

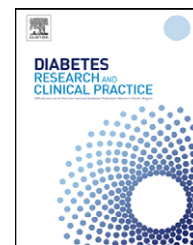


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Correlations of sleep disturbance with the immune system in type 2 diabetes mellitus

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ABSTRACT

Aims: The circadian rhythm and immune system are thought to be associated with the pathological state of diabetes. The aim of this study was to examine the correlation of circadian rhythm disturbance including sleep disturbance with the immune state in patients with type 2 diabetes compared to healthy controls.

Methods: Nineteen patients with type 2 diabetes (11 males and 8 females; aged 46–85 years) and 19 healthy controls (7 males and 12 females; aged 45–85 years) were recruited, and the presence of circadian rhythm disturbance including sleep disturbance was examined using an actigraph. Immunological parameters were also measured.

Results: Sleep and circadian rhythm disturbances were more frequently noted in diabetic patients than in healthy controls. Higher fasting plasma glucose and hemoglobin A1c levels were correlated with stronger sleep and circadian rhythm disturbances. The levels of B lymphocytes, helper T lymphocytes, natural killer cells, natural killer activity, and several cytokines were increased in diabetic patients compared to healthy controls. Correlations were shown among sleep disturbance (circadian rhythm disturbance), immunological measures, and diabetic indices.

Conclusion: The exacerbation of diabetes was related to the level of sleep disturbance, circadian rhythm disturbance, and activation of the immune system.

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1. Introduction

It is well known that diabetes is accompanied by sleep disturbance. A habitually short or long sleep duration increases the risk of developing diabetes [1–5] by impairing glucose tolerance [6]. Although the mechanism leading to the development of type 2 diabetes caused by sleep disturbance has been not fully clarified, several reasons for the increased risk of diabetes development due to sleep disturbance have

been demonstrated. Sleep duration and quality were reported to be significant predictors of HbA1c, a key marker of glycemic control [7]. Sleep deprivation experiments resulted in decreased insulin sensitivity at peripheral receptor sites, which could eventually lead to insulin exhaustion at pancreatic sites after longer periods of sleep deprivation [8]. In particular, the decrease in insulin sensitivity was strongly correlated with a reduction in slow-wave sleep, which is also known as non-rapid eye movement (non-REM) sleep [9].

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A short sleep duration is associated with decreased leptin levels, increased ghrelin levels, and increased hunger and appetite [10,11]. Furthermore, a sleep debt induces decreased glucose tolerance and thyrotropin levels, but increased cortisol levels and activity of the sympathetic nervous system [12,13].

Changes in the immune system are recognized in diabetes, and the immune system is thought to be involved in the diabetes development. Some authors suggest that, in diabetic patients, metabolic control influences the lymphocyte distribution. Dworacka et al. [14] showed that the mean CD3⁺, CD4⁺, CD8⁺28⁻, and CD8⁺28⁺ lymphocyte counts were significantly higher in diabetic patients than healthy controls. Multiple regression analysis revealed that CD4⁺ and CD8⁺28⁻ lymphocyte counts were primarily dependent on 1,5-anhydro- α -D-glucitol levels. The helper T cell subset showed a negative regression with HbA1c, while the cytotoxic subset revealed a positive regression with HbA1c and FBS. The CD4/CD8 ratio showed a negative regression with HbA1c and the diabetic duration [15].

Several cytokines are considered to be involved in the pathogenesis of diabetes. The frequency of the low interferon (IFN)- γ production allele was significantly higher in those with type 2 diabetes compared to controls [16]. Serum interleukin (IL)-12 levels in type 2 diabetics were also lower than in controls. These results indicate that IFN- γ and IL-12 may contribute to the development of type 2 diabetes and/or its complications [17]. Many pro-inflammatory cytokines cause diabetes-associated abnormalities, such as insulin resistance, impaired insulin secretion, increased capillary permeability, accelerated atherosclerosis, and a procoagulant state [18], and also cause associated complications such as dyslipidemia, cardiovascular disease, and renal failure [14]. Thus, there is a close relationship between the immunological state and development of type 2 diabetes. However, the mechanism of the development of type 2 diabetes has yet to be clarified.

