Video Article

Sectioning Mammary Gland Whole Mounts for Lesion Identification

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Abstract

Normal mammary gland development may be altered by exposure to environmental toxicants and pharmaceutical products, excessive exposure to hormones, and genetic alterations. Mammary gland whole mounts are an inexpensive method to capture the progression of morphological changes that may arise after exposure. However, in later life, when abnormalities are more prone to develop, sole reliance on this one method may not always provide enough information to make a proper diagnosis of the abnormality. Historically, in chemical test guideline studies, a single mammary gland is removed at necropsy and prepared as a hematoxylin and eosin (H&E)-stained section. The incorporation of contralateral mammary whole-mount collection and analysis decreases the likelihood of a false-negative assessment. Evaluation of the whole mount is limited by the presence of one or two entire mammary glands on a slide, and in some cases, the abnormalities observed in the whole mount are not uniformly represented in the H&E section. The goal of this study was to develop a protocol for converting coverslipped mammary whole mounts to H&E-stained sections so that lesions that would otherwise have been missed or that are difficult to diagnose can be identified. Here, we detail a method to produce a high-quality, paraffin-embedded H&E section from a mammary gland that was initially prepared as a whole mount. In comparison to a tissue that was intentionally prepared for H&E sectioning, the whole mount requires additional preparation for tissue removal and processing. However, this method is considered inexpensive, as it requires common lab reagents and little additional time. As a result, this method can provide invaluable information on how chemical and environmental exposures alter normal mammary development, as well as display changes that occur because of genetic modifications.

Video Link

The video component of this article can be found at https://www.jove.com/video/55796/

Introduction

The mammary whole mountis a useful and inexpensive method implemented in many rat and mouse studies to understand both normal and chemically-induced, abnormal development. In general, a mammary gland collected at various stages during rodent development (*i.e.,* adolescence, puberty, mid- to late-gestation, and the involution phase) will show morphological changes in tissue and cellular architecture influenced by paracrine, endocrine, and autocrine factors[1]. In the aging rat, the epithelial and stromal portions of the gland become increasingly dense, which makes measuring morphological parameters difficult in a whole mount. Thus, it is common practice to collect a gland for histological evaluation to identify changes on a microscopic level. Both processes are especially useful in studies involving chemical exposure (*i.e.,* environmental, or pharmaceutical) or to examine the morphological changes accompanied by genetic alterations.

Techniques and capabilities for using the mammary gland in risk evaluation continue to evolve. While whole-mount preparation is routine and standardized, modifications to sectioning continue[2,3]. Many research groups from various backgrounds (*i.e.,* academia, government, and industry) have adapted coronal/longitudinal sections of mammary tissue as a preferred method, while some labs still use cross-cut sections through the skin[4]. The latter method results in an inordinate representation of epidermis (skin), rather than the tissue of interest: the mammary gland epithelium[2]. Coronal or longitudinal sections are more useful for characterizing normal tissue[5] and greatly improve the detection of abnormalities and lesions due to the increased surface area and number of structures present. Overall, this makes the whole mount a suitable method for the comparative histopathological assessment of the mammary gland[1,2]. Whether using a mouse or a rat, retrieval, and preservation of the 4th and 5th inguinal glands is highly recommended for the comparison of the whole mount to a contralateral H&E section.

When used in combination, the H&E section and whole mount provide an accurate account of the cellular and morphological changes induced by environmental exposure. This is especially true in certain rodent strains in which the model possesses a low susceptibility to tumor formation or a tumor is not grossly visible. In some circumstances, obtaining the required tissue may not always be possible using both techniques (*i.e.,* insufficient tissue quantity, resources, or unexpected experimental results). In our case, abnormalities were observed in the mammary whole mount, whereas histopathological findings in the contralateral H&E gland were mostly normal. The overwhelming discrepancy between the

contralateral glands led us to develop an economical and efficient procedure to identify the abnormalities in the whole mounts. Using a modified processing and embedding procedure, high-quality H&E-stained sections were prepared from paraffin-embedded whole mounts. We envision this protocol becoming a powerful detection tool for chemical exposure effects, enhancing inter-lab comparisons.

Protocol

All animal use and procedures for this study were approved by the NIEHS Laboratory Animal

Care and Use Committee and conducted in an Association for Assessment and Accreditation of

Laboratory Animal Care-accredited facility.

