Toll-like receptor-4 genotype influences the survival of cystic fibrosis mice

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Canale-Zambrano JC, Auger ML, Haston CK. Toll-like receptor-4 genotype influences the survival of cystic fibrosis mice. Am J Physiol Gastrointest Liver Physiol 299: G381–G390, 2010. First published June 3, 2010; doi:10.1152/ajpgi.00030.2010.—Toll-like receptor (Tlr) 4 is a lipopolysaccharide (LPS) receptor that contributes to the regulation of intestinal cell homeostasis, a condition that is altered in the intestines of cystic fibrosis mice. Herein, we assessed whether Tlr4 genotype influences cystic fibrosis intestinal disease by producing and phenotyping 12-wk (adult)- and 4-day (neonate)-old mice derived from BALB cystic fibrosis transmembrane conductance regulator, Cft−/− (Cftr−/−) and C3-Tlr4−/−β (Tlr4−/−), progenitors. Intestinal disease was assayed through mouse survival, crypt-villus axis (CVA) length, cell proliferation, bacterial load, bacterial classification, inflammatory cell infiltrate, and mucus content measures. Of the 77 Cftr−/− (Cftr−/−) mice produced, only one Cftlrl4−/− double-mutant mouse lived to the age of 12 wk while the majority of the remaining succumbed at ~4 days of age. The survival of CF Tlr4−/− mice exceeded that of both CF Tlr4+/− and Cftlrl4−/− double-mutant mice. Adult CF mice presented increased Tlr4 expression, CVA length, crypt cell proliferation, and bacterial load relative to non-CF mice, but no differences were detected in Tlr4−/− compared with Tlr4+/− CF mice. The double-mutant neonates did not differ from non-CF mice, but fewer Tlr4−/− CF neonates presented with luminal mucus obstruction in the distal ileum, and the intestinal mast cell increase of CF mice was not evident in double-mutant neonates. We conclude that Tlr4 deficiency reduces the survival, but does not alter the intestinal phenotypes, of extended CVA or increased bacterial load in BALB CF mice.

Cystic Fibrosis (CF) is the most common, fatal, autosomal recessive disease of Caucasians with an incidence of 1 in 2,500 newborns in this population (6). Although it is well documented that the basic defect in CF lies in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, exactly how the encoded epithelial chloride-ion channel deficiency leads to the varied CF symptoms, including intestinal disease, is unclear. The intestinal disease is presumed to be due to reduced water secretion and increased mucus accumulation in this organ, which results in the development of meconium ileus in 10–15% of CF newborns (48) and distal intestinal obstruction syndrome episodes in 25% of CF adults (45).

Several mouse models have been made to investigate the complex CF phenotype (7), and, since most Cftr-deficient mice suffer from intestinal goblet cell hyperplasia, mucus accumulation, and crypt dilation, which ultimately lead to lethal obstruction (7, 15), these mice have been used as a model of clinical meconium ileus. One approach toward elucidating the mechanism leading to intestinal disease in CF mice has been to cross the mice with others deficient in specific physiological pathways such as NHE3 a sodium/hydrogen ion exchanger that alters intestinal content fluidity (2) or chloride channel CICα3, (47) to determine whether such deficiencies alter the CF disease course.

Toll-like receptors (Tlr), which are expressed on innate immune and epithelial cells and coordinate an immune response when bound by ligands, have been investigated as modifiers of cystic fibrosis lung disease (1, 26), but the potential influence of such receptors on cystic fibrosis intestinal disease has not been studied. Rakoffs-Nahoum et al. (37) showed that recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis, and, given that both intestinal homeostasis (4) and gram-negative bacterial content (31), which contain the Tlr4 ligand, are altered in the intestine in CF mice, we hypothesized that mutations in Tlr4 would alter the CF intestinal phenotype. Supporting this hypothesis, we have shown Tlr4 expression to be increased in the intestines of BALB × C57BL/6 F2 Cftr-deficient mice (4) as were components of its signaling pathway CD14 and Ly96 and genes of lipid metabolism, a pathway for which there is cross talk with Tlr4 signaling (10). In addition, the intestinal phenotype of the F2 CF mice featured both increased proliferation and less apoptosis in crypts compared with wild-type mice. These changes are consistent with the influence of increased Tlr4 signaling on the CF intestine, since Tlr4-deficient mice, when challenged with dextran sodium sulfate, have been shown to develop colitis with decreased proliferation and increased apoptosis (11). A deficiency in this gene, in CF, could therefore spare the proliferation and apoptosis alterations in the intestine. Alternatively, a deficiency of Tlr4 in CF could leave the mouse unable to mount an adequate inflammatory response to the increased bacteria, which would result in increased disease such as occurs in the development of sepsis (33).

