Original Article
Silent information regulation 2 homolog 3 expression in different types of adipose tissues of normal-weight and obese individuals

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Abstract: The aim of this study was to investigate the expression and significance of silent information regulation 2 homolog 3 (Sirt3) in adipose tissues of individuals with different degrees of obesity. The subjects were divided into a normal-weight group and an overweight group according to body mass index values, and subcutaneous and omental adipose tissues were obtained for the detection of Sirt3 and long-chain acyl-coenzyme A dehydrogenase (LCAD) messenger RNAs (mRNAs), as well as protein expression with real-time polymerase chain reaction and western blot. Compared with that in the overweight group, the expression of Sirt3 mRNA in the adipose tissue of normal-weight subjects was higher, and the difference was statistically significant ($P<0.05$). However, the difference in LCAD mRNA expression between the two groups was not statistically significant ($P>0.05$). Sirt3 and acetylated LCAD protein expression in the adipose tissue of the normal-weight group was higher than that in the tissue of the overweight group. In both the groups, Sirt3 protein expression in the subcutaneous adipose tissue was higher than that in the abdominal adipose tissue, and the difference was statistically significant ($P<0.05$), whereas the difference in deacetylated LCAD protein expression level between the two groups was not statistically significant ($P>0.05$). The expression of Sirt3 gene differed by degree of obesity and location of adipose tissues, and the difference was closely related to energy metabolism and obesity.

Keywords: Sirt3, obesity, adipose tissue, energy metabolism, silent information regulation 2

Introduction

Silent information regulation 2 homolog 3 (Sirt3) belongs to the Sir2 family [1]. Found in a variety of organisms from bacteria to humans, Sir2 is a highly conserved NAD^+-dependent histone deacetylase identified through yeast cell transcriptional silencing studies [2, 3]. Sir2 protein is involved in the silencing of yeast-coordinated and telomere region genes, as well as the regulation of ribosomal DNA recombination and the maintenance of cellular gene stability. It also plays a key role in activities such as metabolism and cell senescence [4]. The mammalian SIRT family expresses seven sirtuins-namely, Sirt1-7 [5]-among which the deacetylase activity of Sirt3 is the most powerful [4, 6].

The human Sirt3 gene is located on chromosome 11 (11 p15.5) and is 21 kb [7]. According to the report, Sirt3 plays a central regulatory role in mitochondrial energy metabolism, including participation in the mitochondrial respiratory chain, the TAC cycle, and the β-oxidation and ketone generation processes of fatty acids. Moreover, it may protect cells by increasing intracellular ATP levels related to blood oxygen status and energy deficiency. Furthermore, Sirt3 may regulate the FOXO transcription factors and pro-apoptotic protein Bax gene, thus alleviating the effects of anti-oxidative stress, reducing apoptosis, and prolonging life. Currently, research on Sirt3 focuses mainly on its activity in the heart, liver, brain, and kidneys, as well as its involvement in cancer and other disease. However, few studies on its mechanism in fat have been undertaken, and most have been performed in rats. None of these studies have examined human subjects.

In a previous study, we developed a nutritionally obese animal model with Sprague-Dawley (SD)

rats and measured the expression of Sirt3 gene at the messenger RNA (mRNA) and protein levels. The results showed that under the stress induced by various caloric intakes, the expression levels of Sirt3 mRNA and protein in SD rat adipose tissues was significantly different. A high-calorie diet reduced expression, whereas a calorie-restricted diet increased expression. The expression levels of Sirt3 mRNA and protein in obese SD rat adipose tissues were lower than those in normal-weight SD rat adipose tissues [8].

Obesity is a state of overnutrition characterized by the excessive accumulation of body fat, especially triglycerides. Research has shown that Sirt3 acts on acetylated long-chain acyl-coenzyme A dehydrogenase (LCAD) via deacetylation inside the mouse liver, increasing LCAD levels and enhancing the oxidation of fatty acids, thus, reducing the accumulation of triglycerides and oxidative fatty acid intermediates, which improves metabolic syndrome [9-11]. Conversely, LCAD defects may lead to disorders in fatty acid oxidation causing free fatty acid accumulation, inducing oxidative stress, and resulting in hepatic steatosis and insulin resistance. Using the results of our original experimental studies, we detected the expression levels of Sirt3 and LCAD genes in different types of obese adipose tissue to investigate further the expression of Sirt3 in human adipose tissue and its deacetylation effect on the LCAD gene. Our aim was to provide an initial theoretical basis for further research of the role of Sirt3 in regulating energy-metabolic balance and its relationship with the development of obesity.