1. Mammary Whole-mount Preparation[2,3,6,7]

1. Remove the inguinal mammary glands from one side of a non-pregnant CD-1 female mouse, as described in reference[3], and place them on an electrostatically charged slide.
2. Cover the gland with non-stick paper or film and place another slide on top. Add pressure (*i.e.,* water weights) to the slide to spread the gland out flat so that the gland will stick to the slide for fixation.
3. Submerge the slides in fixative (*e.g.,* 100% ethanol, chloroform, and glacial acetic acid in a 6:3:1 ratio) overnight at room temperature.
4. Remove the glands from the fixative and wash in ethanol for 15-30 min. Following the 30-min wash, gradually change to water by pouring out 1/3 of the ethanol and adding water. Let the wash sit for about 5 min each time, and repeat with the addition of water 3 times.
5. Stain (*e.g.,* carmine alum solution) for 12-24 h.
   NOTE: Thicker tissues will require longer staining times. See reference[3] for staining details.
6. Pour off the stain after the allotted time and rinse the slides in water for 30 s. Dehydrate the tissues by washing the slides in 70% ethanol for 15 min, followed by performing a 15 min wash in 95% ethanol and a final wash in 100% ethanol for 20 min.
7. Clear the fat from the tissues by placing them in xylene for a minimum of 24 h, or longer if the tissue is thick, so that any opaque or white areas are removed.
8. Remove the tissue from the xylene and quickly add mounting medium to the slide to avoid tissue dehydration; place a coverslip on top. Allow the slide to dry for at least 48 h.
9. Evaluate the whole-mounted tissue for abnormalities, as described in[2].
   NOTE: When lesions are detected in whole mounts and sectioning is necessary to establish their identity, the following steps should be followed.

2. Removal of the Mammary Whole Mount from a Glass Slide

1. Remove the coverslip and mounting medium by submerging the mammary whole-mount slide in a glass staining jar filled with xylene overnight.
   NOTE: Thicker tissues and slides with excessive mounting solution may require additional time for coverslip removal.
2. Following the initial overnight soak, place the slides in fresh xylene and soak for 6 h. Perform one final soak in fresh xylene overnight.
   NOTE: The coverslip will easily fall off the slide after the last soak.
3. With a gloved hand, hold the slide and carefully remove the coverslip with a pair of forceps to ensure that it is removed in one piece.
   NOTE: In the event that the coverslip is still attached to the glass slide, continue soaking until it can be removed with little effort.
4. Once the coverslip is removed, hold the slide perpendicularly to a glass Petri dish containing xylene, carefully take a sharp, disposable razor blade and slide the blade in a single motion down the slide.
5. Once the tissue is removed, quickly submerge the mammary pad into a xylene-filled glass Petri dish to ensure that the tissue does not air dry.
   NOTE: Be careful with this step. The whole mount is thin (≤ 1 mm), and fragile from xylene processing. Any impressions or tears may change the morphology of the tissue and complicate later evaluations.

3. Tissue Processing

1. Once the tissue is in the Petri dish, carefully transfer it to a labeled histology cassette. Grab the edge of the fat pad with blunt-nosed, serrated-tipped forceps to minimize tissue damage.
   1. Using a razor, cut larger tissues (especially for rats) in half and place them in two separate labeled cassettes that will accommodate the tissue size. Place the tissue right-side up (use the lymph node as a reference). Ensure that the tissue without the lymph node is kept in the same, upright position when it is transferred to the cassette.
2. Place the cassette into a container of xylene for up to 2 h before placing it in the tissue processor.
   NOTE: It is recommended to process the samples on the same day they are placed into the cassette. Extended soaking in xylene has not been tested.
3. Load the maximum number of cassettes into the tissue processor by hand.
4. Prior to beginning, program all the reagents that will be used for this process into the processor's reagent list. To automate movement from one station to the next, use the menu options and create a new program. When all steps have been completed, select 'OK' to proceed.
   NOTE: The processor will allow the review of all station details before starting.
   1. Automate the processor to soak the cassettes in xylene for 30 min at 37 °C, followed by a second soak in fresh xylene under the same conditions. Automate the processor to remove the cassettes from the xylene solution and transfer the tissues to the next station containing a 1:1 xylene:molten paraffin mixture, set at 60 °C, for 30 min.

