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ORIGINAL CONTRIBUTION

Long chain saturated fatty acids increase haptoglobin gene expression in C57BL/6J mice adipose tissue and 3T3-L1 cells

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Abstract
Background Dietary lipids are directly related to the composition of adipose tissue, aetiology of obesity and arousal of obesity-related pathologies, like chronic inflammation states. Haptoglobin is an acute phase protein secreted by the liver and white adipose tissue, and its blood levels vary according to the volume of fat in the body.
Aim of the study To investigate the effect of diets enriched with large amounts of dietary fats, which differ in their fatty acid composition, on the haptoglobin gene expression by visceral and subcutaneous adipose tissue of mice fed for 2 days or 8 weeks. 3T3-L1 cells were treated with fatty acids that are found in those types of dietary fats.
Methods Mice were treated acutely (for 2 days) or chronically (for 8 weeks) with diets enriched with soybean oil, fish oil, coconut oil or lard. 3T3-L1 cells were treated with six different fatty acids. Haptoglobin gene expression was quantified by northern blotting.
Results Both chronic and acute treatment with lard, which is rich in long chain saturated fatty acids, increased the haptoglobin mRNA expression in the retroperitoneal and epididymal white adipose tissues. Chronic treatment with coconut oil, which is rich in medium chain saturated fatty acids, increased the haptoglobin expression in the epididymal and subcutaneous depots. In 3T3-L1, palmitic acid increased the haptoglobin gene expression.
Conclusion The type of lipids in the diet can differently modulate the white adipose tissue gene expression of haptoglobin, and saturated fatty acids play a major role in promoting a pro-inflammatory environment. This response is fat pad specific and dependant on the duration of treatment.

Keywords White adipose tissue · Haptoglobin · High fat diet · Palmitic acid · Lauric acid

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Introduction

The adipose tissue is involved in several systemic processes such as energy homeostasis, glucose tolerance and inflammatory response through the action of adipokines, which are major factors secreted by white adipocytes [1–3]. Adipokines are considered to be directly linked to the pathologies associated with obesity, like heart disease, dyslipidemia and metabolic syndromes, and adipokines

gene expression and blood levels can be correlated to the amount of fat mass in the body [4–6].

Haptoglobin, a haemoglobin-binding plasma glycoprotein with anti-oxidant properties, is an important acute phase protein secreted by the liver as well as the adipose tissue [7, 8]. C-reactive protein and haptoglobin blood levels are increased in obese and diabetic patients, supporting as evidence that these conditions are mild chronic inflammatory states [9, 10]. As angiogenesis is concomitant to chronic inflammatory states, the angiogenic properties of haptoglobin have also been described [11], stimulating endothelial cell differentiation and tissue vascularization.

The type of fat in the diet affects the development of insulin resistance, incidence of heart disease and inflammatory response in both experimental models and humans [12–15]. Studies have shown that the incidence of insulin resistance and heart disease is positively related to the ingestion of saturated fatty acids and negatively related to the ingestion of polyunsaturated fatty acids [13, 16]. In this way, Wigmore et al. [17] observed in cultured human hepatocytes treated with n-6 or n-3 polyunsaturated fatty acids that the production of haptoglobin is decreased.

Experimental studies have demonstrated a straight relation between inflammation mediators and haptoglobin levels. Mice treated with lipopolysaccharide and TNF-α, as well as ob/ob mice, have increased haptoglobin mRNA gene expression [7, 8]. 3T3-L1 adipocytes presented increased haptoglobin gene expression levels when treated with lipopolysaccharide, TNF-α and glucocorticoids, but presented decreased levels when treated with rosiglitazone [18].

In this study, mice were treated with diets enriched with four different dietary fats, namely soybean oil, fish oil, lard or coconut oil, as well as adipocytes in culture were treated with fatty acids of different levels of saturation and chain length which are found abundantly in those dietary fats. The purpose on this study was firstly to characterize the haptoglobin gene expression pattern by the white adipose tissue on these dietetic conditions, and secondly to identify potentially pro-inflammatory dietary fats which in turn could exacerbate already settled inflammation-related pathologies in patients.

