Sequestration of chaperones and proteasome into Lafora bodies and proteasomal dysfunction induced by Lafora disease-associated mutations of malin

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Lafora disease (LD) is an autosomal recessive progressive myoclonic epilepsy characterized by the presence of intracellular polyglucosan inclusions commonly known as Lafora bodies in many tissues, including the brain, liver and skin. The disease is caused by mutations in either $EPM2A$ gene, encoding the protein phosphatase, laforin, or $EPM2B$ gene, encoding the ubiquitin ligase, malin. But how mutations in these two genes cause disease pathogenesis is poorly understood. In this study, we show that the Lafora bodies in the axillary skin and brain stain positively for the ubiquitin, the 20S proteasome and the molecular chaperones Hsp70/Hsc70. Interestingly, mutant malins that are misfolded also frequently colocalizes with Lafora bodies in the skin biopsy sample of the respective LD patient. The expression of disease-causing mutations of malin in Cos-7 cells results in the formation of the profuse cytoplasmic aggregates that colocalize with the Hsp70/Hsc70 chaperones and the 20S proteasome. The mutant malin expressing cells also exhibit proteasomal dysfunction and cell death. Overexpression of Hsp70 decreases the frequency of the mutant malin aggregation and protects from mutant malin-induced cell death. These findings suggest that Lafora bodies consist of abnormal proteins, including mutant malin, targeted by the chaperones or the proteasome for their refolding or clearance, and failure of these quality control systems could lead to LD pathogenesis. Our data also indicate that the Hsp70 chaperone could be a potential therapeutic target of LD.

INTRODUCTION

Lafora disease (LD) is a fatal form of progressive myoclonus epilepsy and inherited as an autosomal recessive disorder (1). The disease usually manifests in early adolescence with stimulus-sensitive grand mal, tonic–clonic, absence, visual and myoclonic seizures and rapidly progresses to severe myoclonic seizures, psychosis, cerebellar ataxia, dementia, muscle wasting and respiratory failure (2–5). Death generally occurs within 10 years of disease onset. One of the common pathological features of LD is the accumulation of glycogen-like intracellular inclusions named Lafora bodies in many organs such as the brain, liver, heart, skeletal muscle and skin. Their histomorphological appearance differs depending on tissues affected (3,4,6). In the brain, Lafora bodies are seen predominantly in neuronal perikarya and dendrites. Periodic acid-Schiff (PAS) staining of neuronal Lafora bodies shows that majority of them have a darkly staining core and lightly staining radiating shell, while these inclusions present in the skin are round or oval shaped and stain uniformly (3,7).

Mutations causing LD have been identified in two genes, $EPM2A$ and $EPM2B$ (NHLRC1), and there is evidence for a third locus (8–10). The $EPM2A$ gene encodes laforin, a dual specificity protein phosphatase with a functional carbohydrate-binding domain (11). $EPM2B$ encodes malin, an E3 ubiquitin ligase having RING finger domain (12). Patients with mutations...
in either EPM2A or EPM2B genes are phenotypically indistinguishable and Lafora bodies are observed in every case indicating strongly that these two gene products regulate some common physiological pathways (11,13). In fact, malin has been found to interact with laforin and malin—laforin complex promotes the degradation of muscle glycogen synthase (MGS) and protein targeting to glycogen (PTG) that are required for glycogen synthesis (12,14–18). Therefore, loss of function of laforin or malin would result in increased levels of MGS and PTG, leading to the formation of Lafora bodies. But how mutations in these two proteins cause disease pathogenesis and possible role of Lafora bodies in this process is largely unknown. Although, enforced accumulation of glycogen promotes apoptosis in primary cultured neurons (19), there are other studies indicating that Lafora bodies might not play a primary role in neurodegeneration and the formation of Lafora bodies and neuronal loss might be two independent consequences (20,21). The possible antiapoptotic function of laforin (22) and the involvement of the laforin—malin complex in the clearance of toxic misfolded proteins indicate that these two proteins play an important role in cell survival pathways (23). Mutations in malin could result in improper clearance and accumulation of its target substrates leading to manifestation of LD.

