**Acute supplementation with grapes in obese subjects did not affect postprandial
metabolism: a randomized, double-blind, crossover clinical trial**

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Abstract

Purpose. The aim of this study was to determine whether grape polyphenols have a “second-meal effect”, modulating glucose and lipid elevations in the postprandial period after two successive meals in subjects with obesity.

Methods. A randomized, double-blind, placebo-controlled, acute clinical trial was conducted. Twenty-five obese subjects ($BMI = \geq 30$ and $< 40 \text{ kg/m}^2$) were randomly divided into two groups. At an initial visit, blood was collected in a fasting state and the subjects received breakfast and 46 g of either grape powder (equivalent to 252 g fresh grapes) or placebo, both solved in water. Lunch was provided 5 h later and then blood was collected after 0, 30, 60, 120, 180, 240, 300, 330, 360 and 420 min. Two weeks later, at a second visit, the subjects received the other powder. The following were determined: glucose, insulin, triglycerides, uric acid, blood count, hemoglobin, viscosity, antioxidant capacity, and satiety perception.

Results. Postprandial increases were observed as expected in, for example, glucose and triglycerides after breakfast and lunch. The grape powder supplementation did not cause any significant modification compared to placebo, in these parameters; nor did it significantly modify plasma antioxidant capacity in the 6 h postprandial period.

Discussion. Single grape powder supplementation did not modify postprandial responses in obese subjects, probably because the polyphenol dose was insufficient to induce such an effect. The result of a combination of grape with other polyphenol-rich products or chronic supplementation with grape powder on postprandial responses remains to be elucidated.

Trial registration number. www.clinicaltrials.gov, NCT03741218

Keywords: obesity; postprandial state; polyphenols; grape

INTRODUCTION

Worldwide, obesity prevalence has reached epidemic proportions [1], even in countries where it was not present until a few decades ago [2]. Obesity is known to be closely related to the main cardiometabolic risk factors, such as hypertension [3], and to the most prevalent non-communicable chronic diseases: cardiovascular diseases and cancer [4, 5]. As with other metabolic alterations, obesity can lead to deregulation of several postprandial changes, from triglycerides increase [6] to modifications in orexigenic and anorexigenic peptides [7]. This is particularly important since good regulation of the postprandial response is key to avoiding cardiometabolic alterations. For instance, a high postprandial glucose level has been reported to be a predictor of cardiovascular events in subjects with type 2 diabetes, while increased postprandial triglyceride concentrations are more closely associated with cardiovascular events than fasting triglyceride levels [8, 9]. For this reason, there is interest in strategies that can modulate postprandial responses in subjects with obesity.

Among food constituents with the potential to regulate postprandial metabolism, a highly promising group is that of polyphenols: a wide family of compounds present in plant foods that are known to have several biological functions [10]. In particular, grapes are among the fruits with the highest polyphenol content [11]. Several preclinical studies have reported a beneficial effect of consuming grape polyphenols on the regulation of postprandial glucose [12,13] or triglycerides [14]. Moreover, some small-scale clinical trials have reported postprandial modulation of glucose, once more, or insulin by grape polyphenols [15]. Indeed, several mechanisms of action have been reported to be involved in these effects: improvements in insulin and adiponectin signaling pathways; inhibition of inflammation signaling pathways; stimulation of endogenous antioxidant systems; repression of intestinal

lipid absorption; and decrease of triglyceride content in skeletal muscle as well as of chylomicron/VLDL secretion [16-24]. However, most such studies have tested grape-derived products such as skin, seeds, pomace or extracts, while not considering whole grapes [12-15, 21, 23]. This may lead to a relevant fraction of polyphenols, the non-extractable polyphenols which are not present in grape extracts or juice but are a relevant constituent of whole grapes [25], being discarded and excluded from the studies. Furthermore, it is still the case that clinical trials addressing the postprandial effects of grape products are scarce.

