ID1 inhibits USF2 and blocks TGF-β-induced apoptosis in mesangial cells

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Diabetes is a leading cause of end-stage renal disease, which is associated with high morbidity and mortality rates (31, 37). The late phase of diabetic nephropathy is characterized by loss of resident glomerular cells, a process that correlates with a decline in glomerular filtration rate (28). Apoptosis leads to the elimination of mesangial cells (MC) associated with progressive glomerulosclerosis (26, 40, 43). It has been shown that loss of glomerular cells through apoptosis occurs in experimental diabetic nephropathy (29), whereas MC apoptosis has been shown to correlate with worsening of albuminuria (22). In overt nephropathy, expansion of the mesangial matrix, loss of MC, and glomerular sclerosis are associated with proteinuria, hypertension, and renal dysfunction (36, 42). In biopsy specimens from patients with IgA nephropathy and systemic lupus erythematosus, the number of apoptotic glomerular cells correlates with the glomerulosclerosis index and loss of renal function (1). The increase in apoptotic glomerular cells in diabetes is not a consequence of inflammatory injury, but of a direct stimulation of proapoptotic pathways in MC (14, 26). It has been shown that transforming growth factor (TGF)-β induces apoptosis in MC (14, 24, 26) and modulates the expression or activity of various components of apoptotic pathways. TGF-β1 induces apoptosis in MC by reducing Bcl-2 and increasing Bax levels in mouse MC (14, 24).

The bone morphogenetic proteins (BMPs) are members of the TGF-β superfamily and antagonists of the profibrogenic effect of TGF-β1 in the kidney (45, 46, 53, 56). Several studies suggest a therapeutic role for BMPs in renal disease. Administration of BMP7 restores, at least in part, renal morphology and function (54). However, while the anti-fibrogenic action of BMPs is well-established, there are no studies on the action of BMPs on MC apoptosis.

MC activation by growth factors, mainly TGF-β, triggers the pathological alterations observed in these cells, such as increase in proliferation, hyperplasia, fibrosis, and death, whereas blockade of specific systems tends to attenuate the glomerular damage (28, 35). However, little is known regarding intermediate and more downstream players, specifically those concerning MC apoptosis.

This study investigates the role of ID1 in MC apoptosis. Here, we show that BMPs rapidly induce ID1 expression in MC. ID1 was responsible for the anti-apoptotic effect of BMPs in MC by inhibiting the transcriptional activity of the TGF-β1-regulated factor, namely USF2, which increases transcriptional activity and expression levels of BAX.

**EXPERIMENTAL PROCEDURES**

Cell culture and treatment. Immortalized human MC (hMCi) were kindly provided by Dr. Bernhard Banas (Ludwig-Maximilians University, Munich, Germany) and grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum. Serum-starved hMCi were treated with TGF-β1 (2 ng/ml), BMP-4 (1 ng/ml), or 7 (5 ng/ml) for various periods of time. Next, cells were collected for ChIP, qPCR, Western blotting, and gene reporter assays.

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) assays were performed as described previously (49, 50). Briefly, after BMP-4 treatment (1 ng/ml for 10 h) to obtain maximal Id1 protein expression, formaldehyde was added at a final concentration of 1% directly to cell cultures. Fixation was carried out at 37°C for 10 min and then stopped by the addition of glycine to a final concentration of 1.25 M. hMCi were collected and rinsed in cold PBS. Cell pellets were resuspended in swelling buffer plus protease inhibitor cocktail (Sigma, St. Louis, MO) and incubated in ice for 20 min. The nuclei were collected and then resuspended in sonication buffer plus protease inhibitors and incubated in ice for 10 min. Cells

