Enhanced insulin sensitivity mediated by adipose tissue browning perturbs islet morphology and hormone secretion in response to autonomic nervous activation in female mice

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Omar BA, Kvist-Reimer M, Enerbäck S, Ahrén B. Enhanced insulin sensitivity mediated by adipose tissue browning perturbs islet morphology and hormone secretion in response to autonomic nervous activation in female mice. Am J Physiol Endocrinol Metab 310: E81–E90, 2016. First published November 3, 2015; doi:10.1152/ajpendo.00296.2015.—Insulin resistance results in a compensatory increase in insulin secretion to maintain normoglycemia. Conversely, high insulin sensitivity results in reduced insulin secretion to prevent hypoglycemia. The mechanisms for this inverse adaptation are not well understood. We utilized highly insulin-sensitive mice, due to adipocyte-specific overexpression of the FOXC2 transcription factor, to study mechanisms of the reversed islet adaptation to increased insulin sensitivity. We found that Foxc2TG mice responded to mild hyperglycemia with insulin secretion in wild-type mice was absent in Foxc2TG mice, suggesting impaired sympathetic signaling to the islet. Basal glucagon was increased in Foxc2TG mice, but they displayed severely impaired glucagon responses to cholinergic and autonomic nervous stimuli. These data suggest that the autonomic nerves contribute to the islet adaptation to high insulin sensitivity, which is compatible with a neuro-adipo regulation of islet function being instrumental for maintaining glucose regulation.

METHODS

Animals. The generation of transgenic mice overexpressing the Foxc2 gene in adipose tissue under the direction of the AP2 promoter has been described previously (15). The AP2 gene promoter is specific for mature adipocytes, and the transgene is active from the time of energy homeostasis, it would stand to reason that an adaptation to high degrees of insulin sensitivity is required to continue to maintain normal blood glucose levels. This would also imply a consequent inverse relationship between insulin sensitivity and insulin secretion such that higher insulin sensitivity would be expected to result in lower insulin secretion (3, 11, 22). This was indeed found in a study of elite athletes with high degrees of insulin sensitivity showing that basal and stimulated levels of insulin were decreased compared with sedentary individuals with lower insulin sensitivity when blood glucose levels were equal (6). Similarly, the improved insulin sensitivity induced by weight reduction after bariatric surgery is associated with reduced insulin secretion (18). The mechanistic basis for this “reverse adaptation” to insulin sensitivity has not been characterized.

In recent years, there has been an interest in the enhancement of insulin sensitivity by transitioning insulin-resistant white adipose tissue into energy-dissipating brown adipose tissue, i.e., the so-called “browning” of white adipose tissue (8). In rodents and humans, the transcription factor FoxC2 has been shown to be one of the key mediators of browning of white adipose tissue (15, 30). Mice with an adipose tissue-specific overexpression of the Foxc2 transcription factor show browning of white adipose tissue, with increased expression of brown fat-associated genes uncoupling protein 1, β3-adrenergic receptor, and others as well as increased oxygen consumption in mitochondria and multilocular lipid droplets in white adipocytes (15). The adipose tissue-specific Foxc2 transgenic mouse is lean and resistant to high-fat diet-induced obesity (28). However, whether and by which mechanism the enhanced insulin sensitivity affects islet function has not been defined in this model. Therefore, we have undertaken an in-depth study to understand the cellular and physiological mechanisms that drive adaptation to increased insulin sensitivity to maintain normal glucose regulation, using multiple metabolic challenges in this unique model. We found that the reverse adaptation to insulin sensitivity is mediated primarily by altered neural signaling to the pancreatic islet such that enhanced systemic insulin sensitivity causes increased sympathetic nervous input to the islet to suppress insulin, enhance basal glucagon secretion, and thus maintain normal glucose levels and avoid hypoglycemia.