Materials and methods

Animals and experimental conditions

The Experimental Research Committee of the São Paulo Federal University approved all procedures for care of the animals used in this study. For this study, 80 mice were used. Half of them (40) were treated since the 30th day of life, for 8 weeks; the other half (40), at the 90th day of life, were treated for 2 days. The animals were kept under controlled conditions of light (12:12 h light-dark cycle with lights on at 07.00 am) and temperature (22 ± 1 °C). During the whole experimental period the animals were maintained in individual cages and received water and specific feed ad libitum. The animals were treated either chronically or acutely with diets enriched with four different types of fat, as follows.

Chronic treatment

Forty mice were randomised into five groups and fed since the 30th day of life, for 8 weeks, with either control diet (C), which contained 4.8 % (w/w) of fat, or one of the high fat diets. The following fats were used: Soybean oil (S); Fish oil (F); Lard (L); or Coconut oil (C), with an average final fat concentration of 22.4%. It was previously described that these diets cause obesity in C57BL/6J male mice [19].

Acute treatment

Forty mice, at the 90th day of life, were randomised into five groups and fed for 2 days with the same diets as in the chronic treatment described above.

Preparation of the diets

The diets were prepared in the Laboratory of Nutrition Physiology—Department of Physiology—São Paulo Federal University, as described previously [20]. The diets were based on the commercial chow for rodents NUVILAB CR-1 (Paraná, Brazil) which was ground and enriched with 17.5% of fat, using one of the following oils: soybean oil, fish oil, coconut oil or lard. These oils were purchased from Brazilian food companies, which produced them from national natural sources. An antioxidant, 0.013% butylated hydroxytoluene (w/w) was added. The coconut oil and lard were warmed up in order to obtain liquid state. Powder casein was added to reach the protein concentration of approximately 22%. Lukewarm water was added to achieve the necessary consistence for perfect homogenization of the mixture. After homogenization, the mixture was passed through a milling machine for the production of pellets, which were dried in forced ventilation oven at 60 °C for 24 h. A sample of each diet was sent for analysis of macronutrient composition to the Laboratory of Bromatology and Microbiology of Foods at the Department of Physiology—São Paulo Federal University. The fatty acid composition of these diets was determined by gas-liquid chromatography with an ionizable flame detector (Perkin Elmer, Wellesley, MA, USA), through techniques already standardized in the literature. The nutrient composition of the diets is described in Table 1. The diets were stored in plastic containers at −20 °C and served to the animals every second day.

Table 1 Total caloric value (energy, Kcal/100 g), protein, carbohydrate, alimentary fibre, mineral residues and lipid contents (g/100 g) of control diet (C) or diets enriched with soybean oil (S), fish oil (F), lard (L) or coconut oil (C)

	C	S	F	L	CC
Energy (Kcal/100 g)	289.2	397.6	396.5	399.6	415.4
Protein	22.4	23.1	22.4	23.6	22.7
Carbohydrates	39.1	26.8	29.7	26.8	25.8
Alimentary fibre	11.4	15.2	14.7	15.1	15.1
Mineral Residues	11.9	9.5	9.4	9	8.8
Lipids	4.8	22	20.9	22	24.6
Fatty acid composition (% total)					
SMCFA %	0.6				53.4
SLCFA %	27.6	15.4	34.8	42	23
MUFA %	27.4	25.1	31.7	45	14
PUFA %	43.9	59.5	33.2	9.2	9.8
Total N-6 %	41	55.1	11.1	9.2	9.8
Total N-3 %	2.91	4.35	22.1		
Ratio N-6/N-3	14	12.7	0.5		

The fatty acid composition of the diets is described as the percent of total fatty acid content. The percent of fatty acids in the omega 6 series and omega 3 series is presented, as well as the ratio of omega 6 series over omega 3

SMCFA saturated medium-chain fatty acids, *SLCFA* saturated long-chain fatty acids, *MUFA* monounsaturated fatty acids, *PUFA* polyunsaturated fatty acids

Collection of samples

The animals were treated with one of the five diets (C, S, F, L or CC) for 2 days in the acute treatment group and for 8 weeks in the chronic treatment group, and then sacrificed by decapitation without sedation. All animals were sacrificed early in the morning to avoid chronobiological variations. Animals were acclimatized in a quiet room next to the laboratory under subdue light for 1 h, and taken to the laboratory individually to minimize stress. Samples of epididymal (EPI), retroperitoneal (RET) and subcutaneous (SUB) adipose tissues were dissected and immediately frozen in liquid nitrogen. The samples were stored at −70 °C and used for haptoglobin mRNA quantification by Northern Blotting.