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were sonicated (Sonics VCX 130, New Town, CT) in ice at a setting of 40% for 10 pulses and 20 s to obtain DNA fragments with a range of length of ~200–1,000 bp. The chromatin solution was precleared with the addition of protein A/G-plus Agarose (Santa Cruz Biotechnology, Santa Cruz, CA) for 15 min at 4°C. Precleared chromatin from hMCi was incubated with 1 μg of anti-Id1 mouse monoclonal antibody (Sigma) or nonimmune IgG (sc-2027, Santa Cruz Biotechnology, Santa Cruz, CA) for 15 min at 4°C. Precleared chromatin with the addition of protein A/G-plus Agarose (Santa Cruz Biotechnology, Santa Cruz, CA) for 15 min at 4°C was incubated overnight with anti-ID1 (WH3397M2, Sigma), anti-USF2 (WH7392M1, Sigma), anti-BAX (AV02020, Sigma), or for 1 h with anti-β-actin (A1978, Sigma), and another 1 h with proper secondary peroxidase-conjugated antibodies (sc-2030; Santa Cruz Biotechnology). Membranes were developed through chemiluminescence (ECL plus, GE Healthcare). Band density was measured using National Institutes of Health ImageJ software.

Western blotting. Cell cultures were scrapped in RIPA buffer with 1% protease inhibitor cocktail (Sigma). Total protein quantity was measured by BCA Protein assay kit (Bio-Rad Laboratories, Hercules, CA). Samples were loaded and analyzed by Glicine SDS-PAGE (15%). Proteins were transferred to nitrocellulose membranes (0.22 μm) and blocked for 1 h with 5% nonfat dry milk TBS buffer, incubated overnight with anti-ID1 (WH3397M2, Sigma), anti-USF2 (WH7392M1, Sigma), anti-BAX (AV02020, Sigma), or for 1 h with anti-β-actin (A1978, Sigma), and another 1 h with proper secondary horseradish peroxidase-conjugated antibodies (sc-2030; Santa Cruz Biotechnology). Membranes were developed through chemiluminescence (ECL plus, GE Healthcare). Band density was measured using National Institutes of Health ImageJ software.

Plasmids. pDsRed1-Mito (Clontech Lab, Palo Alto, CA) transcribes a red fluorescent protein. The PU3ML-Luc luciferase construct was a gift from Dr. Leslie Heckert from University of Kansas with the formal consent of Dr. Michèle Sawadogo from MD Anderson Cancer Center/University of Texas. This reporter plasmid contains three repetitions of consensus binding sites to USF transcription factors (5’-GATCCTTATAAAGTGTATGGCCACGTGACCA-3’). The expression vectors pCDNA3.1, pUSF1, and pUSF2 were kindly donated by Dr. A. Verhoeven (Erasmus MC, Rotterdam, Netherlands). BAX-Promoter luciferase plasmid and its respective control pGL3-basic were kindly donated by Dr. Moshe Oren (Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot, Israel). Briefly, an insert of 370 bp corresponding to BAX promoter was cloned into Kpn I and Sac I restriction sites of the pGL3-basic luciferase reporter vector (Promega, Madison, WI).

Lipid-based transfection. hMCi were seeded in 24-well plates 1 day before transfection. At a confluence between 40 and 80% cells were transfected by nonliposomal lipid-based technique with the plasmids pU3ML-Luc, pGL3-basic, or BAX-Promoter-Luc using the Effectene Transfection kit (Qiagen), according to the manufacturer’s instructions. Twenty-four hours after transfection, medium containing transfection reagents was replaced by fresh medium, and TGF-β1, BMPs, or vehicle were then added to cells. Total duration of all assays was 48 h. Cells were then washed with PBS and prepared for luciferase activity detection.

Construction of ID1 overexpression vector. Id1-overexpressing adenoviral vector was built with the ViralPower Adenoviral Expression System (Invitrogen, Carlsbad, CA). Human full-length coding sequence of ID1 cDNA was cloned into the pAd/CMV/V5-DEST (confirmed by DNA sequencing). Adenoviral stocks were obtained by transfection of 5 × 10^6 293A cells with 5 μg of vector in Opti-MEM-I without antibiotics. Cells were cultured for 72 h and culture medium was collected, centrifuged at 4°C, 3,000 rpm for 15 min, and stored at −80°C. Adenoviral stocks were titrated in 293A cells. Adenoviral vector LacZ-expressing β-galactosidase was used as control. For apoptosis and gene reporter assays, MC were infected (identical time points used for plasmids transfections) with Ad-ID1 or Ad-LacZ at MOI 30, obtained from previous dose-response experiments (data not shown).

