Nephron-specific deletion of the prorenin receptor causes a urine concentration defect

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Ramkumar N, Stuart D, Calquin M, Quadri S, Wang S, Van Hoek AN, Siragy HM, Ichihara A, Kohan DE. Nephron-specific deletion of the prorenin receptor causes a urine concentration defect. Am J Physiol Renal Physiol 309: F48–F56, 2015. First published May 20, 2015; doi:10.1152/ajprenal.00126.2015.—The prorenin receptor (PRR), a recently discovered component of the renin-angiotensin system (RAS), is expressed in the nephron in general and the collecting duct in particular. However, the physiological significance of nephron PRR remains unclear, partly due to developmental abnormalities associated with global or renal-specific PRR gene knockout (KO). Therefore, we developed mice with inducible nephron-wide PRR deletion using Pax8-reverse tetracycline transactivator and LC-1 transgenes and IoxP flanked PRR alleles such that ablation of PRR occurs in adulthood, after induction with doxycycline. Nephron-specific PRR KO mice have normal survival to ~1 yr of age and no renal histological defects. Compared with control mice, PRR KO mice had 65% lower medullary PRR mRNA and protein levels and markedly diminished renal PRR immunofluorescence. During both normal water intake and mild water restriction, PRR KO mice had significantly lower urine osmolality, higher water intake, and higher urine volume compared with control mice. No differences were seen in urine vasopressin excretion, urine Na+ and K+ excretion, plasma Na+, or plasma osmolality between the two groups. However, PRR KO mice had reduced medullary aquaporin-2 levels and arginine vasopressin-stimulated cAMP accumulation in the isolated renal medulla compared with control mice. Taken together, these results suggest nephron PRR can potentially modulate renal water excretion.

prorenin receptor; nephron knockout; urine concentration

THE INTRARENAL RENIN-ANGIOTENSIN SYSTEM (RAS) contains all the components necessary to generate luminal ANG II. In this local system, angiotensinogen is synthesized in the proximal tubule and secreted into the tubular lumen (13, 16, 29, 35); in addition to being filtered, renin is produced by the collecting duct (CD) and secreted into the tubular lumen (17, 31, 34). Furthermore, angiotensin-converting enzyme is found in abundance throughout the apical nephron surface (2, 9). Tubule fluid ANG II can interact with luminal ANG II receptors and stimulate Na+ and water reabsorption (19, 27). Furthermore, CD-derived and/or filtered renin may act on the nephron via an ANG II independent pathway: the prorenin receptor (PRR) (22). While the physiological role of the nephron and CD ANG II type 1 (AT1) receptors has been examined (24, 25, 40), the role of renal PRR in the regulation of Na+ and water excretion is largely unknown.

Within the kidney, the PRR has been localized to the macula densa, mesangial cells, podocytes, proximal tubule, distal convoluted tubule, and luminal membrane of intercalated and possibly principal cells in the CD (1, 10, 11, 32, 33). In the CD, the PRR may exert multiple functions. First, binding of prorenin to the cell surface PRR potentially activates prorenin to full enzymatic activity, whereas renin bound to the PRR has fourfold higher catalytic activity than unassociated renin (22). Second, the PRR can also be cleaved into a soluble fragment that is shed in the urine (3) and is capable of activating prorenin. Third, prorenin or renin binding to the PRR, independent of angiotensinogen catalytic activity, can potentially activate cell signaling mechanisms (binding of prorenin to the PRR in cultured CD cells activates ERK1/2) (22). Finally, the PRR functions as an accessory protein for vacuolar ATPase and may be involved in lysosomal acidification (1).