Cell culture

3T3-L1 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured at 37 °C in a humidified atmosphere of 5% CO_2/95% air. The cells were maintained until confluence in Dulbecco's modified Eagle's medium (DMEM; InVitrogen) containing 25 mmol/L glucose, 1.0 mmol/L pyruvate, 4.02 mmol/L L-alanyl-glutamine, to which 10% fetal calf serum was added. Differentiation of the cells was initiated 24 h after confluence by incubation for 2 days in DMEM, to which 0.25 μmol/L dexamethasone, 0.5 mmol/L 3-isobutyl-1-methyl-xanthine, and 5 μg/mL insulin were added. For the next 12 days the cells were maintained in DMEM with 5 μg/mL insulin added. On the 15th day after differentiation, the first set of cells ($N = 6$ per treatment) was treated with DMEM with 5 μg/mL insulin and one of the following fatty acids: palmitic, linoleic, eicosapentaenoic (EPA), docosahexaenoic (DHA), lauric or oleic, in the final concentration of 250 μmol/L [21]. Fatty acids were conjugated to albumin. The control cells received only the same vehicle used for the treated cells. The second set of cells ($N = 6$ per treatment) was used for the palmitic acid dose response study, where the cells were treated with 50, 100, 250 or 500 μmol/L of palmitic acid. In the control plates the medium was changed at the same times. All the cells were harvested 48 h after treatment. All the chemical products used, apart of DMEM, were obtained from Sigma.

RNA preparation and Northern blot analysis

Total RNA was extracted from tissues and 3T3-L1 cells using Tri-Reagent (Sigma), according to the method described previously by Chomczymski and Sacchi [22]. RNA concentration was determined from the absorbance at 260 nm, and purity was determined from the ratio 260/280 nm. Fifteen μg of RNA from each sample were run on 1.3% agarose–formaldehyde gels, blotted onto a positively charged nylon membrane (Roche) overnight, cross-linked under UV light, and hybridized as previously described [23]. An antisense oligonucleotide (5′-CTTGGCTGACG CCGTCTCGCTGTGGTTCA) based on the mouse haptoglobin cDNA sequence (PubMed) was synthesized as the hybridization probe and end-labelled (5′-end) with a digoxigenin ligand (MWG, USA). Following post-hybridization washes, membranes were incubated with an antibody against digoxigenin (Fab-fragment; Roche) for 30 min and then with CDP-Star (Roche) chemiluminescence substrate (10 min at room temperature). Signals were collected by exposure to film for 40–50 min at room temperature. After probing for haptoglobin mRNA, blots were stripped and re-probed for the housekeeping gene 18S rRNA. The sequences of the anti-sense oligonucleotides used as probe for 18S rRNA were as previously described [24]. Blots were quantified by densitometry using Image J software.

Statistical analysis

The results are presented as mean ± SEM. In the analysis of the haptoglobin gene expression in the cell culture and in the adipose tissue, results were expressed in arbitrary units, stipulating 100 as the control value. One-way ANOVA was used for statistical analysis, followed by the post hoc

Tukey's test. The differences were considered significant where $p < 0.05$.

Results

The high fat diets contained in average 22.4 g of lipids per 100 g of mass, as compared to 4.8 g per 100 g in the control diet. On the other hand, the control diet contained higher amount of carbohydrates (39.1%) as compared to the high fat diets (27.3% in average). The high fat diets had an average caloric value of 113 kcal/100 g higher than the control diet, and were similar to it in amount of protein, minerals and fibres (Table 1).

The coconut diet (CC) contained 76.4% of its lipids as saturated fatty acids, and 53.4% as saturated medium-chain (SMCFA). The lard diet (L) contained 42% as saturated long-chain fatty acids (SLCFA) and 45% as monounsaturated fatty acids (MUFA). The soy diet (S) contained 59.5% of its lipids as polyunsaturated fatty acids (PUFA) and 55.1% as N-6 PUFA. The fish diet (F) contained 33.2% of its lipids as PUFA, where 11.1% as N-6 and 22.1% as N-3. The ratio N-6 to N-3 in the soy diet was nearly the same as the control diet, and this ratio for the fish diet is 0.5 (Table 1).

