SORCS1 contributes to the development of renal disease in rats and humans

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Submitted 3 June 2013; accepted in final form 14 June 2013

Lazar J, O’Meara CC, Sarkis AB, Prisco SZ, Xu H, Fox CS, Chen MH, Broeckel U, Arnett DK, Moreno C, Provoost AP, Jacob HJ. SORCS1 contributes to the development of renal disease in rats and humans. Physiol Genomics 45: 720–728, 2013. First published June 25, 2013; doi:10.1152/physiolgenomics.00089.2013.—Many lines of evidence demonstrate that genetic variability contributes to chronic kidney disease susceptibility in humans as well as rodent models. Little progress has been made in discovering causal kidney disease genes in humans mainly due to genetic complexity. Here, we use a minimal congenic mapping strategy in the FHH (fawn hooded hypertensive) rat to identify Sorcs1 as a novel renal disease candidate gene. We investigated the hypothesis that genetic variation in Sorcs1 influences renal disease susceptibility in both rat and human. Sorcs1 is expressed in the kidney, and knocking out this gene in a rat strain with a sensitized genome background produced increased proteinuria. In vitro knockdown of Sorcs1 in proximal tubule cells impaired protein trafficking, suggesting a mechanism for the observed proteinuria in the FHH rat. Since Sorcs1 influences renal function in the rat, we went on to test this gene in humans. We identified associations between single nucleotide polymorphisms in Sorcs1 and renal function in large cohorts of European and African ancestry. The experimental data from the rat combined with association results from different ethnic groups indicates a role for Sorcs1 in maintaining proper renal function.

kidney; SORCS1; proteinuria

RENAI L FAILURE ASSOCIATED WITH chronic kidney disease (CKD) currently affects more than 2 million individuals worldwide (65). The observation that genetic factors contribute to disease development and progression is supported by linkage analyses and genome-wide association studies (GWAS), which have identified a number of genetic loci associated with CKD (5, 11, 12, 14, 21, 29–31, 33, 34, 45, 58, 61, 64). However, both association and linkage approaches have had limited success to date, accounting for only a small percentage of the genetic variability for complex diseases (44, 50). Use of genetically modified animal models, such as consomic and congenic animals, to study the genetic basis of kidney disease and subsequently test candidate genes in human cohorts offers another strategy for discovering the genetic underpinnings.

Our group has utilized the renal disease susceptible FHH (fawn hooded hypertensive) rat as a genetic model for mapping kidney disease genes. In previous studies we crossed the FHH rat with the renal disease-resistant August Copenhagen Irish (ACI) rat to identify five quantitative trait loci (QTL), named Rf-1 to Rf-5, that contribute to the development of renal disease (4, 37, 42, 43, 56, 60). Subsequent studies demonstrated that gene(s) within the various Rf loci interact with one another to produce a synergistic increase in renal impairment (42, 43, 56, 59, 60). In particular, the Rf-1 locus, which is located on the distal arm of rat chromosome 1, strongly interacted with other Rf QTL, specifically Rf-3 and Rf-4 (42, 43, 59, 60). The Rf-1 QTL significantly exacerbates renal impairment in the FHH rat. Since this genetic region plays a central role in renal disease susceptibility, we sought to identify the underlying gene responsible for producing the Rf-1 phenotype.

In the present study, we used a minimal congenic mapping strategy to identify Sorcs1 as the lead candidate gene mediating renal disease in the Rf-1 locus. Sorcs1 is a member of the vacuolar protein sorting 10 (VPS10) family of receptors (22), a key component required for intracellular trafficking that sorts cargo from the early endosome to the trans-Golgi network (35). Human and animal studies have linked SORCS1 to diseases including Alzheimer’s and diabetes (8, 17, 19, 32, 35, 45, 53, 62); however, little is known about a potential role for the SORCS family of proteins in modulating renal function despite high levels of the Sorcs1 transcript in the adult kidney (23, 25). To test if Sorcs1 is causally driving Rf-1, we knocked this gene out in a rat strain with a sensitized genome background and showed that Sorcs1 knockout (KO) increases proteinuria. We then investigated the subcellular mechanism by which Sorcs1 influences protein handling in the kidney by an in vitro proximal tubular protein trafficking assay.