Patients and methods

Subjects

The patients selected for the study were free of cancer, acute inflammation, pregnancy-selective, and trauma abdominal surgery and had no history of using corticosteroids, sex hormones, insulin, or lipid-lowering drugs. Fatty tissues were obtained from the patients during scheduled abdominal surgeries (for biliary calculi, hemangioma, gastric stromal tumor, and uterine fibroids).

Body mass index (BMI) is one of the diagnostic standards for obesity; therefore, patients were divided into a normal-weight group and an overweight group according to BMI. The normal-weight group comprised 10 patients aged 44.6 ± 10.14 years, and the overweight group included 10 patients aged 42.75 ± 4.79 years. All of the patients were of Han nationality. This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of Fujian Medical University. Written informed consent was obtained from all participants.

Collection of surgical specimens

Fasting venous blood samples were obtained on the day of surgery without the addition of anticoagulant. The serum was separated and stored at -80°C for the detection of blood glucose and lipids. Peri-omental fatty tissue samples were obtained during the abdominal surgeries. Two pieces of fat-subcutaneous adipose and abdominal omental-surrounding tissues were obtained from each patient. Visible blood vessels and blood clots were removed, and the samples were immediately placed in liquid nitrogen (-180°C) for rapid freezing and then refrigerated at -80°C for later RNA and protein extraction.

Measurement indicators

Preoperative body weight, height, and waist-hip ratio were determined for each patient according to standard methods. BMI was then calculated as BMI = body weight in kg/(height in m)^2, and waist-hip ratio was calculated as WHR = W/H. Fasting plasma glucose was measured with the oxidase method, and an automatic biochemical analyzer was used to measure the total cholesterol, triglycerides, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and very low-density lipoprotein cholesterol.

Real-time polymerase chain reaction (PCR)

The Trizol (Takara Biotechnology [Dalian] Co., Ltd., Dalian, China) method was used to extract the total RNA from the adipose tissues of each group, and the RNA concentration was determined with an ultraviolet spectrophotometer (Thermo Fisher Scientific Inc., MA, USA). A PrimeScript™ RT reagent kit (Takara Biotechnology [Dalian] Co., Ltd.) was used to reverse-transcribe the complementary DNA (cDNA), and

Table 1. Physical examination results of study subjects

Groups	Ages (year)	Weight (kg)	Height (cm)	BMI (Kg/cm^2)	Waist (cm)	Hip (cm)	WHP (Waist/Hip)
Control	44.6 ± 10.14	53.6 ± 6.19	1.59 ± 0.04	21.26 ± 2.96	80.1 ± 4.75	90.4 ± 4.67	0.89 ± 0.05
Obesity	42.75 ± 4.79	68.63 ± 11.10*	1.58 ± 0.06	27.26 ± 2.51*	90.25 ± 8.85	97.5 ± 8.50	0.93 ± 0.06

Data were presented as mean ± SEM. *P<0.05 compares to control group.

Table 2. Characteristics of study subjects

Group	Blood Glucose (mmol/L)	TC (mmol/L)	TG (mmol/L)	VLDLC (mmol/L)	LDLC (mmol/L)	HDLC (mmol/L)
Control	4.80 ± 0.52	4.49 ± 1.03	0.97 ± 0.18	0.44 ± 0.08	2.9 ± 1.00	1.37 ± 0.18
Obesity	5.3 ± 0.62	4.7 ± 1.01	1 ± 0.50	0.46 ± 0.23	2.90 ± 0.77	1.51 ± 0.44

Data were presented as mean ± SEM.