Expression studies have suggested that CD PRR may be involved in the regulation of renal Na+ and/or water transport since a low-Na+ diet or excess ANG II increases PRR expression (6, 8, 12, 20, 30). Furthermore, activation of PRR by prorenin in cultured CD cells elicits ANG II-independent signaling (1, 7, 43, 44). Notably, a recent study (18) has demonstrated that arginine vasopressin (AVP)-stimulated cAMP accumulation was reduced in Madin-Darby canine kidney (MDCK) cells transfected with PRR small interfering RNA, suggesting that PRR may modulate AVP-regulated water transport processes. However, no studies to date have determined the functional significance of CD PRR, since knockout (KO) of PRR, whether global or cell specific, causes early lethality or organ malformation, likely due to abnormal lysosomal acidification affecting cell and organ development (15, 26). Recently, a CD-specific PRR KO mouse model was developed using Hoxb7-Cre to study the role of PRR in ureteric bud branching and kidney development (39). These mice demonstrated widespread apoptosis, marked renal hypoplasia, and a malformed CD system. Importantly, however, these mice had polyuria and low urine osmolality. While it is clear that ureteric bud-derived PRR are critical for normal renal development, the physiological role of CD PRR in water homeostasis is uncertain since the polyuria might result from abnormal organ development. Therefore, we developed an inducible nephron-wide KO model of PRR such that induction of PRR ablation occurs in early adulthood, avoiding the effects of PRR on organ development. We hypothesized that the PRR regulates water reabsorption through modulation of AVP actions. The present study describes the renal phenotype of the inducible

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nephron PRR KO model and examines its effects on water excretion.

METHODS

Animal care. All animal experiments were conducted with the approval of the Universities of Utah and Virginia Animal Care and Use Committees in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Generation of inducible nephron-specific PRR KO mice. Details on the generation of floxed PRR mice have already been published (15, 26, 39). In brief, a targeting construct was made wherein exon 2 of the Atp6ap2 (PRR) gene was flanked by two loxP sites (floxed) and electroporated into mouse embryonic stem cells. Floxed mice bearing the PRR gene were bred with mice containing Pax8-reverse tetracycline transactivator (rtTA) and LC-1 transgenes. The Pax8-rtTA transgene contains the Pax8 gene promoter driving expression of rtTA (42). The LC-1 transgene encodes tetracycline-inducible bicistronic Cre recombinase and luciferase (38). In the presence of tetracycline or doxycycline, rtTA binds and activates the LC1 transgene, leading to expression of luciferase and Cre recombinase (38).

Generation of inducible nephron PRR KO mice. The PRR mice without Pax8-rtTA or LC-1 transgenes were given doxycycline drinking water for 12 days followed by 4 wk off doxycycline. Floxed for floxed PRR gene were given 2 mg/ml doxycycline in 2% sucrose. These primers are located in introns 1 and 2 and yield a 1,500-bp product in nonrecombined DNA and a 400-bp product in recombined DNA.

Quantitation of PRR mRNA. The renal cortex and inner medulla were dissected from PRR KO and floxed mice for RNA isolation. Reverse transcription was performed on 2 μg total RNA with oligo(dt) and Superscript III reverse transcriptase according to the manufacturer’s protocol (Invitrogen, Grand Island, NY). The resulting cDNA was then assayed for the relative expression of PRR mRNA in KO and floxed mice using a Taqman Gene Expression assay (PRR probe catalog no. Mm00510396_m1 and GAPDH probe catalog no. Mm03302249_g1, Applied Biosystems, Carlsbad, CA).

Western blot analysis. The renal inner medulla was dissected and homogenized in ice-cold sucrose buffer (10 mM triethanolamine and 250 mM sucrose, pH 7.6) with PMSF (100 μg/ml), leupetin (10 μM), and Complete Protease Inhibitors (Roche, Pleasanton, CA). After samples had been centrifuged at 2,000 g for 10 min at 4°C, the supernatant was collected, and an aliquot was taken for the determination of protein content using the Bradford assay (Bio-Rad, Hercules, CA). The remaining sample was solubilized with Laemmli loading buffer containing 0.5% lithium dodecyl sulfate and heated at 60°C for 10 min. Inner medullary lysates (10 μg/lane) were run on a denaturing NUPAGE 4–12% bis-Tris minigel (Invitrogen), transferred to a polyvinylidene difluoride plus nylon membrane, and visualized with the Advance ECL system (GE Healthcare, Piscataway, NJ). Densitometry was performed with a Bio-Rad gel documentation system. Membranes were incubated with primary antibody against PRR (ab40790, Abcam, Cambridge, MA) or aquaporin (AQP)2 (sc-9882, Santa Cruz Biotechnology, Dallas, TX) and, after visualization, reprobed with anti-β-actin antibody (Cell Signaling, Danvers, MA).

