Original article

Serum level of cathelicidin LL-37 is increased in euthymic patients with bipolar disorder irrespective of their cardio-metabolic status

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Abstract

Background: Antimicrobial peptides are components of the innate immune system. Cathelicidin LL-37 plays an important role in antimicrobial defense, exerts proinflammatory effect and strongly affects the immune system functioning. Our recent study revealed that serum concentration of LL-37 is increased in patients with bipolar disorder. **Objectives:** The aim of this study is to re-evaluate serum LL-37 levels in patients with euthymic bipolar disorder and in healthy controls, matched for anthropometric and body composition parameters. **Methods:** 36 adult patients with euthymic bipolar disorder and 68 non-depressed adults were included into the study. Concentration of LL-37 in serum was assessed using ELISA method. Detailed anthropometric measurements, body composition and biochemical analyses were performed. **Results:** There was a statistically significant difference ($p = 0.01$) in serum LL-37 level between patients with bipolar disorder (4.97 ± 7.98 ng/mL) and control subjects (1.78 ± 2.69 ng/mL). **Discussion:** Results of this study indicate that LL-37 serum level is increased in euthymic bipolar disorder patients. We found that this increase could not be attributed to analyzed anthropometric or body composition parameters.

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Keywords: Cathelicidin, LL-37, bipolar disorder, body composition.

Introduction

Bipolar disorder (BD) is a common, severe, and persistent mental illness. Primary symptoms of BD are recurrent episodes of depression that alternate with periods of an excessively elevated or irritable mood known as mania[1]. While exact lifetime prevalence of BD is not known, it is estimated that about 1% of the general population is affected by this condition, while less than half of bipolar patients receives medical help[2]. There is an increasing body of evidence that immune alterations play a major role in the development and course of BD, for example prevalence of autoimmune diseases, allergic diseases, and systemic inflammatory disorders is higher in BD patients[3-6].

Cathelicidins are antimicrobial peptides synthesized by humans and animals in response to various stimuli. The only one known member of the cathelicidin family expressed in humans is LL-37. Cathelicidin LL-37 exhibits broad spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria, viruses, fungi and protozoa. Moreover, besides its antimicrobial functions, increasing evidence points out that cathelicidin LL-37 influences function of cells involved in adaptive and innate immune response and takes part in the regulation of physiological and pathological processes. Several studies also suggest that LL-37 strongly affects inflammatory processes[7,8]. This peptide can stimulate inflammatory cells to produce and release pro-inflammatory cytokines, mediate production of anti-inflammatory mediators, as well as contribute to host homeostasis[7,9-11].

We have previously found[12] that serum concentration of LL-37 is elevated in euthymic patients with bipolar disorder. This could confirm previously postulated role of inflammation in the development of BD[6]. There is however at least one other alternative explanation of this finding, which requires to be examined: observed changes in LL-37 could result from changes in body weight (and accumulation of body fat) that are secondary to treatment with mood-stabilizer. Patients treated with these medications (valproate, lithium, second generation antipsychotics) may gain a considerable amount of body weight[13] and obesity is linked to increased numbers of immune cells, predominantly macrophages, accumulating in the adipose tissue, where they produce cytokines of pro-inflammatory character[14].

Therefore, a question arises whether observed increase of LL-37 serum concentration in BD could be attributed to treatment induced weight-gain and increased amount of body fat. The aim of this study is to find whether previously found (and confirmed in this study) increase of LL-37 in euthymic BD patients could be attributed to differences in anthropometric parameters or body.

Material and methods

Subjects

This was the extension of our original study, with new subjects (10 patients and 16 healthy subjects) enrolled and included into the analysis. Thirty-six adult European Caucasian patients with bipolar disorder (F31 according to ICD-10) were included into the study (the BD group). Diagnosis was established attending psychiatrist, using formal ICD-10 criteria. All patients were euthymic, which was indicated by normal, stable mood for at least 2 months prior the study and on stable treatment for at least 2 months prior the study. Sixty-eight adult European Caucasian were selected as the control group. The control group was selected from a larger sample to match

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age, gender composition, anthropometric, metabolic and body composition parameters of the BD group. The healthy volunteers had neither self-reported personal or familial psychiatric history nor medication history from semi-structured interview and had normal laboratory findings. All study subjects had a normal blood profile, ALT, AST, urea, creatinine, bilirubin, and electrolytes were in norm. Subjects with acute and chronic inflammatory conditions (e.g. pneumonia, rheumatoid arthritis), immunological disorders (e.g. AIDS, allergy), and cancer were excluded from the study. All patients included in the study have been informed about aims and methods of the study and expressed their written informed consent for participation in this study. The study protocol was approved by the Bioethics Committee of the Medical University of Łódź, Poland. There was no financial involvement from the industry.