Sirt3, LCAD, and β-actin primers were designed and synthesized by Shanghai Biosune Biotechnology Co., Ltd. (Shanghai, China). The primer sequences were as follows: Sirt3: upstream 5'-CTGCTTCTGCGGCTCTACAC-3', downstream 5'-GAACCCTGTCTGCCATCACG-3'; LCAD: upstream 5'-AGCCCAGGATACCGAGAAC-3', downstream 5'-CACATCAGCAATTAACAGCCTTTCC-3'; β-actin: upstream 5'-GCACCACACCTTCTACA-ATGAG-3', downstream 5'-TAGCACAGCCTGGA-TAGCAAC-3'.

The cDNA from each group was matched with the upstream and downstream primers of Sirt3, LCAD, and β-actin and added to the reagents of a SYBR® Premix Ex Taq™ kit (Takara Biotechnology [Dalian] Co., Ltd.), according to the manufacturer's instructions. Real-time PCR instrument (ABI Biosystems, CA, USA) was used for detection, with the following reaction conditions: 95°C for 30 s, 95°C for 5 s, and 60°C for 34 s for 45 cycles. The amplification curve and corresponding dissolution curve were generated after the reaction, and 7500 Fast System SDS software (ABI Biosystems, CA, USA) was used to analyze the results and read the threshold cycle (Ct) value. The housekeeping gene, β-actin, was set as the internal control, and the gene expression differences among groups were analyzed with the $2^{-\Delta\Delta Ct}$ method.

Western blot

Human adipose tissue (100 mg) was homogenized with 0.5 mL ice-cold RIPA lysate (Shanghai Xinran Biotechnology Co., Ltd., Shanghai, China), placed in an ice bath for 30 min, followed by centrifugation at 12,000 r/min for 30 min at 4°C. The concentration of the superna-

tant protein was then measured with a Micro BCA Protein Kit (Pierce Inc., IL, USA), and then 50 μg total protein was denatured in 5-time protein gel electrophoresis sampling buffer at 95°C for 10 min.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis separation was carried out, and the proteins were transferred onto a polyvinylidene difluoride membrane and incubated with 2% skim milk for 1 h. The primary antibodies (rabbit anti-Sirt3 polyclonal antibody [Santa Cruz Biotechnology Inc., CA, USA]; rabbit anti-acetylated LCAD polyclonal antibody [Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China]; rabbit anti-deacetylated LCAD polyclonal antibody [Wuhan Boster Biological Engineering Co., Ltd., Wuhan, China]; and mouse anti-β-actin polyclonal antibody [Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., China]) were added and incubated overnight at 4°C. The membrane was washed with Tris-buffered saline containing Tween-20 (TBST) three times (10 min each), and then secondary antibody was added and incubated at room temperature for 1 h and washed with TBST three times (10 min each). After washing with TBST, chemiluminescence staining was performed. β-actin was used as the reference and the results were analyzed with UVP analysis equipment (UVP, USA). The film was scanned, and each strip was selected by the system for automatic generation of the gray value.

Statistical analysis

SPSS 16.0 software was used for statistical analysis, and the measurement data were expressed as means ± standard deviation.

Table 3. Relative expression of *Sirt3* mRNA in four groups

Group (n = 10)	ΔCt	Mean^{ΔΔCt}	$2^{-ΔΔCt}$
SAT-control	5.711 ± 0.27	0.332 ± 0.82	1
SAT-obesity	5.476 ± 0.23	0.098 ± 0.23	1.879 ± 0.04*
VAL-control	3.446 ± 0.79	-1.932 ± 0.79	5.021 ± 1.85
VAL-obesity	5.405 ± 0.18	0.026 ± 0.18	1.272 ± 0.35#

Data were presented as mean ± SEM. *$P<0.05$, compared to SAT-control. #$P<0.05$, compared to VAL-control.

Table 4. Relative expression of *LCAD* mRNA in four groups

Group (n = 10)	ΔCt	Mean^{ΔΔCt}	$2^{-ΔΔCt}$
SAT-control	3.976 ± 0.80	-1.382 ± 0.80	1
SAT-obesity	4.745 ± 0.38	-0.613 ± 0.38	0.696 ± 0.16
VAL-control	4.935 ± 0.39	-0.423 ± 0.133	0.544 ± 0.20
VAL-obesity	4.302 ± 0.71	-1.056 ± 0.7	0.865 ± 0.41

Data were presented as mean ± SEM.

Intergroup comparisons were performed with the one-sample t-test, and pairwise comparisons were made with the least significant difference method. A *P* value of <0.05 was considered statistically significant.

