Effects of high-intensity interval training and combined training associated with photobiomodulation therapy in type 2 diabetic (T2D) patients: a protocol for a randomized controlled trial

Efeitos do treinamento intervalado de alta intensidade e do treinamento combinado associado à terapia de fotobiomodulação em pacientes diabéticos do tipo 2 (DT2): protocolo para um estudo controlado randomizado

ABSTRACT | INTRODUCTION: Type 2 diabetes (T2D) can be responsible for significant cardiometabolic dysfunction and reduction in quality of life (QOL) due to its negative impact on functional exercise capacity. OBJECTIVE: To investigate the effects of different modes of physical training (high-intensity interval training [HIIT] and combined training [CT]) associated with light-emitting diode (LED) therapy on the cardiometabolic status, functional capacity, and quality of life (QOL) in T2D patients. METHODS: A randomized controlled trial will be conducted in a university cardiology rehabilitation laboratory; the participants will be community-dwelling people with a confirmed diagnosis of T2D, aged ≥ 18 years, and with a sedentary lifestyle in the last six months. They will be randomly allocated to one of six groups: TIAI with and without LED therapy, CT with and without LED therapy, and a control group with and without LED therapy. The training protocol will be performed for 12 weeks, three times a week on alternate days, with a total of 36 training sessions. The primary outcomes will be functional exercise capacity and glycemic control. The secondary outcomes will be QOL, endothelial function, musculoskeletal function, autonomic nervous system modulation, and body composition. The outcomes will be measured before and after 12 weeks of training. SPSS® 19.0 software will be used for statistical analysis. The significance level is set at P <0.05. PERSPECTIVES: The findings of this trial have the potential to provide important insights into the effects of different modes of physical training associated with LED therapy and may support the use of this therapy combination in T2D patients, which may improve their general health.

RESUMO | INTRODUÇÃO: O diabetes tipo 2 (DT2) pode ser responsável por disfunção cardiometabólica e redução da qualidade de vida (QV) devido ao seu impacto negativo na capacidade funcional de exercício. OBJETIVO: Investigar os efeitos de diferentes tipos de treinamento físico (treinamento intervalado de alta intensidade [TIAI] e treinamento combinado [TC]) associado à terapia com diodo emissor de luz (LED) no status cardiometabólico, capacidade funcional e QV em pacientes com DT2. MÉTODOS: Estudo controlado randomizado que será realizado em laboratório universitário de reabilitação cardiopulmonar com pessoas da comunidade com diagnóstico confirmado de DT2, idade ≥ 18 anos e sedentários nos últimos seis meses. Os participantes serão alocados aleatoriamente para um dos seis grupos: TIAI com e sem terapia LED, TC com e sem terapia LED, grupo controle com e sem terapia LED. O protocolo de treinamento deve ser realizado por 12 semanas, 3 vezes na semana em dias alternados, totalizando 36 sessões de treinamento. O desfecho primário será a capacidade de exercício e o controle glicêmico. Os desfechos secundários serão QV, função endotelial, função musculoesquelética, modulação autonômica cardíaca e composição corporal. Os resultados serão medidos antes e após 12 semanas de treinamento. Para análise estatística será utilizado o programa SPSS® 19.0. O nível de significância adotado será p<0.05. PERSPECTIVAS: Os resultados deste estudo têm o potencial de fornecer informações importantes sobre os efeitos de diferentes tipos de treinamento físico associados à terapia com LED e podem apoiar o uso dessa combinação terapêutica em pacientes com DT2, melhorando sua saúde geral.


This study is registered on ClinicalTrials.gov under the number NCT03593746.


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Introduction

Type 2 diabetes (T2D) is a major health problem worldwide due to its high prevalence, morbidity, and mortality. It is a chronic metabolic disorder characterized by hyperglycemia, resulting from relative insulin deficiency due to reduced insulin production, reduced insulin action, or both. Subsequent chronic hyperglycemia causes tissue glycation, which can inevitably lead to acute disturbances in metabolism, serious health complications, and long-term organ damage, especially the blood vessels, heart, and nerves.

Individuals with T2D have reduced aerobic fitness, characterized by lower peak pulmonary oxygen uptake. T2D is also associated with lower baroreflex sensitivity and abnormal chronotropic response, including altered heart rate (HR) regulation. In addition, prolonged hyperglycemia in DT2 causes several pathological changes in the vascular endothelial cells, increasing the production of reactive oxygen species and inflammatory cytokines that can cause mitochondrial dysfunction and oxidative damage.

