

TITLE:

A model of piroxicam-accelerated enterocolitis in interleukin-10 knockout mice for studying inflammation-induced metabolic alterations

AUTHORS AND AFFILIATIONS:

Mihir Ranjan¹, Justin Williams¹, Lan Peng², Ezra Burstein³, Jianyi Yin^{3,*}, Luis Sifuentes-Dominguez^{1,*}

¹Department of Pediatrics, The University of Texas Southwestern Medical Center, Dallas, TX 75390, USA.

²Department of Pathology, University of Texas Southwestern Medical Center, Dallas, TX 7390, USA.

³Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX 7390, USA.

*These authors supervised this work.

Luis Sifuentes-Dominguez: luis.sifuentes-dominguez@utsouthwestern.edu

Mihir Ranjan: mihir.ranjan@utsouthwestern.edu

Justin Williams: justin.williams@utsouthwestern.edu

Lan Peng: lan.peng@utsouthwestern.edu

Ezra Burstein: ezra.burstein@utsouthwestern.edu

Jianyi Yin: jianyi.yin@utsouthwestern.edu

SUMMARY:

This protocol uses piroxicam to accelerate colitis development in the interleukin-10-knockout mouse model. The protocol ensures colitis synchronization, resulting in rigorous, reproducible results independent of sex, background strain, or vivarium conditions. It serves as a useful model for studying metabolic alterations during intestinal inflammation.

ABSTRACT:

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder of the digestive tract affecting over 10 million individuals globally. While substantial research has focused on the immunologic mechanisms and consequences underlying IBD, less is understood about how mucosal inflammation contributes to metabolic dysregulation, including weight loss and reduced appetite. Here, we describe a mouse model of piroxicam-accelerated enterocolitis in interleukin-10-knockout (IL10-KO) mice for the study of inflammation-induced metabolic dysregulation. IL10-KO mice are known to develop spontaneous enterocolitis and have heightened susceptibility to enterocolitis triggered by infections or drugs. However, vivarium conditions and strain background have been reported as confounders in colitis development in this model. Piroxicam, a non-steroidal anti-inflammatory drug (NSAID), has been demonstrated to trigger or accelerate enterocolitis in animal models by increasing mucosal exposure to luminal bacteria. In this protocol, male and female IL10-KO mice were fed a piroxicam-fortified diet in place of a regular chow diet. Food intake and body weight were measured daily to reflect whole-body metabolic

alterations, along with clinical manifestations of enterocolitis such as diarrhea and rectal bleeding. The protocol is efficient and reproducible, inducing enterocolitis simultaneously in multiple mice, and allows assessment of metabolic dysregulation in an inflammatory bowel disease model. Further studies using this protocol may investigate the effects of enterocolitis on other components of metabolic dysregulation such as energy expenditure and body composition, revealing broader connections between inflammatory bowel disease and host metabolism.

INTRODUCTION:

Crohn's disease and ulcerative colitis, collectively known as inflammatory bowel disease (IBD), are chronic autoimmune disorders of the gastrointestinal tract characterized by mucosal inflammation with clinical manifestations including abdominal pain, weight loss, hematochezia, and diarrhea. IBD affects millions of individuals worldwide, with the highest prevalence among westernized societies¹. In the US, it is estimated that over 3 million people are affected^{2,3}, with rising incidence and prevalence, particularly among children and young individuals^{4,5}.

The exact mechanisms underlying IBD pathogenesis remain incompletely understood, but the disease is widely thought to result from an exaggerated immune response to the intestinal microbiota in genetically susceptible individuals. Cytokines that modulate inflammatory responses play critical roles in maintaining gut homeostasis and in the development of IBD. Among these, interleukin-10 (IL10) is a well-studied anti-inflammatory cytokine that helps maintain immunologic homeostasis in humans by regulating both innate and adaptive arms of immune response^{6,7}. Notably, polymorphisms in IL-10 and its receptor (IL-10RA/B) have been identified in infants with very-early onset IBD (VEO-IBD), classically presenting with perianal disease within the first several months of life⁸. This association is further supported by large-scale exome sequencing of adult patients with Crohn's disease, which identified IL10RA as a disease susceptibility locus in non-monogenic IBD⁹. Furthermore, a non-genetic pathway involving IL-10 neutralizing antibodies has been described in VEO-IBD patients, further highlighting the importance of this pathway in maintaining mucosal immune homeostasis¹⁰.

