Prolactin anterior pituitary expression and circulating levels are reduced in obese and diabetic rats: role of TGF-β and TNF-α

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Lemini M, Ruiz-Herrera X, Ledesma-Colunga MG, Díaz-Lezama N, De los Ríos EA, López-Barrera F, Méndez I, Martínez de la Escalera G, Macotela Y, Clapp C. Prolactin anterior pituitary expression and circulating levels are reduced in obese and diabetic rats: role of TGF-β and TNF-α. Am J Physiol Regul Integr Comp Physiol 308: R792–R799, 2015. First published February 25, 2015; doi:10.1152/ajpregu.00327.2014.—The levels of the hormone prolactin (PRL) are reduced in the circulation of patients with Type 2 diabetes and in obese children, and lower systemic PRL levels correlate with an increased prevalence of diabetes and a higher risk of metabolic syndrome. The secretion of anterior pituitary (AP) PRL in metabolic diseases may be influenced by the interplay between transforming growth factor β (TGF-β) and tumor necrosis factor α (TNF-α), which inhibit and can stimulate AP PRL synthesis, respectively, and are known contributors to insulin resistance and metabolic complications. Here, we show that TGF-β and TNF-α antagonize the effect of each other on the expression and release of PRL by the GH4C1 lactotrope cell line. The levels of AP mRNA and circulating PRL decrease in high-fat diet-induced obese rats in parallel with increased and reduced AP levels of TGF-β and TNF-α mRNA, respectively. Likewise, AP expression and circulating levels of PRL are reduced in streptozotocin-induced diabetic rats and are associated with higher AP expression and protein levels of TGF-β and TNF-α. The opposing effects of the two cytokines on cultured AP cells, together with their altered expression in the AP of obese and diabetic rats suggest they are linked to the reduced PRL production and secretion characteristics of metabolic diseases.

Obesity and its associated disorders, such as Type 2 diabetes, constitute the most prominent socioeconomic health-related burden in the developed world and parts of the underdeveloped world. Obesity and diabetes are characterized mainly by alterations in glucose and lipid metabolism but also in the hormonal and cytokine milieu. Understanding how such changes arise and lead to further complications is essential to developing effective therapies against these metabolic diseases. Prolactin (PRL) is a peptide hormone secreted mainly by the anterior pituitary gland (AP) and is known for its fundamental role in lactation; however, PRL exerts a wide variety of actions in reproduction, osmoregulation, brain function, immune response, and angiogenesis (16, 25). A very relevant, but still underappreciated, role of PRL is the regulation of energy homeostasis and, particularly, its possible contribution to the development and progression of metabolic syndrome and diabetes. Recently, large cohort studies have shown that high circulating PRL levels correlate with reduced prevalence of glucose intolerance and Type 2 diabetes in adults (6, 60) and that low PRL serum levels are observed in obese children compared with lean controls and constitute a predictive factor for progression toward metabolic syndrome (15). Therefore, identifying factors responsible for the downregulation of PRL circulating levels in obesity and diabetes has potential value for the treatment of these metabolic disorders.

In the present work, we investigated whether the production of PRL is downregulated in the AP of obese and diabetic rats and evaluated whether alterations in the AP expression of TGF-β and/or TNF-α correlated with these changes. TGF-β and TNF-α increase in the circulation of obese and diabetic patients (15, 19, 29, 61, 63) and play important roles in insulin resistance and diabetes pathophysiology (59, 61). Also, these cytokines and their receptors are ubiquitously expressed in a variety of tissues from rodents and humans, including the AP PRL-producing cell (lactotrope) (17, 65), where TGF-β and TNF-α can stimulate (27, 36), but also inhibit (30, 58), PRL synthesis and release; however, there is little or no information regarding the actions of TNF-α or TGF-β on PRL secretion in the context of metabolic diseases.