The body weight gain was similar among C, F, L and C after 8 weeks of treatment; however it was lower in the S (8.02 ± 0.51) as compared to C (10.57 ± 0.73) (data not shown). The daily caloric intake was similar among C, L, and CC groups, and this parameter was higher as compared to S and F (data not shown). The carcass lipid content was increased in all high fat diet groups (data not shown). It was previously described that these diets cause obesity in C57BL/6J male mice [19].

In vivo Northern blot study

The chronic and acute treatment with lard increased the haptoglobin mRNA expression in both EPI (2.56 and 1.5 fold, respectively) and RET (1.8 and 1.76 fold, respectively) fat depots, and also in the SUB of chronically treated ones (2.33 fold). The chronic treatment with coconut oil increased this parameter in the EPI (2.2 fold) and SUB (2.5 fold) fat depots (Fig. 1).

In vitro Northern blot study

The in vitro treatment with 250 μM of different fatty acids revealed that the palmitic acid increased the haptoglobin gene expression (1.9 fold) (Fig. 2), but the other fatty acids produced no effect. The dose response study revealed that the haptoglobin gene expression is overexpressed in doses higher than 100 μM of palmitic acid (100 μM, 1.78 fold; 250 μM, 1.8 fold; 500 μM, 2.0 fold) (Fig. 3).

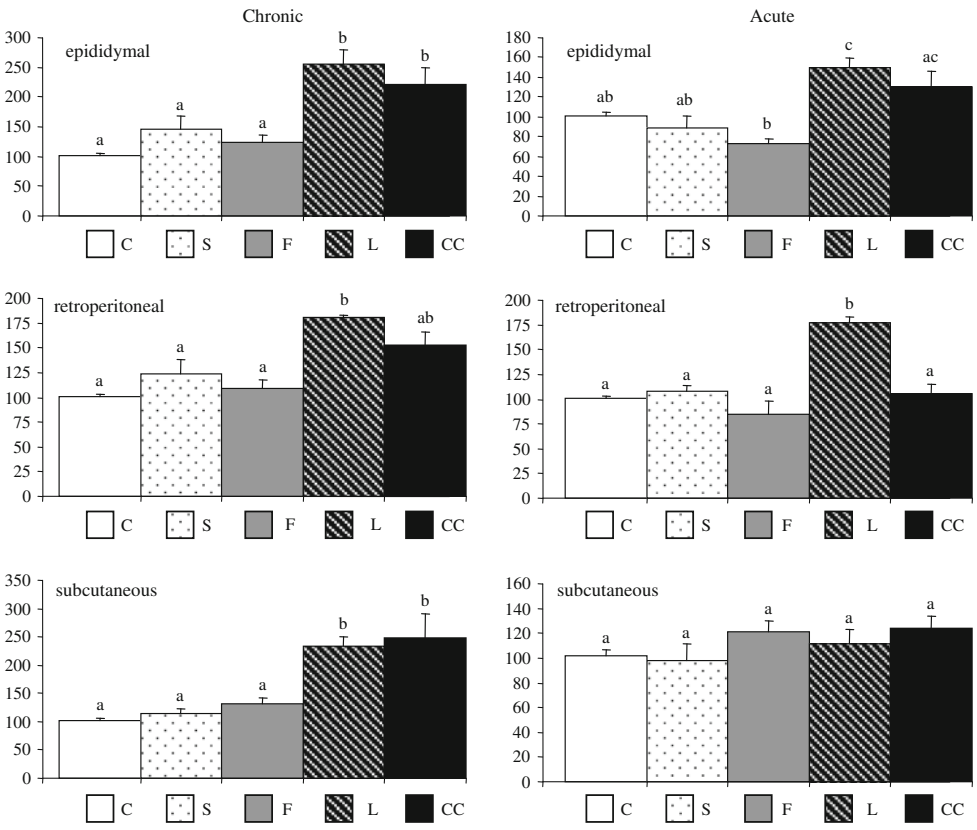

Fig. 1 Haptoglobin mRNA quantification of epididymal, retroperitoneal and subcutaneous adipose tissue of mice treated for 8 weeks (chronic) with control diet (C) or diets enriched with soybean oil (S), fish oil (F), lard (L) or coconut oil (C). Values are expressed as mean ± SEM. Values are arbitrary units as 100 of the control group. Number of animals used per group ranged from 6 to 8. Values with different superscript letters are significantly different from one another at $p < 0.05$

