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Original Article

N-3 polyunsaturated fatty acids (PUFAS) dietary improves the metabolic syndrome induce by high fat food

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Abstract: Objective: Metabolic syndrome is affected by the interaction of multiple genes and diverse environmental factors. Among environmental factors, food composition is a major one in modulating the development of metabolic diseases. Using diabetic hamster model, in the present study, we investigated the effects of different n-3 fatty acids diet (n-3 PUFAs) on metabolic disease. Methods: 40 healthy male depuratory hamsters were divided into 4 groups: control group (10% lard), aerobic exercise (EX) group (10% lard), high α-linolenic acid (HALA) group (n6/n3, 12.4:1) and low α-linolenic acid (LALA) group (n6/n3, 18.4:1). 10 weeks later, the hamsters were fasted for 12 hours and then anaesthetized. After blood samples were collected from femoral artery, all animals were sacrificed, and tissue samples, including the internal organs, skeletal muscles as well as amphi-organs adipose, were harvested. Serum glucose, insulin and lipids were determined. The levels of free fatty acids (FFA), and triacylglycerol (TG) in serum and tissues were also measured. Hepatic lipase (HL) and lipoprotein lipase (LPL) were determined in the tissues of liver, skeletal muscle and adipose. Furthermore, we measured the levels of leptin and TNF-α in adipose tissue, PPARs in liver by RT-PCR. Results: N-3 PUFAs did not affect body weight gain induced by high-fat food. However, it significantly reduced the mass of intermuscular adipose, not other fat tissues. More importantly, n-3 fatty acid dramatic decreased the fasting glucose (FPG) and fasting insulin (FINS), alleviating insulin resistance index (HOMA-IR). We further found that, after supplementation with n-3 PUFAs, hepatic LPL dramatically elevated. PPARs, including PPAR-α, γ and δ, significantly increased in n-3 PUFAs groups, especially in HALA group. Conclusion: N-3 fatty acids is able to reduce body fat masses and improve insulin resistance (IR) possibly through increasing hepatic LPL and elevating the expression levels as well as activity of PPARs. Supplement of n-3 fatty acids in dietary might help prevent diseases associated with metabolic syndrome, including diabetes, coronary heart disease, stroke etc.

Keywords: N-3 fatty acids, insulin resistance, high-fat diet, dyslipidemia-diabetic

Introduction

Metabolic syndrome (MS) is a common disorder worldwide. Its broad clinical spectrum includes glucose intolerance, central obesity, insulin resistance and dyslipidemia [1, 2]. During the last decades, the incidence of metabolic syndrome rise dramatically. According to the 2005 survey in China, the age-adjusted rate of MS was 9.8% in men and 17.8% in women [3]. MS is an independent risk factor for many metabolic diseases, such as diabetes, cardiovascular disease and stroke etc. Therefore, MS management is an efficient way to lower the risk of metabolic diseases.

Recently, some cross-sectional surveys found that higher levels of n-3 polyunsaturated fatty acids (PUFAs) are associated with a lower incidence of metabolic syndrome in both men and women [4-6]. Moreover, several reports indicate the direct association between MS and n-3 PUFAs, such as α-linolenic acid (ALA; C18:3n-3), eicosapentaenoicacid (EPA; C20:5-n-3), docosahexaenoic acid (DHA; C22:6n-3), supporting the notion that n-3 PUFAs protect against MS. Poudyal H *et al* elucidated that PUFAs, especially ALA, lead to lipid redistribution away from the abdominal area, and favorably improve glucose tolerance, insulin sensitivity, dyslipidemia, hypertension and heart func-

tion [7]. N-3 PUFAs exert their antagonism against MS through activating the adenosine monophosphate activated protein kinase alpha2 (AMPKα2) [8]. In addition, n-3 PUFAs is also able to activate proliferator-activated receptor gamma coactivator 1-alpha (PPAR1-α), therefore alleviating insulin resistance [9]. The activation of AMPK and PPAR1-α increases hepatic and skeletal muscle fatty acid oxidation, and decreases lipogenesis and cholesterol synthesis [10]. However, how n-3 PUFAs activate AMPK and PPAR1-α is not fully appreciated.

