Sex Differences in the Renal Changes Elicited by Angiotensin II Blockade During the Nephrogenic Period

Fara Saez, M. Teresa Castells, Adelina Zuasti, Francisco Salazar, Virginia Reverte, Analia Loria, F. Javier Salazar

Abstract—The renin–angiotensin system plays an important role in renal development. However, it is unknown whether reduction in angiotensin II effects during the nephrogenic period leads to different renal alterations in males and females during the adult age. The aim of this study was to evaluate whether the role of angiotensin II on renal development is sex dependent and whether there are sex differences in blood pressure, renal hemodynamics, and severity of renal damage during adult life when nephrogenesis is altered by blocking angiotensin II effects. Newborn Sprague–Dawley rats were treated with an angiotensin II type 1 receptor antagonist (L-158.809; 7 mg/kg per day) during the first 2 weeks of life. At 3 months of age, changes in blood pressure, albuminuria, and renal hemodynamics were assessed, and stereological and histopathologic studies were performed. Blood pressure increased (127±0.5 versus 115±0.7 mm Hg in control rats; \( P<0.05 \)) and nephron number decreased (37%; \( P<0.05 \)) similarly in treated males and females. However, only males had an elevation in albuminuria (5.92±1.65 versus 0.33±0.09 mg per day in control rats; \( P<0.05 \)), a fall in glomerular filtration rate (12.6%; \( P<0.05 \)), and a significant decrease in papillary volume (42%; \( P<0.05 \)). Mean glomerular volume, glomerulosclerosis, arteriolar hypertrophy, and tubulointerstitial damage in cortex and medulla were also higher (\( P<0.05 \)) in angiotensin II type 1 receptor antagonist–treated males than in treated females. The results of this study suggest that females seem to be more protected than males to the renal consequences of reducing angiotensin II effects during renal development. (Hypertension. 2007;49:1429-1435.)

Key Words: nephrogenesis n sex n angiotensin II n glomerulosclerosis n fibrosis n renal hemodynamics

The risk of developing a renal disease and hypertension is enhanced when nephron number decreases.1–3 The decrease in glomeruli number has been reported in cyclooxgenase-2–deficient mice4,5 and has been induced by several experimental manipulations that reduce the renin–angiotensin system (RAS) activity6–9 and lead to renal alterations that may be accompanied by an elevation in blood pressure (BP).5,6,8

Recent studies have demonstrated that there are sex differences in the renal response to the alteration of some mechanisms involved in renal development.5,10 It was found that cyclooxygenase-2 deletion in mice induces an elevation in proteinuria and BP only in males8 and that a modest maternal protein restriction leads to a reduction in glomeruli number in the adult male but not in the adult female offspring.10

The role of angiotensin II (Ang II) in regulating nephrogenesis is supported by studies showing an upregulation of the RAS during renal development1,11,12 and that the administration of either a converting enzyme inhibitor or an Ang II type 1 (AT1) receptor antagonist (ARA) produces renal alterations that may lead to the development of hypertension.8,9 Despite the significant number of studies evaluating the renal effects elicited by the blockade of the RAS during the nephrogenic period, it is unknown whether there are sex differences in the BP and renal abnormalities found in the adult life when Ang II effects have been blocked during renal development. The main aim of this study was to evaluate whether Ang II plays a different role in males and females on normal renal development and whether there are sex differences in BP, renal function, and renal structure when nephrogenesis is altered during its late phase. To accomplish our objective, an ARA was administered to rats during the first 2 weeks of life, and changes in BP, albuminuria, renal function, and renal structure were examined when the rats were 3 months old.

Methods

Sprague–Dawley rats were purchased from the experimental animal laboratory of the University of Murcia. Female Sprague–Dawley rats (250-g body weight) were placed with a male, taking day 0 of pregnancy the morning that sperm evidence was found. Newborn rats were treated from postnatal day 1 to postnatal day 14 with vehicle or an ARA (L-158.809; Merck Sharp & Dohme) at an oral dose of 7 mg/kg per day. Rats had free access to normal rat chow and tap water and were kept in rooms with a controlled temperature...
(24°C) and a 12:12-hour dark–light cycle. Protocols were designed according to the “Guiding Principles for Research Involving Animals and Human Beings” of the American Physiological Society.