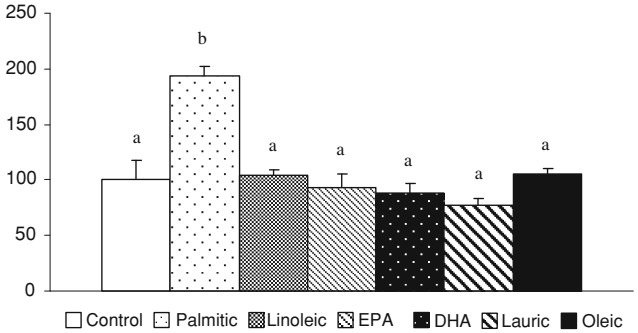

Fig. 2 Haptoglobin gene expression of 3T3-L1 cells after 48 h treatment with 250 μM of Palmitic, Linoleic, EPA, DHA, Lauric or Oleic acids. Values are expressed as mean ± SEM. Values are arbitrary units as 100 of the control group. Number of samples is 6 for all treatments. Values with different superscript letters are significantly different from one another at $p < 0.05$

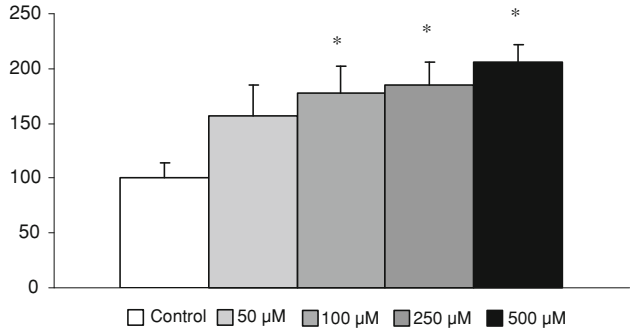

Fig. 3 Haptoglobin gene expression of 3T3-L1 cells after 48 h treatment with 50, 100, 250 or 500 μM of palmitic acid. Values are expressed as mean ± SEM. Values are arbitrary units as 100 of the control group. Number of samples is 6 for all treatments. *$p < 0.05$ as related to the control group

Discussion

Haptoglobin is an acute phase protein synthesized by the liver and its production is increased in inflammation-related conditions [25]. Haptoglobin has antioxidant properties, due to its capacity to bind haemoglobin and protect from oxidative stress that free haemoglobin can cause [26]. Recent studies have revealed that it is also expressed and secreted by the adipose tissue and its production is directly related to the level of adiposity [5, 27].

Fatty acids are the major metabolic fuel reservoirs due to their high energy density and also play a fundamental role in the composition of cell membrane. In addition, fatty acids are precursors of several hormones and cytokines and can act as ligands for transcription factors. The activation of transcription factors, like the peroxisome-proliferator activated receptor (PPAR) and nuclear factor kappa β (NF-kβ) can respectively inhibit or activate the expression of several genes involved in the inflammation response in adipocytes [28]. Several studies have revealed that treatments with different fatty acids not only change cell membrane fluidity [29, 30] but also alter the production of citokynes [15, 31].

In the present study, the effects of four different high fat diets, enriched with either soybean oil, fish oil, lard, or coconut oil, on the haptoglobin gene expression by subcutaneous and visceral (epididymal and retroperitoneal) adipose tissue of mice fed ad libitum were analysed. The high fat diets contained on average 17.5% more lipid (mass) and 12% less carbohydrate than the control diet. The diets were otherwise similar to the control diet and adequate in protein, fibre and minerals (Table 1).

In this study, the chronically treated CC mice showed increased haptoglobin gene expression in the epididymal and subcutaneous adipose tissues (Fig. 1). The coconut oil is rich in medium chain fatty acids, especially lauric acid, which are ketogenic [32]. Studies have shown that hyperketonemia increases TNF-α serum concentration [33] and IL-6 [34], important inflammation-related cytokines. In addition, IL-6 directly stimulates haptoglobin gene expression in 3T3-L1 cells [18].