It is well established that metabolic syndrome is driven by the interaction of multiple genes with diverse environmental factors. Among of environmental factors, food composition is a major one in modulating the progression of metabolic diseases. In the present studies, using hamster model, we supplemented food with different amount of n-3 PUFAs, and test its efficacy in modulating MS induced by high fat diet. We further dissect the underlying mechanism of the phenotypes observed at the whole body level.

Materials and methods

Animals and experimental treatment

Animal protocols were reviewed and approved in advance by the Peking University Institutional Animal Care and the Use Committee. All animals received humane care to minimize the pain and any discomfort.

Fortyty male depuratory hamsters weighing 75-85 g were purchased from Beijing Vitalriver Experimental Animal Technique Ltd (China), maintained at 22°C with a 12-hour light/dark cycle, and fed with rodent chow as well as tap water. After accommodating to environment for one week, the hamsters were changed to the food containing 10% lard, provided by Peking University health science center, and then randomly assigned into 4 groups (n = 10): control group (10% lard), aerobic exercise (EX) group (10% lard), high α-linolenic acid (HALA) group (n6/n3, 12.4:1) and low α-linolenic acid (LALA) group (n6/n3, 18.4:1). EX animals were treadmill trained 5 days per week. The food for the animals in LALA and HALA groups contained different fatty acids, which are listed in supplementary table. Animals' body weights were documented once a week. Ten weeks later, the hamsters were fasted for 12 h and then anaesthetized by pentobarbital. Blood sample was collected from femoral artery and the serum were stored at -80°C for further biochemical test. After blood collection, hamsters were sacrificed by cervical dislocated. The skeletal muscle, gastrocnemius muscle and visceral adipose tissue around the major organs were harvested, weighted and stored at -80°C for further experiment.

Biochemical analysis

The fasting glucose (FPG), fasting insulin (FINS), lipids, free fatty acids (FFA), and triacylglycerol (TG) in serum were determined with an automatic biochemistry analyzer (OLYMPUS AU-600, Tokyo, Japan). Hepatic TG and TC, as well as FFA in liver and skeletal were determined as described previously [11]. The fasting insulin in serum and tumor necrosis factor α (TNF-α) in adipose tissue was measured with ELISA kit (Rapid Bio Lab, California, USA) according to the manufacturer's instruction. Leptin in adipose tissue was assessed by enzyme immunoassay (MARKET INC, California, USA). The index of insulin resistance was calculated using the following equation: Insulin resistance index (HOMA-IR) = Serum fasting glucose (mM) X Serum fasting insulin (mU/l)/22.5 [12]. Apoprotein A-1 (ApoA1) and B (ApoB) in liver were determined by immunonephelometry as previously described [13].

Determination of lipase (HL) and lipoprotein lipase (LPL) in liver, skeletal muscle and adipose tissues

Tissue homogenate was prepared with an ice-cold buffer (pH 8.5) containing sucrose (0.25 mol/L), Tris-HCl (50 mmol/L) and heparin (80 IU/mL). After centrifugation at 3000 rpm for 10 min at 4°C, the homogenate supernatant was collected. The levels of lipoprotein lipase (LPL) and hepatic lipase (HP), as well as the activity of HL and LPL was determined as described previously [14]. Lipase activity was calculated according to the following formula:

LPL activity (U/mgprot) = $(OD_{test}/OD_{standard})$ × $FFA\ concentration_{standard}$ (500 μmol/L) × dilution ratio × (60 min/20 min)/1000/homogenate-protein (mgprot/ml)