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Screening for PRR gene recombination. DNA was isolated from a variety of organs and PCR amplified to examine the organ-specific expression of the recombined targeted renin gene. PCR was performed for 40 cycles at 98°C for 30 s, 68°C for 30 s, and 72°C for 50 s using the following primers to amplify the transgene: forward 5′-GGGGGGTA-CAATTTGATGATGATTCATGACGC-3′ and reverse 5′-TGCTTACCTAGTGATTGATTTGCTTACGC-3′. These primers are located in introns 1 and 2 and yield a 1,500-bp product in nonrecombined DNA and a 400-bp product in recombined DNA.

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Fig. 1. Gene targeting strategy used to generate inducible nephron prorenin receptor (PRR) knockout (KO) mice. A: Pax8 promoter drives expression of the reverse tetracycline transactivator (rtTA), which requires doxycycline (Doxxy) to activate the bicistronic Cre recombinase/luciferase-expressing transgene. Cre is expressed specifically in the nephron but not in glomeruli. TRE- tetracycline response element. B: representative blot of PRR gene recombination in various organs in nephron PRR KO mice (n = 4 mice/genotype). The top 1,500-bp band is the unrecombined allele, and the bottom 400-bp band is the recombined allele.
Histology. Kidneys were fixed overnight in 10% formaldehyde and embedded in paraffin, and 4-μm sections were obtained. Kidney sections were rehydrated with xylene and ethanol and stained with hematoxylin and eosin or Masson trichrome to assess morphological changes and fibrosis.

For immunofluorescence, deparaffinized kidney sections were treated with 1% SDS for 10 min to enhance antibody staining, blocked with 1% BSA in PBS for 1 h, and incubated with primary antibody against PRR (1:50, Abcam) and AQP2 (1:100, Santa Cruz Biotechnology) overnight. After three consecutive washes of 5 min each with PBS, kidney sections were incubated with secondary donkey anti-rabbit Alexa fluor 488 (1:50) and donkey anti-goat Alexa fluor 488 555 (1:400) antibodies for 60 min. After three wash-rinse steps of 5 min each with PBS, slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA) and sealed with a coverslip. Tissue sections were examined and photographed with a Nikon FXA epifluorescence microscope. In a separate experiment, kidney sections from PRR KO and control mice were stained with antibody against the V-ATPase B1 subunit (1:50, generously provided by Dr. Dennis Brown, Massachusetts General Hospital, Boston, MA) and AQP2 to evaluate cell number and the ratio of principal to intercalated cells to assess differences in CD morphology between PRR KO and control mice.

Metabolic balance experiments. Control and PRR KO mice were placed in metabolic cages for 4 consecutive days for measurements of food and water intake, body weight, and 24-h urine collection. Mice were given 9 ml of a normal Na+/K+ diet composed of 62 g of rodent food (TestDiet, St. Louis, MO), 7 g of gelatin, and 110 ml of water with free access to water for the first 3 days. On day 3, 35 μl of blood was collected from the dorsal pedal vein in chilled polypropylene tubes containing heparin lithium. Plasma was separated and chilled at −80°C for the assay of blood urea nitrogen (BUN). On day 4, access to drinking water was discontinued while mice continued to be fed the gel diet (so water was obtained from the gel); this was termed mild water restriction. At the end of 24 h of water restriction, blood was obtained for AVP measurements. In some experiments, mice were fed only dry pellets or a 4× concentrated gel diet (1/4 as much water in the gel per calorie ingested) for 1 day to achieve more severe water restriction.

Urine samples were centrifuged at 15,000 rpm for 15 min, and the supernatant was frozen in aliquots at −80°C until assay. Urinary Na+ and K+ were determined using an EasyVet Analyzer (Medica, Bedford, MA). Plasma and urine osmolality were measured using Osmett H9262 (Osmett Systems, Farmingdale, NY). Plasma urea was measured using a commercially available antibody for PRR (1:1,000 dilution, anti-ATP6IP2/ab40790, Abcam). PRR expression was normalized to β-actin, and densitometry analysis was performed using ImageJ software (NIH, Bethesda, MD).