Using the mutation in Tlr4 originally identified in C3H/HeJ mice (36), which renders the gene product nonfunctional, herein to investigate the hypothesis that Tlr4 genotype alters the CF intestinal phenotype we bred and phenotyped a population of Cftlrl4−/− double-mutant mice and their littermates. We demonstrate that the survival of CF mice is dependent on Tlr4 genotype but that the histology and bacterial load of surviving mice are not. Characterization of a population of 4-day-old mice suggests this survival difference to be affected by mucous and mast cell levels in the intestine.

MATERIALS AND METHODS

Mice. To create Cftlrl4−/− double-mutant mice in one genetic background, we bred C3-Tlr4−/−β mice obtained from Jackson Laboratories (Bar Harbor, ME), which have the C3H/HeJ point mutation in Tlr4, in the BALB background (44), with BALB Cft−/−Unc mice and genotyped the offspring for Cftr (4) and the C3H/HeJ-derived Tlr4 donor region (44). Congenic BALB Cft−/−Unc mice, which had previously been generated by backcrossing the original Cft−/−Unc mice to BALB/c mice for 20 generations

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Cftr and mate controls were then derived from filled by mucus plugs in PAS-stained sections using image analysis. Were measured only from complete and intact CVAs using image measured. The entire depth of the crypt and the length of the villus (Sigma) and stored at 10% buffered formalin and submitted for standard histological processing. Also at dissection, a 1-cm portion of the terminal ileum was removed and immediately homogenized in 1 ml of Trizol reagent (Life Technologies). The intestinal homogenate (tissue in 5 ml of cold sterile PBS) at 37°C for 10 min. The homogenate was then bated on LB agar plates overnight at 37°C. Sixty colonies from each were identified with Assays-on-Demand TaqMan probes using the Applied Biosystems' 7500 Real Time PCR System. A standard curve relating the number of bacteria present to the amount of DNA was created by extracting and quantifying DNA from suspensions of Escherichia coli (17). The number of colony-forming units evident on agar plates (LB Agar, Invitrogen) incubated with serial dilution of the entire intestinal homogenate (tissue in 5 ml of cold sterile PBS) at 37°C for 24 h.

**Phenotypic data analysis.** Tests for differences in mouse weight, gene expression, and ileal morphology between groups were done by Student’s t-test or by ANOVA in the case of multiple groups. Survival curves were generated by using the Kaplan-Meier method and were compared by the log-rank test.

**RESULTS**

Survival of Cftr-deficient mice is dependent on Tlr4 genotype. To determine the effect of Tlr4 genotype on cystic fibrosis intestinal phenotype, a population of mice was bred from a cross of Cftr+/− and Tlr4+/− or Tlr4−/− progenitors. Of a total of 302 mice produced, 77 were homozygous for the Cftr mutated allele. The survival of these CF mice was dependent on Tlr4 genotype, as shown in Fig. 1, where double-mutant mice (Cftr−/− Tlr4−/−) showed lower survival compared with that of Tlr4+/− or Tlr4+/− CF mice. The survival of these mice was independent of Tlr4 genotype (P = 0.10; data not shown).

Seventeen CF mice (16 females) lived to the age of 12 wk, and, at this age, the CF female mice were smaller than the non-CF mice (CF, 17.9 ± 0.78 SE g; non-CF, 22.1 ± 0.28 g; P = 0.0002), in agreement with previous data of this strain (17), but no difference in body weight by Tlr4 genotype in CF (P = 0.4) or non-CF mice (P = 0.1) was evident (data not shown).