The genetic region homologous to Rf-1 in humans has been linked to renal disease in multiple linkage analyses, yet the gene underlying this locus has not been identified (15, 27). We tested Sorcs1 association with renal function in human cohorts and found significant association with estimated glomerular filtration rate (eGFR) and albumin-creatinine ratio phenotypes. The present study demonstrates that Sorcs1 contributes to renal function by influencing protein trafficking in the proximal...
tubule in a pig cell line and inferred to play the same role in rats and humans.

MATERIALS AND METHODS

Generation and phenotyping of congenic sublines. All animal protocols were approved by the local Animal Care and Use Committee at the Medical College of Wisconsin. RF-1a (ACI/FHH[D1Rat74-D1Rat90]) congenic rats were backcrossed to ACI rats to initiate the generation of RF-1 congenic sublines as described previously (37, 49). The F2 generation was genotyped using a fluorescent genotyping protocol (described previously) (40) to select offspring that had inherited a desirable recombination within the RF-1 region. These selected offspring were backcrossed and intercrossed to produce homozygous congenic sublines used for phenotyping (Fig. 1). Male rats used for phenotyping underwent unilateral nephrectomy at 5 wk of age. After surgery, 150 mg/l of -NAME (Sigma Aldrich, St. Louis, MO) was added to the drinking water to induce hypertension. Animals were phenotyped for protein excretion at 13 wk of age as described previously (42).

Genomic sequencing and analysis. Isolated genomic DNA from FHH/Eur/Mcwi and ACI/Eur rats was used to generate libraries containing 200 base inserts. Libraries were sequenced using the Illumina HiSeq 2000. The paired-end reads were aligned to BN (Brown Norway) (renal disease-resistant) reference genome (rn4) with Burrows-Wheeler Aligner v1.6.9 (39). Analysis of FHH, ACI, and BN genome can be accessed on the RGD website (http://rgd.mcw.edu).

Protein reuptake assay. Porcine kidney epithelial LLC-PK1 (PK-1) cells were transduced using lentivirus containing Sorcs1 shRNA (Open Biosystem) and a puromycin resistance gene for selecting positive cells. The reuptake experiment was conducted with 10 nm 1[25S]GTP (Open Biosystem) and a puromycin resistance gene for selecting positive cells. The reuptake experiment was conducted with 10 nm 1[25S]GTP (Open Biosystem) and a puromycin resistance gene for selecting positive cells. The nonserum medium was removed, and serum containing BGT was added (0.8 –1 ml/well) and incubated for 30 s, 5 min, and 10 min, respectively. After each incubation BGT was immediately removed, cells were washed with 3 × 3 ml prewarmed PBS, and then 1 ml fixative buffer (2.5% glutaraldehyde) was added and incubated for 10 min at room temperature. Sheets of cells were mechanically detached, postfixed with 1% osmium tetroxide, dehydrated in a series of graded ethanol, and embedded in epoxy resin. Thin sections were examined with a Hitachi 600 transmission electron microscope (Nissan Sangyo, San Jose, CA) operated at 75 kV. The number of BGT granules per vesicle was quantified in a blinded manner.

Development of Sorcs1 KO rat. The KO strain, produced using a zinc-finger nuclease (ZFN) strategy as previously described (16), was made on the sensitive genomic background of the FHH-1BN consomic strain (38). Briefly, ZFN reagents were designed and assembled by Sigma Aldrich (St. Louis, MO) to target the following genomic sequence AAATACCTTCCCCAGGATcattGACCGGATTC in exon 7 of Sorcs1. The underlined sequences are the target site recognition sequence for each molecule of the ZFN pair. The ZFN mRNA was diluted in microinjection buffer (1 mM Tris·Cl pH 7.4, 0.1 mM EDTA) at a concentration of 10 ng/μl and injected into the pronucleus of 117 newly fertilized FHH-1BN consomic strain eggs. In the founder generation a tail biopsy was performed, and DNA was extracted as described before (40). PCR product was amplified using primers flanking the mutation site Sorcs1 F: 5’-GGGATTTAAC-AAAACTCCCAA-3’ and R: 5’-TTGCTACCCTTCATCTCTT-3’ and was subjected to the Surveyor Nuclease Cel-I assay (Transgenic, Omaha, NE) as previously described (16). Genomic DNA from founders (as identified from the Cel-1 assay) was PCR amplified using the same primer as for the Cel-1 assay and sequenced by Sanger sequencing (55). The sequence was analyzed using Consed (18). Two founders were identified, and genomic sequencing revealed the same 14 bp deletion of atacattgcccg in both animals leading to a frameshift and truncation of the protein. The founders were backcrossed and sibling carriers were identified by genotyping before intercrossing to establish a colony for the studies reported here. The Sorcs1 KO line is officially called FHH-1Rf1Sorcs1em1. Male Sorcs1 KO animals were phenotyped at 12 wk of age for protein excretion as described previously (42). Animals used for phenotyping were maintained on normal drinking water throughout the duration of the protocol.