Albuminuria and renal hemodynamics were examined in 4 groups of rats: vehicle-treated males (n=8), ARA-treated males (n=8), vehicle-treated females (n=7), and ARA-treated females (n=7). At 3 months of age, rats were kept individually in metabolic cages and had free access to powdered rat chow and drinking fluid. After 2 days of adaptation, 24-hour urine samples were obtained during 3 days to examine the urinary excretion rate of albumin by a commercially available ELISA (Bethil Laboratories, Increference E110-125). Three days after finishing the study, rats were anesthetized with sodium pentothal (5 mg/100 g of body weight, IP) and tracheotomized with a polyethylene catheter, maintaining constant their body temperature. Left femoral artery and vein, as well as urinary bladder, were catheterized. Glomerular filtration rate (GFR) and effective renal plasma flow were measured by clearances of tritiated inulin and sodium p-aminohippurate, respectively. After a 60-minute equilibration period, 2 consecutive 20-minute clearances were obtained, and blood samples were withdrawn to determine the inulin and sodium p-aminohippurate concentrations.

BP and renal histology were analyzed in 4 experimental groups: vehicle-treated males (n=7), ARA-treated males (n=7), vehicle treated females (n=7), and ARA-treated females (n=7). Systolic BP was measured in conscious rats by a tail-cuff method using a LE 5002 Storage Pressure Meter (Letica, Panlab). To obtain an accurate BP reading, rats were acclimated to the manipulation in a warm dark chamber, and a traditional tail-cuff occluder was placed on the rat’s tail for an initial series of inflation–deflation cycles. Definitive measurements began when rats remained unperturbed in the chamber during the inflation–deflation cycles. The BP value in each rat is the mean value of 10 measurements performed during 3 days.

One week after BP measurement, rats were anesthetized with sodium pentothal (5 mg/100 g of body weight), and the left kidney was removed. Kidneys were sliced into 2-mm-thick slices using a razor blade cutting device and were then fixed in 10% formaldehyde in PBS (pH=7.4). All of the kidney slices were placed in cassettes, dehydrated through a series of graded alcohols, and embedded in paraffin. Blocks were sectioned at 7 μm using a HM310 microtome (Microm). Every first section was viewed on a personal computer screen at a magnification of ×10. Areas from cortex, outer medulla, and renal papillae were examined with the MIP 4.5 image analysis software (Consulting Image Digital). Total volumes of cortex, outer medulla, and inner medulla were estimated by the Cavalieri principle.

Two sections 21-μm apart were used for the dissector calculations. The glomerular profiles sampled by an unbiased counting frame of the sampled section, which were not present in the look-up section, were counted (Q), and to double the efficiency, glomerular profiles sampled by an unbiased counting frame of the look-up section, which were not present in the sampled section, were also counted. Total glomerular number was estimated using the formula:

\[ N_i = \frac{Q}{V_{ref}} V_{ref} / \sum V_{dis} \]  

where \( V_{ref} \) is the volume of the counting frame, and \( V_{ref} \) is the renal cortex volume.

Glomerular volume was obtained by using the 2 arbitrary parallel sections technique. Approximately 20 glomeruli were measured in each rat to obtain the mean glomerular volume in every experimental group.

To determine glomerular damage, each glomerular profile was graded and assigned to 1 of 4 groups with respect to the degree of damage. Profiles without pathological changes were assigned to grade 0; grade 1 (moderated changes) was composed of glomerular profiles with an increase of mesangial matrix, thickening of glomerular basement membrane, and podocytes with pseudocysts; grade 2 (advanced changes) consisted of sclerotic glomerular profiles; and grade 3 was global sclerosis. Approximately 500 glomerular profiles were examined for each experimental group.

Tubulointerstitial injury was scored using the MIP 4.5 image analysis software (Consulting Image Digital) in sections stained with Masson trichrome and viewed in a personal computer screen at ×285 magnification. Tubulointersitial injury was defined as tubular dilatation, tubular atrophy, thickening of the tubular basement membrane, mononuclear infiltration, and increase of collagen. The percentage area of damaged cortex and medulla was estimated in square fields of 468×468 μm. From each kidney zone and experimental group, 50 fields were examined.