In general, sleep disturbance (circadian rhythm disturbance) influences the immune system. For example, aged populations who show increased amounts of REM sleep and a relative loss of slow wave sleep may exhibit elevated nocturnal concentrations of IL-6 [19]. An insomnia group showed decreases in CD3⁺, CD4⁺, CD8⁺, and CD16⁺ cells in peripheral blood [20].

Although diabetic changes are thought to be closely associated with circadian rhythm disturbance including sleep disturbance and the immune system, it has been independently recognized in the studies published so far that there are correlations between the following:

- (1) Sleep (circadian rhythm) disturbance and diabetic indices.
- (2) Immunological measures and diabetic indices.
- (3) Sleep disturbance and immunological measures.

In the present study, circadian rhythm disturbance including sleep disturbance, was determined using an actigraph, and NK cell activity and cytokine levels in the blood and peripheral lymphocyte subpopulations were also measured in patients with type 2 diabetes and compared to healthy controls at the same time, in order to analyze

effectively the correlations among diabetic indices, circadian rhythm including sleep disturbance, and immune system function in a comprehensive manner.

2. Subjects

Nineteen patients with type 2 diabetes and 19 healthy controls were recruited from Clinic Grandsoul Nara located in Nara Prefecture.

This study was conducted after obtaining approval from the Ethics Committee of Clinic Grandsoul Nara. Detailed explanations of the study were given to each subject, and all subjects gave informed consent. Type 2 diabetes mellitus was diagnosed according to the World Health Organization criteria [21].

The patients with type 2 diabetes consisted of 11 males and 8 females, aged 46–85 years (mean: 63.00, SD: 9.821 years). Age-matched healthy controls consisted of 7 males and 12 females, aged 45–85 years (mean: 57.37, SD: 9.166 years). There was no significant differences in age between the patients with type 2 diabetes and healthy controls ($p = 0.076$). The mean duration from the first diagnosis of diabetes was 7.40 years (range: 1–20 years). The patients were being administered the following drugs: sulphonyl urea (5 patients), α -glucosidase (1), insulin (2), thiazolidine derivatives (1), glimepiride + α -glucosidase (5), sulphonyl urea + α -glucosidase (1), insulin + α -glucosidase (2), and no medication (2).

3. Materials and methods

3.1. Actigraph

The actigraph is a simple, noninvasive method of measuring levels of day- and nighttime activity, and can be used for an accurate estimation of the levels of both day- and nighttime sleep [22,23]. Therefore, the objective and exact circadian rhythm disturbance including sleep disturbance was judged using an actigraph, and compared to a sleep diary alone.

Subject activity or lack thereof was sampled by wearing the actigraph on the non-dominant arm for a continuous 1-week period employing the Mini-Motionlogger Actigraph (Ambulatory Monitoring, Ardsley, NY, USA), and recorded once per minute during this time period. The variables were generated by the manufacturer's companion analysis program, Action-W2 (AW2: Ambulatory Monitoring, Ardsley), which separates the day into an 'up' or 'out-of-bed' portion of the day and a 'down' or 'in-bed' portion. The names and significance of the variables used in this study are shown in Table 1. The *rhythm pattern* was calculated using the following formula: $\text{rhythm pattern} = (\text{mean activity} \times \text{duration out-of-bed}) / (\text{mean activity} \times \text{duration in-bed})$.

The data obtained from actigraphs were analyzed by the maximum entropy method using MemCalc/Win software (V. 1.2), and the power of peaks of the spectrum cycles between 20 and 28 h was calculated. Then, the ratio of the specific to the total spectrum power was also calculated.

The subjects maintained sleep logs during actigraphic measurement.

Table 1 – Variables, their definition, and significance on actigraphic measurement.