Building on human studies implicating IL10 in IBD, interleukin-10-knockout (IL10-KO) mice have been developed as a model to study the role of IL10 in IBD. IL10-KO mice are known to develop spontaneous enterocolitis with an exaggerated CD4⁺ Th1 response to stimuli, typically beginning at 8-12 weeks of age¹¹. However, the development of spontaneous enterocolitis in IL10-KO mice can be unpredictable in the absence of an accelerating agent¹²⁻¹⁴, depending on genetic strain, microbiota composition, and housing conditions^{15,16}. The use of a reliable protocol to induce colitis is critical for the IL10-KO model to have practical experimental utility. Prior studies have utilized non-steroidal anti-inflammatory drugs (NSAIDs) such as piroxicam, to induce enterocolitis in IL10-KO mice^{17,18}. However, these studies have primarily focused on immunologic alterations, with relatively little emphasis on the effect of enterocolitis on metabolism or the role sex plays in colitis severity¹⁹. We herein describe the use of piroxicam-fortified diet to induce enterocolitis in IL10-KO mice and characterization of metabolic dysregulation and mucosal inflammatory response in this model using mice from both sexes. Our protocol measures body weight and food intake to reflect whole body metabolic alterations, as well as clinical manifestations of colitis and histologic and transcript changes to reflect mucosal inflammation. This efficient and reproducible

model provides a valuable tool for investigating the connection between IBD and host metabolism.

PROTOCOL:

All animal experiments were performed in compliance with the protocol (APN 2017-102011) approved by the Institutional Animal Care and Use Committee (IACUC) at University of Texas Southwestern Medical Center, in accordance with the "Guide for the Care and Use of Laboratory Animals". Euthanasia was performed in accordance with institutional guidelines. No anesthesia was required for the procedures performed.

NOTE: Specific details for materials are described in the Table of Materials.

1. Establishing the piroxicam-accelerated enterocolitis model in IL10-KO mice

1.1. Maintain IL10-KO mice in a specific pathogen-free (SPF) facility on a 12-hour light cycle with *ad libitum* access to chow and water.

1.2. Individually house 7–8-week-old, age and sex-matched mice in microisolator cages. Allow mice to acclimate to single housing and daily handling for at least one week prior to the start of the experiment.

NOTE: Ensure that body weights are >20 g for males or >17 g for females, and that mice are free from fight wounds.

1.3. Assign mice to an experimental group and a control group. For the experimental group, replace the standard chow with piroxicam-fortified chow. For the control group, provide fresh standard chow. Add 50 g chow to each cage hopper.

NOTE: Allocate at least five mice per sex to each group.

NOTE: The piroxicam-fortified chow (200 ppm) was prepared by Teklad using piroxicam (Sigma-Aldrich, #P0847) supplied by the investigators for the production of a custom laboratory animal diet. To minimize batch-to-batch variability, it is recommended that all animals within a given experiment receive chow from the same production batch. When possible, the same batch should also be used across experiments to improve reproducibility.

2. Monitoring of body weight and feeding behavior

2.1. Using an analytical scale, record the body weight of each mouse. Weigh each mouse individually at the same time each day, daily for 7 consecutive days. Report daily body weight as a percentage of the baseline weight on day 0 (set as 100%).

NOTE: A weight loss greater than 20% indicates excessive stress and risk of imminent death. Mice should be euthanized if this threshold is reached.

2.2. Using an analytical scale, record the initial weight of the piroxicam-fortified chow and the standard chow in each cage. Then, measure the remaining chow in each cage hopper at the same time each day, daily for 7 consecutive days. Calculate daily chow consumption by subtracting daily chow weight to the remaining chow from the prior day.

NOTE: Routinely check the amount of chow and refill if lower than 10 g. Palatability of piroxicam chow does not influence intake and should closely resemble that of standard chow.

3. Monitoring of disease activity

3.1. On days 0 and 7, visually inspect fecal pellets from individual mice and determine the presence of blood in stool. If no blood is visible, employ a fecal occult blood test by smearing a pellet onto the test card followed by adding the supplied developer reagent. Score results according to **Table 1**.