To find out whether the vascular hypertrophy in cortical arteries was different in treated males and females, immunolabeling of α-actin in vascular media was examined. Five-micrometer sections were immunostained with the monoclonal antibody anti-human α-actin (DakoCytomation). Luminal area, wall area, luminal diameter, outer diameter, and wall thickness/luminal diameter ratio were measured in 25 cortical arteries in every rat by means of the image analysis system MIP 4.5 software.

### Statistical Analysis

All of the values are presented as mean±SE. One-way ANOVA was used to evaluate the differences between groups (GB Stat, Dynamic Microsystems, Inc). A P<0.05 was considered statistically significant.

### Results

Table 1 shows that albuminuria was similar in control males and females and that it increased (P<0.05) only in male treated rats. Albuminuria was lower in female than in male treated rats (P<0.05). Plasma creatinine and effective renal plasma flow were similar in the 4 groups examined. As also shown in Table 1, GFR was not significantly different in both control groups and in female treated rats. However, GFR was lower (P<0.05) in male treated rats than in control males and in treated females. Systolic BP increased similarly (P<0.05) in males and females at 3 months of age when treated with the ARA during the first 14 days of age (Table 2).

Kidney weights were similar in vehicle- and ARA-treated rats, but they were greater (P<0.05) in males than in females.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control Males</th>
<th>RA Males</th>
<th>Control Females</th>
<th>ARA Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma creatinine, mg/100 mL</td>
<td>1.18±0.10</td>
<td>1.16±0.08</td>
<td>1.20±0.09</td>
<td>1.23±0.06</td>
</tr>
<tr>
<td>Renal plasma flow, mL/min per g</td>
<td>4.20±0.20</td>
<td>3.85±0.12</td>
<td>4.10±0.16</td>
<td>3.99±0.15</td>
</tr>
<tr>
<td>g km</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GFR, mL/min per g kg</td>
<td>1.43±0.06</td>
<td>1.25±0.05</td>
<td>1.46±0.06</td>
<td>1.39±0.05†</td>
</tr>
<tr>
<td>Albuminuria, mg per day</td>
<td>0.33±0.09</td>
<td>5.92±1.65†</td>
<td>0.20±0.08</td>
<td>0.78±0.28†</td>
</tr>
</tbody>
</table>

Data are mean±SE. kw indicates kidney weight.

*P<0.05 vs control rats.
†P<0.05 vs ARA male rats.
No significant differences were found in the renal medulla-cortex volume ratio between control and ARA-treated rats (Table 2). With respect to the total glomeruli number, it was similar in both control groups, and ARA treatment elicited a significant decrease that was similar in males and females (Table 2). However, when glomeruli number was calculated considering body weight, it was greater (P<0.05) in ARA-treated males and females by 44% and 39%, respectively. The percentage of papillae volume (with respect to total kidney volume) decreased by 42% (P<0.05) in ARA-treated males but did not change in treated females (Table 2).

Mean glomerular volumes in each experimental group are shown in Table 2. Glomerular volume is similar in vehicle-treated males and females, and ARA treatment during the nephrogenic period leads to a glomerular hypertrophy that was greater in males than in females (P<0.05). To examine whether this glomerular hypertrophy is secondary to a volume increment in all of the glomeruli, glomerular volumes in each group of rats were distributed in 5 groups. Glomerular volume distribution was similar in males and females treated with vehicle (Figure 1). Most of the glomerular volumes in both control groups were in a range between 250 000 and 650 000 μm³, and 4% of glomeruli had a volume <250 000 μm³. Only 1% of the total glomeruli were clearly hypertrophied (>850 000 μm³) in these groups (Figure 1). In contrast, in the ARA-treated males, 13% of the glomeruli had a volume <250 000 μm³, and glomeruli with a volume in the range between 250 000 and 650 000 μm³ were significantly diminished (18% versus 91% in the control group; P<0.05; Figure 1a). The percentage of glomeruli hypertrophied in treated males was significantly elevated (58%; P<0.05). ARA-treated females showed a decrease (P<0.05) in the glomeruli with a volume between 250 000 and 450 000 μm³ (12% versus 57% in control females) and an elevation in the glomeruli with a volume >850 000 μm³ (37% versus 1% in control females; P<0.05; Figure 1b).