Luciferase assay. Luciferase activity was measured in cell lysates through a nonhomogeneous assay using Glo-Lysis Buffer and Bright-Glo Luciferase assay System (Promega), as recommended by the manufacturer. pDsRed1-Mito was cotransfected as an internal control. FARCyte Plate Reader (Amersham/GE) was used for fluorescence.
(544-nm excitation and 595-nm emission wavelengths) and luminescence quantitation.

Electroporation. hMCI were transfected by electroporation with expressor plasmids pcDNA3.1, pUSF1, or pUSF2 using the Multiporator Eppendorf (Hamburg, Germany). Briefly, hMCI cells were detached and resuspended in 1 ml of hyposmolar electroporation buffer (Eppendorf). This suspension was then transferred to 1.5-ml microtubes and DNA was added to a final concentration of 10 μg/ml. Cells were transferred to electroporation cuvette and submitted to 3 pulses of 150 V, during 100 μs. Next, cells were seeded in six-well plates. Twenty-four hours after electroporation, medium was replaced by medium containing 0.5% serum. After a total of 48 h, cells were collected and total RNA was extracted for further analysis.

Quantitative RT-PCR. Total RNA from cell pellets was extracted (Illustra RNAspin MiniRNA Isolation Kit, GE) and reverse transcriptase reaction (ImpromII, Promega) was performed. qRT-PCR was carried out using the ABI7500 thermocycler (Applied Biosystems, Foster City, CA) and the Quantitect SYBR Green I kit (Qiagen, Düsseldorf, Germany), according to manufacturer’s recommendations. Primer sequences and PCR parameters are provided upon request. Expression of target genes was normalized by GAPDH mRNA levels measured concurrently.

Chromatin morphology analysis. hMCI overexpressing ID1 were cultured in six-well plates and deprived from serum for 24 h in a subconfluent stage, when Hoescht 33342 (0.5 μg/ml; Molecular Probes, Eugene, OR) was added to the medium. Adherent and floating cells were collected and analyzed under UV fluorescence microscopy (×400). Apoptosis was evaluated according to chromatin morphology (30). Two hundred cells per sample were counted. Results were expressed as percentage of apoptotic cells.

Caspase-3 activity assay. Cells were collected as described above (see chromatin morphology analysis). A Caspase-3 Fluorometric Assay Kit (Sigma) was used. Cells were grown in six-well plates in DMEM, collected, and lysed. Protein concentration was normalized by BCA Protein Assay Kit (BioAgency, Sao Paulo, Brazil). Cell lysates were incubated with the same amounts of reaction buffer and 50 mmol/l DEVD-AFC substrate for 2 h at 37°C. Fluorescence was

Fig. 2. ID1 Interacts with the transcription factor USF2. A: representation of ChIP-ID1 clones from hMCI treated with BMP-4, with binding motifs for transcription factors. Four USF2 hits (2 of them in clone #6) in 5 clones were identified. No USF2 binding site was detected in negative controls. RE and dissimilarity levels observed (inset) indicate statistical significance and specificity of the finding. B: hMCI were treated with BMP4, transforming growth factor (TGF)-β1, or vehicle, and immunoprecipitations (IP) of ID1 and USF2 from hMCI protein extracts were performed. Proteins were resolved by SDS-PAGE and analyzed by Western blotting (WB) with anti-USF2 or anti-ID1 antibodies. Successful immunoprecipitation of ID1 and USF2 (positive controls) were shown by Western blot analysis of the precipitates with the monoclonal anti-ID1 or anti-USF2 antibodies. Equal amounts of protein were used for IP. Results with nonimmune IgG are presented as negative controls.
measured with an excitation wavelength of 360 nm and an emission wavelength of 460 nm.

Statistical analysis. Comparisons were performed through Student’s t-test or ANOVA. Results were presented as means ± SE. At least three different samples were analyzed in each experimental group. P < 0.05 was considered significant.

RESULTS

BMPs 4 and 7 induce potent upregulation of ID genes in MC. BMPs 4 and 7 induce concentration-dependent upregulation of all ID genes analyzed (Fig. 1, A and B). ID1 presented the highest fold-changes in mRNA expression levels in all cases. Of note, a concentration as low as 1 ng/ml could produce significant increments in ID mRNA levels. Next, we also evaluated protein expression. As depicted in Fig. 1C, ID1 protein levels were markedly elevated after stimulation with BMP4 and BMP7.