NOTE: The processor will then transfer the cassettes into a new chamber that contains molten paraffin for 1 h. After 1 h, the samples will automatically be transferred to a new chamber of fresh molten paraffin and will be processed for an additional 2 h. Both steps are carried out at 60 °C.

2. The processor then transfers the cassettes to the station containing molten paraffin for 1 h. Then, the samples are automatically transferred to the final station of fresh molten paraffin and processed for an additional 2 h. Both steps are carried out at 60 °C.

4. Embedding the Processed Mammary Tissue

1. Place the cassettes in the 58 °C paraffin holding tank of the embedding station until ready to embed the tissue in the mold.
2. Remove the cassette cover to determine the best mold size for the tissue. Make sure that the mold is big enough to accommodate the tissue, and leave enough room to have a tissue-free border of paraffin around the perimeter.
3. Add 3-4 mm of molten paraffin to the mold and orient the mammary whole-mount surface, which was adjacent to the bottom of the glass slide and the bottom of cassette, facing up in the mold.
4. Transfer the mold to a cooling plate and quickly adjust the tissue as needed so that it is parallel to the mold bottom.
   NOTE: Once the paraffin hardens, the tissue will besecured in place.
5. Using warm forceps, place the labeled, bottom half of the cassette on top of the mold; press firmly using the forceps.
6. Add additional molten paraffin to the mold in a continuous motion to cover the entire cassette. Move the mold from the warm plate to a cold plate to complete hardening of the block.
7. Remove the block from the mold when the paraffin completely solidifies to avoid cracks or air bubbles.
   NOTE: Store the paraffin-embedded tissues at room temperature until ready to section.

5. Sectioning the Paraffin-embedded Mammary Tissue on the Microtome

1. Prior to sectioning, incubate the paraffin blocks at -20 °C for 1 h.
   NOTE: There will be residual mounting medium in the block from the original coverslipped whole-mount tissue. Chilling the block improves block sectioning and ribboning.
2. Prepare the microtome by turning on the water bath, containing fresh distilled water, and adjust the temperature to 42-45 °C. Place a fresh, low-profile blade onto the microtome and set it at 4 µm.
   NOTE: Section quality is reduced with sections thicker than 4 µm because of the presence of residual mounting medium in the tissue.
3. Insert the block into the microtome with the wax facing the blade and aligned with the vertical plane. Then, moisten a section of gauze pad in cold water and place it onto the block for several minutes.
4. Section the block by turning the large wheel in a clockwise motion, in combination with the coarse advanced wheel, until a full face or representative section of block is obtained.
   NOTE: The tissue is thin because of xylene clearing during the whole-mount process. Align block properly with the blade to minimize the number of cuts in obtaining a representative section.
5. Pick the ribbon section up by hand, carefully float the ribbon in the warm (45 °C) water bath (prepared in advance), allow tissue wrinkles to dissipate and carefully float a section onto a clean glass slide.
6. Place the slides upright on a drying rack to remove excess water. Incubate the slides in a slightly opened slide box overnight at 37 °C.

6. Automated and Manual H&E Staining of Sectioned Mammary Tissues

1. Prior to staining, the slides are stored at room temperature.
2. For automated staining, select H & E from the stain options on the main menu. Deparaffinization, staining, and dehydration are all completed on the automated stainer. Use mounting medium and a coverslip to mount the sections.
3. If manually staining the slides, deparaffinize the sections by immersing the slides in xylene for 5 min. Transfer to fresh xylene and repeat for 5 min.
4. Rehydrate the slides by immersing them twice in 100% ethanol for 3 min each, followed by two repetitions in fresh 95% ethanol and two separate washes in 1X wash buffer at 5 min each.
5. Rinse the slides in distilled water.
6. Add the slides to the hematoxylin for 1-2 min. Remove the slides and rinse them with running tap water for 5 min.
   NOTE: Filter hematoxylin prior to each use.
7. Place the slides in 1% glacial acetic acid for 30 s to promote color differentiation, and then rinse in running tap water for 1 min.
8. Add slides to 1X PBS for 30 s to 1 min to blue the tissue sections, followed by a 5 min rinse in tap water and then in 95% alcohol for 15 - 20 s.
9. Counterstain with eosin solution for 30 s to 1 min. Following this, dehydrate the slides twice in fresh 95% ethanol, followed by two fresh repetitions in 100% ethanol. Perform each wash for 5 min.
10. Clear the slides in xylene, with two changes in xylene for 5 min each.
11. Coverslip the tissue section with mounting medium and dry flat overnight at room temperature.