Differences in glomerulosclerosis as a consequence of ARA treatment are presented in Table 3. Control groups only had a few glomeruli with sclerosis, and ARA-treated rats have a significant increment in moderate glomerulosclerosis with an elevation in mesangial matrix, thickening of glomerular basement membrane, and podocytes with pseudocysts. The percentage of glomeruli with moderate sclerosis was greater in treated males than in treated females (P<0.05). Advanced sclerosis was also observed in a few glomeruli in treated males and treated females (Table 3).

Tubulointerstitial damage index is shown in Table 4 and Figure 2. As expected, no interstitial damage was seen in cortex (Figure 2) and outer and inner medulla of the vehicle treated males and females. Postnatal ARA treatment leads to a significant elevation of tubulointerstitial damage in the kidney zones examined in males and females. However, tubulointerstitial damage index was significantly greater in males than in females (Table 4 and Figure 2).

### Table 2. Body Weights, Systolic BP, and Stereological Variables in Control and ARA-Treated Rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control Males</th>
<th>ARA Males</th>
<th>Control Females</th>
<th>ARA Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arterial pressure, mm Hg</td>
<td>116±0.5</td>
<td>127±0.5*</td>
<td>114±1</td>
<td>127±0.4*</td>
</tr>
<tr>
<td>Kidney weight, g</td>
<td>1.04±0.02</td>
<td>1.04±0.06</td>
<td>0.65±0.01†</td>
<td>0.64±0.03†</td>
</tr>
<tr>
<td>Medulla/cortex ratio</td>
<td>0.62±0.05</td>
<td>0.62±0.03</td>
<td>0.63±0.01</td>
<td>0.67±0.04</td>
</tr>
<tr>
<td>Glomeruli No.</td>
<td>31315±899</td>
<td>19302±1490*</td>
<td>28395±1593</td>
<td>18289±466*</td>
</tr>
<tr>
<td>Glomeruli No. per gram of body weight</td>
<td>196±6</td>
<td>109±10*</td>
<td>266±20†</td>
<td>162±10†</td>
</tr>
<tr>
<td>Papillae/kidney volume, %</td>
<td>2.6±0.2</td>
<td>1.5±0.1*</td>
<td>3.7±0.3</td>
<td>2.9±0.3†</td>
</tr>
<tr>
<td>Mean glomerular volume, μm³</td>
<td>43520±1477</td>
<td>1049903±57292*</td>
<td>443614±13413</td>
<td>834922±44872*†</td>
</tr>
</tbody>
</table>

Data are mean±SE.
*P<0.05 vs control rats.
†P<0.05 vs ARA male rats.

### Figure 1. Percentage of glomerular volumes distribution in kidneys from male and female rats treated with vehicle (Control) or an AT1 receptor antagonist during the late phase of the nephrogenic period (n=7 in each experimental group). *P<0.05 vs Control.
TABLE 3. Glomerulosclerosis Index

<table>
<thead>
<tr>
<th>Groups</th>
<th>% Grade 0</th>
<th>% Grade 1</th>
<th>% Grade 2</th>
<th>% Grade 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control males</td>
<td>89±4</td>
<td>11±4</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td>ARA males</td>
<td>44±4*</td>
<td>51±2*</td>
<td>5±2</td>
<td>0±2</td>
</tr>
<tr>
<td>Control females</td>
<td>91±3</td>
<td>9±3</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td>ARA females</td>
<td>69±4*†</td>
<td>29±4*†</td>
<td>2±1</td>
<td>0±1</td>
</tr>
</tbody>
</table>

Grade 0 indicates normal; grade 1, moderated changes; grade 2, advanced changes; grade 3, global sclerosis. Data expressed as percentage of area.

*P<0.05 vs control rats.
†P<0.05 vs ARA male rats.

The α-actin immunohistochemistry studies of cortical arteries showed that luminal diameter was similar in male and female vehicle-treated rats. However, wall thickness and wall thickness/luminal diameter ratio were greater (P<0.05) in vehicle-treated males than in vehicle-treated females (Figures 3 and 4). Wall thickness and wall thickness/luminal diameter ratio increased (P<0.05) similarly in ARA-treated males and females, and the sex differences in both parameters remained significant (Figures 3 and 4).