Statistical analysis. All results are expressed as means ± SE. Student’s unpaired t-test was used to compare differences between KO and control animals. For parameters requiring more than one comparison, ANOVA with a Scheffé post hoc test was used, as indicated. The criterion for significance was P ≤ 0.05.

RESULTS

Verification of nephron-specific KO of PRR. At 1 mo of age, mice homozygous for the floxed PRR gene and hemizygous for Pax8-nTα and LC-1 transgenes were treated with doxycycline (2 mg/ml) in 2% sucrose drinking water for 12 days to induce nephron-wide PRR disruption followed by a recovery period of

**Fig. 2. Efficiency of PRR ablation in control and PRR KO mice.** A: kidney PRR mRNA expression by RT-PCR (n = 5–7/group). All values are adjusted to control medullary PRR mRNA levels (100%) since cortical levels of the PRR are much lower than in the medulla. B: PRR protein by Western blot analysis and densitometry (n = 3/group). *P < 0.05 vs. control mice.
at least 4 wk. Equal numbers of male and female mice aged 2–3 mo were examined in all experiments. After doxycycline induction, some PRR KO mice were followed to evaluate if nephron-specific PRR disruption affected survival. PRR KO mice followed until 8 mo of age showed normal growth and survival, confirming that ablation of PRR in adulthood did not affect viability.

PCR of DNA isolated from various organs from nephron PRR KO mouse demonstrated strong recombination in the kidneys with minor recombination in other organs (Fig. 1B). Previous studies (4, 21, 41) have demonstrated that the Pax8-rtTA/LC-1 system is complete and highly specific within the kidney for renal tubular epithelial cells, does not target glomeruli, and minimally targets other organs.

Fig. 3. Immunostaining of kidney sections with anti-PRR (green) and anti-aquaporin (AQP)2 (red) antibodies from control [wild type (WT)] mice (A and C) and PRR KO mice (B and D). Images are representative of four different mice. Magnification: ×200 in A and C and ×400 in B and D.

Fig. 4. Renal tissue structure in control and PRR KO mice. A and B: hematoxylin and eosin-stained kidney sections from control (A) and PRR KO (B) mice. Magnification: ×200. C and D: immunostaining of kidney sections from PRR KO and control mice with anti-V ATPase B1 subunit (green) and anti-AQP2 (red) antibodies to assess cell number and the distribution of principal and intercalated cells. Images are representative of four different mice.
Within the kidney, PRR mRNA levels were similar in the cortex of nephron PRR KO and control mice, presumably due to the presence of PRR in non-nephron structures (1). In contrast, PRR mRNA levels were 65% lower in the inner medulla of nephron PRR KO mice compared with control mice (Fig. 2A). Furthermore, the amount of PRR in the cortex was markedly lower compared with the medulla, consistent with a previous study (1). Additionally, medullary PRR protein levels were also significantly reduced in nephron PRR KO mice (Fig. 2B).

To further evaluate PRR expression within the kidney, dual immunofluorescence with PRR and AQP2 was performed in paraffin-embedded kidney sections. As shown in Fig. 3, control mice demonstrated PRR staining predominantly in the CD with minimal staining in other tubules. Furthermore, PRR staining did not colocalize with AQP2 and appeared to be luminal in distribution, consistent with previous reports (1, 6) showing that the PRR localized to the luminal membrane of intercalated cells. Compared with control mice, PRR immunostaining was markedly reduced in nephron PRR KO mice (Fig. 3), confirming targeting of the PRR.

**Histology.** Since deletion of the PRR from the ureteric bud during embryogenesis causes renal hypoplasia, renal cysts, and reduced CD numbers (39), renal histology was evaluated in nephron PRR KO mice using hematoxylin and eosin and Masson’s trichrome staining. Compared with control mice, PRR KO mice demonstrated no morphological abnormalities in the cortex or medulla (Fig. 4). Similarly, no differences in interstitial fibrosis or nephron number were noted between control and nephron PRR KO mice with trichrome staining (data not shown).