3.2. On days 0 and 7, visually inspect freshly voided fecal pellets to assess stool consistency. Utilize a flat forceps to aid in the determination of consistency. Score results according to **Table 1**.

3.3. On days 0 and 7, determine the degree of rectal prolapse by inspecting the perianal region. Score results according to **Table 1**.

3.4. Measure body weight as described above. Score results according to **Table 1**.

Note: DAI scoring should be performed in a blinded manner by the same researcher throughout the experiment to ensure consistency, minimize observer bias, and improve the reliability of the assessment.

4. Harvesting of colonic tissue

4.1. Euthanize mice by CO₂ asphyxiation and cervical dislocation as reported elsewhere²⁰.

4.2. Place the mouse in supine position and spray 70% ethanol on the ventral abdomen.

4.3. Lift the skin with forceps and use surgical scissors to cut along the midline. Expose the peritoneal cavity and locate the colon.

4.4. Expose the colon and cecum using blunt dissection. Remove the colon *en bloc*, including the cecum and the anal cuff.

4.5. Place the colon on a flat surface and remove any connective tissue.

4.6. Measure and record colon length.

4.7. After measurement of colon length, remove the cecum by cutting at the ceco-colonic junction.

4.8. Prepare a 10 mL syringe filled with PBS with an attached 22-gauge ball tip reusable steel gavage needle. Thread the needle into the colon and flush out intestinal contents.

4.9. Place the flushed colon in a petri dish containing PBS and rinse to remove residual contents. Once cleared, use for downstream assays.

5. Histology analysis

5.1. Lay the colon flat on a glass slide. Use forceps to wrap the tissue around itself starting from the distal end to form a “Swiss roll”. In this fashion, the distal/anal end will remain in the inner roll with the more proximal regions extending to the outer roll.

5.2. Using a 27-gauge needle, pin the rolled colon to maintain the roll and place it inside an embedding tissue cassette.

5.3. To fix tissues, submerge tissue cassettes in 10% buffered formalin solution for 16 hours at 4 °C.

NOTE: 10% formalin is harmful if in contact with skin and can cause severe skin burns and eye damage. Handle cautiously with protective gloves and scientific goggles. Residual formalin should be properly disposed following institutional guidelines.

5.4. After formalin fixation, process tissues for paraffin embedding and sectioned for mounting on glass slides.

NOTE: Samples should be submitted to a histology laboratory with necessary equipment for processing tissue cassettes in paraffin, scroll sectioning, slide mounting and hematoxylin and eosin staining.

5.5. Stain tissue slides with hematoxylin and eosin (H&E), as per established protocols²¹.

5.6. Using the histological scoring system described in **Table 2**, evaluate the following histologic features in each section: tissue damage from enterocolitis, lamina propria inflammatory cell infiltration, and percentage of area involved. Combine the score in each section to generate a total score (**Table 2**). Representative histopathological images of tissue damage and cell infiltration can be found in the publication by Erben et al²².

NOTE: Histologic scoring should be performed in a blinded manner, preferably by a trained pathologist, to ensure consistency and reliability of the assessment.

6. Immunofluorescent staining of formalin fixed paraffin embedded tissue

6.1. Place tissue slides in a slide rack and into a 55°C slide dryer for 1 hour to melt paraffin.

6.2. Place slides in staining rack and sequentially submerge rack for 3 minutes into staining jars with xylene. Repeat this step on 3 occasions.

6.3. Submerge staining rack for 3 minutes into staining jars with 100% ethanol. Repeat this step on 3 occasions.

6.4. Submerge staining rack for 3 minutes into staining jar with 95% ethanol.

6.5. Submerge staining rack for 3 minutes into staining jar with 80% ethanol.

6.6. Submerge staining rack for 5 minutes into staining jars with DI water.

NOTE: All the prior steps are to be performed in a ventilated hood due to the fumes produced by xylene. Handle cautiously with protective gloves and scientific goggles. Residual xylene should be properly disposed following institutional guidelines

6.7. Transfer slides to a slide rack and submerge in staining jar with 1x citrate retrieval solution.

6.8. Place staining jar in staining dish support.

6.9. Place staining dish support in pressure cooker.

6.11. Cook for 15 minutes in pressure cooker.

6.12. Recover slides from pressure cooker and allow them to cool down to room temperature.

6.13. Incubate slides with 300µl blocking buffer (3.5% goat serum diluted in PBS) for 1 hour in a humidified chamber. Ensure the tissue is completely covered by blocking buffer.