In this context, it is important to recall the so-called second-meal effect: the capacity of a meal to diminish the glucose response to carbohydrates eaten during the following meal. This effect has been attributed to dietary fiber through its microbial-derived metabolites, once absorbed in the colon and transferred to the bloodstream [26]. Nevertheless, dietary fiber is not the only food constituent extensively transformed by colon microbiota; it also occurs in the case of polyphenols [27]. Indeed, polyphenol metabolites are known to circulate in the bloodstream for prolonged periods after intake [10], something especially relevant for the non-extractable fraction of polyphenols that are strongly associated with dietary fiber [28]. The second-meal effect has not been specifically explored for polyphenols, although a study with orange pomace, which is rich in both dietary fiber and polyphenols, reported that orange pomace consumption during breakfast significantly decreased the maximal change in glucose concentration after both breakfast and the subsequent lunch, in men with a high cardiometabolic risk [29, 30].

Therefore, the aim of this study was to establish whether grape powder has such a second-meal effect, modulating glucose and lipid increases during the postprandial period after two

successive meals, in subjects with obesity. To this end, a randomized, double-blind, placebo-controlled, acute clinical trial was conducted in obese subjects.

METHODS

Study population

The randomized, crossover, controlled clinical trial was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. It was approved by the Ethics Committee of the Spanish National Research Council (CSIC), Madrid, Spain (2018/09/24, report 15.18) and the Ethics Committee for Clinical Research of the University Hospital Puerta de Hierro-Majadahonda, Majadahonda, Spain (2018/10/02, report 074/2018). It was registered in the Clinical Trials database with the identifier: NCT03741218. The study was conducted in May 2019. All subjects signed an informed consent form, expressing their agreement to participate in the study.

Subjects were recruited via poster advertisements and a direct mailing sent to academic, social, sanitary, and research institutions; in addition to by personal contacts in Madrid, Spain. Inclusion criteria for the study were: age, 18-65 years and obesity ($\text{BMI} \geq 30$ and $< 40 \text{ kg/m}^2$). Exclusion criteria were: previous diagnosis of cardiovascular pathologies or type 2 diabetes; adherence to any weight-control diet; participation in a clinical trial in the previous year; systolic blood pressure $> 150 \text{ mmHg}$ or diastolic blood pressure $> 100 \text{ mmHg}$; blood glucose $> 115 \text{ mg/dL}$; blood triglycerides $> 350 \text{ mg/dL}$; blood cholesterol $> 280 \text{ mg/dL}$.

Study design

It was an acute study in which the subjects received two different treatments, separated by a 2-week wash-out period: grape powder (46 g) and placebo powder (46 g). Details of both

products are provided below. Since it was a crossover design, the subjects were randomly allocated to one of two groups, each starting with one or other of the products. The volunteers were instructed to follow their habitual diet and daily activities between visits. However, in order to limit the potential effect of high dietary polyphenol consumption around the time of the visits, 72 h prior to each one, the subjects were required to refrain from consumption of polyphenol-rich foods such as wine, coffee, tea, cocoa, whole-wheat bread, virgin oil olive, nuts, legumes and certain fruit and vegetables such as berries or artichoke. A detailed list was provided to the volunteers.

On both visit days, subjects attended the Unit of Human Nutrition, ICTAN-CSIC, and a basal blood sample was collected via a venous catheter, which was kept in place for the whole day. Then, the subjects received a high-fat high-sugar breakfast (250 mL of juice, 250 mL of milk, 140 g of pastry and 20 g of honey, with a total caloric value of 949 kcal), together with either the grape or placebo powder. Five hours after basal time, they also received a high-fat high-sugar lunch (115 g of white bread, 40 g of Cheddar cheese, 56 g of a processed red meat (*chorizo*), 40 g of chips, 12 g of white chocolate and 250 mL of juice, with a total caloric value of 846 kcal). Details of the nutritional composition of the breakfast and lunch are provided as Electronic Supplementary Material (**Table S1**). The subjects were instructed to consume both breakfast and lunch in about 20 min each.

Blood samples were then collected after 0, 30, 60, 120, 180, 240, 300, 330, 360 and 420 min. Serum and plasma were obtained after centrifugation at 1000 g for 15 min, and stored at -80°C for further analysis. At 0, 120, 240, 360 and 420 min, the subjects also filled in appetite and satiety reports (see details below).