Laboratory tests

Blood samples were collected between 8 am and 9 am after ensuring at least 8 h overnight fasting. The samples were collected directly into serum separator tubes and centrifuged (10 min, 3,500 rpm). Fasting plasma glucose and lipids (total cholesterol, HDL cholesterol, LDL cholesterol and triglycerides) were measured using automatic Dirui CS-400 analyzer (Dirui, China). Serum concentration of cathelicidin LL-37 was assessed using commercial ELISA kit (MyBIOSource) (the sensitivity of this assay was 0.938 ng/mL), according to instructions provided in the manual. Serum was collected and stored at -80 °C until assayed (up to 6 months). All samples were compared to the standard curve.

Anthropometry

Height was measured with a wall-mounted height measure to the nearest 0.5 cm. Weight was measured with a seca 955 (seca, UK) digital chair scale that was kept on a firm horizontal surface, with subjects undressed. Waist and hip circumferences were measured using a non-stretchable fiber measuring tape. Body mass index (BMI) was calculated as body weight in kilogram divided by the height in meter squared (kg/m^2). Waist-to-hip ratio (WHR) was calculated as waist circumference divided by hip circumference.

Body composition

Body composition was measured using two methods: bioimpedance analysis (BIA) and dual-energy X-ray absorptiometry (DXA). For BIA we used Maltron BIOSCAN 920-2-S Body Fat Analyzer (Maltron, UK), multi-frequency (5 kHz, 50 kHz, 100 kHz, 200 kHz) bioelectrical impedance analyzer. For DXA we used Lunar iDXA scanner (GE Healthcare, UK) with latest version of CoreScan software. Standard operating conditions (including preparation of the participants, electrodes placement and measurement procedures) were monitored by a trained operator. The measurement using DXA and BIA was taken immediately prior to anthropometry measurements with participants lying supine, resting. Briefly, BIA determines the electrical impedance, or opposition to the flow of an electric current through body tissues which can then be used to calculate an estimate of total body water, which can be used to estimate fat-free body mass and, by difference with body weight, body fat. In DXA two X-ray beams, with different energy levels, are aimed at the patient's body and different tissue types (bone, muscle, fat) can be determined from the absorption of each beam by tissues.

The following body composition parameters were measured using DXA method: TBF (total body fat), LBM (lean body mass), VAT (visceral adipose tissue) mass, VAT volume. TBF and LBM are expressed both in kilograms and as percentage of total body mass. SAT (subcutaneous adipose tissue) and VAT areas are measured at the level of the umbilicus, using data input from Maltron BIA analyzer (with special electrodes placement for these measurements) and Maltron software, which converts raw data (impedance and phase angle).

BMI ranges were defined according to WHO recommendations (normal: 18.5-25 kg/m^2, overweight: 25-30 kg/m^2, obesity > 30 kg/m^2). Fat mass index (FMI) was calculated as total body fat in kilogram (measured using DXA method) divided by the height in meter squared (kg/m^2).

Statistical analysis

Statistical procedures were performed with STATA 15.0 (StataCorp, USA). Simple descriptive statistics (means and standard deviations) were generated for all continuous variables. For discrete variables number of patients and percentages are given. Normality of distribution was tested with Shapiro-Wilk test. Variables with normal distribution were analyzed using two-tailed t-test or ANOVA, otherwise Wilcoxon rank sum and Kruskal-Wallis tests were used. Associations were tested by Pearson's (for variables with normal distribution) or Spearman's (for other variables) correlation coefficients. Linear regressions were performed to examine the relationship between serum LL-37 and anthropometric parameters and body composition. Adjusted effect sizes, P values, F values and R^2 were computed for the linear regression models. The level of significance was set at $p < 0.05$ (two sided).