Physical activity optimizes blood glucose control and can prevent or delay T2D development. It also reduces lipids, blood pressure, risk of cardiovascular events, and mortality and improves the quality of life. Although physical activity is currently a key element in preventing and managing T2D, the most effective exercise strategy (intensity, duration, and type of exercise) to improve glucose control and reduce cardiometabolic risk in individuals with T2D has not yet been defined.

Light-emitting diode (LED) therapy, known as low-intensity photobiomodulation, is the application of a laser or light-emitting diode in the range of 1–500 mW in a pathological condition and has been investigated in several populations. Recent studies have demonstrated its use in pain relief, improvement in muscle and cardiopulmonary performance, reduction of muscle fatigue, and stimulation of wound healing. However, in relation to patients with T2D who have prolonged hyperglycemia, no studies have been done investigating the impact of photobiomodulation associated with physical training.

Therefore, the main objective of this study is to investigate the effects of different types of physical training [high-intensity interval training (HIIT) and combined training (CT)] associated with LED therapy on cardiometabolic status, functional capacity, and quality of life (QoL) in patients with T2D. We hypothesize that photobiomodulation therapy associated with physical training optimizes therapeutic effects, such as improving aerobic fitness, which can lead to better glycemic control, functional capacity, and QoL. In addition, the scientific community needs more clinical trials involving human beings to define the parameters of LED therapy application, the mechanisms of action involved, and the long-term effects on the skeletal muscle and cardiometabolic performance.

Methods

Study location and design

A randomized, controlled trial will be carried out at the Laboratory of Cardiopulmonary Rehabilitation at Universidade Nove de Julho. Volunteers will be publicly recruited through flyers and electronic social media. All potential participants will be interviewed in person to verify the inclusion and exclusion criteria. After screening, the participants who meet the eligibility criteria will be randomly allocated to one of the six groups. Subjects will be evaluated before and after 12 weeks of training (three times a week on alternate days, a total of 36 training sessions). The study flowchart (Figure 1) is based on Consolidated Standards of Reporting Trials (CONSORT) recommendations.
Participants

Volunteers will be selected according to the following criteria:

- **Inclusion criteria:** age ≥ 18 years; diagnosis of type 2 diabetes confirmed by a physician (fasting plasma glucose ≥ 126 mg/dl and/or glycated hemoglobin ≥ 6.5%); physical inactivity in the last six months according to the criteria established by the American Heart Association (AHA)\(^2\); and cognitive level sufficient to understand procedures and follow instructions.

- **Exclusion criteria:** confirmed diagnosis of any (1) heart disease, (2) musculoskeletal disorder, (3) respiratory disease, (4) uncontrolled arterial hypertension, (5) peripheral neuropathy, or (6) factors that limit the performance of any study assessment and/or training. During the study, individuals with less than 80% attendance at the training sessions will be excluded.

Randomization

Participants will be randomly allocated through a randomization process using sealed opaque envelopes. The six groups are as follows: 1) LED therapy followed by HIIT, 2) simulation of LED therapy followed by HIIT, 3) LED therapy followed by CT, 4) simulation of LED therapy followed by CT, 5) control group (does not perform any training) with LED therapy, and 6) control group with LED therapy simulation. Block randomization will be performed using www.randomization.com.

T2D - Type 2 diabetes; DMF - flow-mediated dilation; SWTI - Shuttle Walking Test Incremental; SWTE - Shuttle Walking Test Endurance; SF36 - Medical Outcomes Study 36 - Item Short - Form Health Survey; IPAQ - International Physical Activity Questionnaire; HR - heart rate; RM - maximum repetition; HIIT - High Intensity Interval Training; LED - light emitting diode.
Intervention

The training protocol proposed for all study groups will be carried out for 12 weeks, three times a week on alternate days, for a total of 36 training sessions. The HR, blood pressure (BP), and blood glucose measurements for all groups will be monitored before and after the training sessions to identify any adverse events. The session will be canceled if the blood glucose is > 13.9 mmol/l (250 mg/dl) before training.