BLOOD GLUCOSE HOMEOSTASIS is one of the most tightly regulated processes in mammalian physiology. In healthy humans, glucose levels vary by only a few percentage points throughout a 24-h period (40). During feeding, this tight regulation is mediated mainly by interplay between insulin levels and the induction of glucose uptake in insulin-sensitive tissues (9). In contrast, during fasting, glucose regulation is mediated primarily by glucagon-induced release of stored glucose from the liver (21). In overweight and obese individuals, the sensitivity of the liver, muscle, and adipose tissue to insulin decreases; however, the pancreatic β-cells adapt by secreting more insulin to maintain normal glucose uptake in these tissues and maintain normal blood glucose (41). Consequently, the loss of the ability of the β-cells to adapt to systemic insulin resistance is a primary defect causing type 2 diabetes (22). Because tight glucose regulation is such a fundamental factor for normal

adipocyte maturity. Transgenic mice heterozygous for the Foxc2 transgene on a C57BL6/J background were back-crossed for multiple generations onto the C57BL6/JBomTac background. Four- to five-month-old female mice were used for all experiments. We have shown previously that the glucagon response to 2-deoxyglucose (2-DG) is more pronounced in female than in male mice (27). Approval for all animal experiments was received from the regional ethics committee in Lund, Sweden. 

Euglycemic hyperinsulinemic clamp studies. For euglycemic hyperinsulinemic clamp experiments, mice were anesthetized with a single intraperitoneal injection of fentanyl (0.78 mg/kg)-fluanisone (25 mg/kg) (Hypnorm Vetpharma) and midazolam (12.5 mg/kg) and rested for 30 min prior to receiving a primed, continuous infusion of insulin (30 μU·kg−1·mouse−1); Actrapid Novo Nordisk, Bagsvaerd, Denmark) and variable glucose (40% d-glucose in saline) for 90 min. Blood glucose was monitored every 5 min with a hand-held glucose monitor (HemoCue, Angelholm Sweden). Plasma samples were taken at time 0, 60, and 90 min for the measurement of insulin.

Hyperglycemic clamp studies. Anesthetized mice were given a single intravenous injection of d-glucose in the tail vein to elevate blood glucose to the target level and then infused with variable glucose (30–40% d-glucose in saline) to maintain blood glucose at the target level for 90 min. Plasma samples were taken at 0, 1, 5, 20, 50, 70, and 90 min for measurement of insulin.

Intravenous glucose, 2-DG, arginine, and carbachol challenges. Anesthetized mice were fasted for 5 h and then given a single intravenous injection of d-glucose (0.35 or 1.0 mg/kg), 2-DG (50 mg/mouse), l-arginine (5 mg/mouse), or the muscarinic receptor agonist carbachol (30 μg/kg) in the tail vein. Blood was sampled from the intraorbital retrobulbar sinus plexus at 0, 1, 5, 10, 20, 30, and 50 min for glucose, 0, 2, 10, and 30 min for 2-DG, and 0, 1, 5, 10, 20, and 50 min for arginine and carbachol injections. Plasma was separated by centrifugation, and glucose, insulin, and glucagon were measured.

Ex vivo insulin and glucagon secretion from isolated islets. Pancreata were perfused in situ with Hanks’ balanced salt solution (HBSS) containing 2.7 U collagenase P (Roche, Mannheim, Germany), removed, and incubated at 37°C for 19 min. Digested pancreata were washed with HBSS, and islets were hand-picked in an inverted microscope. Freshly picked islets were preincubated in HEPES buffer containing 125 mmol/l MgCl2, 25 mmol/l HEPES (pH 7.4), 5.6 mmol/l glucose, and 0.1% fatty acid-free BSA (Boehringer Mannheim). Islets were then incubated in batches of three per well in eight replicates and incubated with varying glucose concentrations with or without parasympathetic (carbachol) or sympathetic (clonidine) neurotransmitter analogs (Sigma-Aldrich, St. Louis, MO) for 60 min. Media samples are taken at 60 min for measurement of insulin and glucagon.

Immunohistochemistry. Pancreata were extracted and fixed in 4% paraformaldehyde in 0.1 mmol/l PBS containing 0.1% picric acid prior to cryopreservation in 20% (vol/vol) sucrose in PBS. Pancreata were frozen in optimal cutting temperature compound, and 10-μm sections were cut and mounted. Sections were immunostained using antisera against rat proinsulin (1:280 dilution) and rabbit glicentin for glucagon detection (1:10,560 dilution) (both from Eurodiagnostica, Malmö, Sweden). Glucose transporter 2 (GLUT2) was detected using rabbit anti-rat GLUT2 antiserum (1:200 dilution) (Chemicon International, Temecula, CA). Islet areas were quantified as described previously (36, 37). Briefly, Alexa fluor-coupled fluorescent secondary antibodies (goat anti-guinea pig Alexa 546 for insulin and goat anti-rabbit Alexa 488 for glucagon) were used in double-staining experiments. Fluorescent images were captured in an Olympus BX 51 fluorescent microscope with an Olympus DP-50 digital camera. Islet size was determined manually by interactively defining the outline of each individual islet, and the area covered was determined in the program National Institutes of Health Image 1.06 after calibration. A total of five to seven sections from each pancreas were analyzed.