Results

Demographic and clinical characteristics are shown in Table 1. Detailed analysis of cardio-metabolic anthropometric and body composition parameters is also shown in Table 1. There were some inter-group differences for smoking, WHR, lipid lowering treatment, HDL- and LDL-cholesterol, FMI and total body fat (expressed as % of total body weight). Mean treatment duration of the BD patients was 140.4 ± 126.3 months, with mean number of hospitalizations 4.6 ± 4.3. Treatment in the study group was heterogeneous and included both mood stabilizers, antipsychotics and antidepressants. Detailed treatment for each study subject is shown in Table 2.

We have found a significant difference ($z = -2.56$, $p = 0.01$) in serum LL-37 level between BD patients (4.97 ± 7.98 ng/mL) and control subjects (1.78 ± 2.69 ng/mL). This difference remained significant (ANCOVA analysis, $p < 0.05$ for all comparisons) after adjusting for cardio-metabolic parameters (including smoking, WHR, lipid lowering treatment, HDL- and LDL-cholesterol, FMI and total body fat). In the BD group, but not in the control group, level of LL-37 was higher in women (5.92 ± 8.89 vs. 2.51 ± 4.34 ng/mL, $p = 0.02$). In both study groups there were no correlations between LL-37 level and age. Also, in the BD group there were no correlations between LL-37 level and treatment duration. Due to heterogeneity of pharmacological treatment, we were not able to evaluate effect of individual medications on LL-37 level. We have found that subjects taking lipid lowering medications (statins) had significantly lower LL-37 level (1.31 ± 0.88 vs. 3.44 ± 6.11 ng/mL, $p = 0.004$); no such differences were found for subjects treated with anti-hypertensive or anti-diabetic medications. Analysis of the association between serum LL-37 and anthropometric parameters or body composition was performed using linear regression. Since LL-37 levels may be affected by age and smoking, linear regression models were adjusted for sex, age and smoking. Results of regression analysis are shown in Table 3.

Discussion

This is the first study that analyzed the association between LL-37 level and anthropometric parameters or body composition in BD subjects. Previously, our group found that compared with healthy controls, level of LL-37 is increased in patient with BD[12] or unipolar depression[15] (these studies were based on a different samples but used the same LL-37 methodology as in this study). In unipolar depression this increase might be explained by the pro-inflammatory effect of cytokines produced in the visceral adipose tissue. In this study our main objective was to find whether previously found (and confirmed in this study) increase of serum LL-37 in euthymic

BD patients could be attributed to any of anthropometric or body composition parameters. In order to do so, we have compared two groups of subjects (euthymic BD and healthy controls) controlled for anthropometric and body composition parameters. In these groups of subjects, we have confirmed that LL-37 was higher in the BD group. Finally, we found no associations between serum LL-37 and any of analyzed anthropometric or body composition parameters. Results of this study indicate that the differences in LL-37 serum levels could not be attributed to anthropometric or body composition abnormalities (e.g. increased body weight, abdominal obesity, increased total body fat), which are common in patients with BD.

Growing body of evidence points to the significance of inflammation in bipolar disorder. In BD patients increased levels of pro-inflammatory cytokines, such as tumor necrosis factor (TNF), IL-1beta, IL-6 and IL-33 were observed[16-19]. Furthermore, BD patients have significantly elevated serum concentration of anti-inflammatory mediators, such as sTNFR1[18,20,21], sIL-6R[21,22], IL-1Ra[23,24], and IL-10[16,25] as compared with healthy controls. Likewise, in bipolar disorder patients level of IL-4 (known as immunoregulatory cytokine) is raised[26]. In bipolar depressed and manic patients serum concentration of CRP is elevated. Also, Significantly higher serum levels of CRP in bipolar disorder in partial remission were noted[24,27-30]. It should be emphasized that determination of immune and inflammatory parameters in mental disorder need to be considered in the context of metabolic parameters and body composition, since these parameters may significantly affect the immune system functions.

