Zinc-α2-glycoprotein promotes skeletal muscle lipid metabolism in cold-stressed mice

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Abstract. Skeletal muscle is the most abundant tissue in the adult body and plays an essential role in maintaining heat production for the entire body. Recently, muscle-derived non-shivering thermogenesis under cold conditions has received much attention. Zinc-α2-glycoprotein (ZAG) is an adipokine that was shown to influence energy metabolism in the adipose tissue. We used ZAG knock-out (ZAG KO) and wild-type (WT) mice to investigate the effect of ZAG on the lipid metabolism of skeletal muscle upon exposure to a low temperature (6°C) for one week. The results show that cold stress significantly increases the level of lipolysis, energy metabolism, and fat browning-related proteins in the gastrocnemius muscle of WT mice. In contrast, ZAG KO mice did not show any corresponding changes. Increased expression of β3-adrenoceptor (β3-AR) and protein kinase A (PKA) might be involved in the ZAG pathway in mice exposed cold stress. Furthermore, expression of lipolysis-related proteins (ATGL and p-HSL) and energy metabolism-related protein (PGC1α, UCP2, UCP3 and COX1) was significantly enhanced in ZAG KO mice after injection of ZAG-recombinant plasmids. These results indicate that ZAG promotes lipid-related metabolism in the skeletal muscle when the animals are exposed to low temperatures. This finding provides a promising target for the development of new therapeutic approaches to improve skeletal muscle energy metabolism.

Key words: Zinc-α2-glycoprotein (ZAG), Lipolysis, Energy metabolism, Skeletal muscle, Cold stress

THERMOSTASIS is essential for maintaining physiological homeostasis in mammals. In homeothermic animals, low environmental temperatures can activate the body’s regulatory mechanisms aimed at maintaining vital basal metabolism and activities [1]. Brown adipose tissue (BAT) is thought to be active in producing heat by uncoupling mitochondrial respiration under cold stress [2]. This tissue is essential for classical non-shivering thermogenesis [3]. In addition, there is accumulating data supporting the idea that skeletal muscle is also an important thermogenic organ during cold exposure in humans [4, 5]. Moreover, studies have identified brown-fat progenitors in the skeletal muscle [6], and the efficiency of browning in the muscle improves with cold acclimation [7].

Zinc-α2-glycoprotein (ZAG) is a 43 KD soluble glycoprotein. It was identified as an important adipokine, expressed in the adipose tissue, liver and skeletal muscle [8]. It has been shown that ZAG expression is lower in obese patients [9]. ZAG can activate the cyclic adenosine monophosphate-protein kinase A (cAMP-PKA) signal transduction pathway, and β3-adrenergic receptor (β3-AR) to participate in lipid metabolism in adipocytes [10, 11]. It was shown that ZAG promotes lipolysis, evidenced by a decrease in fat deposition in obese (ob/ob) and normal mice [11]. As a new adipocytokine, more and more attention is being paid to ZAG, aiming at the prevention of obesity and energy metabolism disorders [12, 13]. In a previous study, we found that ZAG decreases skeletal muscle lipid content and, therefore, alleviates insulin resistance in high-fat mice [14]. However, whether ZAG exerts its function in the skeletal muscle energy metabolism during cold stress, and whether it is involved in the acclimation to low temperatures is still unknown.

Therefore, in the present study we used wild type (WT) and ZAG knockout (KO) mice to investigate the role of ZAG in lipid metabolism during cold stress, using the gastrocnemius muscle as a model. The results provide a promising target for the development of new therapeutic approaches and improve skeletal muscle energy metabolism.
Materials and Methods

Ethical statement

The Animal Ethics Committee of Nanjing Agricultural University, China approved this study. Euthanasia and sampling procedures followed the “Guidelines on the Ethical Treatment of Experimental Animals” (2006) No. 398 published by the Ministry of Science and Technology, China, and with the “Regulations Regarding the Management and Treatment of Experimental Animals” (2008) No. 45, published by the People’s Government of Jiangsu Province.

Zinc-α2-glycoprotein knockout mice

ZAG KO mice with a C57BL/6J background were generated at the Nanjing Biomedical Research Institute (Nanjing University, Nanjing, China), using the CRISPR/Cas9 technology. The length of the knockout fragment was 2968 bp (AACAATCTCT---GTCAGAAA AG). The two points of the knockout were, respectively, located in introns 1 and 3; exons 2 and 3 were completely deleted.