On the contrary, we observed that the addition of lauric acid into the culture medium of 3T3-L1 adipocytes did not change the haptoglobin gene expression (Fig. 2). This shows lack of direct effect of lauric acid on this adipokine. It may be suggested that the effect of coconut oil enriched diet on haptoglobin gene expression by the adipose tissue could be dependent of ketone body production from medium chain fatty acids by the liver. These fatty acids could possibly indirectly lead to a mild inflammatory state, through an increase in TNF-α and IL-6 blood concentrations, which in turn stimulate the expression of haptoglobin by fat depots.

In this study it was found that mice fed with lard-enriched diet, both acutely and chronically, presented increased haptoglobin gene expression (Fig. 1). Lard is very rich in palmitic acid, which was found in this study to increase the haptoglobin gene expression in 3T3-L1 cells (Fig. 2). It was also found that 3T3-L1 respond to palmitic acid in a dose dependant manner, where the haptoglobin gene expression is increased in doses higher than 100 μM (Fig. 3).

Ajuwon and Spurlock [21] found that treatment with palmitate induced the NF-kβ transcription factor and the expression of IL-6 and TNF-α mRNA in 3T3-L1 cells. Araki et al. [35] demonstrated that treatment with TNF-α induced the production of IL-6 in a dose-dependant manner, in the same cell line. Furthermore, previous studies found that haptoglobin stimulates angiogenesis [11], and that in ob/ob mice treated with angiogenesis inhibitors, their adiposity is reduced [36], which demonstrates the importance of angiogenesis on the development of adipose

tissue. In this way, it has also been described that the adipose tissue of rats expands more intensely after treatment with saturated fat enriched diet as compared to unsaturated fat enriched diet [37], revealing a obesogenic effect of saturated fatty acids. From the evidence found in the literature and in this study, it is suggested that palmitic acid stimulates haptoglobin gene expression probably by increasing tissue adiposity and angiogenesis, as well as through the stimulation of inflammatory pathways, like NF-kβ activation and induction of pro inflammatory citokynes.

In our study, the epididymal adipose tissue of mice treated with either lard or coconut oil, the richest sources of saturated fatty acids, showed increased haptoglobin gene expression (Fig. 1). Shi et al. [38] demonstrated that in RAW264.7 macrophages the fatty acids lauric, mirystic, palmitic, and stearic effectively increase IL-6 mRNA levels, while unsaturated fatty acids only slightly increase this parameter. In the same cells, palmitate increased TNF-α expression in a dose-dependant response, and combined treatment with palmitate and DHA blocked this effect. In that study, it was also found that toll-like receptor 4 (TLR-4), one of the receptors that initiate immune responses, is over expressed in epididymal adipose tissue of ob/ob, db/db and diet-induced obese mice. It has been recently described that saturated fatty acids induce inflammation predominantly through the activation of TLR-4 in the hypothalamus [39].

It is suggested that saturated fatty acids are stimulating a pro-inflammatory environment. This effect could be direct, through the activation of TLR by palmitic acid, subsequently causing the activation of transcription factors in adipocytes; could be indirect, via metabolization of medium chain fatty acids by the liver with subsequent production of ketone bodies; or could be through a local effect on angiogenesis related to adipogenesis due to the obesogenic effect of saturated fatty acids. Haptoglobin gene expression might have been increased as a result of that. Other adipokines may also be changed as well.

In conclusion, this study demonstrates that the type of lipids in the diet can differently modify the white adipose tissue gene expression of haptoglobin. Saturated fat, found in great concentrations in lard and coconut oil, has increased haptoglobin gene expression by the adipose tissue of mice. This response is fat pad specific and depends on the duration of treatment. The composition of the fatty acids in the diet is, thus, a central factor for the modulation of the secretory function of adipose tissue. We corroborate with previous studies that demonstrate metabolic disorders and mild chronic inflammation states as directly linked to each other, and subsequently linked to life style and nutritional status. It can be of importance to consider a specific dietetic intervention on the non-pharmacological treatment of obesity, metabolic syndrome and inflammation-related conditions.

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Conflict of interest statement The authors of this research disclose any potential conflict of interest.

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