Id1 interacts with the transcription factor USF2. ID genes are classically described as dominant negative modulators of E-proteins in a variety of systems. However, our group failed to demonstrate that Id1 interacts with E-proteins E47 and Mash-1 in MC (data not shown). To investigate alternative interactions between Id1 and/or DNA/proteins, ChIP, sequencing, and bioinformatics analysis were performed. A total of 155 clones obtained from DNA fragments immunoprecipitated with Id1 and 65 clones from immunoprecipitation with nonimmune IgG were screened. ChIP showed that Id1, but not nonimmune IgG, interacts with conserved binding sites for the transcription factor USF2, present (four hits) in three out of five distinct clones (Fig. 2A), with significantly high specificity (dissimilarity ≤ 0.52%; hit by chance ≤ 0.001). In addition, we performed Id1 immunoprecipitations in protein extracts from hMCi pretreated with BMP, TGF-β1, or vehicle followed by Western blot to USF2 and vice versa. These experiments, presented in Fig. 2B, confirm data obtained from ChIP and also demonstrate that Id1 interacts physically with USF2.

BMP inhibits USF2 activity through overexpression of ID1 while TGF-β1 increases it. To better characterize the interaction between Id1 and USF2, we performed gene reporter assays by transfecting hMCi with the plasmid pU3ML-Luc, with repeats of USF2 consensus sequences. Cells were previously infected with adenoviruses overexpressing ID1 or LacZ, and, 24 h later, treated with TGF-β1 (2 ng/ml) and/or BMP-4 (1 ng/ml). As shown in Fig. 3, treatment with BMP-4 significantly reduced USF2 transcriptional activity, whereas TGF-β1 increased it. Also, BMP4 blocked TGF-β1 stimulatory effect. Similarly, Id1 overexpression, even in the presence of TGF-β1, significantly decreased USF2 transcriptional activity in hMCi, confirming the interaction between Id1 and USF2.

Antagonistic roles of TGF-β1 and BMPs on MC apoptosis. Previous studies described the induction of apoptosis in MC by TGF-β (26). We investigated the effect of BMP-7 and ID1 on TGF-β1-induced hMCi apoptosis. Cell death was evaluated by nuclear morphology and caspase-3 activity. Figure 4, A and B, shows that, while TGF-β1 increased apoptosis, BMP7 acted as an anti-apoptotic factor in hMCi. As we witnessed minimal levels of apoptosis in the presence of serum, we were not able to detect differences in death rates when BMP-7 was added on top of 10% FBS. In a separate series of experiments, ID1 was overexpressed in hMCi and 24 h later treated or not with TGF-β1, in serum-free condition. Our results show that the infection process did not affect death rate in serum deprivation, as can be seen in Fig. 5A. The control and Ad-LacZ groups presented similar apoptotic rates. On the other hand, we observed a protective effect by ID1 in cells infected with Ad-ID1. This protective role of ID1 was confirmed through caspase-3 activity assay (Fig. 5B). In addition, we also demonstrate that ID1 overexpression attenuates apoptosis TGF-β1-induced (Fig. 5C), suggesting that the BMP-7 anti-apoptotic action is through ID1.

TGF-β1 increases BAX expression and apoptosis through mediation of USF2. According to qPCR experiments, TGF-β1 (2 ng/ml, 24 h) significantly increased USF2 expression (×2.24; Fig. 6A). Bioinformatics analysis using the Alggen-PROMO tool revealed three USF2 consensus binding sites in the promoter region of BAX, a well-known proapoptotic gene. Accordingly, TGF-β1 also upregulated BAX expression compared with controls (×1.82; Fig. 6B). On the other hand,
TGF-β1 treatment failed to modify Bcl2 mRNA levels (data not shown). To test whether TGF-β1-induced BAX upregulation was mediated by USF2, gene reporter assays were performed. hCMi were cotransfected with BAX-Promoter-Luc and pUSF2 and treated with TGF-β1 or vehicle. BAX transcriptional activity was significantly stimulated (×7.42) when USF2 was overexpressed. Similarly, TGF-β1 treatment also increased the BAX promoter activity (×3.25; Fig. 7). Next, pUSF2 or pcDNA3.1 expression vectors were transfected by electroporation in hMCi cells. Note that USF2 stimulated BAX expression (×1.40; Fig. 8A). It is known that USF2 forms homodimers or heterodimers with USF1 (6, 20, 51). To verify the role of USF1 on BAX expression, we transfected cells with pUSF1, pUSF2, or a combination of both at equivalent concentrations. Figure 8A shows that USF1, alone or combined with USF2, did not modify BAX expression, indicating that the