Sorcs1 expression analysis. Total RNA was prepared from ZFN wild type (FHH-1BN) and ZFN Sorcs1 KO whole kidneys using Trizol reagent (Invitrogen, Carlsbad, CA). The first strand of cDNA was generated using SuperScript III First-Strand Synthesis System (Invitrogen). The level of expression of Sorcs1 in KO and wild-type kidneys was determined by quantitative (q) PCR as described before (43). Exon spanning primers (forward 5’-AGGCCAACAGAAAA-TAACCTTTCC and reverse 5’-TAAATGGTGTCCTCCTCCTGATGT) were designed upstream of the deleted region to amplify Sorcs1 mRNA. Quantitation of the relative expression levels of Sorcs1 was normalized against GAPDH.

Statistical analysis. The data are presented as means ± SE and were analyzed by t-test or a one-way ANOVA followed by the Holm-Sidak multiple comparison test using Sigma Plot 11.0 software.

Human populations. A genome-wide association meta-analysis in 67,093 Caucasian individuals from 20 general population-based cohorts within the CKDGen consortium was performed as published, previously (34). HyperGen study participants were recruited from FBPP Hypertension Genetic Epidemiology Network (HyperGEN) (63). Recruitment criteria required that participating sibships had ≥2 siblings who had been diagnosed with hypertension before age 60 yr. Hypertension was defined as currently taking antihypertensive medication or having an average systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg measured at two separate clinic visits. Average blood pressure was calculated using the second
and third measurements of three readings using an oscillometric blood pressure monitor (Dinamap 1846 SX; GE Healthcare, Waukesha, WI). Creatinine was measured by a thin film adaptation of the amidohydrolase enzymatic method using Vitrolyzer analyzer (Johnson & Johnson Clinical Diagnostics, Rochester, NY). The SPQ Test System (Diasorin, Stillwater, MN) was used for microalbumin measurement as a quantitative determination of human albumin through the use of an automated immunoprecipitation analysis on the Roche/Hitachi 911 (Roche Diagnostics). Albumin was measured by immunoprecipitation analysis system (Diasorin). To prevent antigen excess errors, total protein was assayed for each sample. Urine values for albumin were directly measured if the total protein value was <100 mg/dl, the urine was diluted and the albumin measurement repeated. Individuals with a history of Type 1 diabetes, severe renal disease, or valvular heart disease were excluded. This study was approved by the centers’ institutional review boards and all subjects gave informed consent.

Human genotyping and statistical analysis. Genetic data for HyperGEN samples were obtained via the Affymetrix Genome-Wide Human 6.0 SNP Arrays. Subjects with a call rate <95% or with a sex mismatch were excluded. HyperGEN genotyped 1,264 self-reported African-American participants who initially had echocardiography data available.

First, we tested for Hardy-Weinberg equilibrium (HWE) in founders only as a first screen and subsequently tested HWE on the whole sample set assuming independence of all individuals. $\chi^2$ goodness-of-fit tests were used to test HWE except when the minor allele frequency was $\leq 0.1$, in which case we performed Fisher’s exact test. We flagged the SNPs at two levels, namely, 1) if the HWE $P$ value was between $10^{-4}$ and $10^{-6}$ and 2) if the $P$ value was $<10^{-6}$.