To evaluate if there were differences in the cell number or distribution of principal and intercalated cells, numbers of CD cells were counted in sections using dual immunofluorescence to label principal cells with AQP2 and intercalated cells with the V-ATPase B1 subunit. There were no differences in the absolute number of principal and intercalated cells per high-powered field between control and PRR KO mice. Similarly, there was no difference between genotypes in the ratio of

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<th>Table 1. Food intake, weight, and renal function parameters in control and PRR KO mice fed a normal water and salt diet</th>
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<td>Control mice</td>
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Values are means ± SE; n = 6–8 mice/group. PRR, prorenin receptor; KO, knockout.
principal to intercalated cells (2:1 principal to intercalated cells; Fig. 4), confirming that there was no evidence of differences in the ratio or abundance of these two cell types between control and PRR KO mice. Collectively, these findings, along with the PRR mRNA, protein, and immunostaining results, support the notion that the nephron PRR KO mouse is a potentially useful model to study the functional significance of nephron-derived PRR.

Effects of nephron PRR KO on renal water transport and transport pathways. During normal water intake, nephron PRR KO and control mice had similar food intake, body weight, and kidney weight (Table 1). Furthermore, no differences were noted in BUN levels or 24-h urine Na\(^+\) or K\(^+\) excretion (Table 1). However, compared with control mice, nephron PRR KO mice demonstrated increased water intake and urinary volume and decreased urine osmolality (Fig. 5). With mild water restriction for 1 day (access to normal Na\(^+\) gelled diet without water), nephron PRR KO mice continued to have increased urine volume despite decreased water intake (the latter possibly related to decreased activity due to volume contraction) compared with control mice (Fig. 5). Although urinary AVP excretion tended to increase in both groups in response to water restriction (nephron PRR KO mice, baseline: 731 ± 134 pg/day vs. water restriction: 1,859 ± 461 pg/day; control mice, baseline: 682 ± 136 pg/day vs. water restriction: 1,051 ± 333 pg/day), no detectable differences were observed between nephron PRR KO and control mice at baseline or after water restriction. Urinary AVP tended to be higher in PRR KO than control mice after water restriction (Fig. 5). No differences were noted in renal cortical PRR protein levels between the two groups.

### DISCUSSION

The present study describes, for the first time, a viable renal-specific KO of PRR. Nephron PRR KO mice have normal growth and survival, do not demonstrate any renal morphological or histological abnormalities, have normal kidney weights, and show no generalized alteration in kidney function (as evidenced by normal BUN and urinary Na\(^+\) and K\(^+\) excretion). These findings are in contrast to previous studies (15, 26, 39); cardiomyocyte-specific PRR KO resulted in a urine concentration defect. No differences were observed between male and female control and PRR KO mice with respect to the above parameters.

To determine if AVP responsiveness was altered, the effect of AVP-stimulated cAMP was measured in the isolated inner medulla from control and nephron PRR KO mice. At baseline, no cAMP was detectable in the isolated inner medulla in both groups. Upon stimulation with 10 nM AVP, cAMP levels were significantly lower in nephron PRR KO mice (54 ± 14.5% of control mice; Fig. 7). Pretreatment with PD-98059 (ERK1/2 inhibitor) did not prevent the differences in AVP-stimulated cAMP accumulation between control or PRR KO mice (Fig. 7). Pretreatment with indomethacin (COX inhibitor) increased AVP-stimulated cAMP levels; however, it did not prevent the differences in AVP-stimulated cAMP between PRR KO and control mice (Fig. 7).

To evaluate whether nephron PRR KO had the ability to maximally concentrate urine, more severe water restriction experiments were attempted using a fourfold concentrated gelled diet or a pellet diet and no access to water. Nephron PRR KO mice were unable to tolerate this degree of water restriction, with 30% mortality occurring within the first 24 h.