Biochemical analyses. Plasma glucose was determined by the glucose oxidase method with 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) as the substrate. Plasma insulin was determined by radioimmunoassay (Linco, St. Louis, MO). Plasma glucagon was determined by radioimmunoassay (Linco).

Statistical analyses. All data are presented as means ± SE. The acute insulin and glucagon responses are calculated as the mean of the 1- and 5-min values after baseline subtraction during an IVGTT or hyperinsulinemic clamp, whereas acute insulin response (ΔAIR) is the baseline-subtracted 1-min value only. For 2-DG challenges, the acute insulin and glucagon responses were calculated as the baseline-subtracted mean 2- and 10-min values, whereas ΔAIR was the baseline-subtracted 2-min value only. Area under the curve was calculated using the trapezoidal rule. Statistical significance was determined by the nonparametric Mann-Whitney U-test unless otherwise stated. Statistical calculations were made using the GraphPad Prism 6.0 software package.

RESULTS

Foxc2 transgenic mice have lower basal glucose and insulin and are highly insulin sensitive. Body weight and basal plasma parameters were obtained to assess the basal metabolic state of Foxc2 transgenic mice. Foxc2 transgenic mice had a slightly higher body weight compared with wild-type mice (Fig. 1A). Four- and 16-hour fasting plasma glucose were significantly lower in Foxc2 transgenic mice (Fig. 1B), as was 4-h fasting insulin (Fig. 1C), suggesting an increased insulin sensitivity. However, there was no significant difference in plasma insulin after an overnight fast. To assess whole body insulin sensitivity, euglycemic hyperinsulinemic clamps were performed. Blood glucose was clamped at 6.5 mmol/l under a 30-min steady-state period (Fig. 1D). During the steady-state period, the glucose infusion rate needed to maintain euglycemia, a direct measure of insulin sensitivity, was threefold higher in Foxc2 transgenic mice compared with wild-type mice (Fig. 1, E and F).

Foxc2 transgenic mice respond to high insulin sensitivity with a glycemia-dependent reduction in insulin secretion in vivo. To determine the effect of the notable increase in insulin sensitivity on insulin secretion, Foxc2 transgenic mice were subjected to hyperglycemic clamp experiments at three different levels of hyperglycemia. The acute insulin response to glucose during the hyperglycemic clamps showed a distinct pattern that was dependent on the degree of hyperglycemia. When clamped at 11.1 mmol/l glucose, the acute insulin response was significantly lower in Foxc2 transgenic mice and the second phase of insulin secretion nearly absent, suggesting adaptation to increased insulin sensitivity with decreased insulin secretion (Fig. 2, A, D, and G). When clamped at 16.7 mmol/l glucose, approximately twofold higher than normal nonfasted blood glucose, there was no difference in the acute insulin response or second-phase insulin secretion between the wild-type and Foxc2 transgenic mice (Fig. 2, B, E, and H). When clamped at 33 mmol/l glucose, to establish maximal insulin secretory responses to glucose in vivo, Foxc2 transgenic mice exhibited significantly greater acute insulin responses than wild-type controls, with no differences in second-phase insulin apparent (Fig. 2, C, F, and I).

Foxc2 transgenic mice have normal insulin responses to arginine and cholinergic agonists but impaired responses to autonomic nervous activation by 2-DG. In addition to glucose, we also determined the insulin secretory responses to nonglu-
due to parasympathetic nerve activation (24). It was found that the acute suppression of insulin by 2-DG was completely absent in Foxc2 transgenic mice (Fig. 3, F and I). In contrast, the subsequent insulin secretion from 2 to 30 min was not different between wild-type and Foxc2 transgenic mice (Fig. 3F).