Fig. 4. TGF-β1 promotes while BMP-7 inhibits apoptosis in human mesangial cells (MC). A: hMCi were treated with TGF-β1 and maintained in the presence or absence of serum for 24 h. Morphological analysis of chromatin was performed by staining cell nuclei with Hoechst 33342 and expressed as percentage of apoptotic cells. n = 6, **P < 0.05, *P < 0.05 vehicle vs. TGF-β1. B: hMCi were pretreated with BMP-7 or vehicle for 6 h and serum was removed from the medium for additional 24 h. BMP7 was readded to cell cultures by the time 10% FBS medium was replaced by serum-free medium (total BMP-7 incubation time = 30 h). Analysis was performed as in A. n = 6, *P < 0.01 vehicle vs. BMP-7.

Fig. 5. ID1 protects human MC from apoptosis. A: hMCi overexpressing ID1 or LacZ (MOI30) were deprived from serum for 24 h. Morphological analysis of chromatin was carried out by staining cell nuclei with Hoechst 33342 and expressed as percentage of apoptotic cells. As controls, cells were cultured in the presence or absence of serum without contact with adenoviral vectors. n = 6, P < 0.01 ID1 vs. LacZ. B: hMCi overexpressing ID1 or LacZ (MOI30) were deprived from serum for 24 h. Caspase-3 activity was determined by as described in EXPERIMENTAL PROCEDURES and expressed as arbitrary units of fluorescence. *P < 0.01 ID1 vs. LacZ. C: hMCi overexpressing ID1 or LacZ (MOI30) were treated with TGF-β1 (2 ng/ml) or vehicle. Serum-free conditions were maintained for 24 h. Morphological analysis of chromatin was carried out as described above. *P < 0.01 vs. control. #P < 0.01 vs. TGF-β1.
regulation observed was mediated by USF2 only. In Fig. 8B, we show that electroporation of hMCi with pUSF2 plasmids induced marked elevations in its protein levels and significant increments also in Bax protein. Finally, we tested whether USF2 was capable of increasing apoptosis in hCMi. As seen in Fig. 8C, USF2 overexpression potentiated serum deprivation-induced cell death by 23%.

**DISCUSSION**

Apoptosis is one of the mechanisms of glomerular cell deletion, essential for the development and progression of glomerular diseases (9, 33). Although apoptosis of glomerular cells (such as MC and podocytes) has been described (43), most studies focused mainly on extracellular matrix overproduction/reduced degradation by MC as the primary cause of glomerular capillary destruction (11, 16, 44). Hence, the characterization of downstream mediators of MC apoptosis is crucial for a better understanding of the pathophysiological processes that affect the glomerulus.

The results presented here show that BMPs act as antiapoptotic agents on hMCi through upregulation of ID1, which interacts with the transcription factor USF2, inhibiting its activity. On the other hand, we identified increases in USF2 activity, BAX expression levels, and TGF-β1-mediated apoptosis in MC, whose levels are often elevated in injured glomeruli.

In this study, we show that TGF-β1 is capable of inducing apoptosis in MC. In agreement with our data, other studies already showed that TGF-β1 induces death by apoptosis in various cell lines, including MC (13, 24, 26). In a condition mimicking diabetic nephropathy, the high-ambient glucose sensitizes MC to the effects of TGF-β1, which subsequently decreases NF-κB activation and in turn alters the expression ratio of BAX:Bcl2. The latter favors caspase-3 activation and increases apoptosis (13). Also, authors proposed a pathway for glucose-induced apoptosis in MC characterized by increased BAX:Bcl2 ratio through redox-dependent mechanisms (3, 12). This signaling cascade also involves the activity of the heterodimeric transcription factor NF-κB. In addition, other studies showed that TGF-β-induced apoptosis in MC is mediated by Smad7 and consequent caspase-3 activation (26), or by casein kinase-2, which phosphorylates and activates the pro-apoptotic protein p53 (24).