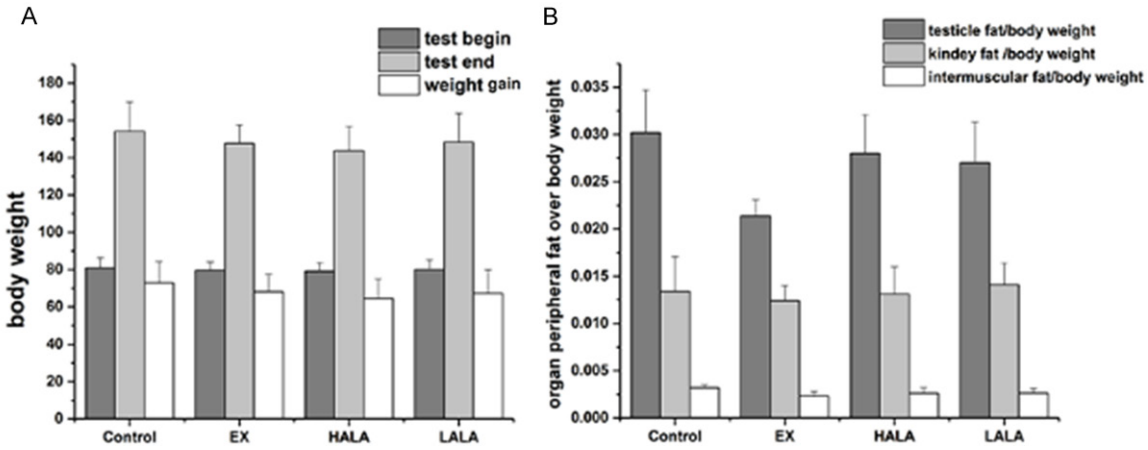

Figure 1. Effects of the n-3PUFAs diet on the weight of body and adipose tissues. A: n-3PUFAs did not affect body weight gain caused by high fat diet. B: n-3 PUFAs supplement dramatically reduced the intermuscular adipose-to-body weight ratio. However, it did not affect the adipose tissues around testis and kidney.

Table 1. Effects of the n-3 PUFAs diet on FPG, FINS and HOMA-IR of hamsters

Items	Control	EX	HALA	LALA
FPG	19.23 ± 2.45	12.89 ± 3.35[a]	11.08 ± 2.75[a]	13.12 ± 2.11[a]
Fins	31.70 ± 6.51	20.95 ± 6.96[b]	20.59 ± 7.11[b]	22.41 ± 9.50[b]
HOMA-IR	6.39 ± 0.14	2.47 ± 0.35	5.35 ± 0.51[c]	5.59 ± 0.40[c]

Note: [a]$P<0.05$ compared with control group; [b]$P<0.01$ compared with control group; [c]$P<0.01$ compared with control group.

HL activity (U/mgprot) = $(OD_{test}/OD_{standard}) \times FFA\ concentration_{standard}$ (500 μmol/L) × dilution ratio × (60 min/20 min)/1000/homogenate-protein (mgprot/ml)

Gross activity = LPL activity + HL activity

RNA extraction and reverse transcription polymerase chain reaction

Total RNA was extracted using the Trizol extraction protocol provided by the manufacturer (Applygen Technologies Inc, Beijing, China). For cDNA synthesis, total RNA (5 μg) was added to 20 μl reaction mix containing avian myeloblastosis viral reverse transcriptase, random hexamers and deoxyribonucleotide triphosphates (Promega Biotech, USA). The sequence of PCR primer (PPARα: Upper Primer 5'TCAATGCCCTAGAACTGGATG 3', Lower Primer 5'CTGCGTCAGACTCGGTCTTC 3'; PPARγ: Upper Primer 5'GGAGCCCAAGTTTGAGTTTGC 3', Lower Primer 5'TGACGATCTGCCTGAGGTCTG 3'; PPARδ: Upper Primer 5'GTACTGCCGCTTCCAGAAGTG 3', Lower Primer 5'GTTCACCAGCTGCTTCCACAC 3') were designed on Primer 3 website and synthesized by SBS Biotechnology (Shanghai, China).

PPARs was amplified with an initial denaturation at 94°C for 3 min, followed by 33 cycles, each of which is consisted of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 60 s in GeneAmp PCR system 9700 (PE, USA). β-actin was amplified concomitantly under the same amplification conditions as an internal control. PCR products (5 μL) were electrophoresed in 2.0% agarose gel. Band intensity was quantified under UV light using the Sygene Bio-ID system (USA). The mRNA expression level was expressed as the ratio of band intensity of the target gene relative to that of β-actin.

Statistical analysis

All data are presented as the mean values ± SD. The difference among groups was compared via one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. Statistical evaluation was performed using SAS 9.1 (SAS Institute Inc., Cary, NC) software. Differences were considered to be statistically significant when $P<0.05$.