Apart from glycogen, Lafora bodies also have been found to contain 6–28% protein depending on tissues, although the natures of these proteins are mostly unknown (24,25). Glycogen synthase and glycogen synthase kinase-3B are known to be associated with Lafora bodies (14,26,27). Interestingly, Lafora bodies are also ubiquitinated, indicating that these inclusion bodies consist of abnormally folded proteins targeted for clearance by the proteasome (14,27). A protein to be degraded through the ubiquitin—proteasome system (UPS) is first covalently attached with multiple ubiquitin molecules and then the multi-ubiquitinated proteins are degraded through the 26S proteasome (28). Several LD-associated mutations of either laforin or malin are found to be very unstable and form aggregates in mammalian cell culture (29,30). However, localization of these mutant proteins in Lafora bodies or other cellular compartment in the LD patient has not been reported so far, and the involvements of these mutant proteins in the disease pathogenesis are not yet explored. In the present investigation, we found that the Lafora bodies are ubiquitinated irrespective of mutations in either laforin or malin and are associated with the molecular chaperones and the proteasome component. The mutant malin that is misfolded also localizes in Lafora bodies. Transient expression of several LD-associated mutations of malin into Cos-7 cells results in their aggregation and induction of apoptosis probably through proteasome dysfunction. Over-expression of Hsp70 reduces the mutant malin aggregation and associated cell death. These observations provide insight into evolution of the pathological lesions and the disease.

RESULTS

Lafora bodies are ubiquitinated and colocalize with 20S proteasome and Hsc70/Hsp70 chaperones

To investigate the involvement of the UPS and the chaperones in LD, we have first studied the distribution of the ubiquitin, 20S proteasome and various chaperones into Lafora bodies. As Lafora bodies are formed not only in the brain but also in the skin, we took advantage of studying easily accessible axillary skin biopsy samples from clinically and genetically confirmed cases of LD and were subjected to immunohistochemical staining using antibodies against the ubiquitin, 20S proteasome and various chaperones. The PAS staining of these skin biopsy samples confirmed the presence of Lafora bodies in the apocrine sweat glands and ducts (Fig. 1). These Lafora bodies were also strongly labeled with the ubiquitin, 20S proteasome and the Hsc70/Hsp70 chaperones, and observed in both EPM2A (laforin) and EPM2B (malin) mutant patients (Fig. 1 and Supplementary Material, Fig. S1 for appropriate negative controls). These Lafora bodies were not recognized by amyloid β antibody that clearly detected amyloid plaques in the AD brain (Supplementary Material, Fig. S1). The LD patient who does not have any known mutations in either laforin or malin also exhibited similar staining for ubiquitin in Lafora bodies of the skin biopsy samples (data not shown). We have also confirmed the localization of the ubiquitin, 20S proteasome and the Hsc70/Hsp70 chaperones into Lafora bodies present in the brain biopsy samples of a clinically confirmed LD patient of unknown mutation (Fig. 2).
LD-associated malin mutants form aggregates in cell culture and associate with Lafora bodies

The ubiquitination of Lafora bodies and recruitment of the 20S proteasome and the Hsc70/Hsp70 chaperones into Lafora bodies indicate that these inclusion bodies consist of abnormal proteins that are targeted for refolding and clearance. Since, several deletions and missense mutants of either laforin or malin are misfolded and aggregate in cell culture, we suspected that mutant malin or laforin might be aggregating in Lafora bodies and other cellular compartments. To confirm this hypothesis, we tested for the presence of mutant malin in Lafora bodies. First, we found that all the LD-associated mutants of malin that we have tested were aggregated in Cos-7 cells upon overexpression (Fig. 3A). More than 75% of the mutant malin-transfected cells showed aggregates within 24 h of transfection. In similar experimental conditions, wild-type malin formed aggregates in approximately 2–4% transfected cells. The expression of the mutant malin plasmids at very low concentration (500 ng/well of the six-well tissue culture plate) for 24 h also showed an aggregate formation in 40–50% of transfected cells. The expression of the mutant malin plasmids at very low concentration (500 ng/well of the six-well tissue culture plate) for 24 h also showed an aggregate formation in 40–50% of transfected cells. The expression of the mutant malin plasmids at very low concentration (500 ng/well of the six-well tissue culture plate) for 24 h also showed an aggregate formation in 40–50% of transfected cells. The expression of the mutant malin plasmids at very low concentration (500 ng/well of the six-well tissue culture plate) for 24 h also showed an aggregate formation in 40–50% of transfected cells. The expression of the mutant malin plasmids at very low concentration (500 ng/well of the six-well tissue culture plate) for 24 h also showed an aggregate formation in 40–50% of transfected cells.