Results

The subjects in the two groups were selected in strict accordance with the experimental design, and their general characteristics are shown in **Tables 1** and **2**. No significant differences were found between the two groups in age, waist-hip ratio, blood glucose, cholesterol, triglycerides, high-density lipoprotein, low-density lipoprotein, or very low-density lipoprotein (*P*>0.05), whereas body weight and BMI in the overweight group were significantly higher than those in the normal-weight group (*P*<0.05).

Expression of Sirt3 and LCAD mRNA

The total RNA of the 40 adipose tissue samples from the 20 subjects was measured. The corresponding ΔCt value was calculated according to the Ct value of real-time PCR, and β-actin was used as the internal control. The relative gene expression levels among the groups were calculated by using the $2^{-ΔΔCt}$ method (**Tables 3** and **4**), and the relative quantitative results are shown in **Figure 1**. Sirt3 mRNA expression level in the normal-weight group was higher than that in the overweight group, and the difference was statistically significant (*P*<0.05). However, no significant difference was found between the groups in the LCAD mRNA expression levels

(*P*>0.05). The expression levels in subcutaneous adipose tissue in the normal-weight group were recorded as SAT-control, whereas that of the overweight group was recorded as SAT-obesity. The expression level in the abdominal adipose tissue of the normal-weight group was recorded as VAL-control, and that of the overweight group was recorded as VAL-obesity.

Expression of Sirt3 and LCAD protein

β-actin was set as the internal control, and the analysis was performed according to system-generated gray values. The relative protein expression in each group was then standardized with the target protein gray value/internal reference protein gray value (**Figure 2**). The results revealed that in the subcutaneous fat, Sirt3 and acetylated LCAD protein expression levels in the normal-weight group were higher than that of the overweight group. Similarly, in the abdominal fat, these expression levels were higher in the normal-weight group than in the overweight group, and the differences were statistically significant (*P*<0.05). The protein expression of deacetylated LCAD in the normal-weight group was not significantly different from that of the overweight group (*P*>0.05). In the normal-weight group, Sirt3 protein expression levels in the subcutaneous fat were higher than those in the abdominal fat, and in the overweight group, Sirt3 protein expression levels in the subcutaneous fat were higher than those in the abdominal fat. These differences were statistically significant (*P*<0.05).