It is known that obesity is more prevalent in the population of patients with mental disorders. In addition, obesity may directly or indirectly change immunological parameters and is a chronic low-grade inflammation, in which there is a two-way interaction between adipokines and cytokines, the innate (macrophages, neutrophils, eosinophils, mast cells, NK cells, MAIT cells) and the

Table 1. Demographic and clinical characteristics

	Bipolar disorder (n = 36)	Control (n = 68)	p
Age (years)	54.9 ± 13.5	57.8 ± 13.9	0.29
Men	10 (27.8%)	10 (14.7%)	0.11
Smokers	16 (44.4%)	9 (13.2%)	**< 0.001**
Smoking (pocket-years)	13.9 ± 24.1	5.1 ± 9.7	0.06
Antihypertensive treatment	15 (41.2%)	17 (25.0%)	0.08
Lipid-lowering treatment	4 (11.1%)	23 (33.8%)	**0.01**
Antidiabetic treatment	3 (8.3%)	4 (5.9%)	0.63
Laboratory tests			
Fasting glucose (mg/dL)	96.5 ± 22.8	92.9 ± 13.0	0.89
Total cholesterol (mg/dL)	190.1 ± 45.1	213.5 ± 43.1	0.05
HDL cholesterol (mg/dL)	53.7 ± 15.7	50.7 ± 12.2	**0.02**
LDL cholesterol (mg/dL)	120.9 ± 32.2	138.6 ± 39.3	**0.04**
Triglycerides (mg/dL)	114.3 ± 50.3	121.3 ± 61.8	0.81
Anthropometric measurements			
Weight (kg)	77.6 ± 16.3	73.0 ± 14.3	0.17
BMI (kg/m^2)	27.1 ± 4.5	27.3 ± 4.7	0.80
BMI category Normal Overweight Obesity	 12 (33.3%) 13 (36.1%) 11 (30.6%)	 21 (30.9%) 27 (39.7%) 20 (29.4%)	0.37
FMI (kg/m^2]	9.4 ± 3.8	11.8 ± 5.2	**0.02**
Waist circumference (cm)	94.0 ± 11.5	89.7 ± 12.7	0.09
Hip circumference (cm)	103.3 ± 9.1	102.5 ± 9.6	0.71
WHR	0.99 ± 0.06	0.87 ± 0.07	**0.01**
Body composition			
Total body fat (kg)	26.6 ± 10.5	31.1 ± 13.0	0.14
Total body fat (% of total body weight)	35.3 ± 14.9	41.7 ± 15.5	**0.01**
Lean body mass (kg)	46.0 ± 10.8	43.1 ± 9.3	0.29
Lean body mass (% of total body weight)	61.4 ± 16.2	58.8 ± 10.1	0.63
VAT area (cm^2)	94.4 ± 53.9	111.5 ± 81.3	0.51
SAT area (cm^2)	97.8 ± 37.3	120.6 ± 55.4	0.07
VAT mass (g)	1061.5 ± 864.0	1003.6 ± 736.6	0.91
VAT mass (% of total body weight)	1.2 ± 0.7	1.3 ± 0.8	0.58
VAT volume (cm^3)	1125.1 ± 915.8	1063.8 ± 780.7	0.91

Data given as mean±standard deviation or n (%).

BMI: body mass index; FMI: fat mass index; WHR: waist to hip ratio; VAT: visceral adipose tissue; SAT: subcutaneous adipose tissue.

Table 2. Detailed pharmacological treatment in the bipolar disorder group

Subject n^o	Treatment
1	LIT 875, LAMO 300, QUE 400, TIAN 37.5
2	CLOM 150, CBZ 450
3	LIT 875, LAMO 300, QUE 400
4	AMI 600
5	VAL 1100
6	OLA 20, VAL 1800
7	QUE 350
8	QUE 350
9	CBZ 600
10	CBZ 600
11	CBZ 600
12	CBZ 600
13	QUE 325, LAMO 200
14	QUE 300, LAMO 200
15	LAMO 100, VEN 187.5, ARI 15
16	VEN 225, LAMO 200
17	SUL 100, LAMO 50
18	LAMO 100, QUE 750
19	LAMO 250, LIT 1000, ESCIT 5
20	QUE 300, LIT 1000, LAMO 250, ESCIT 5
21	QUE 100, LAMO 25
22	QUE 150
23	LAMO 200, QUE 600, SUL 50, OLA 15
24	LAMO 125, QUE 600, OLA 15
25	QUE 400, VAL 1500
26	QUE 400, VAL 1500
27	OLA 12.5, ESCIT 10
28	RIS 2, VAL 600, CLO 75
29	VAL 1500, QUE 300
30	VAL 1500, QUE 300
31	LAMO 100, MIAN 30
32	QUE 500, LAMO 300
33	QUE 500, LAMO 300
34	FLX 20, LAMO 50, QUE 100, OLA 7.5
35	SERT 150, MIRT 30, QUE 100
36	QUE 550