Name of Variables	Definition	Significance	
		Sleep disturbance	Circadian rhythm disturbance
Duration	Minutes from start to end of interval	ib: shorter	
Activity mean	Mean activity score (counts/epoch)	ob: lower, ib: higher	
Wake minutes	Total minutes scored as wake	ib: longer, ob: shorter	
Sleep minutes	Total minutes scored as sleep	ib: shorter, ob: longer	
%Sleep	Percent minutes scored as sleep (100×(sleep + light sleep)/duration)	ib: lower, ob: higher	
Sleep efficiency	(100× sleep minutes/(O–O duration))	ib: lower	
Wake after sleep onset	Wake minutes during O–O interval	ib: longer	
Activity index	Percentage of epochs with >0 activity score	ob: lower, ib: higher	
Wake episodes (WE)	Number of blocks of contiguous wake epochs	ib: more	
Mean wake episode	Mean duration of WE (minutes)	ib: longer	
Long wake episodes	WE ≥5 minutes	ib: more	
Sleep episodes (SE)	Number of blocks of contiguous sleep epochs	ob: more	
Mean sleep episode	Mean duration of SE (minutes)	ob: longer	
Long sleep episodes	SE ≥5 minutes	ob: more	
Wake minutes/ out-of-bed duration	Wake minutes when the subjects were out-of-bed	ob: lower	
Rhythm pattern	(Mean activity × duration out-of-bed)/ (mean activity × duration in-bed)		Lower
24-h correlation	Autocorrelation at 24 h		Lower

ib: when the subjects were in-bed; ob: when the subjects were out-of-bed.

3.2. Biochemical and hematological measurements of peripheral blood

Peripheral blood was drawn for the biochemical analysis of liver and pancreas functions, and lipid and carbohydrate metabolism including fasting plasma glucose, insulin, and HbA1c at Falco Biosystems, Ltd., Kyoto, Japan. Blood cell counts including numbers and proportions of leukocytes were also examined at Falco Biosystems, Ltd.

3.3. NK cell activity assay

Natural killer (NK) cell activity was measured by employing the calcein-AM release assay using NK-sensitive K-562 cells as a target [24]. Assays were performed in triplicate.

3.4. Differentiation of lymphocyte subpopulations by flow cytometric analysis

Immunofluorescence staining was performed using the following antibodies: fluorescein isothiocyanate (FITC)-conjugated goat anti-human immunoglobulin (Ig) chains specific for γ and μ heavy chains (Tagoimmunologicals, Camarillo, CA, USA), monoclonal antibodies: FITC-conjugated CD3, CD4, CD8, and CD20, R-phycoerythrin (R-PE)-conjugated CXCR3 and CCR4 (BD Biosciences, San Jose, CA, USA), FITC-conjugated CD16 and CD56 (Coulter Corporation, Miami, FL, USA). All monoclonal antibodies were employed in direct immunofluorescence tests using whole blood according to the manufacturer's instructions (BD Biosciences). Immunofluorescence-positive cells were determined by flow cyto-

metry (BD FACSCalibur, BD Biosciences). Determinations of CXCR3 and CCR4 expression on CD4⁺ cells were performed using a two-color immunofluorescence staining technique with the combination of FITC-conjugated CD4 and R-PE-conjugated CXCR3 monoclonal antibodies and FITC-conjugated CD4 and R-PE-conjugated CCR4 monoclonal antibodies, respectively, according to the manufacturer's instructions (BD Biosciences).

Here, CD3⁺ lymphocytes were considered to be T lymphocytes, CD4⁺ lymphocytes: helper T lymphocytes (Th), CD8⁺ lymphocytes: cytotoxic T lymphocytes, CD20⁺ lymphocytes: B lymphocytes, CD4⁺CCR3⁺ lymphocytes: type 1 helper T lymphocytes (Th1), CD4⁺CXCR4⁺ lymphocytes: type 2 helper T lymphocytes (Th2), surface Ig⁺ lymphocytes: B lymphocytes.

3.5. Cytokine single cell analysis of CD4⁺ T cells

Peripheral blood mononuclear cells were isolated by Ficoll-Paque density gradient centrifugation, and the cells were activated for 4 h with 25 ng/ml phorbol 12-myristate 13-acetate (PMA: Sigma-Aldrich, St Louis, MO, USA) and 10 μ M calcium ionophore, A-23187 (Sigma-Aldrich), in the presence of 10 μ g/ml brefeldin A (Sigma-Aldrich). Activated cells were washed twice with phosphate-buffered saline (PBS) and processed for flow cytometry. Three-color immunofluorescence staining was performed using PerCP-Cy5.5-conjugated anti-CD4 monoclonal antibodies (BD Biosciences), FITC-conjugated anti-interferon (IFN)- γ antibodies, and PE-conjugated anti-interleukin (IL)-4 (BD Biosciences) antibodies.