• Combined training (CT)

Supervised exercise will last for 1 h and 10 min in each session (5 min warm-up, 30 min of aerobic exercise, 30 min of resistance exercise, and 5 min of cool-down/relaxation). Aerobic exercise will be performed on a treadmill at an HR corresponding to 60-80% of the pre-test’s VO2 peak (peak pulmonary oxygen uptake). The treadmill’s incline can be increased gradually during training sessions (once the individual completes two consecutive sessions at the specified exercise intensity level).

Resistance exercise will be performed at 60-80% of one-repetition maximum (1-RM) and three sets of 10 repetitions with a 2-3 minute rest interval between each set. The main muscle groups, including the upper and lower limbs, will be trained as recommended by the American Diabetes Association. For resistance training of the biceps and triceps muscles, dumbbells will be used. Resistance training for the hamstring muscles will be performed with a flexor chair and the quadriceps femoris with an extension chair. Workload (weight) will progressively increase through the sessions to maintain consistent training repetitions. To avoid a biased effect during the training program, the subjects will alternate the order of exercises (aerobic training first, followed by resistance training, and subsequently, resistance training followed by aerobic training) in each session.

• High-Intensity Interval Training (HIIT)

Supervised exercise will last for 30 min in each session (5 min warm-up, 20 min HIIT, and 5 min cool-down/relaxation). The exercise will be performed on a treadmill, and the HIIT protocol will be as follows: run of 10 × 60 s, separated by 60 s of rest (20 min of training). The training intensity must reach HR values ≥ 85% of the pre-test peak VO2 or 85% of the maximum HR for age. Individuals are encouraged to run as much as possible during the sprint phase (Figure 2). The treadmill’s incline can be increased gradually during training once the individual completes two consecutive 20-minute HIIT sessions at the set intensity level. HR and perceived exertion using the modified Borg scale will be monitored before, during, and after each training session.
Photobiomodulation therapy with LED

The application will be carried out using a device containing 50 LEDs with a wavelength of 850 ± 20 nm (infrared), which was developed especially for research by the Universidade Federal de São Carlos and the Universidade de São Paulo (Figure 3). This device’s parameters are initially calibrated using a Thorlabs® optical meter (Dachau, Germany), model PM100D.

The LED therapy parameters will be based on literature reports and are shown in Table 1. LED therapy will be applied to the main muscle groups (biceps brachii, triceps brachii, hamstrings, and quadriceps femoris). The treatment duration for each muscle group will be 30 s. LED therapy will be performed before physical training according to the procedures and randomization parameters described above. As the therapy will be infrared, the patient will not be able to identify whether the LED will be active or not, which guarantees concealment.
Data collection and variables

- **Primary outcome:** The primary outcome will be exercise capacity (determined by cardiopulmonary testing and the incremental shuttle walking test) and glycemic control (determined by the percentage of glycated hemoglobin).

- **Secondary outcome:** Our secondary outcomes include QOL, endothelial function, musculoskeletal function, cardiac autonomic modulation, and body composition.

The variables are measured before and after 12 weeks of training. Therefore, assessments will be performed on three alternate days, as shown in Figure 4.

**Figure 4.** Assessment before and after 12 weeks of training

- Demographic data
- Blood collection (biochemical analysis)
- Heart rate variability
- Cardiopulmonary test

**DAY 1**

- Endothelial function (FMD)
- Shuttle Walking Test Incremental (2 tests)
- Quality of life questionnaire (SF36)
- International Physical Activity Questionnaire (IPAQ)

**DAY 2**

- Shuttle Walking Test Endurance
- Body composition (BMI and bioimpedance)
- Maximum strength test (1RM)
- Isokinetic dynamometry

**DAY 3**

FMD - flow-mediated dilation; SF36 - Medical Outcomes Study 36 - Item Short - Form Health Survey; IPAQ - International Physical Activity Questionnaire; BMI - body mass index; RM - maximum repetition.
Body composition

Height and weight will be determined using conventional methods (light clothing and no shoes) with a stadiometer and electronic scale, respectively, and body mass index (BMI in kg/m²) will be calculated by dividing weight by height squared. Body composition will also be assessed using the tetrapolar bioimpedance method (Biodynamics® Model 450, TBW). The procedure will be performed with the individual lying on a nonconductive surface in the supine position, with arms and legs abducted at 45°. One emitting electrode will be placed close to the metacarpophalangeal joint on the dorsal surface of the right hand and another one distal to the transverse arch of the upper surface of the right foot. In addition, according to the recommendations of the manufacturer, the detector electrodes will be placed between the distal prominences of the radius and ulna of the right wrist and between the medial and lateral malleolus of the right ankle.