Sample size calculation

The primary outcome variable for which we calculated the required sample size was glucose maximum concentration in the period 300-420 min. In particular, our power calculations were based on detecting a decrease of 0.18 mM in this parameter, based on a previous nutrition clinical trial focused on the second-meal effect derived from dietary fiber [29]. A sample size of 19 was calculated as sufficient to detect this change with 80% power and an alpha value of 0.05, using published variances in this parameter [29]. We increased this number to 25, to ensure that the statistical power would be sufficient, even if an important proportion of the subjects failed to complete the trial.

Grape powder

The grape powder was provided by the California Table Grape Commission. It consisted of a composite of fresh red, green, and black California grapes (seeded and seedless varieties), frozen, ground with food-quality solid carbon dioxide, freeze-dried, and re-ground, always using good manufacturing practices for food products throughout. The powder included 1% silica as a flow agent. The powder was processed and stored to preserve the integrity of the biologically active compounds found in fresh grapes. Detailed composition of the grape powder is provided as Electronic Supplementary Material (**Table S2**). The 46 g of grape powder provided to each participant in the study was equivalent to the consumption of 252 g of fresh grapes; it was provided as powder merely in order to make handling the product easier.

The placebo was formulated to closely match the freeze-dried grape powder in terms of dietary fiber, sugar profile and organic acid profile, as well as of its sensory characteristics: sweetness, tartness, mouthfeel, and viscosity. It contained fructose and glucose (as dextrose), the two main sugars in the grape powder, as well as organic acids (tartaric, malic and citric acids) and dietary fiber. Artificial colors were used to replace the natural color

components and avoid any addition of phenolic compounds via natural colorants. All the flavorings used were free of phenolic compounds and antioxidants. The placebo also contained modified food starch and tapioca maltodextrin, two potassium salts, and silicon dioxide (at the same level used in the grape powder).

Outcomes

Hematological blood count and hemoglobin

Blood erythrocytes, hemoglobin, hematocrit, MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), MCHC (mean corpuscular hemoglobin concentration), platelets, and MPV (mean platelet volume) were measured using an automatic analyzer (DXH 800 from Beckman Coulter; Brea, CA, USA).

White blood cells, neutrophils, lymphocytes, monocytes, eosinophils and basophils were measured using the same automatic analyzer (the Beckman Coulter DXH 800)

Biochemical determinations

During the screening process, fasting blood glucose was determined by applying the enzyme electrode method, using a Free Style Optimum Neo blood glucose meter from Abbott (Chicago, IL, USA). Serum total cholesterol, HDL cholesterol, LDL cholesterol, and triglycerides were measured using an automatic analyzer (ADVIA Chemistry XPT System from Siemens; Munich, Germany).

During the clinical trial, both fasting and postprandial blood glucose were determined as described above. Insulin was measured using a commercial ELISA kit (Merck-Millipore; Burlington, MS, USA). The areas under the curves (AUCs) for glucose and insulin were estimated using the trapezoidal function. Uric acid was measured in plasma using a commercial kit (Spinreact, S.A.; Sant Esteve de Bas, Girona, Spain). Triglycerides were

measured in plasma using a commercial kit (Cromakit SL; Maracena, Granada, Spain). Plasma antioxidant capacity was measured using the spectrophotometric FRAP (ferric reducing/antioxidant power) assay, following procedures reported elsewhere [31, 32]. Plasma viscosity was measured in a Kinexus pro rheometer (Malvern Instruments Ltd.; Malvern, Worcestershire, UK) equipped with a cone-plate of 60 mm diameter, 1° angle and a gap of 0.030 mm. One hundred microliters of plasma (1:15 dilution) was placed with a pipette on a preheated plate (37°C). The temperature was controlled to within 0.1°C by Peltier elements in the lower plate. A cover was used to maintain the samples at the specified temperature (37°C) and prevent evaporation. To homogenize them to a mechanical equilibrium before measurements, all the samples were put through a pre-shearing test for one minute at 100 s⁻¹ and 37°C. Flow curves were then plotted as a function of shear rate, ranging from 1000 to 2000 s⁻¹. For statistical analysis, viscosity was obtained as the average of the viscosities recorded at four different ascending shear rates (1000, 1259, 1595 and 1995 s⁻¹). At least three replicates were performed for each plasma sample.

Blood pressure

During the screening process, blood pressure was measured using an automated digital oscillometric device (Omron M6 Comfort from Omron Corporation; Tokyo, Japan), and the mean of two readings was taken.