Materials and Methods

GH4C1 cell culture. GH4C1 cells are a subclone of the cell line GH3 isolated from an AP rat tumor that produces both growth hormone and PRL (56). The stock line was maintained between passages 3 and 10. GH4C1 cells were cultured in F10 medium (Sigma Chemicals, St. Louis, MO) supplemented with 15% heat-inactivated horse serum (Gibco, Invitrogen, Carlsbad, CA), 2.5% heat-inactivated FBS (Gibco), and 1% penicillin-streptomycin at 37°C in 5% CO2. Subconfluent GH4C1 cell cultures (5 × 10⁵ cells/well in 24-well plates or 10⁶ cells/well in 12-well plates) were plated and incubated for 48 h. Medium was then changed to high-glucose DMEM-containing 0.5% FBS and antibiotics for 24 h, and the cells were treated with different concentrations of TGF-β and/or TNF-α for 48 h. Recombinant human TGF-β and TNF-α were purchased from R&D Systems (Minneapolis, MN). The concentrations of both cytokines were comparable to those used previously in similar in vitro studies (18, 27). At the end of the incubation, the cells were counted, and their viability was evaluated by the dye-exclusion method.

Animal models of obesity and diabetes. Male Wistar rats were housed under standard laboratory conditions (22°C, 12:12-h light-dark cycle, free access to food and water). All animal procedures were approved by the Bioethics Committee of the Institute of Neurobiology of the National University of Mexico and comply with the U.S. National Research Council’s Guide for the Care and Use of Laboratory Animals (8th ed., National Academy Press, Washington, D.C.). To induce obesity, 4-wk-old rats were fed with a 60% high-fat diet for 24 weeks and had free access to chow (40% of total calories) and water. After 24 weeks, rats were humanely killed at 26–28 wk of age, and blood and tissues were collected. The weight of the anterior pituitary gland was determined after removal and rinsing in PBS. Glucose tolerance tests were performed after a 6-h fast. Rats were intraperitoneally injected with 1 g/kg of glucose, and blood samples were collected at 0, 15, 30, 60, and 120 min after injection. Insulin tolerance tests were performed after a 6-h fast. Rats were intraperitoneally injected with 0.75 U/kg of insulin, and blood samples were collected at 0, 15, 30, 60, and 120 min after injection.
(HFD) (Open Source Diet D12492; Research Diets) or a standard chow (CD) in which 13% of calories comes from lipids (Laboratory rodent Diet 5001, LabDiet, Richmond, IN). To induce diabetes, male Wistar rats (250–300 g) were injected with a single intraperitoneal dose of streptozotocin (STZ; 60 mg/kg in citrate buffer, at pH 4.5) (Sigma-Aldrich, St. Louis, MO) or vehicle (citrate buffer) after an overnight fast. Rats with a blood glucose concentration ≥250 mg/dl were considered diabetic. After 10 wk on the HFD or 6 wk after the STZ injection, animals were anesthetized by CO2 inhalation followed by decapitation between 1000 and 1400 to determine the AP mRNA and circulating levels of PRL, TGF-β, and TNF-α. TGF-β and TNF-α protein levels were also evaluated in the AP of control and diabetic rats 6 wk after STZ injection. To avoid stress-induced PRL release, animals were handled daily for 7 days before death.

Blood glucose and insulin tolerance test. After rats were either fasted overnight or not fasted, blood glucose levels were measured with a glucometer. Blood was collected from the tip of the tail using a lancet needle. ITT was performed by intraperitoneal injection of 0.75 U/kg insulin (Humulin R, Eli Lilly, Indianapolis, IN) after a 1-h fast, and blood glucose was evaluated before (time 0) and at 15, 30, 60, and 120 min after insulin injection.

Real-time quantitative RT-PCR. Total RNA was extracted from frozen AP or cultured GH4C1 cells using the guanidine isothiocyanate method, and cDNA was synthesized using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Warrington, UK). PCR products were detected and quantified with Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, Auburn, AL) in a 10-μl final reaction volume containing template and 0.5 μM of each of the primer pairs for rat PRL: forward 5′-TGG CAC AGA AGG TTT GA-3′, reverse 5′-CCA TGA ACA GCC AAG TGT CA-3′; rat TNF-α: forward 5′-GGG CTT GTC ACT CGA GTT TT-3′, reverse 5′-TGG CTC AGC TTC TCA TT-3′; and rat TGF-β: forward 5′-CAC CAT CAT GTT GGA CAA CTG CTCC-3′, reverse 5′-CTT CAG CTC CAC AGA GAA GAA CTGC-3′. Amplification performed in the CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA) included a denaturation step of 10 min at 95°C, followed by 40 cycles of amplification (10 s at 95°C, 30 s at the primer pair-specific annealing temperature and 30 s at 72°C). The PCR data were analyzed by the 2^−ΔΔCT method, and cycle thresholds were normalized to the housekeeping genes GAPDH (in AP) or 18S (in GH4C1 cells) to calculate the expression levels of genes of interest. Different housekeeping genes were used because the expression of GAPDH was very stable in the APs from the different groups of rats, but not in GH4C1 cells, where its expression varied among treatments. 18S was a good constitutive expression gene in the cell line.