Our results show for the first time that TGF-β1-induced, BAX-mediated death of MC depends on USF2. USF2 belongs to the myc family of transcription factors characterized by a bHLH leucine zipper domain, which is responsible for dimerization and DNA binding. Through binding to specific regions called E-boxes (CACGTG), USF2 regulates the expression of many target genes (39).

It is already known that USF2 is involved in the progress of diabetic kidney injury. USF2 is upregulated by high-ambient glucose through CREB-dependent transactivation of USF2 promoter (38), and also by glycated albumin through NF-κB-dependent transactivation (54). USF2 increases the expression of profibrotic genes, among them fibronectin and thrombos-
Fig. 8. Overexpression of USF2, but not USF1, upregulates BAX expression and promotes apoptosis in hMCI. qPCR for BAX gene. A: hMCI (1 x 10^6 cells) were transfected by electroporation with pcDNA3.1 (10 μg/ml), pUSF1 (10 μg/ml), pUSF2 (10 μg/ml), or both (5 μg/ml each). After 48 h, total RNA was extracted. Data were normalized by GAPDH expression levels and expressed as fold-change. n = 4; *P < 0.01 vs. pcDNA3.1. B: Western blot for hMCI electroporated with pUSF2 (10 μg/ml) showing USF2 and BAX protein expression increased. Band intensity was quantified densitometrically. Results are expressed as means ± SE. *P < 0.05. C: hMCI were transfected by electroporation with pcDNA3.1 or pUSF2 (10 μg/ml). After 24 h, cells were deprived of serum. Morphological analysis of chromatin was carried out by staining cell nuclei with Hoechst 33342 and expressed as percentage of apoptotic cells. n = 6, *P < 0.02.
pondin-1, the major regulator of TGF-β activation in renal and cardiac complications of diabetes (19, 47, 48). However, until now there was no evidence on the role of USF2 regarding MC apoptosis.

The profibrotic role of TGF-β in the kidneys is well-established (5, 15, 27, 41a), while BMPs are described as antagonists of these effects (18, 34, 55). BMPs are best known for their role as morphogens during embryonic development, but they also regulate growth, differentiation, chemotaxis, and apoptosis of various adult cell types, including epithelial, mesenchymal, hematopoietic, and neuronal cells (23). However, the role of BMPs on MC death, particularly in pathological settings, is yet to be defined. A recent study using mouse MC suggests that the maintenance of BMP-7 activity by silencing gremlin (a BMP antagonist) protected cells from high-ambient glucose-induced abnormalities, such as apoptosis and increased collagen IV overproduction (55). Another study indicates that BMP-7, through Smad-5 activation, is a differentiation and survival factor for podocytes, reducing diabetic podocyte apoptosis that occurs early on the course of diabetic renal damage (23), as opposed to MC apoptosis, which seems to occur in a later stage (12, 13). Our study supports the notion that BMP, through ID1 upregulation, exerts a protective action against TGF-β1-induced glomerular damage not just by preventing ECM deposition, but also by reducing death.

Despite the potential beneficial effects of BMPs on the diabetic nephropathy, pleiotropic actions of BMPs in various systems do not allow them to be considered suitable therapeutic targets. Similar remarks could be made concerning TGF-β1. Thus, the identification of novel, downstream mediators of BMPs and/or TGF-β1 pathways in the glomerulus seems to be critical for the development of tools for the treatment of diabetic nephropathy. USF2 appears to be a potential target for manipulation in that scenario. The consequences of silencing USF2 in vivo remain to be evaluated and the success of this endeavor will largely depend on demonstrating that its action is cell-specific and selective to EMC production and glomerular cell apoptosis.

Here, we describe a novel regulatory pathway in which ID1 plays a protective role against MC apoptosis by inhibiting USF2 activity (Fig. 9), and thereby potentially contributing to halting the progression of glomerular damage. The role of USF2 as a marker of disease or therapeutic target in diabetic nephropathy is promising, but yet to be tested. Complementary in vivo studies, beyond the scope of this manuscript, are necessary to define the full implications of the findings described here.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

REFERENCES