**Ileal expression of Tlr4 is increased in BALB Cftr-deficient mice.** To determine whether the ileal expression of Tlr4 is altered in 12-wk-old BALB CF mice, as it is in (BALB × B6)
F₂ CF mice (4), real-time PCR analysis was performed. As depicted in Fig. 2A, CF mice had higher expression levels of Tlr4 compared with levels in non-CF mice, and the expression of Tlr4 was not altered by the Tlr4 mutation in CF (P = 0.44) or non-CF (P = 0.11) mice, as has been shown in C57/BL/J mice (36). The expression level of Tlr4 did not differ between Cfr+/− and Cfr−/− mice (P = 0.15, data not shown); thus, the data of these groups were combined in Fig. 2. Second, the expression level of Cfr was greater in non-CF mice than in CF mice, and this difference was independent of Tlr4 genotype (Fig. 2B).

We used immunohistochemistry to confirm the increased expression of Tlr4 in the CF intestine and to identify the cells expressing this protein. As shown in Fig. 2C, Tlr4 staining was evident in mononuclear cells in lamina propria and submucosa with faint staining in enterocytes and epithelial crypt cells. Quantification of this staining revealed the number of Tlr4-positive mononuclear cells to be increased in the CF mice compared with non-CF littermates, as shown in Fig. 2D. This increase in the number of Tlr4-positive cells was evident for both Tlr4+/− and Tlr4−/− CF mice, as shown in Fig. 2D.

Intestinal phenotypes unaltered by Tlr4 haploinsufficiency in 12-wk-old CF mice. We (4) and others (9, 14, 24, 41) have shown the intestinal phenotype of Cfr-deficient mice to include an extended CVA and increased proliferation compared with non-CF mice. To determine whether this phenotype was evident in BALB CF mice and was influenced by Tlr4 haploinsufficiency, histological measures were completed. Comparison of the intestinal phenotypes demonstrated CVA length and proliferative cell number to differ between BALB CF and non-CF mice but not to be affected by Tlr4 haploinsufficiency either in CF or non-CF mice, as shown in Fig. 3, A–D. CVA length and cell proliferation counts did not differ between Cfr+/− and Cfr−/− mice (P = 0.17 and P = 0.34, respectively; data not shown); thus, the data of these groups were combined in Fig. 3.

Next, bacterial loads in the small intestines were recorded to determine whether Tlr4 genotype influenced this phenotype. As shown in Fig. 3E, and in agreement with Norkina’s (31) report of C57/BL/J CF mice, more bacteria colonized the small intestine of 12-wk-old CF mice compared with that of non-CF littermates, but no difference in bacterial counts was found in mice grouped by Tlr4 genotype.

To determine whether the intestinal bacterial composition differed among animals grouped by Cfr and Tlr4 genotype, species identification was completed by sequence analysis. As shown in Table 1, the bacteria in the intestines of non-CF, Tlr4+/− mice was 68% gram positive and 32% gram negative. In Tlr4 haploinsufficient and insufficient non-CF mice, the percentage of gram-negative bacteria increased to 47 and 62%, respectively.

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**Fig. 2. Expression of Tlr4 and Cfr in ileal tissue of 12-wk-old mice derived from BALB/c Cfr+/−tm1Unc and C3-Tlr4Lps-d/J progenitors. A 1-cm section of the distal ileum was collected at autopsy, and the expression levels of Tlr4 (A) and of Cfr (B), relative to the ataxia 10 control gene, were measured by real-time PCR analysis (n = 3–10 mice/group). C: representative immunostaining for Tlr4. Note the localization of positive mononuclear cells in the lamina propria of cystic fibrosis (CF) mice (arrows). Original magnification of ×200; inset: higher magnification of area in box. D: average ± SE number of Tlr4-positive cells in the lamina propria and submucosa of 12-wk-old mice (n = 4–5 mice/group). *Significant difference between groups, P < 0.05 and, in D, between CF and non-CF groups.**
respectively. In contrast to non-CF Tlr4/H11001/H11001/H11001 mice, the bacteria in the intestines of CF/Tlr4/H11001/H11001/H11001 mice were 60% gram negative while in CF/Tlr4/H11001/H11001/H11002 mice the gram-negative proportion of 42% is similar to that of non-CF, Tlr4/H11001/H11002/H11002 mice (47%). The predominant gram-positive bacterial classification identified in both CF and non-CF mice was Lactobacillales, whereas gram-negative bacteria were Bacteriodales in non-CF mice and -Proteobacteria in CF mice.