6.14. Expose slides to 300µl of primary antibody diluted in blocking buffer for 16 hours at 4°C, inside a humidified chamber. Ensure the tissue is completely covered by primary antibody. The following primary antibody was used: Anti-S100A8 + S100A9 antibody (1:1000 dilution).

NOTE: Dilutions of antibodies will be reagent specific and should follow recommendations from the supplier.

6.15. Rinse slides by serially submerging them for 5 minutes on 3 occasions in PBS in a staining jar.

6.16. Expose slides to 300µl fluorescent secondary antibody diluted in blocking buffer for 1 hour at room temperature, inside a humidified chamber. Ensure the tissue is completely covered by primary antibody. The following fluorescent secondary antibody was used: goat anti-Rabbit Alexa Fluor 555 (1:150 dilution).

NOTE: From this point on slides should be protected from light to prevent photobleaching of fluorophores. Dilutions of secondary antibodies will be reagent specific and should follow recommendations from the supplier.

6.17. Rinse slides by serially submerging them for 5 minutes on 3 occasions in PBS in a staining jar.

6.18. Expose slides to nuclear staining by submerging in Hoeschst solution diluted 1:5000 in PBS for 20 minutes in a staining jar.

6.19. Rinse slides by serially submerging them for 5 minutes on 3 occasions in PBS in a staining jar.

6.20. Carefully wipe off any PBS from slides using a non-abrasive tissue. Add 50µl of antifade mounting medium and coverslip.

7. RNA extraction

7.1. Snap freeze the entire colon or a fragment in liquid nitrogen and then store at -80°C degrees. Alternatively, submerge in 1.5 mL of RNAlater preserving solution and store according to the manufacturer's instructions.

7.2. Thaw previously frozen colon and place on a flat surface. Disrupt and homogenize the tissue with a handheld homogenizer.

7.3. Isolate RNA using the RNeasy mini kit, following the manufacturer's instructions.

7.4. Measure RNA concentration using a nanodrop instrument. A_{260}/A_{280} ratios >1.8 and A_{260}/A_{230} ratios of 2.0-2.2 indicate acceptable RNA purity.

7.5. Proceed with reverse transcription utilizing the superscript system followed by qPCR with SYBR green intercalating dye.

7.6. Analyze results using the QuantStudio 7 Pro platform or similar instrument.

REPRESENTATIVE RESULTS:

Male and female IL10-KO mice develop enterocolitis after a short exposure to a piroxicam-fortified diet (**Figure 1A–B**). By day 2, piroxicam-exposed mice exhibited statistically significantly greater weight loss than their corresponding same-sex regular chow-fed controls, and comparable weight loss was appreciated independent of sex (**Figure 1B**). Apparent daily food intake remained unchanged throughout piroxicam exposure and enterocolitis development, and resembled intake of standard chow, indicating that weight loss in this model is not primarily driven by reduced intake but rather by other factors that promote metabolic expenditure (**Figure 1C**).

To further investigate the progression of intestinal inflammation, disease activity was assessed by a combined score of weight loss, blood in stool, stool consistency, and degree of rectal prolapse. This modified disease activity index (DAI) was recorded on the initial and last day of piroxicam exposure to reflect multiple clinical manifestations of enterocolitis. On day 7, piroxicam-exposed mice demonstrated higher DAI scores when compared to non-exposed controls (**Figure 1D**). In addition, the development of enterocolitis observed in piroxicam-exposed mice was associated with a strong trend to increased mortality, although not statistically significant, $p = 0.12$ (**Figure 1E**). In this model, early mortality is secondary to mice reaching humane endpoints (weight loss >20%) and preferentially affecting males (**Figure 1E**). If mortality is a sought-after experimental endpoint, a larger experimental animal number than used here may be considered. In this study, we utilized the minimum number of animals required to achieve statistical power through study completion, after accounting for expected mortality.