Discussion

The major finding of this study is the existence of important sex differences in the renal damage elicited by the blockade of Ang II effects during the late phase of the nephrogenic period. Females have a greater renal protection in response to a similar fall in glomeruli number, because albuminuria increases and GFR decreases only in males, and glomerular hypertrophy, glomerulosclerosis, and interstitial fibrosis are significantly more important in males than in females treated with the ARA. The other relevant sex difference is that only treated males had a significant papillary atrophy that occurred simultaneously with an increment in fibrosis in the inner medulla.

The importance of RAS in regulating normal renal development has been reported by studies showing that its activity is enhanced during the nephrogenic period11,12 and showing that the administration of a converting enzyme inhibitor or an ARA elicits an important renal deterioration.17,18 However, it remains unknown whether there are sex differences in the responses of renal structure and renal function to reducing Ang II effects during the nephrogenic period. Our hypothesis that males would be more affected than females by reducing Ang II effects was supported by previous studies. It is important to examine the renal consequences of reducing the nephron number without changes in kidney weight, because many studies have examined the renal adaptations to a decrease in renal tissue, but these adaptations without changes in renal mass are not well known.

As reported previously, ARA administration in our study led to a significant elevation in BP that was similar in conscious males and females. In contrast to our results, it has been reported that converting enzyme inhibitor during nephrogenic period does not modify BP in the adult age. One possible explanation for these different results is that BP values were obtained in conscious rats in our studies and in anesthetized rats in other studies. In support of this explanation, it has been reported that BP is different when measured in conscious and anesthetized animals.

No sex differences were found in the wall thickening of cortical arteries in the ARA-treated rats. Wall thickness/luminal diameter ratio was greater in treated males than in treated females, but the increment was similar in both sexes when compared with the values found in control rats. This hypertrophy of the arterial wall seems to be because of an expansion of the vascular media, which corresponds with a vascular smooth muscle cell hyperplasia.

Sex differences in the histopathologic alterations cannot be attributed to the increments in arterial pressure or to the fall in total glomeruli number, because both parameters changed similarly in males and females. The significant decrease in papillary volume in treated males was expected, because previous studies5–9 have shown that the RAS blockade during the nephrogenic period elicits a papillary atrophy and reduces the renal ability to concentrate urine in males. However, as far as we know, our study is the first showing that this RAS blockade does not modify papillary volume in females and most probably has no effect on papillary function. The mechanisms responsible for papillary atrophy in males but not in females are unknown. However, this sex difference could be explained by the fact that only females have Ang II type 2 receptors in the papilla and that these receptors are activated by estrogens. Therefore, it is proposed that the papillary atrophy in males could be secondary not only to the different hormonal production but also to the absence of Ang II type 2 receptors in the renal papillae in males.

As expected, glomerular volume was enhanced as a consequence of the decrease in glomeruli number in males and females. However, this increment was significantly greater in males than in females (Table 2). Most probably, the elevation in glomerular volume is an adaptive response trying to maintain constant GFR by increasing filtration rate in each nephron. In support of this hypothesis, it was found that the calculated single nephron GFR (SNGFR=GFR/gomeruli number) was greater (P<0.05) in ARA-treated males (to 71±3 nL/min) than in ARA-treated females (to 58±2 nL/min). The SNGFR increment was enough to maintain GFR at control levels in females but not in males, because GFR only decreased in treated males. To evaluate whether all of the glomeruli increased their volume to compensate for the fall in glomeruli number and, therefore, to maintain total GFR, glomerular volumes were distributed in 5 sizes in each...
The marked dispersion of volumes in control rats was expected. It is important that the amount of glomeruli with a lower volume (<250,000 μm³) was elevated (P<0.05) in treated male rats despite the fact that their glomeruli number was significantly diminished. It is also important that only 58±6% and 37±7% of the total glomeruli were clearly hypertrophied (>850,000 μm³) in males and females, respectively, when glomeruli number was diminished by blocking Ang II effects during the nephrogenic period. The mechanisms responsible for the increment in glomerular volume in some but not in all nephrons when their total number is diminished remain to be determined. It is also unknown why glomerular volume increases more in males than in females in response to a similar decrease in glomeruli number. On the other hand, it is probably important that glomeruli number by body weight is lower in males than in females. It is possible that the lower glomeruli number by body weight in treated males versus treated females could be related to the greater renal injury found in these male rats. Although clearly speculative, the greater glomeruli number by body weight in control females may partly explain why kidney function is more preserved in females than in males during aging.