Here, IFN- γ -expressing CD4⁺ lymphocytes were considered to be Th1, and IL-4-expressing CD4⁺ lymphocytes Th2.

Table 2 – Actigraphic measures in diabetic patients and healthy controls, when they were in-bed.

			N	Mean	SD	p
DM	+	Activity index	18	50.390	15.023	<0.01
			19	36.710	11.297	
	–	Long wake episodes	18	3.246	1.523	
			19	2.259	1.398	
FPG	≥126	Sleep efficiency	15	88.908	7.093	<0.05
	<110		18	93.472	5.204	
	≥126	Activity index	15	50.181	16.724	
	<110		18	37.896	11.943	
	≥126	Long wake episodes	15	3.360	1.507	
	<110		18	2.227	1.386	
	≥126	Rhythm pattern	15	27.739	15.616	
	<110		18	43.440	24.662	

3.6. Cytokine assay

Prior to the measurement of cytokine levels, serum samples were stored at -20°C until processing. A Cytometric Bead Array (CBA) system (BD Biosciences) [25] was utilized to measure serum cytokine levels of IFN- γ , IL-2, IL-4, IL-10 and IL-12. The procedures were followed as described in the manufacturer's instructions (BD Biosciences).

3.7. Statistical analysis

Differences in data between the patients and healthy controls were examined using Student's *t*-test. A *p*-value less than 0.05 was considered significant. Pearson correlation coefficients were calculated for indices related to diabetes, actigraphic measures and immunological measures. All statistical analyses were conducted using SPSS software (Advanced Models 16.0)).

4. Results

4.1. Actigraphic measures in diabetic patients and healthy controls

Actigraphic measures were analyzed using AW2 to evaluate sleep and circadian rhythm disturbance, and the differences between diabetic patients and healthy controls were examined using Student's *t*-test.

The results showed that the activity index when the subjects were in-bed was significantly higher and long wake episodes in-bed were more frequent in diabetic patients than in the controls (Table 2).

When the subjects were in-bed, the activity index and long wake episodes were significantly greater in the subjects showing a fasting plasma glucose (FPG) level of 126 mg/dl or higher than in those with level below 110 mg/dl, while the sleep efficiency was significantly lower in the former compared to the latter.

The rhythm pattern score (sleep/wake pattern) was significantly lower in the subjects showing an FPG level of 126 mg/dl or higher than in those with values below 110 mg/dl (Table 2).

4.2. Immunological measures in diabetic patients and healthy controls

The percentages of CD20⁺ and IFN- γ -expressing CD4⁺ lymphocytes were significantly higher in diabetic patients than in controls (Table 3). The percentage of IFN- γ -expressing CD4⁺ lymphocytes was also significantly higher in subjects showing FPG levels of 126 mg/dl or higher and HbA1c of 6.5% or higher than in those below FPG of 110 mg/dl and below HbA1c of 6.5%.

The percentages of IL4-expressing-CD4⁺ lymphocytes and CD4⁺CCR4⁺ lymphocytes were also significantly higher in the subjects showing HbA1c of 6.5% or higher than in those with values below 6.5%. Surface IgG⁺ lymphocytes were significantly more abundant in subjects showing abnormal (beyond normal range) compared to those with normal insulin concentrations (5–15 $\mu\text{U/ml}$).

4.3. Correlation between diabetic indices and actigraphic measures

When the subjects were in-bed, there were positive correlations between the activity mean, wake minutes, wake minutes after sleep onset, and long wake episodes and FPG, while negative correlations were noted between %sleep and sleep efficiency or the rhythm pattern and FPG. There were positive correlations between the activity mean and mean wake episode and HbA1c level (Table 4).

4.4. Correlation between diabetic indices and immunological measures

FPG and HbA1c showed positive correlations with IFN- γ -expressing CD4⁺ lymphocytes, and HbA1c revealed a positive correlation with IL-4 expressing CD4⁺ lymphocytes (Table 4).

4.5. Correlation between actigraphic and immunological measures

When the subjects were out-of-bed, there were positive correlations between the sleep minutes, %sleep, sleep episodes, mean sleep episodes, and long sleep episode, and the level of IL-10, and between the %sleep, sleep episodes, and long sleep episode and the level of IL-4. In addition, when the subjects were in-bed, positive correlations were noted

Table 3 – Immunological measures in diabetic patients and healthy controls.