Anthropometric measurements

During the screening process, height, body weight and both abdominal and hip circumference were measured.

Satiety

Satiety was evaluated by applying a validated test with several questions [33, 34], which we gave to the volunteers at different times during each visit; each time, they received the questions in a random order, to avoid automatic responses. The subjects' feeling of thirst was also recorded. The list of questions is provided as Electronic Supplementary Material. From this test, three different indexes were calculated: 1) CSS (composite satiety score), obtained from an overall evaluation of satiety/appetite at each time; 2) satiety, obtained by evaluating questions related to this aspect and the caloric content of each meal; and 3) appetite, obtained by evaluating questions related to this aspect and the caloric content of each meal. The values for these indexes were obtained as follows:

$$\text{CSS} = [(\text{fullness} + \text{desire to eat}) + (100 - \text{desire for sweet}) + (100 - \text{desire for savory}) + (100 - \text{hunger}) + (100 - \text{amount subjects desires to eat})] / 6$$

$$\text{Satiety} = [(\text{Satiety at } 0' - \text{Satiety at } 120') / \text{energy content of last intake}] \times 100$$

$$\text{Appetite} = [(\text{Hunger at } 0' - \text{Hunger at } 120') / \text{energy content of last intake}] \times 100$$

Statistical analysis

The data were analyzed using the SPSS IBM Statistics 25 package for Windows. First, the possible effect of randomization (group assignment) was evaluated. To this end, Student's t-test for independent variables was performed for basal values for each of the biochemical parameters between the two groups (grape powder and placebo). We observed no statistical differences between groups and therefore, the data for all the subjects, independent of the assignation order, were processed together. Subsequently, a normal data distribution was evaluated based on kurtosis coefficient (between -1 and +1) and, when data were discovered not to be normally distributed, we performed a log or square-root transformation. Finally, Student's t-test for paired samples was applied for each parameter and each time point. For comparing the increment in glucose and insulin values at the

different time intervals, since the variables were not-normally distributed and transformation was not possible because negative values were generated, the Wilcoxon test was applied.

RESULTS

Subject recruitment

The detailed CONSORT diagram of this clinical trial is shown in **Figure 1**. Briefly, from a total of 243 subjects interested in the study, 54 consented and attended the screening visit. Of those, 16 did not meet the inclusion criteria and 13 declined to participate, resulting in a total of 25 subjects. Nobody withdrew during the study, due to sensory reasons or any adverse effects, so all 25 participants completed the two phases of the study. The meals were adapted for two of the participants: one who did not like milk; and for another, the *chorizo* was replaced by ham.

Subject characteristics

Basal values for the subjects are shown in **Table 1**. Their age range was 22-57, with a mean value of 35 ± 2 . Fifteen participants were women. Mean BMI was $34 \pm 0.6 \text{ kg/m}^2$. Values for cardiometabolic markers (blood pressure, blood glucose, total cholesterol, HDL cholesterol, LDL cholesterol and triglycerides) were all within normal ranges.

Hematological and immunological basal values (**Table 2**) were measured at the beginning of each visit, in order to rule out possible physiological alterations. Moreover, these data were used to determine whether the subjects were in comparable physiological situations at both visits, and no significant differences observed.

To obtain a clearer image of the characteristics of the subjects participating in the clinical trial, Pearson correlations were calculated between basal parameters and they were

represented as a heat map (**Figure S1**). Parameters that lacked any significant associations with any other were not included in this representation. Significant associations were found between markers of cardiometabolic risk, such as LDL cholesterol and uric acid, or insulin and BMI.

Glycemia control

Mean values for blood glucose and plasma insulin during the two interventions are provided in **Table 3**, while the AUCs obtained after their graphical representation are shown in **Table 4**. For both glucose and insulin, the postprandial metabolism after placebo consumption was as expected, with a peak after breakfast and another after lunch. However, no significant differences were found between treatments at any time after supplementation, either in absolute values for glucose and insulin (**Table 3**), in the AUCs for the different intervals (**Table 4**) or in the increments between time intervals (data not shown).