Representative Results

This method is effective at assisting with diagnoses that may have otherwise been missed if the original contralateral H&E section of the mammary pad did not show any histological changes. However, the outcome will only be useful if care is taken during the initial whole-mount preparation, as well as during the preparation of the tissue for the histological evaluation. Paraffin embedding will provide protection and will help to preserve the tissue for future sectioning.

To determine the ideal thickness of the mammary tissue, 4-µm and 6-µm sections were prepared. We tested depths that were greater than the margin of error of ± 1 µm on the microtome. Sections of a 6-µm thickness (**Figure 1A**) were very dense with compact cells . Overall, the slides lacked distinct cellular details that would be necessary to make a proper identification. The optimal thickness was 4 µm (**Figure 1B**). These tissues provided the best results, where epithelial and stromal areas and corresponding cell types were easily distinguished.

Slides from a large, ongoing study were used to illustrate the ease and utility of this method. In several cases, the original H&E section from 14-month-old virgin female CD-1 offspring were found to have conflicting findings compared to the contralateral mammary whole mount from the same animal. Lesions were evident in the whole mount, but could not be identified without further sectioning and staining. Samples from two different animals were chosen as representative cases. In both cases, a single section was used for staining, but several cuts were made until a representative section was obtained. Very few cuts were necessary to obtain a representative section, since previous whole-mount preparations cleared most of the fat pad, leaving the gland very thin compared to a mammary gland originally prepared for paraffin embedding, which is surrounded by a thick fat pad. **Figure 2A** and **Figure 3A** illustrate histological sections of glands that were assessed as normal. Both glands show ductal structures surrounded by a robust and homogenous adipocyte-rich population. Each duct is lined by a single layer of simple cuboidal epithelial cells and is maintained by a second layer of basal cells, mainly composed of myoepithelial cells, but also encompassing stem and progenitor cell populations. The representative contralateral whole mounts (**Figures 2B** and **Figure 3B**) demonstrated ducts and stroma with increased opacity. However, it was difficult to determine if the opaqueness was the result of hyperplastic, inflammatory, or neoplastic alterations without having an H&E section that could provide distinct cellular details.

Contralateral whole mounts from the same animals were used to implement the method described here and can be observed in **Figure 2C and D** and **Figure 3C and D**. The samples in **Figure 2C & D** were diagnosed as perivascular inflammation due to the increased number of lymphocytes that were present around a large blood vessel in the mammary gland section. For the second case, the mammary gland lobular architecture was maintained but was enlarged multifocally by the increased number and size of normal alveoli and ducts (lobuloalveolar hyperplasia). Alveolar epithelial cells were well-differentiated, round, often-vacuolated, and formed one concentric layer around a lumen that typically contained proteinaceous fluid. Ducts were lined by columnar cells and were similar to alveolar cells, with a well-differentiated epithelium that formed one concentric layer. This phenotype is most often observed in the mammary glands of mid-pregnant mice and rats. This should not be confused with lobuloalveolar structures in the mammary glands of adult male rats[8]. In the adult male mammary gland, alveoli are prominent and ducts are infrequent, but the alveoli and ducts are lined by stratified epithelium, with tall, vacuolated cuboidal to short columnar epithelial cells.

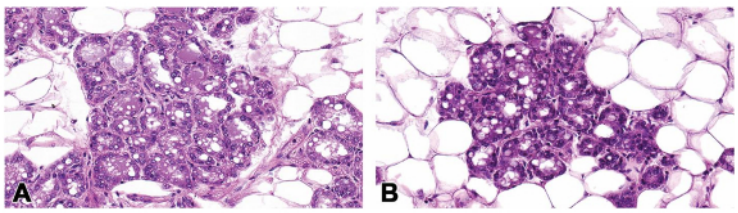

Figure 1:**Recommended thickness for mouse mammary gland whole mount to H&E sections**. A) 6 µm and B) 4 µm. The resolutions of mammary gland structures were not optimal for histopathological evaluation using the thicker (6-µm) sections, so the 4-µm section is recommended. This figure has been modified from Tucker *et al.*[9]. Please click here to view a larger version of this figure.