Finally, Shang et al. (40) reported transgenic mice created with the Tlr4 gene constitutively active in the intestinal epithelium to have increased B cell numbers in the proximal region of this tissue compared with control mice. Because Tlr4 was of higher expression in CF mice, we investigated the presence of intestinal B cells in our 12-wk-old CF and non-CF mice. The numbers of CD20 positive cells did not differ in mice grouped by CF status, and this result did not depend on Tlr4 genotype (data not shown).

Phenotypic assessment of Cftr/Tlr4 neonates. Because Cftr/Tlr4 double-mutant mice had low survival to the age of 12 wk, we assessed the influence of Tlr4 genotype on the intestinal phenotype of CF mice at the age of 4 days. A population of mice was bred from a cross of Cftr/H11001/H11002/H11002 and Tlr4/H11001/H11001/H11002 progenitors, and, of the 166 mice produced, 42 were homozygous for the Cftr mutated allele, which is consistent with expected Mendelian ratios. CF mice, independent of Tlr4 genotype, had lower body weight compared with non-CF mice (CF, 2.1 ± 0.08; non-CF, 3.5 ± 0.05, P < 0.0001).

To determine whether phenotypic alterations are present in the intestines of Cftr/Tlr4 double-mutant neonates, which
might account for their reduced survival to 12 wk of age, we measured the CVA length, bacterial content, goblet cell count, intestinal mucus obstruction, and inflammatory infiltration of this tissue. Histological assessment of the intestine revealed the distended phenotype of CF mice to be evident at this age, as shown in Fig. 4, A and B, but that the greater crypt depth in CF mice was not dependent on Tlr4 genotype ($P = 0.81$). Similarly, the intestinal bacterial load in the neonates revealed higher counts for the CF mice compared with non-CF littermates, and this was independent of Tlr4 genotype in CF ($P = 0.28$) and non-CF ($P = 0.58$) mice, as shown in Fig. 4C.

Inflammatory cell phenotyping revealed the number of CD3$^+$ cells to be greatest in the small intestine of Cft/rTlr4 double mutants, but no statistically significant differences in lymphocyte count by Cfr genotype ($P = 0.15$ by t-test) or Tlr4 genotype within CF mice ($P = 0.22$ by ANOVA) were evident (Fig. 5A). As shown in Fig. 5B, and in agreement with previous reports on adult CF mice (8, 32), the number of mast cells was increased in the small intestine of CF compared with non-CF mice ($P = 0.025$ by t-test), and, within the CF mice, the double knockouts had the fewest mast cells ($P = 0.068$ by ANOVA, $P = 0.033$ compared with Tlr4$^{+/+}$ CF). An increase of mucosal F4/80$^+$ cells was evident in the non-CF compared with the CF mice ($P = 0.016$ by t-test), but no difference in this phenotype by Tlr4 genotype was observed within the CF mice ($P = 0.64$ by ANOVA) (Fig. 5C). The findings in submucosal macrophages, however, did not reveal a significant difference in cell numbers by either Cfr or Tlr4 genotype ($P = 0.33$ by ANOVA) (Fig. 5D).

Goblet cell hyperplasia is a feature of CF intestinal disease in mice (41), and our assessment of the neonates showed this trait to be evident in the proximal intestine of CF mice ($P = 0.003$), independent of Tlr4 genotype (ANOVA, $P = 0.50$), whereas, in the distal half, which includes the terminal ileum, the number of goblet cells did not differ by CF status ($P = 0.46$; Fig. 6A). Most CF neonates also had histological evidence of dilated mucus-filled crypts and the accumulation of mucus material in intervillous spaces and in the intestinal lumen, while a few presented with a nonobstructed ileum (Fig. 6B). By Tlr4 genotype, the incidence of intestinal obstruction was eight of nine double mutants: 5/5 Tlr4$^{+/+}$ CF mice and 3/6 Tlr4$^{-/-}$ CF mice. To assess whether the obstructed area was dependent on Tlr4 genotype, we measured the total obstructed area in the ileum of PAS-stained sections of these mice. As expected, CF mice had a greater obstructed area compared with the level in non-CF, and we observed a nonsignificant decrease expected in the affected area of CF Tlr4$^{+/+}$ compared with CF Tlr4 homozygous mice (Fig. 6C).