Lipidemia control

Plasma triglyceride values at the different times, together with their AUCs, are shown in **Table 5**. Although basal values in the placebo group were slightly higher than in the grape treatment group (140 ± 19 vs 103 ± 10 mg/dL), our statistical analysis showed that they are equivalent. As expected for the placebo treatment, a peak was observed after breakfast and another one after lunch (both delayed, compared to the peaks in blood glucose reported above). Nevertheless, no significant differences between treatments were found.

Table 6 shows the evolution over time of plasma uric acid values. No significant differences between the treatments were observed at the different times.

Viscosity evaluation

Results for plasma viscosity are shown in **Table 7**. This parameter proved to be almost constant over time in the placebo treatment. This same tendency was also observed in the grape treatment, with no significant differences between the two. We should emphasize that fasting plasma viscosity showed an inverse association ($p < 0.05$) with plasma insulin.

Satiety perception

Table 8 shows the results for CSS and thirst. As expected for the placebo treatment, two increases in CSS were observed, corresponding to the periods after breakfast and after lunch. For thirst, values were high at the beginning and remained similar during all the study, in accordance with the free access to water provided to the volunteers. No significant differences were observed between treatments. The determinations of postprandial appetite and satiety (two hours after breakfast and lunch) are provided in **Table 9**; no significant differences were observed between the treatments.

Plasma antioxidant capacity

Plasma antioxidant capacity values are shown in **Table 10**. No significant differences were found for this parameter between the treatments at the different times evaluated. Indeed, no increase in plasma antioxidant capacity was observed after grape consumption, which was an unexpected result, as discussed below.

DISCUSSION

This study was designed to evaluate the potential effect of grape intake on the postprandial state after two successive meals, in contrast to other studies that have focused on the postprandial response tested early in the morning, i.e., after overnight fasting. The approach adopted here aimed to closely reproduce a real situation in Western countries, defined by some authors as a continuous postprandial state [35]. Indeed, the combination of such periods needs to be tightly regulated to ensure proper glycemic control and also to avoid

alterations in other physiological processes concomitant with a postprandial state. These include increased inflammation and oxidative stress, which are altered in obese subjects [36, 37]. Thus, the starting hypothesis of this clinical trial was that grape intake could modulate postprandial response after two successive meals. Based on previous knowledge of the metabolic fate of both polyphenols and dietary fiber, this effect might be a consequence of two different mechanisms. It could be a result of the direct contribution of microbial-derived polyphenol metabolites to the regulation of insulin metabolism. Alternatively, it could result from the enhancement of the fermentation of dietary fiber, which increases the levels of short-chain fatty acids derived from the fermentation of dietary fiber and which in turn are involved in second-meal tolerance.

Subjects participating in this study may be considered to be the so-called metabolically healthy obese, since they did not exhibit altered values for the most common cardiometabolic markers. Nevertheless, it has been pointed out that such subjects also have increased cardiovascular risk, compared to lean subjects [38]. Furthermore, the participants exhibited the expected associations between the different cardiometabolic risk markers, and they showed physiological postprandial glucose and triglyceride responses after breakfast and lunch. Therefore, they may be considered representative of such a population.

In contrast to our initial hypothesis for this clinical trial, supplementation with 46 g grape powder did not cause any significant variation in the measured outcomes, compared to placebo. This was evident as the responses of carbohydrate metabolism (measured by blood glucose and plasma insulin), fat metabolism (measured by plasma triglycerides and uric acid) and satiety perception (evaluated by a validated self-report test) were similar when the subjects consumed either the grape powder or the placebo. This finding is in disagreement with a previous study that reported an improvement in satiety, glucose and insulin after two

successive meals when supplemented with orange pomace [29]. Since the population in that study (men at high cardiometabolic risk) was rather similar to the one studied here, the differences may be related to the product provided; phenolic compounds in orange are rather different to those of grape and dietary fiber content in orange pomace is higher than that of grape powder. Similarly, our results are at odds with another study that observed a significant amelioration of insulin response after two meals in a pilot clinical trial based on supplementation with peel from the tropical fruit *Myrciaria jaboticaba*, although the population was rather different to the one in our study, since they were young, normal-weight healthy subjects [39]. In contrast with these studies suggesting a beneficial role of polyphenols in second meal response, a dietary supplement of grape seed extract did not improve satiety in normal-weight subjects after two successive meals [40]. And a recent study did not find any effect on postprandial glucose or insulin of acute supplementation to subjects with abdominal obesity with grape and pomegranate pomace together with an oral glucose overload [41]. Together, these results show that the effects of polyphenols on postprandial regulation is controversial and it is a topic that deserves further research in order to elucidate such divergent findings.