Blood Tests

Blood samples, such as plasma glucose, plasma insulin, glycated hemoglobin, total cholesterol (C-total), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and triglycerides, will be collected from the ulnar vein before and after 12 weeks of training, after a minimum period of 12 h overnight fasting. An experienced nurse will collect the blood sample. Glycated hemoglobin will be measured using high-performance liquid chromatography (Variant II, BioRad, Berkeley, CA, USA), together with a fluorescence detector method certified by the National Glycohemoglobin Standardization Program. Fasting plasma glucose will be measured by an enzymatic method, using an AU 680® (Beckman Couter, Suarlée (NAMUR), Bélïgica) and plasma insulin by a chemiluminescent assay (UniCel® DxI 800, Pasadena, CA). Total C, LDL-C, HDL-C, and triglycerides will be measured by the AU 680® enzymatic method (Beckman Couter, Suarlée (NAMUR), Belgium).

Cardiopulmonary Test

A symptom-limited incremental stress test will be performed using a cycle ergometer (Corival®, LODE BV Medical Technology Groningen, Netherlands) connected to a system consisting of a gas analysis module coupled to a flow module/wave analyzer and Breeze CardiO2 (Medical Graphics Corporation-MGC, St. Paul, Mo, USA). Incremental exercise will begin after a two-min warm-up at 15 W. The load increment will be defined after the clinical evaluation of the patient. It will be adjusted so that the test is limited by symptoms between 8 and 12 min. Each individual will be asked to maintain a cycling speed of 60 ± 5 rpm until exhaustion is reached.

Oxygen consumption (VO2, mL/min), carbon dioxide production (VCO2, mL/min), minute ventilation (VE, L/min), tidal volume (CV), respiratory rate, and ventilatory equivalents for oxygen and carbon dioxide will be evaluated continuously using a pre-calibrated breath-by-breath analysis system. In addition, electrocardiographic tracing, HR, and pulse saturation (SpO2) will be constantly recorded, and BP will be checked by the auscultatory method every two minutes of exercise. Dyspnea and lower limb fatigue perception scores, using the modified Borg scale, will be evaluated at rest, every 2 minutes of exercise, and immediately after the end of the exercise.

The cardiopulmonary test will be symptom-limited and will end when the subjects meet two of the following criteria: VO2 reaches a plateau with a change of less than 150 mL.min⁻¹; heart rate reaches the maximum level predicted by age (220-age); respiratory exchange ratio (RER)>1.10; and/or the individual presents respiratory or muscular exhaustion according to the assessment of the perceived exertion scale (Borg). After the test, there will be a 5 min recovery at 10 W.

Isokinetic Dynamometry

Concentric isokinetic strength of the knee extensor on the dominant side will be performed at a speed of 60°/s using an isokinetic dynamometer (Biodex Medical Systems 3, Shirley, NY, USA) before and after 12 weeks of training. Volunteers will perform five maximal efforts to determine peak torque (in N m) at a low angular velocity (U) of 60 °/s. During the entire test, the subjects will receive verbal stimuli from the examiner to perform the contractions. The dynamometer will be calibrated according to the manufacturer’s guidelines, and the subjects will be properly positioned and stabilized using straps to avoid compensatory movements. The axis of rotation of the dynamometer will be aligned with the axis of movement of the knee.
evaluated at the level of the lateral epicondyle of the femur. The height and base of the chair, the distance of the support, and the base of the dynamometer will be adjusted to the needs of each individual. The volunteers will perform a 5 min warm-up, consisting of pedaling a cycle ergometer (Inbramed®, Porto Alegre, RS, Brazil) at 100 rpm without load before the isokinetic test on the dynamometer.

**One-Repetition Max Test (1-RM)**

To determine the training protocol loads, the 1-RM test will be performed by gradually increasing the load until the volunteer cannot perform more than one repetition. As recommended by the American Diabetes Association, the 1-RM test will be conducted for major muscle groups, including the upper limbs (biceps and triceps brachii) and lower limbs (hamstrings and quadriceps).