Animals

In this study, we performed two experiments. In the first animal experiment, the effects of ZAG deficiency on skeletal muscle energy metabolism were investigated, using a cold-stressed mouse model. The experiments used ten ZAG KO and ten WT eight-week-old C57BL/6J male mice, that were fed standard chow (Table 1). The mice were acclimated for one week, before being randomly assigned to 6°C (ZAG KO + C, WT + C) and 22°C (ZAG KO, WT) conditions (five mice per cage). Treatment lasted one week, during which the mice were kept in a controlled environment (12 h light/dark cycle, 60%–70% humidity) and fed standard chow ad libitum.

At the end of the treatment week, the mice rectal temperature was measured using a thermometer and then were euthanized by intraperitoneal injection of sodium pentobarbital (40 mg/kg), the eyeball was removed with tweezers, and blood was collected into an Eppendorf tube with a syringe. The gastrocnemius muscle was also collected, and its weight was recorded.

In the second animal experiment, we used, again, eight-week-old ZAG KO mice. For this experiment, we used a pcDNA3.1-ZAG plasmid that purchased from Bioworld Technology, Inc. (No. PPL50018-2a). The ZAG recombinant plasmid (25 μg) or pcDNA3.1 negative control plasmid in 150 μL OPTI-MEM medium was mixed thoroughly with 110 μL OPTI-MEM medium supplemented with 40 μL Lipofectamine 2000 (Invitrogen). The resulting 300 μL mixture was incubated at room temperature for 30 min. ZAG KO mice were randomly divided into a control group (injection of pcDNA3.1) and ZAG group (injection of ZAG recombinant plasmid). All mice were housed at 6°C for one week. The plasmid was injected into the tail vein two days before the start of the cold stress treatment and then once every other day for a total of five injections. Mice were given free access to food and water while exposed to the 6°C cold stress. The gastrocnemius muscles were collected and weighed after the one-week cold stress treatment.

Triglycerides and total cholesterol detection

Plasma concentrations of triglycerides (TG) and total cholesterol (Tch) were detected with an automatic biochemical analyzer (Hitachi 7020; HITACHI), using commercial assay kits (995-33093 and 999-33493, respectively; Wako Pure Chemical Industries, Ltd. Wako). Gastrocnemius muscle concentration of TG was measured using a tissue TG assay kit (E1013; Applygen Technologies, Inc.) following the manufacturer’s instructions.

RNA isolation and quantitative real-time PCR

Total RNA was extracted from frozen gastrocnemius muscle samples (40 mg) using the TRIzol reagent (15596026, Invitrogen, CA, USA) according to the manufacturer’s protocol. The extracted RNA was quantified using the NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, USA). The ratios of absorption (260/280 nm and 260/230 nm) of all samples was between 1.8 and 2.0, which indicates that their purity was adequate. Two micrograms of RNA were used to generate cDNA by PrimeScript® 1st Strand cDNA Synthesis Kit (D6110A, Takara, Dalian, China). Peptidylprolyl isomerase A (PPIA), which could not be affected by the experimental conditions, was chosen as the reference gene and used as

| Table 1 | The formula of standard chow |
|---------|-----------------------------|
| Energy Composition | 100 |
| Protein (%) | 20 |
| Carbohydrate (%) | 70 |
| Fat (%) | 10 |
| Composition of fatty acid | 100 |
| Saturated (%) | 28.7 |
| Monounsaturated (%) | 32.7 |
| Polyunsaturated (%) | 38.6 |
| Type of fat (gm) | 45 |
| Lard (gm) | 20 |
| Soybean Oil (gm) | 25 |
an internal control. The resulting cDNA was diluted 1:20 and 5 μL of diluted cDNA was used as the template in the real-time PCR reactions on a real-time PCR system (Mx3000P, Stratagene, USA). The primer sequences of the target genes are detailed in Table 2.