The higher degree of glomerulosclerosis found in our treated males as compared with females may be explained by the fact that SNGFR needs to increase more in males than in females in response to a similar decrease in glomeruli number. On the other hand, it is probably important that glomeruli number by body weight is lower in males than in females. It is possible that the lower glomeruli number by body weight in treated males versus treated females could be related to the greater renal injury found in these male rats. Although clearly speculative, the greater glomeruli number by body weight in control females may partly explain why kidney function is more preserved in females than in males during aging.

The higher degree of glomerulosclerosis found in our treated males as compared with females may be explained by the fact that SNGFR needs to increase more in males than in

Figure 2. Tubulointerstitial damage in the renal cortex of rats treated with an AT₁ receptor antagonist or vehicle during the nephrogenic period was examined in Masson Trichrome-stained sections. Interstitial damage is greater in male (c) than in female treated rats (d). Male (a) and female rats treated with vehicle (b) did not show signs of tubulointerstitial damage. Arrows show damage areas. Hypertrophied glomeruli were found in both ARA-treated males and females (star). Calibration bar=100 μm.

Figure 3. Vascular wall thickness and vascular wall thickness/luminal diameter changes in males and females treated with an AT₁ receptor antagonist during nephrogenic period (n=7 in each experimental group). *P<0.05 vs Control. †P<0.05 vs males.
females trying to maintain GFR. The increment in SNGFR when nephron number decreases may be secondary to an elevation in glomerular capillary pressure. It has been proposed that glomerulosclerosis development is directly related to an elevation in SNGFR that is probably because of a dilatation of the afferent arteriole. The hypothesis that the sex difference in glomerulosclerosis is secondary to a greater increment in glomerular capillary pressure and, therefore, in SNGFR is supported by the fact that albuminuria only increases in male treated rats. The greater glomerulosclerosis in males may also be attributed to the effects of androgens and to the absence of estrogens.

Tubulointerstitial damage found in the ARA-treated rats was expected, because it is well established that blockade of RAS during nephrogenesis results in tubular dilatation, chronic interstitial inflammation, and fibrosis in the adulthood and that tubular dilatation is an early event followed by a secondary inflammatory response. It is clear that tubulointerstitial injury in the renal cortex and renal medulla was also greater in males than in females, with a similar decrease in nephron number. In support of our results, Mulroney et al reported that tubular lesions in rats are greater in males than in females 8 to 10 weeks after uninephrectomy. The mechanisms responsible for the significant sex difference in tubulointerstitial injury as a consequence of reducing Ang II effects during renal development are unknown, and further studies are required to determine these mechanisms. However, the higher degree of tubulointerstitial damage in males in our study may be partly secondary to the greater glomerulosclerosis and proteinuria in males than in females and to the well known beneficial effects elicited by estrogens in females and the detrimental effects of androgens in males.

In summary, the results of this study show that blockade of AT1 receptors during the nephrogenic period induces an increment in arterial pressure and a decrease in glomeruli number that is similar in males and females. However, it is important that only males have a decrease in GFR, an important elevation in albuminuria, and a significant papillary atrophy. This is also the first study showing that the renal histopathologic changes associated with the decrease in glomeruli number during the nephrogenic period are significantly more important in males than in females.

**Perspectives**

Based on studies showing that human kidney development is completed before birth, whereas nephrogenesis continues in the rat into the second postnatal week, the results of this study suggest that a stimulus that reduces the Ang II effects during the third trimester of pregnancy in humans may elicit the development of hypertension and a significant alteration of the renal structure and renal function that would be much more important in males than in females. The evaluation of the mechanisms involved in the development of renal damage as a consequence of a reduction in nephron number may help in improving the treatment of these alterations.

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**Disclosures**

None.

**References**


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