Results

Effects of the N-3 PUFAS diet on the weight of body and adipose tissues

There was no difference of the body weight among hamsters at the starting and ending point of experiment. This result demonstrated

Table 2. Effect of n-3 PUFAs diet treatment on serum biochemical items, tissue lipid contents, and tissue lipase activity in different group

Items	Control	EX	HALA	LALA
Serum				
TG	1.40 ± 0.55	2.10 ± 0.90	1.89 ± 0.84	1.94 ± 1.06
TC	3.69 ± 0.73	3.86 ± 0.61	3.30 ± 0.44	3.59 ± 0.96
LDL-C	0.64 ± 0.68	0.91 ± 0.45	0.65 ± 0.31	0.75 ± 0.72
HDL-C	1.05 ± 0.24	0.91 ± 0.07	0.95 ± 0.16	0.91 ± 0.11
FFA	1388.68 ± 779.55	1504.58 ± 360.01	1291.98 ± 541.99	1300.00 ± 611.22
Liver				
TG	4.39 ± 1.85	4.04 ± 1.49	3.11 ± 0.80	3.98 ± 1.23
TC	3.76 ± 0.80	5.49 ± 0.47[a]	4.66 ± 0.92	5.50 ± 1.40[a]
FFA	792.38 ± 236.07	759.63 ± 67.15	808.13 ± 97.35[c]	790.67 ± 153.12
ApoA1	0.775 ± 0.232	0.984 ± 0.079[a]	0.883 ± 0.079[d]	1.047 ± 0.073[b]
ApoB	1.847 ± 0.571	1.923 ± 0.336	1.569 ± 0.266[d]	2.161 ± 0.374
LPL	0.096 ± 0.011	0.123 ± 0.052[a]	0.107 ± 0.026	0.096 ± 0.017
HP	0.093 ± 0.003	0.130 ± 0.047	0.098 ± 0.131	0.112 ± 0.022
Perirenal fat				
TG	1228.63 ± 403.01	1405.62 ± 396.21	1389.45 ± 435.72	1230.43 ± 321.03
FFA	50.75 ± 19.85	57.00 ± 20.65	70.44 ± 30.56	64.57 ± 23.37
Muscle				
TG	735.58 ± 118.62	1106.99 ± 431.87[b]	864.545 ± 209.88	786.57 ± 143.59
FFA	107.00 ± 17.92	115.13 ± 20.61	99.00 ± 12.02	101.35 ± 15.19

Note: [a]$P<0.05$ compared with control group. [b]$P<0.01$ compared with control group. [c]$P<0.01$ compared with control group. [d]$P<0.01$ compared with LALA group.

that n-3PUFAs did not affect body weight gain caused by high fat diet (**Figure 1A**).

In order to observe the effects of the n-3 PUFAs on lipid deposits, we harvested intermuscular adipose and the adipose tissues around major organs, such as testis and kidney etc. Then we measured tissue mass and calculated the adipose-to-body weight ratio. Compared with lard only group, n-3 PUFAs supplement dramatically reduced the intermuscular adipose-to-body weight ratio. However, it did not affect the adipose tissues around testis and kidney (**Figure 1B**).

Effects of the N-3 PUFAS diet on FPG, FINS and HOMA-IR of hamsters

N-3 fatty acids diet reduced FPG levels (**Table 1**). The FPG in LALA and HALA groups was significantly lower than that in lard only group (P<0.05). We further found that N-3 fatty acids dietary also dramatically decreased the FISN levels (P<0.01) (**Table 1**). More importantly, Both LALA and HALA diet led to the significant reduction of HOMA-IR (P<0.01) (**Table 1**). These results indicated that 3-n PUFAs ameliorate insulin resistance.

Effects of the N-3 PUFAS diet on LDL-C and HDL-C levels in serum, TG and TC levels in serum and tissues

There are no obvious difference of serum TG, TC, LDL-C and HDL-C among groups (P>0.05) (**Table 2**).

No significant difference was observed among different groups in terms of the levels of hepatic TC (**Table 2**). However, the hepatic TC in LALA group was elevated and has the significant difference compared with lard only group (P<0.01). TG in the intramuscular and perirenal fat did not change after supplementation with n-3 PUFAs.

Effects of the N-3 PUFAS diet on FFA in serum, liver, muscle and perirenal fat

The free fatty acids in serum, liver, muscle, and perirenal fat shows no difference among different groups (P>0.05) (**Table 2**).