**PRR expression and water deprivation.** Finally, to determine whether water deprivation regulated PRR expression, wild-type mice were treated with 24-h water restriction. Mice treated with water restriction had 60% higher renal medullary PRR protein levels compared with mice with free access to water (Fig. 8). No differences were noted in renal cortical PRR protein levels between the two groups.
in fatal heart failure (15), whereas podocyte-specific PRR KO lead to severe proteinuria, renal failure, and death within 4 wk of birth (26, 32). Moreover, CD-specific PRR ablation caused renal hypoplasia and medullary cysts due to abnormal renal development (39). Collectively, these studies indicate that KO of PRR during embryogenesis leads to early lethality, possibly from abnormal lysosomal acidification (1). Therefore, using a mouse model involving inducible KO during adulthood allows examination of the functional relevance of nephron-derived PRR while avoiding abnormal organ development.

Nephron PRR KO mice showed a urine concentration defect with increased water intake and urine volume and decreased urine osmolality. Although these results have been previously reported in CD-specific PRR KO mice (39), it was unclear if this was due to abnormal CD development. Furthermore, a recent study (18) has demonstrated that AVP-stimulated increases in cAMP accumulation were abolished in Madin-Darby canine kidney. C11 cells transfected with PRR small interfering RNA. We also found that, on a normal water diet, nephron PRR KO mice had normal urinary AVP excretion, reduced AVP-stimulated cAMP accumulation in the acutely isolated inner medullary CD, and reduced inner medullary total AQP2 protein content; these findings suggest a nephrogenic diabetes insipidus. Consistent with this, nephron PRR KO mice had normal urinary AVP excretion, reduced AVP-stimulated cAMP accumulation in the acutely isolated inner medullary CD, and reduced inner medullary total AQP2 protein content; these findings suggest a nephrogenic diabetes insipidus. Consistent with this, nephron PRR KO mice had normal urinary AVP excretion, reduced AVP-stimulated cAMP accumulation in the acutely isolated inner medullary CD, and reduced inner medullary total AQP2 protein content; these findings suggest a nephrogenic diabetes insipidus. Consistent with this, nephron PRR KO mice had normal urinary AVP excretion, reduced AVP-stimulated cAMP accumulation in the acutely isolated inner medullary CD, and reduced inner medullary total AQP2 protein content; these findings suggest a nephrogenic diabetes insipidus. Consistent with this, nephron PRR KO mice had normal urinary AVP excretion, reduced AVP-stimulated cAMP accumulation in the acutely isolated inner medullary CD, and reduced inner medullary total AQP2 protein content; these findings suggest a nephrogenic diabetes insipidus. Consistent with this, nephron PRR KO mice had normal urinary AVP excretion, reduced AVP-stimulated cAMP accumulation in the acutely isolated inner medullary CD, and reduced inner medullary total AQP2 protein content; these findings suggest a nephrogenic diabetes insipidus.

Fig. 8. PRR expression in WT C57BL6 mice during normal water intake and after 24-h water deprivation. A: representative Western blot analysis of the PRR from the cortex and medulla. B: densitometry of PRR. n = 4/group. *P < 0.05 vs. normal water intake.
excess AVP (40). Therefore, if PRR regulation of water transport was mediated via AT$_1$ receptor, one would not expect to see a urinary concentration defect at baseline, as seen in the present study. Another possibility is that the PRR, independent of ANG II, might modulate intracellular signaling pathways (23). Activation of PRR has not been shown to modify intracellular Ca$^{2+}$ mobilization (22), a key mediator of vasopressin signaling. However, recent studies (5, 22, 37) have demonstrated PRR activation of ERK1/2/Akt pathways, both of which have been implicated as components of vasopressin signaling, albeit likely downstream of cAMP generation (28, 45). In addition, activation of the PRR can lead to upregulation of COX2 (7, 14) and activation of p38 MAPK/heat shock protein 27 and phosphoinositide 3-kinase pathways (36, 37). In the present study, pretreatment with an ERK1/2 inhibitor or a COX inhibitor did not abolish the differences in AVP-stimulated cAMP levels between control and PRR KO mice. Clearly, further studies are needed to delineate the exact mechanism by which nephron PRR might modulate water reabsorption.

In summary, the present study demonstrates the initial characterization of a viable, nephron-specific PRR KO and identifies a role for PRR in renal water excretion. These data suggest that the nephron PRR may be important in water regulation by modulating the AVP/V2R/AQP2 pathway. Key remaining questions include the mechanisms by which PRR modulates water reabsorption and whether this is dependent or independent of ANG II.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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