Figure 3B showed the immunoblot analysis of wild-type and mutant malins using Myc and malin antibodies. Malin antibody specifically detected wild-type as well as all the mutants of malin that we have tested. Since malin antibody detected wild-type as well as various mutants of malin, we further analyzed the localization of wild-type and mutant malins in the skin biopsy samples of LD patients. The delF216_D233 and L126P mutants of malin were localized both in cytoplasm and nucleus and frequently associated with Lafora bodies in the respective LD skin biopsy sample (Fig. 4). This malin antibody also detected wild-type malin predominantly in nucleus of the skin sample of the LD patient having laforin mutation (Fig. 4). This finding suggests that some of the mutant forms of malin aggregate in Lafora bodies or in other cellular compartment in the LD patient.

Overexpression of mutant malin induces proteasomal dysfunction and cell death

The redistribution of the proteasome into Lafora bodies and their ultimate inability to clear Lafora bodies prompted us to investigate possible proteasomal dysfunction in the mutant malin expressing cells. Several LD-associated malin mutants were also found to be very unstable and rapidly aggregate when overexpressed in the mammalian cells (31,32), which could ultimately affect the cellular proteasomal function. To study the proteasome dysfunction in the mutant malin expressing cells, we have used a model substrate of the proteasome, a destabilized enhanced green fluorescence protein (d1EGFP) containing PEST (proline, glutamate, serine and threonine) sequence at its C-terminus. The half-life of this protein is approximately 1 h. Proteasomal malfunction will increase the stability of d1EGFP leading to enhanced fluorescence intensity of GFP. Cos-7 cells were transfected with wild-type malin or its LD-associated mutants along with pd1EGFP plasmid for different time periods and then subjected to immunofluorescence staining to detect the fluorescence intensity of GFP in various malin-transfected cell or immunoblotting using the GFP antibody. The expression of various mutants of malin for 48 h leads to increased fluorescence of GFP indicating the proteasomal dysfunction (Fig. 5A). These mutant malin-transfected cells also exhibited increased levels of d1EGFP protein (Fig. 5B). The proteasome activity was also significantly decreased in the mutant malin expressing cells (Fig. 5C). The mutant malin expressing cells also demonstrated increased accumulations of ubiquitinated proteins (Fig. 5D). Overexpression of wild-type malin also results a mild increase in the accumulation of d1EGFP protein and a decrease in the proteasome activity. These observations indicate the proteasomal dysfunction markedly occurs in the mutant malin expressing cells. Since the proteasomal dysfunction induces apoptosis, we tested the effect of the expression...
of malin mutants on induction of cell death. Expression of mutant malins for 72 h was found to significantly increase cell death (Fig. 6A and B) and activation of caspase-3 (Fig. 6C).

Chaperone overexpression reduces aggregation of mutant malins and protects mutant-induced cell death

The recruitment of the Hsc70/Hsp70 chaperones into Lafora bodies implies that cells might be trying to correct the folding or clearance of some abnormal proteins present in these inclusion bodies. Since, Lafora bodies also consist of aggregated mutant malin, we studied the role of the Hsp70 chaperone in suppression of aggregation of mutant malins and mutant-associated cell death. We recorded the recruitment of the Hsp70 chaperone in the mutant malin aggregates and its expression also dramatically induced in the mutant malin expressing cells (Fig. 7A). Transient co-expression of Hsp70 along with mutant malin significantly reduced the aggregation of mutant malins (Fig. 7B and C). Overexpression of the Hsp70 chaperone also significantly protected mutant malin-induced cell death (Fig. 7B and D).