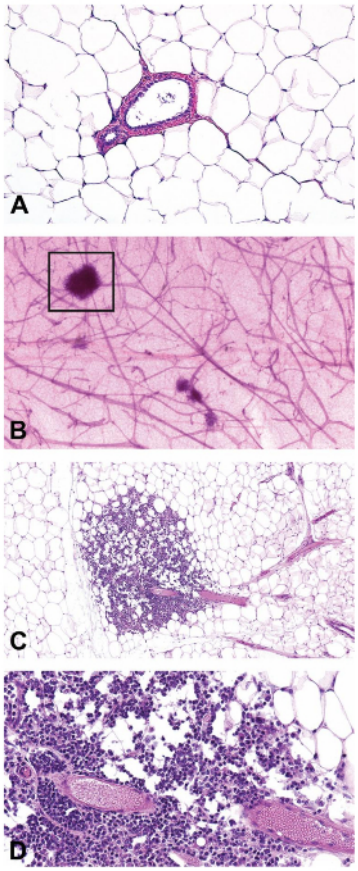

Figure 2: Whole mount to H&E section of perivascular inflammation. This image is a mouse mammary gland that was collected in diestrus. A) Formalin-fixed H&E mammary gland section with no histopathogical alterations. B) Contralateral whole-mount section, with areas of increased opacity (boxed area). C) 20x magnification of an H&E section from a mammary whole mount (boxed area). Clusters of mononuclear cells surrounded the blood vessel and extended into the surrounding adipose tissue. D) The 40x magnification of an H&E section from a mammary whole mount (boxed area) shows in greater detail that most of the mononuclear population consists of lymphocytes and highlights the severity of the perivascular inflammation. This figure has been modified from Tucker *et al.*[9]. Please click here to view a larger version of this figure.

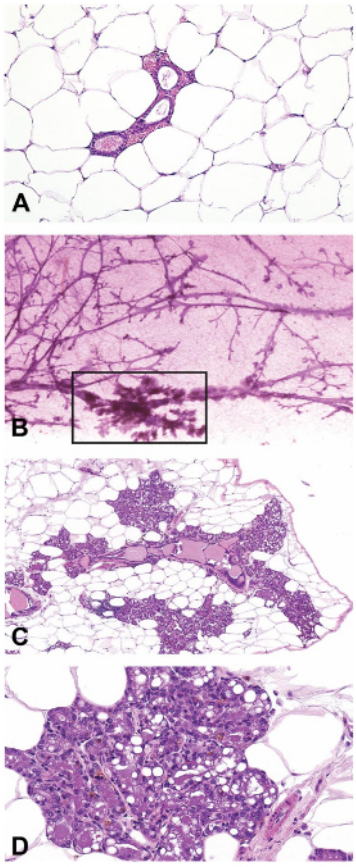

Figure 3: **Whole mount to H&E section of lobular alveolar hyperplasia**. This image is a mouse mammary gland that was collected in diestrus. A) Formalin-fixed H&E mammary gland section with no histopathological alterations. B) Contralateral whole-mount section with increased opacity in the ductal and stromal areas (boxed area). C) 20x magnification of an H&E section from a mammary whole mount (boxed area). Lobular architecture was maintained but was enlarged multifocally by the increased number and size of normal alveoli and ducts (lobuloalveolar hyperplasia). D) The 40x magnification of an H&E section from a mammary whole mount (boxed area) reveals that the enlarged lobules contain an increased number of alveoli and ducts that are lined by well-differentiated, often-vacuolated epithelial cells, which form lumens that contain proteinaceous fluid. This figure has been modified from Tucker *et al.*[9]. Please click here to view a larger version of this figure.

Discussion

The mammary gland whole mount is a powerful tool that may be used to illustrate the normal mammary development and morphological alterations that may arise and persist following exposure to chemicals, including endocrine disruptors. When a whole mount and H&E section from the same animal are assessed together, they can provide an accurate visual snapshot of early alterations, which can progress into a more diseased state.