### Western blot

Frozen gastrocnemius muscles samples (50 mg) were homogenized in 500 μL of pre-cooled RIPA buffer containing a protease inhibitor cocktail (complete EDTA-free and PhosSTOP, Roche, Penz-berg, Germany). Protein concentrations were determined using a Pierce BCA Protein Assay kit (Pierce, Rockford, IL, USA). The extracted proteins were then separated by SDS-PAGE in 5%, 10% or 15% polyacrylamide gels. The bands were visualized by enhanced chemiluminescence using the LumiGlo substrate (Super Signal West Pico Trial Kit, Pierce, USA). An imaging system (Bio-Rad, USA) recorded the signals analyzed using the Quantity One software (Bio-Rad). Information on the antibodies is presented in Table 3.

### Statistical analysis

For the first animal experiment, the results were analyzed by one-way ANOVA, followed by the LSD post-hoc test, using the SPSS 20.0 software. For the second animal experiment, in which ZAG recombinant plasmids or empty plasmids were injected into the mice, the results were analyzed using the Student’s t-test. The results are presented as the means ± standard error of the mean (SEM). Differences were considered statistically significant when \( p < 0.05 \). The numbers of replicates used in each experiment are noted in the legends to the Tables and Figures.

### Results

#### The effects of ZAG knockout on plasma biochemical, gastrocnemius muscle indexes and body temperature in the mice

The cold stress treatment significantly reduced the content of total cholesterol (Tch) and triglyceride (TG) in the plasma of ZAG KO mice (\( p < 0.05 \), Table 4). In addition, the content of Tch was significantly reduced in the

| Table 2 | The primer sequences of the target genes |
|---------|------------------------------------------|
| Gene    | Forward | Reverse |
|----------|---------|---------|
| UCP1     | GTGTGCGAGGTGCATTG | ACTCTGTAAGCATTGTAGGTT |
| CD137    | TCATTACCTCCTGTTCTCTG | TCATAGCCTCCTCCTCCT |
| PPIA     | GCAAGACCAGCAAGAGA | CAGTGAGAGCAGAGATTACA |

| Table 3 | Antibodies in this study |
|---------|-------------------------|
| Antibody description | Company | Item No. | Dilution ratio |
|-----------|--------|---------|---------------|
| p-HSL     | bioworld | BS4234  | 1:1,000       |
| ATGL      | bioworld | BS7989  | 1:1,000       |
| ACS21     | santa cruz | sc-373847 | 1:1,000       |
| FASN      | bioworld | BS6050  | 1:1,000       |
| β2-AR     | santa cruz | H-20   | 1:200         |
| β3-AR     | santa cruz | M-20   | 1:200         |
| PKA       | bioworld | BS1576  | 1:1,000       |
| PGC1α     | proteintech | 66369 | 1:1,000       |
| CPT1a     | bioworld | BS7744  | 1:1,000       |
| UCP2      | proteintech | 11081-1-AP | 1:1,000       |
| UCP3      | bioworld | BS2849  | 1:1,000       |
| COX1      | bioworld | BS1636  | 1:1,000       |
| COX4      | bioworld | AP0705  | 1:1,000       |
| UCP1      | abcam   | ab10983 | 1:1,000       |
| CD137     | bioworld | BS2039  | 1:1,000       |
| GAPDH     | bioworld | AP0063  | 1:5,000       |
plasma of WT mice exposed to the cold stress ($p < 0.05$, Table 4). However, the two groups did not differ in plasma Tch and TG content ($p > 0.05$, Table 4).

After the cold stress treatment, the weight of the gastrocnemius muscle and TG content significantly decreased in WT mice ($p < 0.05$, Table 4), yet there were no demonstrated changes in the ZAG KO mice compared with the mice maintained under normal temperature ($p > 0.05$, Table 4).

The body temperature of normal and cold stress mice was $36.4 \pm 0.11^\circ C$ and $34.4 \pm 0.08^\circ C$ in the WT mice and $36.3 \pm 0.10^\circ C$ and $34.1 \pm 0.16^\circ C$ in the ZAG KO mice, respectively. The body temperature was significantly decreased after cold stress in both WT and ZAG KO mice, yet there was no significant difference between WT and ZAG KO mice under cold condition.