DISCUSSION

The data presented in this study demonstrate the involvement of the molecular chaperones and the UPS in LD. The Lafora body in the affected neurons and in the myoepithelial cells of sweat glands in the skin is strongly labeled by antibodies to the ubiquitin, 20S proteasome and the chaperones Hsc70/Hsp70. The recruitment of the molecular chaperones or the UPS components into Lafora bodies indicates that these poly-glucosan inclusions consist of abnormal proteins that are targeted for refolding or degradation. We have identified at least two LD-associated mutants of malin (delF216_D233 and L126P) localized with Lafora bodies. In fact, several LD-associated missense and deletion mutations of malin including delF216_D233 are found to be very unstable and aggregate rapidly in mammalian cell culture (31). Therefore, aggregation and association of mutant malin with Lafora bodies is not surprising. In addition to poorly branched glycogen, Lafora bodies are composed of 6–28% protein depending on the tissues and so far only glycogen synthase, glycogen synthase kinase-3β and laforin interacting protein-1 are found to be associated with Lafora bodies (14,24–26). We are reporting for the first time the presence of disease-associated misfolded mutant malin in the Lafora body. The mutant proteins encoded by disease-linked deletions and missense mutations of laforin are also found to be unstable, insoluble, ubiquitinated and accumulated in aggresome-like structures (30). Although, the presence of mutant laforin in the Lafora body has not been reported so far, the ubiquitination of Lafora bodies in the LD patient with laforin mutant indicates that abnormal mutant laforin might also be a component of Lafora bodies. Earlier reports have indicated that Lafora bodies are ubiquitinated in a malin-dependent manner, being absent in the brain of the malin mutant LD patient (14). On the contrary, our findings suggest that ubiquitination of Lafora bodies is a generic feature that can be observed in any LD patient. We have also observed ubiquitinated Lafora bodies in the skin biopsy of the respective LD patient.
sample of a LD patient who was not having any known mutation in either laforin or malin.

Although the redistribution of the chaperones and the proteasome could be the adaptive response of the cell to clear Lafora bodies or abnormal proteins present in the Lafora body, their apparent unsuccessful attempt could overwhelm the chaperone and proteasome system leading to deleterious consequences. The proteasome dysfunction is well known to induce apoptosis (33,34). Accordingly, we have found that transient overexpression of mutant malins in the similar manner and then the collected cells were subjected to immunoblot analysis using GFP antibody. Values are mean ± SD of three independent experiments. (C) and D) Cells were transfected with various constructs of malin and 72 h later cell lysates were made and the cytosolic fractions were either subjected to the proteasome activity assays (C) or immunoblot analysis using ubiquitin antibody (D). In (D), quantification of band intensities was performed using NIH Image analysis software and normalized against GAPDH. Values are mean ± SD of three independent experiments. *P < 0.01 in comparison with the pcDNA3.1 or wild-type malin-transfected group.

Figure 5. Overexpression of LD-associated mutations of malin induces the proteasomal dysfunction. (A) Cos-7 cells were transiently transfected with plasmids encoding wild-type and mutant malins (each 1 μg/well of two-well chamber slide) along with pd1EGFP plasmid (500 ng/well). Forty-eight hours of post-transfection, cells were processed for immunofluorescence staining with Myc and FLAG antibodies to detect wild-type and malin mutants as described in Figure 3A. Arrow indicates the mutant malin-transfected cell showing the proteasome dysfunction. (B) Cells were transfected with the above-mentioned plasmids in the similar manner and then the collected cells were subjected to immunoblot analysis using GFP antibody. Values are mean ± SD of three independent experiments. (C and D) Cells were transfected with various constructs of malin and 72 h later cell lysates were made and the cytosolic fractions were either subjected to the proteasome activity assays (C) or immunoblot analysis using ubiquitin antibody (D). In (D), quantification of band intensities was performed using NIH Image analysis software and normalized against GAPDH. Values are mean ± SD of three independent experiments. *P < 0.01 in comparison with the pcDNA3.1 or wild-type malin-transfected group.

ER stress (30). Moreover, various ER stress markers were also found to be up-regulated in the brain sample of a LD patient having laforin mutation as well as in laforin knockout mice (39). Since the laforin–malin complex involved in the degradation of cellular misfolded proteins, it is postulated that the loss of laforin function could cause accumulation of abnormal proteins in the cell leading to ER stress (23). Equally, the induction of ER stress markers observed in the LD patient brain sample could also be due to the proteasome dysfunction, because proteasomal inhibition is known to induce ER stress. In fact, the proteasome dysfunction was noticed in the liver (but not in the brain) of laforin knockout mice, which could be due to redistribution of the proteasome into Lafora bodies (39). All these findings suggest that the proteasome dysfunction might be playing an important role in the pathogenesis of LD.