			N	Mean	SD	p
DM	+	CD20	19	13.165	6.245	<0.05
			19	9.918	2.782	
	–	Th1	18	29.382	13.124	<0.01
			19	16.605	11.164	
FPG	≥126	Th1	15	30.441	13.538	<0.01
	<110		18	17.089	11.478	
	≥126	NK	15	32.454	14.838	<0.05
	<110		18	20.363	13.454	
HbA1c	≥6.5	CD4/CCR4	18	18.233	4.570	<0.05
	<6.5		20	14.722	5.545	
	≥6.5	Th1	17	28.088	11.707	<0.05
	<6.5		20	18.345	13.794	
	≥6.5	Th2	17	4.473	2.485	<0.01
	<6.5		20	2.278	1.110	
Insulin	Abnormal	IgG	16	18.917	9.358	<0.05
	Normal		18	11.637	7.839	

Insulin: 'normal' means 5–15 μU/ml, 'abnormal' means beyond normal range.

Table 4 – Correlations between diabetic indices and actigraphic or immunological measures, when the subjects were in-bed.

	Actigraphic measures									Immunological measures	
	Activity mean	Wake minutes	%Sleep	Sleep efficiency	Wake after sleep onset	Activity index	Mean wake episode	Long wake episodes	Rhythm pattern	Th1	Th2
FPG	0.353(*)	0.349(*)	–0.343(*)	–0.374(*)	0.350(*)	0.377(*)	0.299	0.364(*)	–0.331(*)	0.426(**)	0.190
HbA1c	0.356(*)	0.317	–0.286	–0.298	0.295	0.290	.399(*)	0.245	–0.287	0.407(*)	0.499(**)

* Pearson correlation coefficient: $p < 0.05$.
** Pearson correlation coefficient: $p < 0.01$.

between the activity index and CD4⁺ lymphocytes, between the mean wake episodes and the level of CD16⁺ or CD56⁺ lymphocytes, and between the 24-h correlation and the level of IL-4-expressing CD4⁺ lymphocytes or NK cell activity. Furthermore, when the subjects were out-of-bed, negative correlations were identified between the activity mean, wake minutes, and 24-h correlation and level of IL-10, between the wake minutes/duration and the level of IL-4, and between the activity index and level of IL-12p70 (Table 5).

5. Discussion

The associations of pathological states of type 2 diabetes with sleep disturbance including circadian rhythm disturbance and immunological parameters have recently been clarified.

In general, sleep disturbance is closely associated with the development of diabetes, as reported by several investigators [1–7,9]. In these studies, sleep disturbance was investigated using questionnaires or polysomnography for one night. However, we employed actigraphy to examine sleep disturbance and the circadian rhythm accurately during a longer period, because actigraphy is a simple, noninvasive method of measuring the levels of day- and nighttime activity and can be used for a precise estimation of the levels of both day- and

nighttime sleep. In addition, activity patterns over several consecutive days can be analyzed with autocorrelational techniques to provide estimates of circadian rhythms [22,23].

First, differences between diabetic patients and healthy controls were noted in several variables. Namely, the activity increased at night in diabetic patients, and sleep disturbance was more frequently noted in diabetic patients than healthy controls. Sleep and circadian rhythm disturbances were also identified in the subjects showing high FPG values.

Then, correlations were examined between actigraphic measures and diabetic indices, and the results showed that higher FPG and HbA1c values were correlated with more pronounced sleep and circadian rhythm disturbances. These results are in agreement with those reported in many studies.

The reasons for the increased risk of diabetes development due to the presence of sleep disturbance were estimated based on the results of several studies [8,10–13], focusing on several molecules (leptin, ghrelin, thyrotropin, cortisol, etc.), or carbohydrate metabolism (glucose tolerance, insulin sensitivity, etc.). In the near future, we plan to examine the relationship between leptin and ghrelin and sleep and circadian rhythm disturbances in diabetic patients.

There have been studies suggesting that the immune system is involved in the development of type 2 diabetes. Namely, specific subpopulations of peripheral lymphocytes

Table 5 – Correlations between the actigraphic and immunological measures.