**The effect of ZAG knockout on the expression of lipid metabolism-related proteins in the gastrocnemius muscle of the mice**

We tested the levels of lipolysis-related proteins in the mice gastrocnemius muscle. After cold stress, the expression of the proteins adipose triglyceride lipase (ATGL) and phosphorylate hormone-sensitive lipase (p-HSL) has increased significantly in the WT group was higher than in the ZAG KO group ($p < 0.05$, Fig. 1A). These data show that ZAG could promote lipolysis-related protein expression in the gastrocnemius muscle during cold stress, and that break down of TG to fatty acids participates in energy metabolism. To investigate the effect of ZAG on energy metabolism, we tested the expressions of energy metabolism-related proteins in the gastrocnemius muscle of the mice. After the cold stress treatment, expression levels of the proteins peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) and carnitine palmitoyltransferase-1a (CPT1a) in the WT mice were higher than in the ZAG KO mice ($p < 0.05$, Fig. 1B). Also, expression levels of the energy metabolism-related proteins uncoupling protein 2 (UCP2) and uncoupling protein 3 (UCP3) as well as that of cytochrome c oxidase 4 (COX4) were higher in WT mice compared with ZAG KO mice after the cold stress treatment ($p < 0.05$, Fig. 1C).

**The effect of ZAG knockout on the expression of browning-related genes and proteins in the gastrocnemius muscle of the mice**

Cold stress promotes the transformation of white fat cells into brown fat cells. We, therefore, tested the expression of browning-related proteins in the gastrocnemius muscles of the mice. After the cold stress treatment, expression of both uncoupling protein 1 (UCP1) and tumor necrosis factor receptor superfamily member 9 (CD137) mRNA and protein has increased in the WT mice and was higher than in the ZAG KO mice ($p < 0.05$, Fig. 2A and 2B).

**The effect of ZAG knockout on the expression of lipid synthesis-related proteins in the gastrocnemius muscle of the mice**

After the cold stress treatment, the levels of the protein β3-adrenoceptor (β3-AR) and protein kinase A (PKA) in the ZAG KO mice were significantly lower than in the WT mice ($p < 0.05$, Fig. 3A). The expression of β2-AR protein did not differ between WT and ZAG KO mice. The lipid synthesis-related protein expression, including acyl-CoA synthetase short-chain family member 1 (ACSS1) and fatty acid synthase (FASN), showed no detectable difference between the two groups after the cold stress treatment ($p > 0.05$, Fig. 3B).

**The effect of ZAG plasmid injection on plasma biochemical and gastrocnemius muscle indexes in the mice**

When ZAG plasmids were injected into the tail vein of ZAG KO mice who were exposed to the cold stress conditions, the plasma TG and Tch levels and the gastrocnemius muscle TG content have significantly decreased ($p < 0.05$, Table 5), but there was no changed in the gastrocnemius muscle weight ($p > 0.05$, Table 5).

### Table 4  Plasma biochemical and gastrocnemius indexes of ZAG KO mice

|            | WT       | ZAG KO   | WT + C   | ZAG KO + C |
|------------|----------|----------|----------|------------|
| Plasma     |          |          |          |            |
| TG (mmol/L)| 0.25 ± 0.03 ab | 0.32 ± 0.08 a | 0.15 ± 0.01 b | 0.16 ± 0.03 b |
| Tch (mmol/L)| 2.72 ± 0.12 a | 2.52 ± 0.32 ab | 1.92 ± 0.18 bc | 1.82 ± 0.19 c |
| gastrocnemius |          |          |          |            |
| weight (g) | 0.26 ± 0.01 a | 0.23 ± 0.02 ab | 0.22 ± 0.01 b | 0.26 ± 0.01 a |
| TG (nmol/g)| 4.69 ± 0.23 a | 6.08 ± 0.91 a | 3.65 ± 0.17 b | 6.46 ± 1.13 a |

The results were expressed as Means ± SEM ($n = 5$). Mean values not sharing the same letters are significantly different, $p < 0.05$. 

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The effect of ZAG plasmid injection on the expression of lipid metabolism-related proteins in the gastrocnemius muscle of the mice

A previous study indicated that ZAG could influence lipid metabolism-related protein expression under cold conditions. When ZAG plasmids were injected into the tail vein of the ZAG KO mice exposed to the cold stress treatment, the expression of the proteins ATGL, p-HSL, β3-AR and PKA has significantly increased (*p* < 0.05, Fig. 4A and 4B).