The phenotype was log-transformed to normalize the distributions. HyperGEN used Eigenstrat (47) to generate principal components to adjust for population substructure. We selected the first 30 principal components to adjust for finer population stratification, if such stratification existed. Since prior research has suggested that association analyses are not sensitive to the number of principal components selected, we conducted a sensitivity analysis to determine the impact of using a smaller number of principal components (i.e., 5 and 10), and found that the correlation of the $P$ values from the GWAS results within each SNP category were $>0.99$ across the models tested. Therefore, we retained the 30 principal components in our models. GWAS analyses were conducted in two steps using two sets of adjustment for each trait using the GRAMMER method introduced by Aulchenko et al. (3).

RESULTS AND DISCUSSION

A 1.5 Mb region of RNO1 is responsible for the Rf-1 phenotype. To refine the Rf-1 region to a reasonable number of candidate genes, we generated a panel of overlapping congenic lines. A map of the introgressed regions in the congenic strains and a summary of the protein and albumin excretion (urinary protein excretion and urinary albumin excretion) data are presented in Fig. 1. We found that the Rf-1 congenic sublines 3, 4, and 7 excreted significantly higher levels of protein and albumin compared with the ACI control strain. Thus, by comparing the genetic region common to all three of these lines we reduced the candidate interval of Rf-1 to a 1.5 Mb region (254,755,671–256,269,030). There is one known gene in this congenic interval, Sorcs1, and three pseudogenes, LOC690193 (similar to NAD synthetase 1), LOC100360227, and RGD1561154 (similar to heat shock protein).

The entire refined candidate interval was sequenced in the FHH and compared with the ACI and BN renal disease resistant strains to identify variants potentially responsible for the Rf-1 phenotype. We found no exonic variants in the genomic region of Sorcs1, despite the large size of the gene (26 exons, 1,168 amino acids spanning 3,504 nt). The high degree of similarity found between FHH and BN suggests that Sorcs1 is highly conserved between the strains since FHH and BN are distally related strains (54). To confirm the high conservation of this gene, we compared the SS (salt sensitive, SS/JHsdMcwi) (unpublished) and SHR (spontaneously hypertensive rat, SHR/Olalpcv) (2) Sorcs1 sequence to the BN. There were a total five unique exonic variants, all of which were synonymous, giving further evidence that this gene is highly conserved. The entire interval contains 1,182 intergenic and 1,310 intronic sequence variants between FHH and BN. Moreover seven SNPs reside in the three pseudogene regions. We identified only 17 SNPs that are unique to FHH compared with both BN and ACI (Table 1). Since these sequence variants are unique to the disease-susceptible strain, we hypothesized that they are more likely to influence renal disease susceptibility. Three SNPs are intergenic, and 14 are residing in Sorcs1 intronic regions and can potentially affect transcription factor binding site as assessed by TFSearch (http://www.cbrc.jp/research/db/TFSEARCH.html). Specifically, a G→A variant at position 256,245,290 in the FHH rat is the most highly conserved (conservation score = 1) unique variant in the Rf-1 candidate interval. Several predicted transcription factor binding sites span this position; therefore, the FHH variant could alter transcriptional regulation of Sorcs1. The recently published Encyclopedia of DNA Elements (ENCODE) data (13) indicates that regulation of gene expression is more complex than once thought: the ENCODE consortium suggests that 80% of the genome can be functional. Although this percentage has been disputed (20), it is well known that noncoding DNA can affect gene function and expression. Despite the lack of SNPs in coding or promoter regions, or near splice sites, one cannot rule out potential functional impact of intronic or intergenic SNPs. Whole kidney quantitative (q) PCR did not reveal Sorcs1 RNA expression differences between ACI and FHH (data not shown). However, it is possible that Sorcs1 expression differ-

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Reference (Ref) and variant (Var) position and allele are provided. FHH, fawn hooded hypertensive; ACI, August Copenhagen Irish; BN, Brown Norwegian.
ences at the RNA or protein level exist in a specific cell type(s) within the kidney but could not be detected by whole kidney qPCR analysis. Future studies could be aimed to decipher cell type-specific expression levels of Sorcs1 between ACI and FHH.