Colon shortening is a feature associated with the severity of colitis in murine models. At the time of harvest, both male and female IL10-KO mice exposed to piroxicam diet had shorter colons than those with regular chow (**Figures 2A and 2B**). Upon histological assessment, colons from the piroxicam-fed group showed epithelial damage and increased immune cell infiltration, changes associated with colitis (**Figure 2C**). These findings translated into higher histological score (**Figure 2D**). Additionally, transcriptional levels of pro-inflammatory cytokines involved in human IBD and murine models of colitis were evaluated²³. In agreement with prior reports, colons from piroxicam-fed IL10-KO mice displayed a significant upregulation of pro-inflammatory cytokines at the transcript level (**Figure 3 A–D**). Of note, this transcriptional change was not observed in IL10-KO mice receiving regular chow, or WT mice receiving piroxicam-fortified diet, indicating that the transcriptional signature was driven by a combination of IL10 deficiency and piroxicam, rather than either factor alone. Finally, we confirmed that the transcriptional signature observed in colon from piroxicam treated mice resulted in increased expression of calprotectin, a neutrophil cytoplasmic protein implicated in innate immune responses that is clinically utilized as a biomarker for intestinal inflammation in patients with IBD (**Figure 3E**)^{24,25}.

FIGURES AND TABLE LEGENDS:

Figure 1: Piroxicam-accelerated enterocolitis in interleukin-10-knockout (IL10-KO) mice. (A) Schematic overview of experimental protocol. (B) Body weight, (C) food intake, (D) day 7 disease activity index (DAI), (E) survival curve in 8-week-old male and 8-week-old female IL10-KO mice

receiving piroxicam-fortified diet or regular diet. Male control: n = 5, male piroxicam: n = 5, female control: n = 6, female piroxicam: n = 6 in (B), (C), and (D). Male control: n = 6, male piroxicam: n = 10, female control: n = 6, female piroxicam: n = 7 in (E). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ by pairwise comparison of male (top asterisks) and female (bottom asterisks) mice in (B). Statistical analyses were performed using two-tailed unpaired t test in (B), (C), and (D), and log rank test in (E). Mean values and error bars representing standard error of mean (SEM) are depicted. In bar charts, individual measurements are depicted by jitter plots.

Figure 2: Colon shortening and histological assessment. (A) Representative images of colon from 8-week-old male and female interleukin-10-knockout (IL10-KO) mice receiving piroxicam diet (top) or regular diet (bottom) for 7 days. Scale bar: 1 cm. (B) Colon length on day 7 (male control: n = 5, male piroxicam: n = 5, female control: n = 6, female piroxicam: n = 6). (C) Representative images of H&E staining of colon. Scale bar: 100 μ m. (D) Histology score on day 7. Two-tailed unpaired t test in (B) and (D). Mean values and error bars representing SEM are depicted. In bar charts individual measurements are depicted by jitter plots. Not significant (NS), * $p \leq 0.05$, *** $p \leq 0.001$, **** $p \leq 0.0001$ by pairwise comparison.

Figure 3: Inflammatory changes in piroxicam-accelerated enterocolitis. qRT-PCR results of (A) *Tnf*, (B) *Il6*, (C) *Il1b* and (D) *Ifng* in colonic tissue from 8-week-old wild type (WT) and interleukin-10-knockout (IL10-KO) male mice at time of harvest. WT piroxicam: n = 7, KO piroxicam: n = 6, KO control: n = 3. (E) Calprotectin fluorescent immunohistochemistry from colons of 8-week-old IL10-KO mice at time of harvest. Statistical analyses were performed using two-tailed unpaired t test. Mean values and error bars representing standard error of mean (SEM) are depicted. In bar charts individual measurements are depicted by jitter plots. Scale bar in (E) = 200 μ m. Not significant (NS), * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ by pairwise comparison.

Table 1: Disease activity score (DAI). Scoring system to determine disease activity by weight loss, stool consistency, blood in stool, and rectal prolapse. Adapted from Sifuentes-Dominguez, et al²⁶.

Table 2: Histologic scoring system. Scoring system to determine histologic changes and damage. Adapted from Sifuentes-Dominguez, et al²⁶.