PRL, TGF-β, and TNF-α levels. PRL was measured in serum of CD- and HFD-fed rats by conventional radioimmunoassay (RIA) using standard procedures and reagents provided by the National Hormone and Pituitary Program and by Dr. A. F. Parlow (Harbor University of California, Los Angeles Medical Center) or in control and STZ-induced diabetic rats by the Nb2 cell bioassay, a standard procedure based on the proliferative response of the Nb2 lymphoma cells to PRL (55). Serum TGF-β and TNF-α were quantified by ELISA from R&D Systems (Minneapolis, MN) and BD Biosciences (San Diego, CA), respectively. AP TGF-β and TNF-α levels were measured by ELISAs from Cloud-Clone (Houston, TX) and Sigma-Aldrich (St. Louis, MO), respectively, that were designed to evaluate tissue lysates.

Statistics. All data were replicated in three or more experiments. The statistical analyses were performed using the Sigma Stat 7.0 (Sigma Stat 7.0; Systat Software, San Jose, CA) and the GraphPad Prism (GraphPad Software, La Jolla, CA) software. Statistical differences were determined by the Student’s t-test (between two groups) or by ANOVA (for more than two groups). Differences comparing a combination of two factors (TGF-β or TNF-α) were analyzed by a two-way ANOVA and Bonferroni’s post hoc test. The effect of different doses of only TGF-β were evaluated using one-way ANOVA followed by Bonferroni’s post hoc test. The threshold for significance was set at 5%.

RESULTS

Opposing effects of TGF-β and TNF-α on PRL expression and release by GH4C1 cells. Consistent with the effects shown on AP primary cultures (36, 49), TGF-β inhibits (18, 24), while TNF-α stimulates (27), PRL expression by the GH3 or GH4 lactotrope cell lines. Here, we confirm these findings in GH4C1 cells, a cell line derived from the somatomammotrophic GH3 cells, which in contrast to their precursors primarily produce PRL (56). We show that treatment with different concentrations of TGF-β reduced the levels of PRL mRNA in cell lysates (Fig. 1, A and B) and of PRL in the cell-conditioned media (Fig. 1, E and F). Both effects were prevented and, in some cases, turned into stimulation by the coaddition of a high concentration of TNF-α (50 ng/ml) (Fig. 1, B and F); moreover, stimulation by this TNF-α concentration was reduced in the presence of the higher concentrations of TGF-β (Fig. 1, B and F). Similarly, we found that TNF-α, at various concentrations, stimulates PRL mRNA and secreted protein levels, and showed that stimulation was blocked by the coinubation with a maximal inhibitory concentration of TGF-β (10 ng/ml) (Fig. 1, C and G). The antagonism between the two factors was further illustrated when cells were treated with high concentrations of both cytokines simultaneously, in which case, the levels of PRL mRNA and secreted protein were significantly different from those observed when the cells were treated with each cytokine alone and similar to those of the control, untreated cells, essentially cancelling each other’s effects (Fig. 1, D and H). Neither TGF-β nor TNF-α at their high inhibitory (10 ng/ml) or high stimulatory (50 ng/ml) concentration modified the viability or the number of cells (Fig. 1, I and J).

PRL, TGF-β, and TNF-α AP mRNA and serum levels are modified in HFD-fed rats. Next, we investigated whether PRL levels were altered in rats fed a HFD, a widely accepted model of obesity and metabolic syndrome (1). After 10 wk on a HFD, the rats showed a 30% increase in body weight (Fig. 2A), and they were hyperglycemic in fasted and fed states (Fig. 2B) and insulin-resistant, as evaluated by the insulin tolerance test (Fig. 2C). While both groups responded similarly to a high bolus of exogenous insulin, glucose levels remained higher at all time points in HFD-fed rats compared with controls. Similar curves are commonly observed in this type of experiment and imply that, because of insulin resistance, endogenous insulin is not able to maintain normoglycemia.

