there is no possibility of the production of wildtype CEP290 in those patients. Studies to rescue the phenotype in a mouse model achieved significant and stable rescue by introducing a C-terminal fragment (amino acid 1491 to the C-terminal – 2479).[37] The truncated protein in the cat terminates at 2322 following 2 altered amino acids and thus lacks the last 157 amino acids. The successful rescue in the mouse model with the 988 amino acid C-terminal portion of the protein suggests that this region of the protein may be sufficient for retinal function. However, the mouse study used *rd16* mice that also had the neural retina leucine zipper (*Nrl*) gene knocked out. Absence of *Nrl* results in all photoreceptors being cone-like. The $Cep290^{rd16/rd16};Nrl^{-/-}$ mice have a slower photoreceptor degeneration than $Cep290^{rd16/rd16}$ mice that have rods as well as cones.[37] The study was not repeated with $Cep290^{rd16/rd16}$ mice, so it is not known whether that truncated version of Cep290 can also preserve rods. Thus, extrapolation to the *rdAc* cat cannot be made.

The presence of the truncated CEP290 in the *rdAc* cat holds interesting implications for nonsense-mediated decay (NMD) in the retina as it shows that a significant amount of truncated transcript is translated. Future studies investigating avoidance of NMD may hold additional insights into retinopathy mechanisms and treatments against these diseases.

In summary, we present here evidence that translation of a low level of wildtype protein, or a combination of wildtype protein plus a truncated protein which perhaps has some residual function, may lead to the comparatively mild phenotype seen in the *CEP290* mutant feline.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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