Foxc2 transgenic mice have enhanced basal glucagon but impaired glucagon secretion in response to cholinergic and autonomic stimuli. Because carbachol is a mimetic of acetylcholine and 2-DG stimulates both cholinergic and adrenergic nervous activity, the diminished glucose excursions in response to these stimuli suggested impaired glucagon secretion and/or action in response to autonomic nervous activation in Foxc2 transgenic mice. We next investigated glucagon secretion in the basal and stimulated states in Foxc2 transgenic mice. Foxc2 transgenic mice had twofold greater basal plasma glucagon than wild-type mice after a 5-h fast (Fig. 4A). Interestingly, there was no increase in basal plasma glucagon in response to a prolonged fast in Foxc2 transgenic mice (Fig. 4B).

We then proceeded to investigate glucagon secretion in response to the nonglucose secretagogues. Despite the higher basal plasma glucagon in Foxc2 transgenic mice (Fig. 4C), the acute glucagon response to arginine was not significantly different between wild-type and Foxc2 transgenic mice (Fig. 4F).

Whereas adrenergic receptor activation in the islet is inhibitory for insulin secretion and stimulatory for glucagon secretion, cholinergic receptor activation is stimulatory to both (1,
Carbachol activates cholinergic muscarine 3 receptors on α-cells (17) as well as β-cells, whereas 2-DG stimulates both sympathetic adrenergic and parasympathetic cholinergic neurotransmission to the islet. Intravenous injection of carbachol resulted in a significantly blunted acute glucagon response in Foxc2 transgenic mice compared with wild-type mice despite having higher basal glucagon (Fig. 4, D and G). 2-DG administration resulted in significantly impaired acute glucagon responses in Foxc2 transgenic mice (Fig. 4, E and H) as well as impaired total glucagon secretion (Fig. 4E).

Foxc2 transgenic mouse islets have normal responses to parasympathetic and sympathetic neurotransmitter analogs ex vivo. Because the insulin and glucagon secretory responses to 2-DG were impaired in Foxc2 transgenic mice in vivo but the insulin secretory response to carbachol was normal, it was unclear as to whether the defect in hormone secretion was due to altered transmission of neural signals to the islet, altered response of the islets to neurotransmitter stimuli, or both. If neural signaling to the islet is impaired in vivo, then isolated islets should respond normally to stimulation by neurotransmitters in vitro if post-receptor signaling and function are unaltered. Therefore, we next performed in vitro insulin secretion experiments in islets isolated from wild-type and Foxc2 transgenic mice. Similar to the insulin secretion in vivo during hyperglycemic clamps, islets from Foxc2 transgenic mice had slightly but not significantly lower insulin secretion in response to 11.1 mmol/l glucose (Fig. 5A). However, more importantly, islets from wild-type and Foxc2 transgenic mice displayed similar increases in insulin secretion in response to the parasympathomimetic carbachol (Fig. 5B). Furthermore, glucose-stimulated insulin secretion was suppressed to similar extents by the α2-receptor agonist clonidine in wild-type and transgenic mice (Fig. 5B). Arginine stimulates insulin secretion independently of any receptor activation, and in the presence of arginine, carbachol stimulated and clonidine inhibited insulin secretion to similar extents in both wild-type and Foxc2 transgenic mice (Fig. 5B).

Foxc2 transgenic mice have smaller islet size compared with wild-type mice. Although it was clear that there was low basal and glucose-stimulated insulin secretion and impairments in glucagon secretion in response to autonomic neurotransmission, it was unclear whether this was due to alterations in islet morphology. Therefore, we next investigated islet morphology in Foxc2 transgenic mice. Immunohistochemical analysis of

Fig. 2. Dynamic insulin secretion in vivo determined by hyperglycemic clamp. Plasma glucose (A–C) and insulin (D–F) in mice fasted for 5 h and given a bolus injection of 2-glucose followed by variable infusion of glucose to reach target blood glucose of 11.1 (A, D, and G), 16.7 (B, E, and H), or 33.3 mmol/l (C, F, and I) and which was maintained at a steady state for 90 min. The acute insulin response (ΔAIR) to glucose was calculated as described in METHODS; n = 7–11/group. Data are presented as means ± SE. *P < 0.05 and **P < 0.01, probability level of random difference between the WT and Foxc2TG mice.
pancreatic sections showed that the core-mantle structure, typical of rodent islets, was unaltered in Foxc2 transgenic mice (Fig. 6A). However, evaluation of islet areas in wild-type and Foxc2 transgenic mice revealed significantly smaller total areas of islets from Foxc2 transgenic mice (Fig. 6B). This was associated with a significantly lower insulin-positive area of islets of Foxc2 transgenic mice (Fig. 6C) but not the glucagon-positive area (Fig. 6D).