The observations in the present study along with others suggest that the deletions or missense mutations in either malin or laforin not only cause loss of function of the native
proteins but also could lead to toxic gain of function due to their aggregation. The concept of toxic gain of function in disease pathogenesis has been widely implicated in many age-related neurodegenerative disorders involving protein misfolding and aggregation (40,41). Since LD is inherited as an autosomal recessive fashion and heterozygous individuals are asymptomatic, it is difficult to explain gain-of-function mechanism of disease pathogenesis. In heterozygous individual, the laforin–malin complex could be functional and cellular quality control systems are able to prevent the accumulation of the mutant protein aggregates.

Finally, we have shown that overexpression of the molecular chaperone Hsp70 suppresses the mutant malin aggregation and its associated toxicity. The expression of Hsp70 is also induced in the mutant malin expressing cells. These findings along with the recruitment of Hsc70/Hsp70 within Lafora bodies in the LD patient indicate that these chaperones could be a potential therapeutic target for LD. The molecular chaperones Hsc70/Hsp70 are not only involved in the refolding of abnormally folded proteins but also enhance their clearance through the UPS or autophagy (42–45). The wild-type malin is also a natively unfolded protein and its stability is shown to be regulated by the chaperones Hsc70/Hsp70, CHIP as well as by its substrate laforin (19,31). The molecular chaperones Hsc70/Hsp70 could keep the aggregation prone malin or its LD-associated mutants in a functionally competent state and thereby reduce the formation of Lafora bodies and rescue the loss-of-function-associated phenotype. Additionally, these chaperones could also enhance the clearance of the toxic aggregates of mutant malin or laforin and correct their possible gain-of-function-associated abnormalities. Taken together, this study provides evidence for the first time that Lafora bodies are composed of abnormal proteins including LD-associated mutant malin. Our studies also highlight the importance of the molecular chaperones and the UPS in the pathogenesis of LD and implicate that the molecular chaperones Hsc70/Hsp70 could be a potential therapeutic target of LD.

**MATERIALS AND METHODS**

**Materials**

All cell culture reagents, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and mouse monoclonal anti-FLAG were purchased from Sigma. Lipofectamine 2000, OptiMEM, mouse monoclonal V5 antibody were purchased from Invitrogen. NBT and BCIP were purchased from Roche Applied Science and Bradford reagent was procured from BioRad. Mouse monoclonal anti-Myc and anti-Hsp70, rabbit polyclonal anti-GAPDH and anti-malin, goat polyclonal anti-Hsc70/Hsp70 were purchased from Santa Cruz Biotechnologies. Rabbit polyclonal anti-ubiquitin and anti-20S proteasome were procured from Dako and Calbiochem, respectively. Alkaline phosphatase, HRP and fluorophore conjugated secondary antibodies and Novared kit were purchased from Vector Laboratories. The plasmid pd1EGFP was purchased from BD Clontech. The construction of malin-V5 plasmid and the source of Hsp70 plasmid were described elsewhere (31,46). Mutant malin constructs (C26S with Myc tag and delF216-D233 with FLAG tag) were provided by Dr S. Ganesh of IIT Kanpur, India. The construction of wild-type and various mutants of malin (L126P and L279P) with Myc tag was performed from the C26S malin mutant using GeneTailor site-directed mutagenesis kit (Invitrogen). All the plasmids were sequenced to confirm the expected mutation. The primer sequences are given in Supplementary Material, Table S1.

**Cell culture, transfection and immunoblotting**

Cos-7 cells were regularly maintained in DMEM with 10% fetal bovine serum and antibiotics penicillin/streptomycin. Equal amounts of cells were plated into six-well or 60 mm tissue-cultured plates at a sub-confluent density and transfected after