**Shuttle Walking Test Incremental (SWTI)**

Before and after 12 weeks of training, the functional exercise capacity of the participants will be assessed through the application of SWTI. This test will be carried out according to the original description. A 10 m corridor will be used, where two cones inserted 0.5 m from each end demarcate a distance of 9 m. The patient must travel along this predetermined path according to the rhythm imposed by the sound stimulus. A single beep signals the patient to maintain walking speed, and a triple beep determines the start of a new test level, wherein the patient must walk faster. The total test consists of 12 levels, each lasting for one minute. During the test, HR and SpO2 will be monitored every minute using a portable oximeter (Ohmeda-Biox 3700®). Additionally, BP and perceived exertion variables (modified Borg scale) will be recorded before and after the test. The SWTI will be stopped by the examiner when the patient does not reach the cone at the time of the sound stimulus (the patient cannot keep up with the test rhythm) or when they report discomfort (dizziness, nausea, significant dyspnea, extreme fatigue, or chest pain).

**Shuttle Walking Test Endurance (SWTE)**

The SWTE is applied in the same corridor used for the SWTI. Initially, a warm-up (walking) will be performed for 100 s. At the end of the warm-up, a triple beep will indicate an increase in the speed and will be maintained throughout the test. This speed is controlled by single beeps, which are moments in which the patient must be at the ends of the circuit, where the cones are. The walking speed in SWTE should correspond to 85% of that achieved with SWTI. The maximum walking duration at a specific endurance speed should be 20 min. Vital signs will be measured at the exact moments described for the SWTI, and for the interruption of the test, the same criteria used in the SWTI will be followed.

**Heart rate variability (HRV)**

Each volunteer will receive the following guidelines for the eve and the day of collection: avoid consumption of stimulant drinks (tea, coffee, alcoholic beverages), do not perform physical activity, have light meals, and have an adequate night’s sleep (at least eight hours). The collection will be carried out in an acclimatized laboratory with a temperature of 22º-24ºC, air humidity of 50%-60%, and at the same time of the day (morning or afternoon). RR intervals (iRR) will be recorded continuously using a telemetry system with a Polar S810i heart rate meter (Polar Electro Oy, Kempele, Finland). These data will be used to quantify HRV. Each subject will rest for 10 min before the start of data collection to ensure HR stabilization. Then, the HR will be recorded continuously for 10 min, with the volunteer in the supine position.

The FC signals will be transferred to a microcomputer, and the iRR series will be reviewed by visual inspection. The final analysis will include only segments with > 90% pure sinus beats. For HRV analysis, the data will be transferred to software called Kubios HRV (MATLAB, version 2 beta, The Signal Biomedical and Medical Imaging, Analysis Group, Department of Applied Physics, University of
Kuopio, Finland) and will be analyzed using a series of sequential iRR, after choosing the segment with greater signal stability. The linear and nonlinear properties of the HRV will be analyzed.

**Endothelial Function**

The endothelial function will be evaluated based on the percentage of flow-mediated artery dilation (FMD%); it is an effective, simple, and noninvasive method that allows the measurement of the vascular response. To assess endothelial function, participants will be instructed to abstain from vasoactive drugs, exercise, foods rich in nitrates and caffeine for 24h, and alcohol for 48h before the test to minimize the effect of these confounding factors.\(^\text{31,32}\)

In our study, the change in brachial artery diameter will be measured using color Doppler vascular ultrasound (Philips Medical Systems, ViCare Medical, Denmark) before and after the reactive hyperemia caused by the inflation of a pressure cuff. We will use the protocol described in previous studies.\(^\text{31-33}\) Before the assessment, the participant will rest for at least 15–20 minutes. To evaluate the brachial artery, the individual will be placed in a dorsal decubitus position, with the dominant arm extending laterally (90°) stabilized by foam support. Doppler ultrasound imaging of the brachial artery will be identified using Doppler ultrasound imaging above the cubital fossa. After linear visualization of the vessel, the screen will be frozen to obtain the arterial gauge measurements. The average of three measurements taken at baseline is taken for at least 1 min. Subsequently, the pressure cuff was positioned distal to the brachial artery site. The cuff will be inflated at a pressure of 200-250 mmHg for 5 minutes, after which it will slowly deflate. Further measurements of the vessel size will be carried out for at least 2-5 minutes. The analysis of endothelial function data will be performed using Cardiovascular Suite software (Quipu Group, version 3.4, USA).