DISCUSSION

It has been more than 30 years since a number of studies demonstrated that insulin-resistant subjects have a compensatory increase in insulin secretion (10, 11, 23). Despite much research on this area since then, relatively few studies have demonstrated the logical reverse that increased insulin sensitivity is compensated for by decreased insulin secretion. A few studies have been performed on elite athletes as a model of increased insulin sensitivity, each showing reduced fasting and stimulated insulin secretion in response to oral or intravenous glucose or arginine (6, 35). After bariatric surgery, an increase in insulin sensitivity after weight loss is met with a decrease in insulin secretion (18). This inverse adaptation would functionally be a safeguard against increased risk for hypoglycemia in high insulin sensitivity. It is known that a degree of expansion of functional \( \beta \)-cell mass partly explains the mechanism by which insulin resistance is compensated for by increased insulin secretion. A mechanism for how the body adapts to increased insulin sensitivity has not been described to date.

The findings of this study suggest that the metabolic response to increased systemic insulin sensitivity is a decrease in basal and glucose-stimulated insulin secretion at physiological glucose levels. Therefore, this decreased insulin secretion is appropriate for the ambient level of insulin sensitivity. The islets have smaller insulin-positive areas in response to increased insulin sensitivity, just as insulin-positive islet area increases in response to insulin resistance (7). When challenged with high glucose there was no difference in insulin secretion between wild-type and transgenic mice, but when challenged with supraphysiological amounts of glucose there was a greater insulin secretory response in transgenic mice, suggesting that there is no functional impairment of the \( \beta \)-cell population resulting from the high level of insulin sensitivity,
The regulation of islet hormone secretion by the nervous system involves autonomic nerves, sensory nerves, and neuropeptides (1). Sympathetic nervous signaling results in decreased insulin secretion and increased glucagon secretion, as energy in the form of glucose needs to be rapidly mobilized from stores under conditions of acute stress (24). Parasympathetic nervous signaling to the islet occurs via the vagal nerve (5). Parasympathetic vagal stimulation results in the secretion of the major islet hormones insulin, glucagon, somatostatin, and pancreatic polypeptide (4, 12). In addition to autonomic signals, there are sensory nerves innervating the islet that affect islet hormone secretion (38). Similar to sympathetic nervous signaling, sensory nervous activation and the release of calcitonin gene-related peptide within the islet are inhibitory to insulin secretion and stimulatory to glucagon secretion (2).

As tools for investigating autonomic stimuli in vivo, we used the cholinergic agonist carbachol and the autonomic activator 2-DG, which is a nonmetabolized glucose analog that through competition with glucose uptake and intracellular phosphorylation creates intracellular glycopenia (14, 42). Since the central nervous system is highly dependent on glucose availability, systematic administration of 2-DG results in neuroglycopenia (31), which activates the autonomic nervous system, as evidenced by increased plasma catecholamine levels and pancreatic sympathetic activation (20). This in turn results in increased glucagon and insulin secretion as we have demonstrated previously in mice (24, 26), and since these responses are inhibited by both muscarinic blockade and α-adrenergic blockade (25), it is clear that both the parasympathetic and sympathetic branches of the autonomic nervous system are activated by 2-DG in mice. We observed increased basal glucagon secretion but impaired glucagon secretion in response to cholinergic or autonomic stimuli. There appeared to be a dysregulation between autonomic nervous signaling and the islet, as the suppressive action of adrenergic signals on insulin secretion and the stimulatory effect of adrenergic and cholinergic signals on glucagon secretion were impaired in Foxc2 transgenic mice. This could be due to an increase in the basal sympathetic activity in response to the extreme insulin sensitivity seen in Foxc2 transgenic mice. Foxc2 transgenic mice