		CD4	CD16	CD56	Th2	NK activity	IL-4	IL-10	IL-12p70
Out-of-bed	Activity mean	–0.153	–0.040	–0.068	0.254	0.154	–0.323	–0.331([†])	–0.128
	Wake minutes	–0.100	–0.093	–0.154	0.045	–0.080	–0.208	–0.382([†])	–0.139
	Sleep minutes	0.100	0.103	0.092	–0.162	–0.110	0.393([†])	0.494([†])	0.273
	%Sleep	0.098	0.101	0.092	–0.137	–0.087	0.399([†])	0.494([†])	0.270
	Activity index	–0.068	0.008	0.056	0.069	0.296	–0.284	–0.148	–0.359([†])
	Sleep episodes	0.110	0.111	0.109	–0.218	–0.162	0.394([†])	0.384([†])	0.271
	Mean sleep episode	0.047	0.113	0.092	–0.171	–0.022	0.067	0.335([†])	0.304
	Long sleep episodes	0.144	0.100	0.114	–0.161	–0.095	0.379([†])	0.449([†])	0.252
In-bed	%Sleep	–0.167	–0.112	–0.202	0.035	0.095	–0.008	0.124	0.304
	Sleep efficiency	–0.103	–0.179	–0.278	0.019	0.098	–0.019	0.141	0.298
	Wake after sleep onset	0.108	0.172	0.284	–0.043	–0.123	0.045	–0.097	–0.301
	Activity index	0.328([†])	–0.069	0.073	0.043	0.027	0.113	0.210	–0.049
	Mean wake episode	–0.011	0.336([†])	0.411([†])	0.069	–0.112	–0.026	–0.256	–0.273
	Wake minutes/ out-of-bed duration	–0.105	–0.101	–0.093	0.143	0.092	–0.400([†])	–0.499([†])	–0.269
	24-h correlation	–0.157	0.080	0.131	0.432(^{††})	0.346([†])	–0.319	–0.404([†])	0.016

[†] Pearson coefficient correlation: $p < 0.05$.

^{††} Pearson coefficient correlation: $p < 0.01$.

were associated with type 2 diabetes [14,15]. It is also conceivable that pro-inflammatory cytokines are associated with the development of diabetes and its complications [14,16,17].

Therefore, immunological parameters in diabetic patients and healthy controls were examined in the present study. The results showed that the percentages of CD20⁺ (B lymphocytes) and IFN- γ -expressing CD4⁺ lymphocytes (Th1) were significantly higher in diabetic patients than in healthy controls. The percentages of IFN- γ -expressing CD4⁺, IL-4-expressing CD4⁺ (Th2), and CD4⁺CCR4⁺ (Th2) lymphocytes were also significantly higher in all subjects showing higher levels of FPG and/or HbA1c than in those with normal levels of FPG and/or HbA1c. Surface IgG⁺ lymphocytes were significantly more abundant in subjects showing abnormal compared to those with normal insulin concentrations. These results suggest that B lymphocytes and type 1 and 2 helper T lymphocytes increased in diabetic patients compared to healthy controls.

Furthermore, FPG and HbA1c were positively correlated with IFN- γ -expressing CD4⁺ lymphocytes, and HbA1c revealed a positive correlation with IL-4-expressing CD4⁺ lymphocytes. In addition, there were positive correlations between sleep disturbance and the levels of IL-10, IL-4, CD4⁺, CD16⁺, and CD56⁺ lymphocytes. Thus, not only Th1 and Th2 but also NK cells and several cytokines were also correlated with diabetic indices. Namely, the activation of innate and acquired immunity, including humoral and cellular immunity, may affect the pathological state in type 2 diabetes.

Although the results of the present study are not completely consistent with those of previous studies [14,16,17], together, the data clarify that cytokines are involved in the development of type 2 diabetes.

It was concluded in the present study that sleep disturbance, circadian rhythm disturbance and immunological changes are present in patients with type 2 diabetes, and that the exacerbation of diabetes is related to the magnitude of sleep disturbance, circadian rhythm disturbance, and activa-

tion of the immune system. It is conceivable that the interactions among circadian rhythm and sleep disturbances and immunological factors might influence the progression of diabetes, and *vice versa*.

Conflict of interest

There are no conflicts of interest.

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