Mood stabilizers – LIT: lithium, CBZ: carbamazepine; VAL: valproate; LAMO: lamotrigine. Antidepressants – CLOM: clomipramine; MIRT: mirtazapine; FLX: fluoxetine; ESCIT: escitalopram; MIAN: mianserin; SERT: sertraline; TIAN: tianeptine; VEN: venlafaxine. Antipsychotics – CLO: clozapine; OLA: olanzapine; ZIP: ziprasidone; QUE: quetiapine; AMI: amisulpride; ARI: aripiprazole; RIS: risperidone; SUL: sulpiride.Doses given as: mg/day.

adaptive (CD4 T cells, CD8 T cells, regulatory T cells, and B cells) immune system and adipose tissue[31]. It should be emphasized that this chronic low-grade inflammation takes part in the development of obesity-related diseases (e.g. type 2 diabetes and insulin resistance)[32]. Gonzalez-Curiel *et al.* indicated that patients with diabetes mellitus type 2 have lower LL-37 gene expression[33]. On the other hand, other researchers reported increased serum LL-37 concentration in patients with diabetes[34] and therefore changes of LL-37 production may also mediate these processes.

Data on the influence of body composition on LL-37 expression are still incomplete. There are studies showing that there is an association between metabolic parameters (such as BMI, waist circumference, WHR, blood glucose and lipids) and expression or concentration of cathelicidin LL-37[35,36]. Interestingly, defensins, which together with cathelicidins belong to the family of AMPs, are also affected by metabolic abnormalities, such as abnormal lipid profile[37]. Hoang-Yen Tran *et al.* have found that obese non-diabetic patients have higher levels of cathelicidin LL-37 compared with patients with normal BMI. Even in comparison with obese pre-diabetic patients, obese non-diabetic patients had increased serum LL-37 concentrations[35]. Additionally, this group has found that LL-37 inhibits the CD36 fat receptor and lipid accumulation in adipocytes and hepatocytes, leading to reduction of hepatic steatosis and fat mass. Therefore, likewise some adipokines (such as leptin) LL-37 may restrict obesity by inhibiting accumulation of lipids in the adipose tissue in response to increased body fat mass. It should be noted that this feedback mechanism in pre-diabetes or diabetes seems to be reduced and fails to protect from lipid accumulation in adipocytes, thus leading to further development of obesity. Benachour *et al.* noted that there are numerous positive correlations between expression of LL-37 and anthropometric parameters, such as BMI, waist circumference, systolic blood pressure and WHR[36]. In this study we did not replicate their finding that LL-37 correlates with BMI. One potential explanation for this are differences in BMI distribution, age, medications and life style of the study subjects. The disagreement with a study of Benachour *et al.*[36], which shows that in women LL-37 is strongly correlated with BMI and waist circumference may result from differences in both studies protocols. Benachour *et al.* measured LL-37 expression at the mRNA level in peripheral blood mononuclear cells, while we assessed serum LL-37 levels. Also, the distribution of obesity was different between both studies, with higher percentage of subjects overweight or obese in our study and higher percentage of patients with normal BMI in the study of Benachour *et al.* Lack of correlation between LL-37 and BMI was previously reported by Cakir *et al.* in children with tuberculosis[38].

Another potential explanation for higher level of LL-37 in the BD group is the effect of lipid-lowering treatment. Increasing body of evidence indicates that statins have anti-inflammatory properties[39]. Since there were more subjects in the control group taking statins, this could translate into lower LL-37 concentration in this group. However, while there are no studies evaluating a direct effect of lipid-lowering medications on LL-37 concertation, Huang et al. observed that statins enhance activity of another antimicrobial peptide (beta-defensin 2) in Salmonella-infected intestinal epithelial cells[40]. These authors have suggested that enhanced activity of antimicrobial peptides by statins could protect the host against infections, while modulation of pro-inflammatory responses could prevent the detrimental effects of overwhelming inflammation in the host.