The HFD-fed rats showed a 46% reduction in AP PRL mRNA levels, in line with a similar decrease (45%) in serum PRL levels (Fig. 3, A and D). TGF-β mRNA values increased by 3.5-fold, and those of TNF-α decreased by 56% in the AP of HFD-fed rats compared with CD-fed animals (Fig. 3, B and C). The levels of both TGF-β (P = 0.008) and TNF-α (P = 0.006) increased in the circulation of obese animals (Fig. 3, E and F).

PRL, TGF-β, and TNF-α AP mRNA and serum levels are modified in STZ-induced diabetic rats. We then investigated whether similarly altered PRL, TGF-β, and TNF-α AP mRNA and serum levels found in the HFD-induced obesity model could be observed in other diabetes models, particularly in the STZ-treated rat, a well-characterized model of Type 1 diabetes.
Six weeks after a single intraperitoneal injection of STZ, rats showed a 21% reduction in body weight and marked hyperglycemia, compared with control, vehicle-treated rats (Fig. 4, A and B). PRL AP mRNA and circulating levels were reduced by 26% and 85%, respectively, in STZ-treated compared with control rats (Fig. 5, A and D). Of note, the serum PRL values in control rats were slightly higher than expected for normoprolactinemia and may reflect a mild stress response to the handling procedure. AP TGF-β and TNF-α mRNA values increased significantly in STZ-injected rats ($P < 0.0006$ and $P = 0.03$ vs. control for TGF-β and TNF-α, respectively) (Fig. 5, B and C). Moreover, in line with their mRNA levels, the protein values of both cytokines increase in the AP of STZ-treated rats compared with control animals ($P = 0.048$ and $P = 0.002$ vs. control for TGF-β and TNF-α, respectively) (Fig. 5, E and F).

**DISCUSSION**

PRL functions as a metabolic hormone. It affects metabolic homeostasis by promoting the proliferation, survival, and in-
Insulin production of pancreatic β-cells (26, 57), and by stimulating food intake (11), liver insulin sensitivity (64), and adipose tissue growth and function (8). Large cohort clinical studies showed that reduced PRL serum levels, within the physiological range, are associated with diabetes and impaired glucose regulation (6, 60). Also, lower PRL circulating levels in obese children correlate with increased insulin resistance, inflammation, and body mass index (15). Consistent with the clinical studies, circulating levels of PRL are reduced in STZ-treated diabetic rats (9, 54), in mice rendered obese by chemical means (51), and in the ob/ob (38) and db/db mouse (50) models of diabetes and obesity. The 24-h pattern of plasma PRL levels is also disrupted in HFD-fed male rats (13), and serum PRL levels are reduced in HFD-fed female mice (52).

Here, we confirmed the reduced circulating levels of PRL found in STZ-induced diabetes in rats and found lower PRL serum levels in obese male rats fed a HFD. Moreover, we show that PRL mRNA levels are also reduced in the AP of both STZ-induced diabetic and HFD-fed obese rats, suggesting that downregulation of PRL synthesis contributes to the decrease in systemic PRL. Lower PRL mRNA levels may also reflect a reduction in the number of lactotropes. Along this line, the AP PRL content and the number of AP PRL secretory granules and lactotropes are reduced, and lactotrope apoptosis is increased in STZ-induced diabetic rats (3, 4, 62).

Dopamine (DA) is the main inhibitor of AP PRL synthesis and release (7), and several DA-dependent and independent factors may act centrally and locally to regulate AP PRL synthesis and release in metabolic diseases (37, 43). Increased fat mass, hyperinsulinemia, and hyperleptinemia may modify PRL secretion in obesity and diabetes (37). However, both HFD-fed obese rats and STZ-induced diabetic rats show reduced AP PRL synthesis and release despite their opposite changes in weight gain (Refs. 1 and 33, present results), leptin (5, 53), and insulin (33, 44) levels, suggesting that other factors...
besides increased fat mass, hyperleptinemia, and hyperinsulinemia may affect PRL levels in these animals. In search of PRL regulators common to both models, we evaluated TGF-β and TNF-α.