Discussion

The western blot results showed that compared with expression in the abdominal fat tissue, the expression of Sirt3 and acetylated LCAD proteins in the subcutaneous adipose tissues was significantly higher in all subjects. This site-specific expression of Sir3 and acetylated LCAD proteins indicated that abdominal and subcutaneous adipose tissues may have different characteristics, but the reason was unknown. These
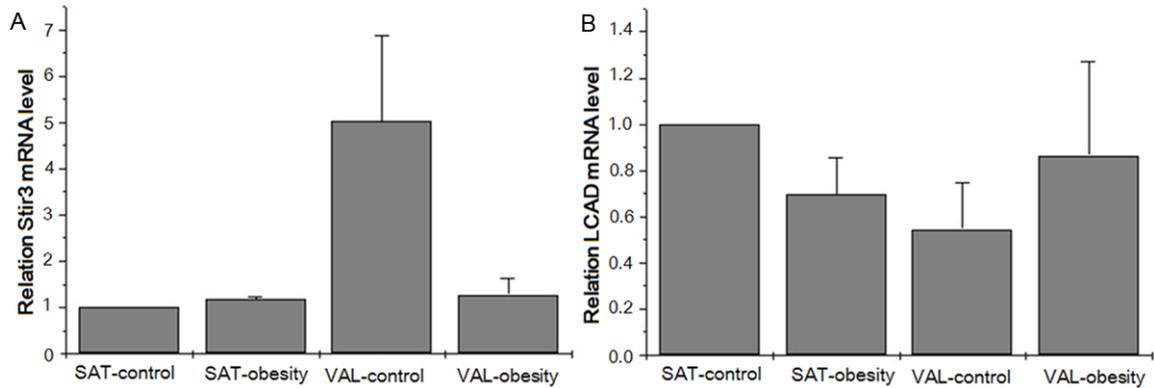
Figure 1. Sirt3 and LCAD mRNA expression in adipose tissue of human obese population.
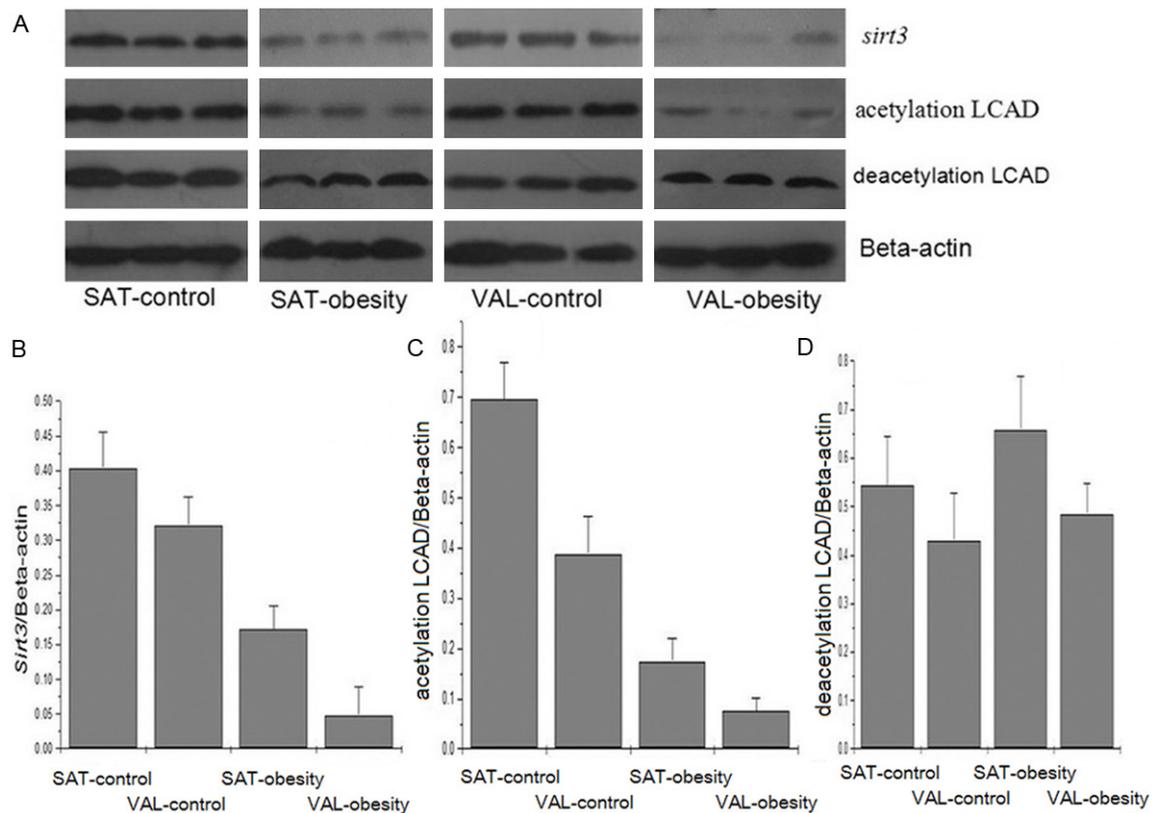

Figure 2. Western blot analysis of protein levels Sirt3 and acetylation LCAD and deacetylation LCAD in adipose tissue of human obese population.

site-specific characteristics might be associated with the nervous and endocrine environments or the nature of the fat cells in the two locations. Insulin and glucocorticoids might also be key regulators of this kind of site specificity.

BMI is currently considered an important indicator of obesity, but it does not completely reflect the distribution of body fat. Waist circumference is an important indicator of abdominal fat accumulation. Therefore, it is reasonable to hypothesize that the expression of Sirt3 in subcutaneous fat tissues might be related to whole-body fat content, whereas Sirt3 expression level in the abdominal adipose tissue possibly, to some extent, reflect the increase in abdominal visceral adipose tissue.