The ability to obtain this useful information lies in ensuring that care is taken to preserve and not compromise glandular morphology when removing the tissue. While the rodent has several mammary gland sites located bilaterally along the dorsal wall, it is recommended to collect the 4th and 5th glands to minimize muscle tissue recovery. The nipple attachment area and lymph node should also be present, as they serve as useful morphological landmarks. The gland should also be spread and stretched across a flat surface (*i.e.,* a glass slide, cardstock, or cloth) to closely mimic the natural structure of the tissue *in situ*. Abnormalities within the mammary gland are usually not visible until after the gland has been defatted in xylene or has been carmine-stained. Unlike mammary tissue that was originally prepared for histological sectioning, a whole mount removed from the glass for paraffin embedding will be extremely thin and fragile. Forceps impressions and tissue tears should be avoided, as they will possibly alter cellular morphology and make histopathological evaluations of the finished product difficult.

Once the whole mount has been paraffin-embedded, prior to sectioning, it is recommended to allow the blocks to incubate at -20 °C for 1 h. This step improves the ability to obtain an optimal representative tissue section in a ribbon. Sectioning of the tissue at 6 µm (**Figure 1A**)[9] produced a dense cellular architecture, with less-than-optimal resolution. By using a thinner section (**Figure 1B**)[9], all cellular features, including epithelial tissue and surrounding stromal infiltrates, were easily identified. It should also be noted that, although these sections were previously carmine-stained, the stain was not visible following sectioning and did not interfere with H&E staining. Therefore, a de-staining step was not necessary in the protocol. Although tissue sections were stained solely with H&E, we expect with minor modifications, other histochemical and possibly immunohistochemical stains might be applicable once sectioning is complete. However, it was beyond the extent of this protocol and will require further investigation to determine the optimal conditions for these stains.

This procedure was developed because we observed discrepant findings between routinely collected whole mounts and contralateral H&E-stained mammary gland sections from the same animal. Similar mammary protocols exist[6,10]; however, the procedures were not detailed or easy to follow. Those methods established a basis for the development of our protocol. Normal gland morphology was observed in the H&E-stained tissue sections (**Figure 2A** and **Figure 3A**)[1], while the complementary whole mounts revealed numerous abnormal morphological features (**Figure 2B** and **Figure 3B**)[9]. By performing histology on the whole mounts, we could classify the abnormal features. For example, perivascular inflammation and lobuloalveolar hyperplasia were identified in two separate cases that were disparate compared to the H&E findings observed in the contralateral sides (**Figure 2C and D** and **Figure 3C and D**)[9].

To the authors' knowledge, there are no known reports of mammary gland discrepancies similar to those observed in our ongoing study. However, this may be due to experimental design issues rather than lack of occurrence, as the mammary gland is often solely collected for one application or analysis that does not involve morphology, such as RT-PCR or Western blots. However, many experiments incorporate multiple applications that require an adequate amount of tissue for each endpoint. The inguinal glands are the preferred choice for whole mounts and downstream applications because they seldom have contaminating surrounding tissue like the thoracic glands (*i.e.,* muscle contamination) and because the inguinal mammary lymph nodes provide valuable landmarks for orientation and comparison across glands. Therefore, deciding which gland and how much of the gland will be dedicated to each application will influence the precision of detection. Prioritization for the use of the mammary gland should be for: 1) a whole mount composed of the entire 4th and 5th glands, 2) histology of the contralateral 4th gland containing some lymph node for use as a landmark, and 3) the contralateral 5th gland (with no contaminating lymph node) for downstream applications (*i.e.,* RNA, DNA, and protein). If an abnormality is visible at necropsy, a whole mount should take preference for histology collection.

As with any protocol, there are accompanying limitations; the need to permanently destroy the whole mount during sectioning and having limited tissue for use in alternative analyses, for example. Thus, we recommend extensively documenting whole mounts with a desktop scanner to produce digital images for future analysis and reference. Also, if staining will not occur within a month, limit the number of sections cut to preserve the tissue for any future analyses. The benefits of this whole mount to H&E section method outweigh the limitations, such that it can be universally applied to mammary studies involving multiple disciplines, especially toxicology. Several studies using chemicals with known endocrine effects have included a modified version of this protocol, demonstrating its applicability[11,12,13]. Findings from these studies may help to reduce the chances of a false negative in chemical testing but may also provide new or additional information that can be used for regulatory and risk-assessment decisions.

Disclosures

The authors have no conflicting interests to declare in relation to the research, authorship, and/or publication of this article.

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