An important aspect that may contribute to understanding the lack of effect observed here after grape supplementation is the fact that no significant increase in plasma antioxidant capacity was observed when the subjects consumed the grape powder. Although this parameter is known to be influenced by several plasma constituents [42], polyphenols are a key contributor to it. In other studies, significant increases in plasma antioxidant capacity have been reported after intake of grape and pomegranate juice [43] or of red cabbage [44]. Since such an effect was not observed here, it seems logical that the lack of increase in the circulation of polyphenol metabolites after grape supplementation (indirectly assessed as

plasma antioxidant capacity) led to the absence of regulation of postprandial responses. This raises the question of whether a higher dose of grape should have been provided. The one we administered in this study (252 g equivalent) was designed to emulate the grapes that would be consumed in a typical meal; higher doses would vary from a typical intake so they were discarded in this study. Moreover, a more realistic approach is to consider that, in a real situation, the polyphenols capable of originating physiological modifications would be provided by the whole ensemble of foodstuffs containing polyphenols [45]: including, but not limited to, the contribution from grapes. In this way, it is possible that, although the acute grape supplementation used here did not modify plasma antioxidant capacity, such an effect would be observed after chronic supplementation, as it was the case with grape and pomegranate juice [43]. Along these lines, chronic intake of onion improved postprandial endothelial dysfunction in healthy men [46]. Finally, we should not rule out the possibility that the lack of effect observed here was due to an altered metabolic fate of polyphenols in obese subjects; this has been reported in rats regularly fed a high-fat high-sucrose diet as compared to rats fed a control diet, when both groups were supplemented with grape polyphenols [47] or with tea polyphenols [48]. Regarding evidence in humans, it was suggested that mango polyphenols were less transformed by obese subjects than by lean subjects [49] and it was found that, in the former ones, the generation of short chain fatty acids after mango supplementation was enhanced [50]. Indeed, the increase in plasma antioxidant capacity after the acute intake of red cabbage at nutritional doses was observed in subjects exhibiting normal weight [44], in contrast to the volunteers in this study.

Another parameter determined in this clinical trial was plasma viscosity, since its potential use for evaluating cardiometabolic risk is current the subject of discussion [51]. Modifications in postprandial viscosity have so far been studied less than fasting viscosity.

In this study, this parameter did not change during the placebo treatment. Previous modifications in postprandial blood viscosity were reported for healthy subjects but not for type 2 diabetic subjects [52]; this agrees with the absence of a modification observed here. So, since no physiological modifications were observed in postprandial plasma viscosity in the control participants in this study, we similarly expected that this parameter would remain constant after grape supplementation, as indeed was observed.

CONCLUSIONS

The results from a randomized, double-blind placebo-controlled, acute clinical trial in obese subjects show that acute grape supplementation at nutritional doses does not lead to an improvement in postprandial metabolism of glucose or lipids, or viscosity, after two successive meals. This is probably due to a low polyphenol dose, while in a real situation, grape polyphenols would be combined with those from other food sources. The potential effects of grape supplementation on postprandial regulation after chronic supplementation remain to be explored.

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Conflict of interest. There are no conflicts to declare.

Authors' contributions. J. Pérez-Jiménez conceived the study, got funding and supervised all tasks; M. Cuesta-Hervás, O. Alonso-Gordo, E. García-Maldonado, J. Pérez-Jiménez and A.M. Veses-Alcobendas performed volunteer recruitment and the development of the clinical trial; E. García-Maldonado, O. Alonso-Gordo and M. Martínez-Suárez processed

biological samples; M. D. Álvarez, M. Cuesta-Hervás, O. Alonso-Gordo, E. García-Díez, B. Herranz and A.M. Veses-Alcobendas performed biochemical determinations in biological samples; E. García-Díez carried out data curation, statistical analysis and visualization; M.D. Álvarez, E. García-Díez, J. Pérez-Jiménez and M.P. Vaquero interpreted the results; J. Pérez-Jiménez wrote the first draft of the manuscript. The manuscript was reviewed and approved by all authors.

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