**DISCUSSION**

In this work, we show the CF intestinal disease traits of extended CVA, goblet cell hyperplasia, and greater bacterial load to be present in the BALB Cfr$^{tm1UNC}$ model of cystic fibrosis and that these changes are evident in 4-day-old CF neonates. By creating and analyzing a population of mice carrying Cfr and/or Tlr4 mutations, we demonstrate the survival of CF mice to be altered by mutations in Tlr4, where, specifically, the incidence of lethality was increased in Tlr4 homozygous mutant CF mice while survival was enhanced in Tlr4 heterozygous CF mice. Finally, our studies revealed the level of Tlr4 expression to be increased in the intestinal tissue of adult BALB CF mice but that haploinsufficiency of Tlr4 did not alter the CVA length, crypt proliferation, or bacterial content in these adult CF mice.

In this population, the survival of CF mice with the Tlr4$^{+/+}$ genotype agrees with that reported for the original Cfr$^{tm1UNC}$ mouse model (41) by both the early wave of perinatal lethality and in that ~46% of mice survived to weaning. In contrast, the CF mice with homozygosity for the Tlr4 mutation succumbed to early lethality in this study, whereas the greater survival to 12 wk of age of Tlr4$^{+/+}$ CF mice was associated with perinatal deaths in only 25% of the population. The mechanisms contributing to this enhanced (for Tlr4$^{+/+}$ CF mice) or reduced (for Tlr4$^{-/-}$ CF mice) survival were most likely Tlr4-dependent development of mucus obstructions and a reduced inflammatory response in double-mutant mice, respectively, since
The mucus in the CF intestine increases the survival of these mice (2, 47) and that the administration of LPS, a Tlr4 ligand, can reduce mucus levels in the lung (38), we speculate that the improved survival of Tlr4+/− CF mice is due to a reduction in intestinal mucus. Elucidation of the mechanism that reduces mucus in Tlr4+/−, but not Tlr4+/*, CF mice, if such exists, will require additional study. Further analysis of the intestinal disease of 4-day-old CF mice revealed a decrease in mast cell infiltration in Tlr4−/−, compared with the level in Tlr4+/+, CF mice. The reduced mast cell infiltration in the double-mutant mice, which may be due to the deficiency in Tlr4 signaling (23, 46) coupled with the reduced inflammatory response of Tlr4-deficient mast cells (42), could result in an inadequate immune response to the gram-negative bacterial burden in the intestines of CF mice (31), such as occurs in sepsis (42), and thus the reduced survival of the Cftr/Tlr4 double-mutant mice.

Established functions for Tlr4 include initiating an innate immune response to bacterial challenge, which ultimately decreases tissue bacterial load (43) and, in the intestine, signaling to maintain epithelial cell homeostasis (37); however, in this CF model, neither the epithelial cell proliferation nor the amount of luminal bacteria was altered by mutant Tlr4. That intestinal bacterial load was not dependent on functional Tlr4 has been reported for related animal models of ileitis and colitis (21, 25) and distinguishes Tlr4 function in this tissue from that in a systemic response where bacterial levels are related to Tlr4 genotype (3, 29, 43). Second, the influence of Tlr4 on intestinal cell homeostasis and disease phenotype in challenge models is not obvious, since the deficiency has been shown to increase (11, 12, 37) or to lessen (21, 25, 27) disease. Regarding the latter, Leaphart et al. (27) reported C3H/HeJ mice to be spared necrotizing enterocolitis due to reduced apoptosis and increased proliferation compared with the levels in wild-type mice. Despite carrying the same C3H/HeJ mutation in Tlr4, the crypts of our Cftr/Tlr4 mutant mice did not display altered levels of apoptosis or proliferation relative to CF Tlr4+/+ animals. The absence of a Tlr4 effect on proliferation/apoptosis in the CF intestine may have occurred, since the level of injury in CF is not of the order of that in the induced colitis models or may indicate this Tlr4-regulated tissue response does not contribute to CF intestinal disease.