**International Physical Activity Questionnaire (IPAQ)**

Physical activity level will be assessed using the IPAQ. This instrument has been validated in 12 countries, including Brazil, and covers issues related to the practice of physical activity in the domains of work, domestic activities, transportation, leisure, and time spent sitting.\(^\text{34,35}\)

**Medical Outcomes Study 36 - Item Short-Form Health Survey (SF-36)**

Before and after 12 weeks of training, the participants' quality of life will be evaluated using the SF-36 questionnaire. The instrument consists of 36 items grouped into eight health dimensions: functional capacity, limitations caused by physical problems, limitations caused by emotional disorders, socialization, body pain, general health, mental health, and vitality. The purpose of the SF-36 is to examine patients' perceptions of health status.\(^\text{36}\)

**Data Monitoring**

An independent researcher blinded to the allocation of groups will be responsible for the database management and statistical analyses. Therapists will monitor doses and comply with the training.

**Sample size**

Statistical power is estimated using the study by Terada et al.\(^\text{37}\) Our primary endpoint is exercise capacity (peak pulmonary oxygen uptake). We will need a sample size of 78 (13 per group) to achieve a 5% Type I error and 80% power to reach statistical significance, assuming a 20% dropout rate. In addition, we will use an alpha level of 0.05 and a two-tailed test to determine the difference between groups in exercise capacity.

**Statistical analysis**

The Shapiro-Wilk test will be used to test the data against the Gaussian distribution. This information will show descriptive data as means and standard deviations or medians and interquartile ranges. Appropriate statistical tests will be used to evaluate and compare the effects of different types of physical training associated with LED therapy on the cardiometabolic status and functional capacity of T2D patients. The significance level is set at 5% (p <0.05) for all comparisons (intergroup and intragroup). Statistical analysis will be performed using Statistical Package for the Social Sciences for Windows® (version 19.0; SPSS Inc., Chicago, IL, USA).
Ethics and confidentiality

The study was approved by the local Human Research Ethics Committee (CAAE number 77531417.5.0000.5511) and registered with ClinicalTrials.gov under the study number NCT03593746. This study will follow the human research standards established by Resolution 196/96 of the National Health Council. The participants' understanding of the study's expectations, procedures, risks, and benefits will be verbally assessed before requesting a written informed consent form.

Anonymous data will be stored in a secure database with daily backup, and hard copies of the data will be stored in an approved secure storage location. In addition, we aim to publish this study's results in an internationally recognized journal, make the unidentified database publicly available through the study registry, and inform all participants of the study's conclusions at the end of the study.

Perspective

Although physical activity is a key element in the prevention and management of T2D, the most effective training strategy (intensity, duration, and type of exercise) to improve glycemic control and reduce cardiometabolic risk in individuals with T2D has not yet been defined. Furthermore, patients with T2D have prolonged conditions of hyperglycemia, and no known studies have investigated the impact of photobiomodulation on physical training. Therefore, this randomized clinical trial aims to evaluate the effects of different types of physical training (HIIT and CT) associated with LED therapy on cardiometabolic status, functional capacity, and QoL in patients with T2D.

The results of this study can provide important information about the effects of different types of physical training associated with LED therapy and may support the use of this therapeutic combination in individuals with T2D, improving their overall health. In addition, the results of this study may help physical therapists make more assertive clinical decisions. As a result, they will be able to develop a more effective training strategy to improve functional capacity and cardiometabolic control in patients with T2D.

Registration in ClinicalTrials

This study is registered with ClinicalTrials.gov under the number NCT03593746.

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Author contributions

Padovani C participated in the conception and design of the study and will analyze and interpret the data and write the manuscript. Arruda RMC, Phillips S, and Parizotto NA will participate in data analysis and interpretation. Sampaio LMM designed the study, critically reviewed the text, and will interpret the data. All authors approved the final wording of this article.

Interest conflicts

No conflicting financial, legal, or political interests with third parties (government, commercial, private foundation) have been disclosed for any aspect of the submitted work (including, but not limited to, donations, data monitoring advice, study design, manuscript preparation, statistical analysis).

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