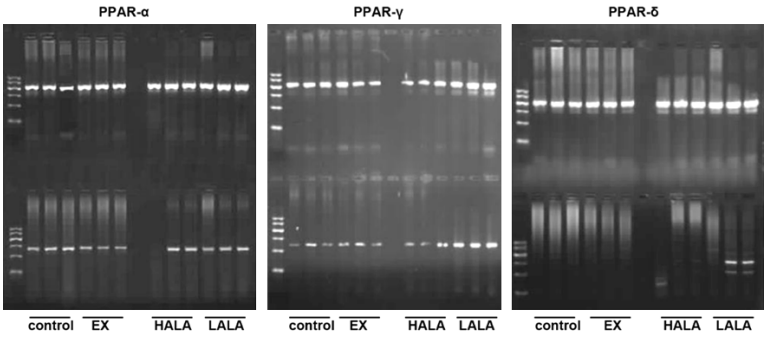

Figure 2. Effects of the n-3PUFAs diet on the expression of PPARs in liver of different group.

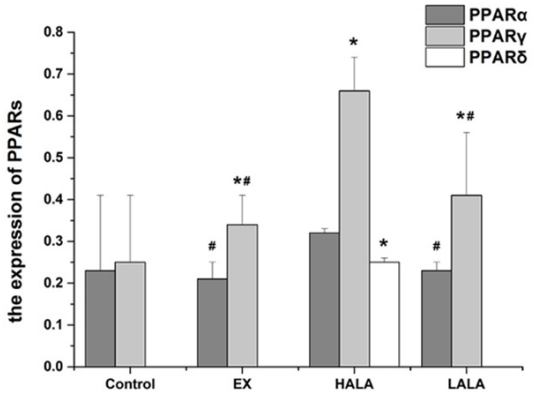

Figure 3. Quantification expression of PPARs in liver of different group. N-3 PUFAs significantly elevated the PPARs, including PPAR-α, γ and δ, in livers compared with lard only group. Furthermore, we found that HALA was more powerful to increase hepatic PPARs *P<0.05 compared with control group. #P<0.05 compared with HALA group.

Effects of the N-3 PUFAS diet on hepatic ApoA1 and ApoB levels

Compared with lard only group, LALA and HALA diet significantly increased liver ApoA1 and ApoB levels (P<0.05) (**Table 2**).

Effects of the N-3 PUFAS diet on the levels of leptin and TNF-A in perirenal fat

No difference was found among the groups (P>0.05) (**Table 2**).

Effects of the N-3 PUFAS diet on hepatic PPARS, LPL and HP

N-3 PUFAs significantly elevated the PPARs, including PPAR-α, γ and δ, in livers compared with lard only group. Furthermore, we found that HALA was more powerful to increase hepatic PPARs (**Figures 2**, **3**). N-3 PUFA did not display any significant effect on the levels of LPL and HP in liver (P>0.05) (**Table 2**).

Discussion

N-3 (or ω-3) fatty acids, a class of polyunsaturated fatty acids (PUFAs), has received a lot of attention over the last 30 years [15, 16], due to its anti-inflammatory and immuno-modulatory properties [17]. Some studies also found that they are able to improve insulin resistance and to lower the risk of metabolic diseases, including cardiovascular disease, obesity and diabetes [18, 19]. Nevertheless, precise mechanisms are not so clear. Using hamster model, which faithfully recapitulates the cholesterol metabolism in human being, the present study demonstrated that n-3 fatty acid enables to improve metabolic syndrome in the whole body level by reducing intermuscular fat mass and ameliorating insulin resistance [20, 21]. N-3 PUFAs exerts these beneficial effects possibly through modulating PPARs-mediating pathway.