General conclusion from these limited studies is that since cathelicidins are associated with inflammatory conditions, one can expect an increase of these peptides in obesity. The mechanism linking obesity with altered LL-37 expression may be located in the adipose tissue, involved in the metabolism of endogenous sex steroid hormones (e.g. progesterone, which influences autoimmune and infectious diseases via adaptive and innate effects, also involving alterations of antimicrobial peptides, such as defensins and LL-37)[41].

In conclusion, our study showed that in euthymic bipolar patients there is increased serum LL-37 level and this difference could not

Table 3. Linear regression analysis (adjusted for sex, age and smoking) of serum LL-37 and metabolic parameters

	Bipolar disorder				Control			
	Beta	p	F	R^2	Beta	p	F	R^2
Weight (kg)	0.01	0.98	0.67	0.08	0.04	0.77	0.28	0.02
BMI (kg/m^2)	0.04	0.81	0.68	0.08	0.02	0.85	0.27	0.02
FMI (kg/m^2)	0.06	0.73	0.70	0.08	-0.05	0.71	0.30	0.02
Waist circumference (cm)	0.04	0.82	0.68	0.08	0.01	0.91	0.27	0.02
Hip circumference (cm)	0.08	0.67	0.72	0.08	0.13	0.30	0.54	0.03
WHR	-0.03	0.88	0.68	0.08	-0.14	0.33	0.51	0.03
Total body fat (kg)	0.04	0.79	0.69	0.08	-0.04	0.77	0.29	0.02
Total body fat (%)†	0.02	0.90	0.67	0.08	-0.07	0.60	0.29	0.02
Lean body mass (kg)	-0.04	0.81	0.68	0.08	0.06	0.60	0.33	0.02
Lean body mass (%)†	-0.06	0.72	0.70	0.08	0.01	0.92	0.22	0.01
VAT area (cm^2)	0.04	0.81	0.68	0.08	-0.09	0.47	0.32	0.02
SAT area (cm^2)	0.14	0.43	0.84	0.09	-0.11	0.41	0.37	0.02
VAT area to SAT area ratio	-0.08	0.68	0.71	0.08	-0.03	0.82	0.20	0.01
VAT mass (g)	-0.11	0.62	0.74	0.09	0.01	0.99	0.26	0.02
VAT mass (%)†	-0.11	0.62	0.74	0.09	-0.01	0.99	0.26	0.02
VAT volume (cm^3)	-0.11	0.62	0.74	0.09	0.01	0.99	0.26	0.02
Fasting glucose (mg/dL)	-0.01	0.98	0.67	0.08	-0.01	0.98	0.26	0.02
Total cholesterol (mg/dL)	-0.07	0.70	0.71	0.08	0.18	0.15	0.80	0.05
HDL cholesterol (mg/dL)	0.01	0.96	0.67	0.08	0.03	0.82	0.28	0.02
LDL cholesterol (mg/dL)	0.27	0.15	1.27	0.14	0.19	0.13	0.87	0.05
Triglycerides (mg/dL)	0.09	0.61	0.74	0.09	-0.02	0.93	0.26	0.02
Lipid-lowering treatment	-0.18	0.35	0.91	0.11	-0.20	0.13	0.87	0.05

Beta: standardized regression coefficient; F: F-statistic; R^2: coefficient of determination.

BMI: body mass index; FMI: fat mass index; WHR: waist to hip ratio; VAT: visceral adipose tissue; SAT: subcutaneous adipose tissue.

† Percentage of total body weight.

attributed to any of analyzed body composition parameters. Our results extend the current knowledge about the role of inflammatory proteins in bipolar disorder. Limitations of the study include: relatively small study groups, heterogeneous treatment and cross-sectional design, which limits its ability to establish a causal relationship. Another potential limitation is that diagnosis was only based on clinical ICD-10 criteria and clinical symptoms were not measured using formal diagnostic tools. It was previously reported that vitamin D may affect LL-37 concentration[42]. Since we have no data for vitamin D status, we could not control our results for this variable. On the other hand, we studied subjects from groups that are very comparable in terms of many confounding factors, thus limiting their impact on the results.

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Conflict of interest

All authors declare no conflict of interest.

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