An assessment of the intestinal bacteria species revealed both Tlr4 genotype- and CF status-dependent profiles to exist in these mice. In detail, sequencing and BLASTN analysis of the 16S rDNA gene in the intestinal contents of the BALB non-CF, Tlr4+/+ mice revealed the majority of the bacteria to be gram-positive, in agreement with findings of Hasegawa et al. (16) who used a similar technique to define the bacterial species present in the small intestines of C57BL/6 (Tlr4+/+) mice. In their study, however, the predominant gram-positive bacteria detected was Erysipelotrichales, and these were not detected in the current work. Compared with the Tlr4-sufficient mice, non-CF mice of the Tlr4+/+ or Tlr4−/− genotype had an increased proportion of gram-negative bacteria in their small intestines, reflecting their deficiencies in the innate immunity receptor for gram-negative bacteria. In CF mice, we observed not just a shift from gram-positive to gram-negative bacteria but a profound overgrowth of certain bacteria that are detected in small numbers in the control group. This shift and over-

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**Fig. 4.** Increased CVA length and bacterial load of the small intestine of 4-day-old CF compared with non-CF mice independent of Tlr4 genotype. Mice were killed at 4 days of age, and CVA length measurements were made by image analysis of histological sections. A: representative sections of ileal tissue, stained with hematoxylin and eosin. Original magnification of ×200. B: the increased CVA length in CF mice is largely due to the presence of elongated and fully developed crypts in these mice (n = 3–6 mice/group). C: bacterial load was measured by plating the tissue homogenate of the entire intestine and counting the number of colony-forming units at 24 h postplating. Each dot represents one mouse, and the dotted line represents the average of the group. *Significant difference between CF and non-CF mice, P < 0.05.
growth agrees with the report of bacterial content in the intestines of C57BL/6 CF mice by Norkina et al. (31). The gram-negative bacteria in the intestines of BALB CF mice were principally classified as \( \beta \)-proteobacteria, a class that includes bacteria known to infect the lungs of CF patients, \textit{Burkholderia cepacia} (28), while Bacteroidales were of increased proportion in the intestines of \( \text{Tlr4} \) genotype-matched non-CF mice. Because commensal bacteria function to supply nutrients to the host, aid in digestion, and can contribute to the development and regulation of the immune system (22, 39), these changes in flora may contribute to the disease phenotype of CF mice. Whether such changes contributed to the altered survival of CF mice by \( \text{Tlr4} \) genotype is not evident, since bacterial phenotyping was completed in mice surviving to 12 wk of age only.

In this work, we confirmed our previous finding of higher levels of \( \text{Tlr4} \) expression in the ileal tissue of CF mice (4) and extended this work to show this increase to be due in...
part to greater numbers of Tlr4-positive cells in the lamina propria in CF mice relative to control. The localization of Tlr4-positive cells to the intestinal lamina propria in mice has been reported previously (13, 34) as has increased Tlr4 expression levels in the intestinal diseases of necrotizing enterocolitis (27) and inflammatory bowel disease (5), indicating a similarity in tissue response across syndromes. Despite the increased expression of intestinal Tlr4 in CF mice, we did not detect an increase in intestinal B lymphocytes as was reported by Shang et al. (40) for mice with the Tlr4 gene constitutively active in the intestine, although in their work this increase was evident in the proximal small intestine, and here we evaluated the more distal intestine. Nevertheless, we report B cell numbers in the intestine to be unaltered in CF mice.

In summary, the results of this study show the increased expression and presence of Tlr4 in the CF mouse small intestine and, significantly, that functional interruption of this protein (through mutation) severely reduced the survival of CF mice. Despite evidence of Tlr4 mutations altering tissue bacterial levels and intestinal cell homeostasis, neither of these traits was identified as Tlr4 dependent in the CF intestine, rather the reduced survival of Cfrtr/Tlr4-deficient mice was associated with decreased mast cell numbers. Finally, we also showed the CF newborn mouse to acquire an almost immediate small intestinal bacterial overgrowth, crypt elongation, and mucus accumulation compared with non-CF neonates, resembling the phenotypes found in CF adult mice.

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DISCLOSURES
No conflicts of interest are declared by the authors.
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