TGF-β and TNF-α increase in the circulation of obese and diabetic humans (15, 19, 29, 61, 63), HFD-fed rodents (20, 61), and STZ-diabetic rats (4, 21, 22). TGF-β promotes adiposity and glomerular pathology in obesity and diabetes, and both the adipose and renal tissues are major sources of TGF-β released into the circulation (14, 22, 23, 61). Also, TNF-α is produced and released by stressed adipocytes from fat depots and by activated macrophages from tissues such as fat, muscle, and liver to have local and systemic effects on metabolism, inflammation, and vascular function (29, 31). Of note, both cytokines have direct effects on PRL secretion. TGF-β inhibits and TNF-α can stimulate PRL synthesis and release by cultured lactotropes (18, 24, 27, 36, 49). Because both cytokines increase in obesity and diabetes, we investigated the outcome of their combined treatment on PRL secretion by the GH4C1 lactotrope cell line. We observed that TGF-β inhibited and TNF-α stimulated PRL synthesis and release in a dose-dependent manner, and these effects did not appear to involve altered cell viability or proliferation. Notably, the two cytokines antagonized the effect of each other, suggesting that their relative concentrations could determine whether PRL synthesis is up-regulated or downregulated. We hypothesized that, if these cytokines were involved in the PRL downregulation seen in obesity and diabetes, TGF-β inhibitory effects would prevail over the stimulatory action of TNF-α.

Our findings measuring the circulating concentrations of both cytokines agree with studies showing increased TGF-β levels in HFD-fed rodents and of TNF-α in HFD-fed and STZ-treated rats. However, in contrast to previous work (22), TGF-β systemic levels were reduced in STZ-treated diabetic rats. The reason for this discrepancy is unclear. TGF-β in serum may represent TGF-β released by the kidney due to diabetic nephropathy (14). It is possible that the earlier stage of nephropathy associated with the shorter exposure to diabetes in our study (6 vs. 10 wk) influenced TGF-β circulating levels. However, TGF-β and TNF-α values affecting PRL secretion in the pituitary milieu may not necessarily mirror their circulating levels. Both cytokines are expressed in the AP. The production of TGF-β appears to be restricted to folliculostellate cells (32) and lactotropes (10). In the latter, TGF-β synthesis is upregu-

![Fig. 4](image-url) Streptozotocin injection results in body weight loss and hyperglycemia in rats. Body weight (A) and blood glucose levels (B) were evaluated in rats 6 wk after a single intraperitoneal injection of vehicle (CTRL) or streptozotocin (STZ; 60 mg/kg). Values are expressed as means ± SE. Numbers inside parentheses indicate n values. P values are provided above each panel.

![Fig. 5](image-url) Levels of PRL, TGF-β, and TNF-α mRNA in the anterior pituitary and the corresponding proteins in serum are altered in STZ-induced diabetic rats. Anterior pituitary (AP) mRNA levels of PRL (A), TGF-β (B), and TNF-α (C) were evaluated by qRT-PCR, serum levels of PRL (D) were evaluated by the Nb2 cell bioassay, and AP and serum levels of TGF-β (E, F) and TNF-α (C, F) were determined by ELISA in rats 6 wk after a single intraperitoneal injection with vehicle (CTRL) or STZ. Values are expressed as means ± SE. Numbers inside parentheses indicate n values. P values are provided above each panel.
lated by dopamine and downregulated by estrogens to modulate PRL secretion and lactotrope proliferation (10, 46, 48). TGF-α is produced by AP macrophages and somatotrophs (2). Therefore, an important question is whether the levels of TGF-β and TNF-α are altered in the AP under the conditions of obesity and diabetes.

To our knowledge, this is the first report showing that the expression of TGF-β increases and that of TNF-α decreases in the AP of HFD-induced obese rats, reciprocal changes that, if translated into opposite protein levels, could together explain the decrease in PRL synthesis and release. We also show that the AP mRNA and protein levels of TGF-β and TNF-α are upregulated in STZ-induced diabetic rats. The increase in TNF-α expression is consistent with a previous report showing elevated levels of the TNF-α protein in the AP of STZ-induced diabetic rats that were investigated as a mechanism inducing the apoptosis-mediated loss of lactotropes occurring in diabetes (4).