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**Figure 6.** Expression of mutant malins induces apoptosis. Cos-7 cells were transfected with various malin constructs and 72 h later cells were processed for MTT assay (A), immunofluorescence staining to detect various malin-transfected cells and the apoptotic features (fragmented nuclei, see Supplementary Material, Fig. S2) (B) and caspase-3 activity assay (C). In (A) and (C), values are mean ± SD of three independent experiments each performed triplicate. In (B), values are mean ± SD of three independent experiments with a minimum of 250 transfected cells scored for each experiment. *P < 0.01 in comparison with respective control.
24 h with Lipofectamine® 2000 according to the manufacturer’s instruction. Transfection efficiency was about 80–90%. After 24 or 48 h of transfection, cells were collected, washed in ice-cold PBS, pelleted by centrifugation and lysed on ice for 30 min using Nonident P-40 (NP-40) lysis buffer (50 mM Tris; pH 8.0, 150 mM NaCl, 1% NP-40, complete EDTA-free protease inhibitor cocktail). Cell lysates were collected after brief sonication and centrifugation at 20,000 g for 20 min. Protein estimation was done using the Bradford method. The total cell lysates were separated through SDS–polyacrylamide gel electrophoresis and processed for immunoblotting as described elsewhere (47). Most primary antibodies were used at 1:1000 dilutions, and anti-V5, anti-FLAG at 1:5000 dilutions and malin antibody at 1:250 dilutions.

**Immunofluorescence staining and counting of aggregates and apoptotic cells**

Cos-7 cells grown in two-well chamber slides were transiently transfected with appropriate plasmids. Twenty-four hours later, cells were washed in PBS, fixed with 4% paraformaldehyde in PBS for 30 min. After fixation, cells were washed again with PBS and permeabilized with 0.3% Triton X-100 in PBS for 10 min and subsequently blocked with 5% non-fat milk in PBS or 3% BSA for 1 h. Primary antibody incubation was performed overnight at 4°C. After several washings with PBS, cells were incubated with fluorophore-conjugated secondary antibody for 1 h. Cells were finally washed with PBS and mounted in DAPI and imaged using Axioplan fluorescence.
microscope/Apomete (Zeiss). Anti-Myc, anti-V5 and anti-FLAG were used at 1:1000 dilution; anti-Hsp70 at 1:200 dilutions; anti-malin at 1:50 dilution.

Transfected cells exhibiting aggregates and apoptotic bodies (fragmented nuclei) were counted at × 20 magnification. Fields were randomly chosen and about 200–300 cells were counted per experiment. Each experiment was repeated at least three times and counts were performed in a blinded manner.

**Immunohistochemistry and PAS staining**

For immunohistochemistry, serial paraffin sections from formalin-fixed and paraffin-embedded the axillary skin (from clinically and genetically confirmed cases of LD) and the brain biopsy (clinically diagnosed case of LD or AD) were obtained from the archives of Human Brain Tissue Repository, National Institute of Mental Health and Neurosciences, Bangalore, India, in accordance with the ethical guidelines of ICMR, protecting the confidentiality of the subjects and with consent of the subjects to utilize the material for research purposes. The tissue sections were dewaxed, rehydrated in graded alcohol and subjected to antigen retrieval in a pressure cooker. The sections were treated with 3% H2O2 in methanol, permeabilized in 0.3% Triton X-100 and blocked with 5% goat serum with 0.1% Triton X-100. The serial sections were incubated with antibodies to the ubiquitin, 20S proteasome, Hsc70/Hsp70, malin and amyloid β, respectively. Color development was carried out using the Nova red kit. One section from each case was stained with PAS to highlight the Lafora bodies as described earlier (27).

**Assays for various protease activities and cell viability**

Cos-7 cells were plated in a six-well tissue culture plate and, on the following day, cells were transfected with various plasmids. Cells were collected at different time points and processed for the proteasome or caspase-3 activity assays (48). The substrates Suc-Leu-Leu-Val-Tyr-MCA and Z-Leu-Leu-Val-Tyr-MCA were used to determine chymotrypsin and post-glycogen synthase, Hsc70/Hsp70, malin and amyloid β, respectively. Color development was carried out using the Nova red kit. One section from each case was stained with PAS to highlight the Lafora bodies as described earlier (27).

**Statistical analysis**

Statistical analysis of the data was performed using analysis of variance. Values were expressed as mean ± SD. The Bonferroni post hoc test was conducted to compare individual means where analysis of variance indicated statistical differences. The level of significance for all analysis was set at \( P < 0.05 \).

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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**Conflict of Interest statement.** None declared.

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