Sorcs1 KO causes increase proteinuria. We have narrowed the list of candidate genes in the Rf-1 region to one known gene (Sorcs1) and three pseudogenes. Because of the high expression of Sorcs1 in the kidney, we hypothesized that this gene is responsible for the Rf-1 phenotype. The majority of published results indicate that members of VPS10 family are important in neurogenesis, plasticity-related processes, and functional maintenance of the nervous system (24, 26, 52). Little is known about a role for Sorcs1 in kidney function besides evidence of its expression in the kidney (23). Recent studies have suggested a role for a member of the VPS10 domain receptor family, sorting protein-related receptor with A-type repeats (SORLA, also known as SORL1 or LR11), in maintaining renal function homeostasis. This study showed that SORLA is expressed in epithelial cells of the thick ascending limb of Henle’s loop, distal convoluted tubule, and connecting tubule and demonstrated that this gene is involved in renal NaCl excretion (51). Because of the known role of VPS10 domain containing proteins in renal homeostasis, we predicted Sorcs1 also plays a role in renal function.

To investigate the potential role for Sorcs1 in maintaining kidney function we chose to knock out Sorcs1 in the FHH-1BN consomic rat strain, which is protected from proteinuria (38). This rat strain is sensitized to renal disease since majority of the genetic background comes from the FHH strain with the exception of RNO1, which comes from BN. We hypothesized that if Sorcs1 mediates renal function, then knocking out Sorcs1 in this sensitized strain would increase proteinuria. A 14 bp deletion in exon 7 of Sorcs1 was introduced via ZFN (see MATERIALS AND METHODS) and verified by Sanger sequencing. The resulting frame shift produced a premature stop codon at 364aa. This gene modification completely disrupted the transmembrane domain and eliminated the cytoplasmic domain of Sorcs1 (Fig. 2A). Furthermore, the mutation disrupted potential for Sorcs1 isoforms, which have been previously reported (23).

We found that proteinuria was markedly increased in the homozygous KO and unchanged in heterozygotes or wild-type littermates (Fig. 2B). This pattern of inheritance would be expected considering the recessive mode of inheritance found in the genetic mapping studies for Rf-1 (4, 56). The increased proteinuria on an otherwise protected strain demonstrates that Sorcs1 plays an important role in maintaining proper renal function.

Real-time PCR was performed to analyze the level of Sorcs1 transcript utilizing primer pair upstream of the induced frame-shift mutation, i.e., the same primer pair can be used for KO and wild-type alleles. A threefold decreased of Sorcs1 expression was detected in KO rats compared with wild type (Fig. 3). The decreased Sorcs1 transcript levels in the Sorcs1 KO animals could be due to a mechanism such as negative or compensatory regulation from other members of VPS10-containing proteins. Alternatively, in mammalian cells, newly synthesized transcripts undergo a series of processing steps to become mature templates for protein synthesis. It is possible that the deletion activated multiple decay pathways to eliminate nonfunctional transcripts (6), through “mRNA surveillance” events on the ribosome (57).

Sorcs1 knockdown impairs proximal tubule protein reuptake. Proteinuria is generally regarded as a consequence of two mechanisms: increased permeability of glomerular capillary wall and impaired reabsorption by the epithelial cells of the prox-

Fig. 2. Location, structure, and phenotypic effect of the zinc finger nuclease (ZFN) induced mutation in the FHH-1BN-Sorcs1em1 strain. A: the 14 bp deletion causes a frame shift resulting in a premature truncation at amino acid 364. This frame shift resulted in loss of the cytoplasmic (PKD) and transmembrane (VPS10) domains. B: levels of proteinuria in the Sorcs1 knockout (KO) (−/−), heterozygote (−/+), and wild-type (WT) (+/+).
nal tubuli, but the role of tubuli is still controversial (10, 28). In the normal kidney proteins with a molecular weight <40 kDa are freely filtered across the glomerular membrane and then reabsorbed in the proximal tubules with >99% efficiency (9). Therefore, even a small decrease in tubular function leads to great increases in urinary protein levels. Previous reports from our group demonstrated that Rf-1 congenic animals do not have increased glomerular permeability to albumin (43), thus we hypothesized those genes in the Rf-1 region influence tubular reabsorption of filtered proteins. Many studies involving protein trafficking in the proximal tubule have focused on the megalin/cubilin system (1, 7), as modulation of these receptors results in marked increases in proteinuria. Aside from megalin and cubulin, there are limited reports of protein processing mechanisms in the renal tubule. We recently demonstrated that a mutation in the small GTPase, Rab38, is responsible for increased proteinuria underlying the Rf-2 QTL (48). Interestingly, Rab38 is also involved in tubular reabsorption of proteins, suggesting that there may be many genes and pathways associated with protein trafficking in the renal tubule, besides megalin and cubulin, that remain to be discovered. Neither cubulin nor megalin gene expression was altered in the Rf-1 congenic animals (data not shown), suggesting that Sorcs1 is part of an alternative pathway involved in proximal tubule protein trafficking.