The higher expression of both TGF-β and TNF-α in the AP of diabetic rats, the reduced levels of circulating TGF-β in diabetes, and the systemic rise of TNF-α in obesity and diabetes are difficult to reconcile with the lower expression and circulating levels of PRL. From the cell line data, an increase in TNF-α would cancel out the inhibitory effect of TGF-β on PRL. A possible explanation for a prevailing effect of TGF-β in spite of the upregulation of TNF-α may relate to the endogenous concentrations of the two cytokines and their receptor binding affinities. In diabetic rats, the AP concentrations are similar (122 vs. 72 pg/mg of protein for TGF-β and TNF-α, respectively) but the $K_d$ value (50 pM) of the TGF-β receptor-2 (TGF-β R-2) is 20- and 7-fold lower than the $K_d$ values of the TNF-α R-1 (1.23 nM) and the TNF-α R-2 (0.35 nM), respectively (40, 47). TGF-β R2 forms a heteromeric complex with TGF-β R1 and is essential for all TGF-β-induced signaling (40, 45). Moreover, the circulating levels of TGF-β measured in diabetic (316 pg/ml, i.e., 25.3 pM) and in HFD-fed (674 pg/ml, i.e., 53.9 pM) rats are similar to the $K_d$ concentration of the TGF-β R-2, whereas the systemic levels of TNF-α in diabetic (47 pg/ml, i.e., 2.8 pM) and HFD-fed (39 pg/ml, i.e., 2.3 pM) rats are more than 400- and 100-fold lower than the $K_d$ values of the TNF-α R-1 and TNF-α R-2, respectively. Therefore, it is possible that TGF-β would be more effective than TNF-α at their endogenous levels. Alternatively, it should also be noted that the effect of TNF-α may not occur or may turn into inhibition, depending on complex in vivo interactions. Concentrations of TNF-α similar to those found to be stimulatory in our study, may have either no effect (41), inhibit PRL release (30, 58), or promote lactotrope apoptosis (12), depending on the duration of TNF-α exposure, and on the sex, influence of gonadal steroids, and age of animals from which the cells were obtained. Also, lactotrope apoptosis occurs in STZ-treated rats (4), so it is possible that STZ-mediated upregulation of TNF-α could help lower AP PRL indirectly by promoting lactotrope apoptosis.

The altered pituitary TGF-β and TNF-α expression profiles and circulating levels found in obesity and diabetes, together with the direct reciprocal effects of the two cytokines on AP PRL synthesis and release, suggest that TGF-β and TNF-α play both local and systemic regulatory roles to inhibit PRL secretion in metabolic diseases. Evaluating whether specific, in vivo blockage of the two cytokines in the AP milieu modifies PRL secretion should test this putative causative link.

The functional interaction between TGF-β and TNF-α raises the question of what controls their AP production in obesity and diabetes. TGF-β is upregulated by DA in lactotropes (46, 48) and may be linked to the overactivation of hypothalamic DA occurring in obesity (51, 52) and diabetes (54). Production of TNF-α in the AP could be a stress response (34). TNF-α can affect the secretion of ACTH by AP cells (28), and TNF-α is upregulated in the AP by a relatively weak, systemic inflammatory stress (34), which occurs in obesity (39) and Type 1 diabetes (42). Chronic poor metabolic control also triggers TNF-α expression (21), and hypoglycemia stimulates PRL secretion in poorly controlled diabetic patients (35). Further research is needed to investigate whether the altered expression of TGF-β and TNF-α in the AP could influence metabolic disorders by other mechanisms besides PRL.

**Perspectives and Significance**

The present study demonstrates that reduced AP PRL secretion in obese and diabetic rats correlates with altered changes in the AP expression of two metabolically relevant cytokines, TGF-β and TNF-α. We hypothesize that an imbalance in the AP between the two cytokines, derived from their altered local and systemic levels, favors PRL downregulation in metabolic disorders. These findings help establish the context of TGF-β and TNF-α regulation of PRL, a hormone with recognized influence on metabolic homeostasis that, when downregulated, may aggravate metabolic alterations resulting from obesity and diabetes. However, further studies are needed to prove these hypotheses.

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**DISCLOSURES**

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

**AUTHOR CONTRIBUTIONS**


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