Although n-3 fatty acid diet does not show significant effects on body weight and body-weight gain, the mass of intermuscular adipose in the HALA and LALA group is decreased compared with the lard only group. More importantly, the ratio of intermuscular adipose to body weight is also reduced. These results are corroborated by previous studies, in which oral administration of n-3 fatty acid reduces visceral fat [22, 23]. After it is absorbed into blood, dietary fat is packaged inside chylomicron and transported to extra-hepatic tissues. Most of the exogenous TG in these tissue is hydrolyzed by LPL into FFA for energy generation or fat deposition. Indeed, we found that n-3 fatty acids significantly increased liver LPL levels. However, n-3 PUFAs does not affect lipid absorption, since this diet does not change the contents of TG, TC, LDL-C and HDL-C in serum. Therefore, it can be inferred that n-3 fatty acids decrease triacylglycerol accumulation and suppresses hepatic lipogenesis, possibly due to the increase of plasma activity and gene expression of lipopro-

tein lipase. The other studies also support this hypothesis [24, 25]. Secondly, previous study has shown that n-3 fatty acids has substantial affinity to, but is poor substrates for, the enzymes responsible for triglyceride synthesis, thus inhibiting of acyl coenzyme A (CoA): 1,2-diacylglycerol-O-acyltransferase and reducing triglyceride synthesis in the liver [26]. Finally, n-3 fatty acids are the ligand for PPAR-α and other PPAR isoforms, and increases the expression of PPAR target genes which are involved in fatty acid β-oxidation. The increased expression of PPAR target genes, such as acyl-CoA oxidase, CPT-I and CPT-II etc., decreases triacylglycerol accumulation in the liver [27, 28]. Taken together, n-3 PUFAs reduces the accumulation of intermuscular adipose probably via enhancing the activity of hepatic LPL, inhibiting triglyceride synthesis, and activating PPAR-mediated fatty acid β-oxidation in the liver.

PPARs, the peroxisome proliferator-activated receptors, are a group of nuclear receptors and regulates the transcription of multiple genes involved in generation, transportation and β-oxidation of FFA [29]. PPAR-α mainly expresses in liver, heart, small intestine, skeletal muscle and brown adipose tissues. Polyunsaturated long chain fatty acids, oxidized fatty acids and arachidonic acid are its natural ligands. The physiologic role of PPAR-α is mostly related to fatty acid metabolism [30, 31]. PPAR-γ mainly expresses in adipose tissue, and polyunsaturated fatty acids as well as endocannabinoids are its natural ligands. PPAR-γ is mostly involved in the regulation of the adipocyte differentiation, energy balance, lipid biosynthesis and fat storage. Meanwhile, it also prevents lipotoxicity and allows for optimum insulin signaling [32, 33]. PPARβ/δ participates in fatty acid oxidation, mostly in skeletal and cardiac muscles, but it also improves insulin sensitivity and fatty acid oxidation in adipose tissue [31, 34]. In the current studies, the expression of PPARs are dramatically increased by n-3 fatty acids. The PPAR-γ expression in HALA group even is upregulated by about two times compared with the lard only group. Besides upregulating the expression levels of PPARs, n-3 PUFAs can directly activated the function of PPARs as their ligands. Consistent with this, we found the increased expression levels of apolipoprotein (Apo) A and B, two PPARα target genes, in liver after administration of n-3 PUFAs diet.

Besides decreasing intermuscular fat, n-3 PUFAs significantly improves insulin resistance, manifesting by the decreased FPG, FINS and HOMA-IR in both HALA and LALA groups. Improvement of insulin resistance probably attributes to the activation of PPAR-α and PPAR-γ by n-3 PUFAs, ligands of PPARs. Under the condition of high-fat diet, the activation of PPAR-α enables to markedly improve insulin sensitivity. Intracellular fatty acid can interfere insulin-mediated glucose metabolism. Hence, activation of PPAR-α increases fatty acid β-oxidation, decreases the lipid contents in tissues, especially in liver, and enhances insulin sensitivity. The activation of PPAR-γ also increases tissues sensitivity to insulin and accelerates glucose utilization in an insulin-dependent manner. Thiazolidinediones (TZD), a new class of oral antidiabetic drugs for diabetes mellitus type 2, are the strong and specific activators of PPAR-γ. In current studies, we only measure the changes of PPARs in hepatic tissue. In future, we will further observe these proteins in other tissues, such as adipose and muscles.

In conclusion, n-3 fatty acids are able to reduce body-fat masses and improve insulin resistance (IR) induced by high-fat diet. Therefore, the healthy diet supplemented with n-3 PUFAs appears to play important roles against the adverse symptoms of the metabolic syndrome, possibly preventing coronary heart disease and stroke.

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Disclosure of conflict of interest

None.

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