DISCUSSION:

IBD is a complex polygenic disorder where preclinical models serve as invaluable tools to dissect pathways that contribute to IBD pathogenesis and develop novel diagnostic and therapeutic strategies. Currently, many IBD preclinical models rely on chemical exposure, such as dextran sulfate sodium (DSS), oxazolone, and trinitrobenzene sulfonic acid (TNBS), due to their ease of use. Genetic models that develop spontaneous colitis are also available. Among these, the IL10-KO model is particularly valuable because genetic variations in the IL10 pathway are known to cause intestinal inflammation in humans, and IL10-KO mice develop spontaneous chronic enterocolitis that resembles human disease. However, a major limitation of this model is phenotypic variability. Although enterocolitis develops spontaneously, its onset can be variable depending on strain background and vivarium conditions²⁷.

Combining chemical exposure with a genetic model, the protocol described here represents a reliable and reproducible murine model of experimental colitis. Enterocolitis is accelerated in IL10-KO mice by piroxicam, with disease onset within 7 days following exposure to piroxicam-fortified diet. This protocol allows synchronization of colitis development. The model recapitulates features observed in other commonly used murine models of colitis, including weight loss, blood in stool, diarrhea, colon shortening, histologic damage and transcriptional changes. Furthermore, this model is reproducible in the widely used C57Bl/6J strain, which facilitates interrogation of additional gene defects associated with IBD through the relative ease of generating mice carrying additional mutations in this strain.

The model described here provides a useful tool to study metabolic changes during intestinal inflammation. In this model, mice exposed to piroxicam that develop accelerated enterocolitis, continue to feed normally without a significant decrease in daily intake. This indicates that weight loss in this model is not due to impaired feeding but rather to additional factors. Further studies are needed to determine whether this results from increased caloric expenditure due to inflammation, excessive caloric loss through diarrhea, or impaired thermogenesis. Furthermore, male and female mice exhibit similar patterns of disease severity a finding that contrasts prior observations made on IL10-KO and DSS models²⁸⁻³⁰.

There are several important troubleshooting steps to ensure reproducibility of this model. First, mice should be acclimated to daily handling and observations for one week prior to exposure to piroxicam. This prevents stress-associated weight loss. Second, cages should contain no more than three mice, and only mice that are harmoniously housed should be included in the experiment. Mice exhibiting fighting behavior or fight wounds are more susceptible to colitis development and may succumb prior to harvest^{31,32}. Third, if food intake will be monitored throughout the experiment, mice should be individually housed to allow accurate measurement of chow consumption. In addition, only large intact food pellets should be placed in the feeding hoppers to prevent pellet crumbling into the bedding, which may affect measurements. The methods for quantifying food intake described here are in line with published rodent food consumption guidelines³³. Finally, cage changes should be avoided during the experiment, as sudden environmental changes may lead to decreased food intake for 1-2 days, particularly in young mice.

Our protocol has several limitations. First, assessment of metabolic alterations is limited to body weight and food intake, while other measures of systemic metabolism, such as respiratory exchange ratio (RER), energy expenditure, and physical activity, were not evaluated. In addition, the mechanisms underlying weight loss were not investigated; therefore, the relative contributions of reduced food intake, altered energy expenditure, malabsorption, and other factors associated with intestinal inflammation remain unclear. Second, only colonic tissue was collected and analyzed, whereas other organs involved in metabolic regulation, including adipose tissue, liver, and skeletal muscle, were not examined. Third, this protocol was developed using C57BL/6 IL10-KO mice, which may exhibit different susceptibility to colitis compared with other genetic backgrounds. Despite these limitations, the protocol can readily be adapted to

incorporate additional metabolic phenotyping and analysis of extraintestinal tissues, allowing investigation of the mechanisms underlying weight loss as well as the relationship between intestinal inflammation and systemic metabolic alterations³⁴, or assessment of novel anti-inflammatory nanomaterials³⁵. Furthermore, the approach should be applicable to other IL10-KO mouse strains, although additional validation studies are warranted.

In summary, we report an efficient and reproducible protocol that simultaneously induces enterocolitis in multiple IL10-KO mice and allows evaluation of feeding behavior. Further studies are needed to investigate the effects of enterocolitis on energy expenditure and body composition in this model, which will reveal broader connections between IBD and host metabolism.

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DISCLOSURES:

The authors declare no pertinent disclosures.

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