Fig. 4. Plasma glucagon after fasting or injection of nonglucose secretagogues. Plasma glucagon in mice fasted for 4 (A) or 16 h (B) \((n = 11–14/\text{group})\). Plasma glucagon in mice fasted for 5 h and given single intravenous injections of 1-arginine \((n = 4; C)\), carbachol \((n = 6; D)\), or 2-deoxyglucose \((n = 11; E)\). The acute glucagon responses \((\text{AGR})\) to each secretagogue, 1-arginine \((F)\), carbachol \((G)\), and 2-deoxyglucose \((H)\), were calculated as described in METHODS. Data are presented as means ± SE. *\(P < 0.05\), **\(P < 0.01\), and ***\(P < 0.001\), probability level of random difference between the WT and Foxc2 TG mice.
had decreased basal insulin and increased basal glucagon, which would be expected if sympathetic signaling to the islet was increased. In fact, in response to 2-DG, plasma insulin in wild-type mice was initially suppressed to the same level as that of Foxc2 transgenic mice at baseline, whereas plasma insulin was completely unchanged immediately after 2-DG injection in Foxc2 transgenic mice. This suggests that Foxc2 transgenic mice already had a high level of sympathetic nervous signaling to the islet. When islets from Foxc2 transgenic mice were stimulated with parasympathetic or sympathetic neurotransmitter analogs in vitro, the insulin and glucagon secretory responses were normal. This confirms that altered neural signaling in vivo, not altered islet function, is the main contributor to the secretory phenotype of Foxc2 transgenic mice.

The finding of altered islet responses to nervous inputs in the lean, highly insulin-sensitive Foxc2 transgenic mice should not be a complete surprise. Nearly 30 years ago, Peterson et al. (34) demonstrated an inverse relationship between body fat and autonomic nervous activity. Subsequent studies have demonstrated that caloric restriction and/or exercise result in increased insulin sensitivity in parallel with an increase in autonomic nervous activity (32).

Mechanistically, the overexpression of the FOXC2 transcription factor in adipose tissue results in increases in brown adipose tissue mass and white adipose tissue with brown adipose characteristics (15). There are a number of other transgenic mouse models that show increased brown adipose tissue characteristics and energy expenditure, and these are also associated with increased insulin sensitivity (16, 19, 33). However, the dramatic increase in insulin sensitivity seen in Foxc2 transgenic mice and other models cannot be explained by increased energy dissipation in brown and white adipose tissue alone. Ryu et al. (39) have recently described the neural innervation and signaling not only to the brown adipose tissue but also from the brown adipose tissue to centers in the brain. The signaling from the brown adipose tissue was mediated via sensory nerves and fed back to the autonomic nervous system at the level of the hindbrain (39). This suggests that the increased mass and metabolic activity of brown adipose tissue of Foxc2 transgenic mice could itself be activating autonomic nervous signaling independent of the insulin sensitivity status. There are some limitations to the study that are worth mentioning. This study was carried out only in female mice, as previous studies have demonstrated a significantly greater islet hormone response to autonomic nervous stimulus by 2-DG in females than in males (27). Furthermore, the high degree of insulin sensitivity in the Foxc2 transgenic mouse model is much less pronounced in males (data not shown), making them less suitable for a study on islet adaptation to high degrees of insulin sensitivity. The reasons for this sex difference are not known at present. Another limitation of our study is the lack of definitive information about the absolute β-cell mass in this model of extreme insulin sensitivity.
Fig. 6. Islet morphology and cellular composition in WT and Foxc2TG mice. A: islet fluorescent immunohistochemistry from Foxc2TG and WT mice fed normal kilocaloric (NK) diets. Top: staining with antibodies against GLUT2 (green). Bottom: staining with antibodies against insulin (red) and glucagon (green). Scale bar, 50 µm. B–D: total islet area (B), insulin-positive islet area (C), and glucagon-positive islet area (D); n = 4 mice/group. Data are presented as means ± SE. *P < 0.05, probability level of random difference between the WT and Foxc2TG mice.
To our knowledge, our study is the first to demonstrate that chronically increased insulin sensitivity affects islet hormone secretion by a mechanism involving alterations to islet morphology and the basal activation state of the branches of the autonomic nervous system. Future studies will need to be performed to determine whether autonomic nervous signaling affects islet hormone secretion in highly insulin-sensitive humans.

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DISCLOSURES

The authors have no conflicts of interest to disclose, financial or otherwise, with regard to this study.

AUTHOR CONTRIBUTIONS


REFERENCES