The effect of ZAG plasmid injection on the expression of energy metabolism- and browning-related proteins in the gastrocnemius muscle of the mice

When ZAG plasmids were injected into the tail vein of ZAG KO mice, the mRNA and protein expression of PGC1α and the proteins UCP2, UCP3, COX1, and CD137 have significantly increased (*p* < 0.05, Fig. 5A–5C), but there was no change in the expression of the proteins CPT1a, COX4 and UCP1 after exposure to cold stress (*p* > 0.05, Fig. 5A–5C). In addition, ZAG treatment was significantly increased the CD137 and UCP1 mRNA expression compared with CON group (*p* < 0.05, Fig. 5D).

**Discussion**

In recent years, the energy expenditure of skeletal muscle received much attention in relation to cold stress conditions [15]. As an adipokine, accumulating data suggest that ZAG influences lipid mobilization in the adipose tissue [4, 16]. In the present study, we investigated the action of ZAG in the skeletal muscle of cold stressed
mice. The results demonstrate that ZAG affects the expression of lipid metabolism-related proteins in the skeletal muscle during cold stress.

ZAG is mainly synthesized in the adipose tissue and secreted into the blood to enter other tissues or organs where it fulfills its functions. Our previous study showed that cold stress leads to a markedly higher plasma content of ZAG in WT mice compare to the ZAG KO mice [17]. In the present study, knocking-out ZAG significantly influenced TG content in the gastrocnemius muscle after the cold stress treatment. The plasma TG content, however, showed no obvious difference between the WT and ZAG KO mice. ATGL and HSL are the main enzymes involved in lipid hydrolysis in the skeletal muscle [18, 19]. FASN and ACSS1 are important enzymes in the process of fatty acid synthesis. ACSS1 catalyzes the ATP-dependent conversion of acetate and CoA (coenzyme A) to acetyl-CoA, which is then utilized for the oxidation of acetate within the tricarboxylic acid cycle. FASN participates in the fatty acid synthesis process [20, 21].

Fig. 2  The effect of ZAG knockout on the expression of browning-related genes and proteins in the gastrocnemius muscle of the mice. A: mRNA expression of the UCP1 and CD137; B: protein expression of CD137 and UCP1. Results are expressed as mean ± SEM (n = 5). Mean values not sharing the same letter are significantly different, p < 0.05.

Fig. 3  The effect of ZAG knockout on the expression of lipid synthesis-related proteins in the gastrocnemius muscle of the mice. A: Expression of β2-AR, β3-AR and PKA. B: Expression of ACSS1 and FASN. Results are expressed as mean ± SEM (n = 5). Mean values not sharing the same letter are significantly different, p < 0.05.
Previous studies indicated that ZAG plays a role in the lipolysis of adipose tissue [22, 23]. The present results show that there is no difference in lipid synthesis-related protein expression between the skeletal muscles of WT and ZAG KO mice after exposure to cold stress. However, lipolysis-related proteins have significantly increased in the gastrocnemius muscle of WT mice after the cold stress treatment, and were significantly higher than those in the ZAG KO mice. These results show that ZAG influences lipolysis-related protein levels in the skeletal muscle during cold stress.

PGC1α is a target gene for peroxisome proliferators-activated receptor alpha (PPARα), which maximizes the body’s ability to generate heat [24]. CPT1a is a key rate-limiting enzyme for the transfer of long chain fatty acids from the cytoplasm to the mitochondria for energy supply by oxidation [25]. The UCP family has similar functions, mainly involving oxidative phosphorylation and the generation of ATP and heat. As such, it plays an important role in alleviating obesity and maintaining energy homeostasis [26]. In the present study, the cold stress treatment increased the expression of fatty acid oxidation-related proteins in the WT mice, but there were no corresponding changes in the ZAG KO mice. These results indicate that ZAG might be contributing to the energy expenditure-related protein levels in the skeletal muscle of the mice.