To test the hypothesis that Sorcs1 is involved in tubular processing of proteins, we performed an in vitro albumin reabsorption assay using porcine proximal tubular cells, LLC-PK1 (PK1). PK1 cells were used as it is an established model and offers a cross species test. This assay tests the ability of Sorcs1 to influence protein trafficking as indicated by arrows. D: quantification of reuptake in WT PK1 and shRNA KO Sorcs1 cells. The vesicles were divided into 2 arbitrary groups, i.e., vesicles with <5 or >5 granules for quantification. Arrows indicate endocytotic vesicles.

**SORCS1 is associated with renal function in humans.** The congenic mapping data, in vivo KO and in vitro knockdown studies together suggest that Sorcs1 directly contributes to renal impairment. Since several human genetic studies (15, 27) found evidence for linkage to human disease as Alzheimer and diabetes in the homologous Rf-1 region, we tested if SORCS1 could be associated with kidney disease in two large GWAS cohorts. For the European ancestry cohort, the SORCS1 region was interrogated for eGFR in 67,093 participants (34). We identified a significant association at rs1572456 (minor allele frequency 0.34, median imputation score of 0.93, \( P = 0.0011 \)), which is below the threshold of significance determined by the number of independent blocks queried (0.05/33 blocks). Likewise, there is an association at rs843990 (\( P = 0.00065 \)) for the albumin-creatinine ratio in 1,264 African-American participants of the HyperGen family cohort ascertained initially for hypertension minor allele frequency 0.29. As expected in studies with different ethnic groups, the SNPs were not identical; however, both lie within the SORCS1 gene. Given the strong association evidence from two independent and different racial cohorts, we predict SORCS1 influences renal function phenotypes in humans. Many reports have suggested that susceptibility loci are shared between human and other mammals, so we leveraged the FHH rat to assist identification of renal susceptibility genes in the human and used PK1 cells to test the cellular mechanism. All three species

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**Fig. 4.** Electron microscopy images and quantitative results of the gold-labeled albumin transport study in PK1 cells. A: control cells with endocytic vesicles filled with numerous gold particles. In contrast shRNA KO Sorcs1 (B and C) show only a few gold particles, indicating impairment of protein trafficking as indicated by arrows. D: quantification of reuptake in WT PK1 and shRNA KO Sorcs1 cells. The vesicles were divided into 2 arbitrary groups, i.e., vesicles with <5 or >5 granules for quantification. Arrows indicate endocytotic vesicles.
and all tests performed confirm that Sorcs1 plays a role in the genetic basis of renal disease and appears to be involved in protein trafficking.

### Conclusion

We have used a genetic mapping strategy to localize the Rf-1 gene to a 1.5 Mb region of RNO1, which is only 5% of the original Rf-1 region. This region contains a positional candidate gene, Sorcs1, and three predicted genes. Sorcs1 belongs to a family of mammalian type-I transmembrane receptors with a common VPS10 domain. Knocking out Sorcs1 in vivo in a sensitized strain confirmed our hypothesis that this gene is involved in renal function. We hypothesize that Sorcs1 is part of a new pathway contributing to the regulation of protein trafficking in the proximal tubule. Further studies are required to investigate this potentially new pathway so that we can gain further insight into complex renal disease physiology.

### ACKNOWLEDGMENTS

The authors thank N. Barretto, M. Tschanzen, J. W. Andrea, and B. Schilling for excellent technical assistance and A. Geurts and M. Flister for helpful suggestions. Electron microscopy was performed by C. W. Wells from the Electron Microscopy Facility at MCW.

### GRANTS

This study was performed with financial support from National Heart, Lung and Blood Institute Grant 5RO1HL-069321 to H. J. Jacob.

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### DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

### AUTHOR CONTRIBUTIONS