The mitochondrion is a key location of cellular intermediary metabolism, and mitochondrial dysfunction is associated with a large number of metabolic diseases. Sirt3 is the most important mitochondrial NAD$^+$-dependent histone deacetylase [12], and therefore plays a major role in mitochondrial functions. Hirschey et al. [13] and Jing et al. [9] detected changes in Sirt3 expression in mouse models of excess nutrients. The results of their studies showed that Sirt3 activation plays a regulatory role in high-fat diets as well as a key role in age-related metabolic disorders, including obesity, insulin resistance, diabetes, and a series of metabolic syndromes. Studies by Jing et al. [9, 10] showed that Sirt3 participates in the deacetylation of acetylated LCAD in mouse liver, increasing LCAD levels and enhancing fatty acid oxidation, thereby reducing the accumulation triglyceride and fatty acid intermediate products and improving metabolic syndromes. Other studies [10, 11] have reported that LCAD defects might lead to fatty acid oxidation disorders, cause free fatty acid accumulation, induce oxidative stress, and thus result in hepatic steatosis and insulin resistance. These findings demonstrate the relationship among Sirt3 and LCAD levels, mitochondrial protein acetylation, and key metabolic enzyme activities.

Although the effects of Sirt3 and mitochondrial protein acetylation on the body's complex metabolic system are unknown, Sirt3 has been proven to be a major mitochondrial deacetylase that may regulate the acetylation levels of many proteins. Proteomics studies have shown that acetylation is a common post-translational modification in the mitochondria [11, 14-16]; more than one-third of all proteins are acetylated, and those involved in metabolism undergo acetylation preferentially [17]. The main metabolic pathways, such as glycolysis, tricarboxylic acid cycle, urea cycle, fatty acid metabolism, and glycogen metabolism, include acetylases [18].

Some Sirt3 substrates have been confirmed, including LCAD [13]; superoxide dismutase, an antioxidant enzyme that participates in β-oxidation [19]; 3-hydroxy-3-methyl-coenzyme A, which is involved in the formation ketone bodies [20]; and carbamoyl transferase, which participate in the urea cycle [21]. Sirt3 may also deacetylate 5' adenosine monophosphate-activated protein kinase, peroxisome proliferation-activated receptor γ auxiliary activating factor, and uncoupling protein 1, which play vital roles in the regulation of carbohydrates, 5' adenosine monophosphate-activated protein kinase, peroxisome proliferation-activated receptor γ auxiliary activating factor, and uncoupling protein 1, which play vital roles in the regulation of carbohydrates, amino acids, fat metabolism, and TAC.

The results of the present study showed that the expression levels of Sirt3 mRNA and protein in the normal-weight group were higher than those in the overweight group ($P<0.05$), consistent with the results of our previous animal experiments [8]. At the protein level, the comparison between the normal-weight and the overweight groups revealed that increased Sirt3 expression was accompanied by the increased expression of acetylated LCAD ($P<0.05$), whereas the expression levels of deacetylated LCAD were not significantly different ($P>0.05$). Thus, we hypothesized that the activation level of LCAD in the overweight group was higher than that in the normal-weight group. Our results differed from those of Hirschey et al. [13] and Jing et al. [9]; perhaps because the acetylation level impacts substrate specificity, the increased activity of certain enzymes inhibits the activity of other enzymes, and the changes in the activity of these enzymes helps to restore nutrient deprivation-stress homeostasis.

At the mRNA level, the comparison between the normal-weight and the overweight group revealed that increased Sirt3 expression did not cause significant changes in LCAD expression ($P>0.05$). This result was consistent with our findings at the protein level and might have occurred because Sirt3 acted on LCAD through its deacetylation activity, which increased LCAD levels and enhanced the oxidation of fatty acids at the protein level. The expression levels of Sirt3 and LCAD are affected by multiple factors at the mRNA level; therefore, increased Sirt3 expression may not necessarily have led to the increase in LCAD expression.

In summary, Sirt3 gene is closely related to the development of obesity, a state of overnutrition caused by the excessive accumulation of body fat, especially triglycerides. The roles of Sirt3 in energy metabolism might provide new targets

for obesity treatment. For example, enhancing Sirt3 gene expression in the adipose tissues of obese patients is likely feasible. This study lays the preliminary ground work for further research on the role of Sirt3 in fat cell metabolism, and thus provides novel ideas to design studies for the development and treatment of obesity.

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Disclosure of conflict of interest

None.

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