Previous studies have shown that cold stress promotes browning of adipocytes, and enhances the occurrence of the heat generation process [27]. In our previous study, thermogenesis by the brown and white adipose tissues had significantly increased in WT mice compared to ZAG KO mice who experienced cold stress [17]. Brown adipose tissue and skeletal muscle are two well-known sites of thermogenesis [3, 28, 29]. Both tissues use energy substrates such as fatty acids to generated heat to maintain body temperature. Studies showed that brown-fat progenitors exist in the skeletal muscle [6], and the efficiency of browning changes in the muscle with cold acclimation [7]. Browning adipocytes contain different

![Fig. 4](image_url) The effects of ZAG plasmid injection on the expression of lipid metabolism-related proteins in the gastrocnemius muscle of the mice. A: Expression of p-HSL and ATGL; B: Expression of β3-AR and PKA. Results are expressed as mean ± SEM (n = 5). Mean values not sharing the same letter are significantly different, p < 0.05.

| Table 5 | Plasma biochemical and gastrocnemius indexes of mice were injected with ZAG plasmid |
|---------|------------------------------------------|
|         | CON | ZAG |
| **Plasma** |     |     |
| TG (mmol/L) | 0.30 ± 0.04 | 0.16 ± 0.05* |
| Tch (mmol/L) | 3.23 ± 0.19 | 1.90 ± 0.62* |
| **Gastrocnemius** |     |     |
| weight (g) | 0.27 ± 0.01 | 0.28 ± 0.002 |
| TG (mmol/g) | 7.58 ± 1.06 | 4.98 ± 0.47* |

The results were expressed as Means ± SEM (n = 5). Mean values not sharing the same letters are significantly different, p < 0.05.
specific molecular markers, such as CD137 and UCP1 [30]. The present study show that ZAG KO hinders the increase in UCP1 and CD137 protein expression during cold stress, indicating that ZAG exerts a browning action in the gastrocnemius muscle under cold stress conditions.

ZAG has not been reported to have a specific receptor. It has been reported that the ZAG effect was attenuated by the specific β3-adrenoreceptor antagonist SR59230A, and enhanced by the cyclic AMP phosphodiesterase inhibitor Ro20-1724 [16, 31-33]. This due to its ability to bind to β3-AR and activate the cyclic AMP pathway. Therefore, we investigated the crucial genes involved in this pathway. This study shows that cold stress and ZAG knock-out did not affect β2-AR protein expression. After cold stress, the expression of the β3-AR protein was significantly higher in the gastrocnemius muscles of WT mice than in those of ZAG KO mice. Moreover, a higher PKA protein level was observed in the gastrocnemius muscle after injecting the ZAG plasmid to the ZAG KO mice. These results suggest that ZAG might influence lipid-related metabolism in the skeletal muscle through the β3-AR/PKA signaling pathway.

To confirm the influence of ZAG on lipid metabolism and browning in skeletal muscles, we injected ZAG recombinant plasmids into the tail vein of ZAG KO mice under cold stress conditions. ZAG protein is mainly synthesized in the adipose tissue, and the expression of ZAG in skeletal muscle is relatively less compared to adipose tissue. Our previous study showed that the ZAG protein expression was significantly increased after injection of ZAG plasmids in adipose tissue [17]. ZAG in adipose tissue is secreted into the blood, where it travels to skeletal muscles and acts. The TG content in the gastrocnemius muscle of these mice has significantly decreased, and the expression of lipolysis-related and energy metabolism-related proteins has significantly increased. We detected the expression of UCP1 mRNA and injection of ZAG plasmids actually increased the mRNA

![Figure 5](image.png)

**Fig. 5** The effects of ZAG plasmid injection on the expression of energy metabolism- and browning-related proteins in the gastrocnemius muscle of the mice. A: Expression of PGC1α and CTP1α; B: Expression of UCP2, UCP3, COX1 and COX4; C and D: Expression of CD137 and UCP1. Results are expressed as mean ± SEM (n = 5). Mean values not sharing the same letter are significantly different, p < 0.05.
expression of UCP1, and the results showed UCP1 may be influenced by the ZAG treatment though the detected UCP1 protein in the present study didn’t show the significantly changes. The ZAG plasmid injection treatment trial further demonstrates that ZAG plays an essential role in lipid metabolism of the skeletal muscle when exposed to cold stress conditions.

The present study suggests that ZAG might be involved in lipolysis, energy expenditure and browning in skeletal muscles of mice exposed to cold stress. The results point at a promising approach to dissipate deposited lipids in skeletal muscles as a treatment for obesity and its associated metabolic complications.

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Disclosure Statement

The authors declare that they have no competing interests.

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