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**PAPER**

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# Daily walnut consumption increases 6-sulfatoxymelatonin urinary levels and can improve sleep quality: a randomized crossover trial†

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We aimed to study the potential impact of daily consumption of walnuts on urinary 6-sulphatoxymelatonin (6-SMT) levels and sleep quality parameters. We conducted an open-label randomized crossover trial (NCT04799821) in 76 young adults (24.1 ± 3.4 years; 85.5% women) who either ingested 40 g of walnuts daily during dinner (intervention) or refrained from eating walnuts or any other nuts (control) for 8 weeks, with a 2-week washout period. Outcome variables included the determination of 6-SMT in urine samples collected in two consecutive periods: evening (from 20:00 to 23:00) and nighttime (from 23:00 to 07:00), the measurement of sleep quality parameters (latency, wake after sleep onset, awakenings, and efficiency) and daytime sleepiness (Epworth Sleepiness Scale). Tryptophan and melatonin contents of the walnuts used for the intervention were quantified by high-performance liquid chromatography. The 8-week walnut intervention significantly increased evening urinary 6-SMT concentrations ($p$ = 0.029) and improved sleep latency ($p$ = 0.001), while no differences were found between the baseline and control conditions. Likewise, the walnut intervention resulted in increased global sleep quality ($p$ = 0.002) and lower daytime sleepiness ($p$ = 0.002). Partial correlation analyses indicated that during the intervention, evening urinary 6-SMT concentrations were significantly associated with higher sleep efficiency ($p$ = 0.026) and an improved global sleep quality ($p$ = 0.006). Our findings highlight the potential of walnuts as sleep-promoting foods among young adults. Specifically, we demonstrated that a daily serving of 40 g of walnuts increases urinary 6-SMT levels, reduces sleep latency, and improves global sleep quality. Further research is needed to fully understand the underlying mechanisms involved in the diet–sleep association.

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## Introduction

Poor sleep quality is highly prevalent in the contemporary society and is strongly related to adverse health consequences, including obesity and cardiometabolic diseases.[1,2] Furthermore, evidence indicates a significant correlation between poor sleep quality and unhealthy eating habits.[3–5] On the bright side, recent findings from our group revealed that regular nut consumption is significantly associated with a better sleep quality.[4] This aligns with existing evidence suggesting that healthier diets, particularly those rich in plant-based foods, may enhance sleep quality by providing sleep-promoting nutrients, among which tryptophan stands out.[2,6,7]

Tryptophan, an essential amino acid found abundantly in nuts, is a precursor of melatonin,[2] the main hormonal regulator of the sleep–wake cycle.[8] A crucial factor in tryptophan's sleep-promoting role is its ratio relative to other large neutral amino acids, commonly referred to as competing amino acids (CAAs).[2] Current evidence suggests that foods with a higher tryptophan/CAA ratio (*i.e.* milk) could potentially act as sleep-promoting foods.[9,10] A higher ratio may facilitate the transport of tryptophan across the blood–brain barrier, allowing tryptophan to be converted into melatonin.[2,10] However, little is

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known about the relative tryptophan content of nuts compared to other CAAs, as well as their role as sleep promoting foods.

Another reason why regular nut consumption could improve sleep quality is their content of plant-based melatonin.[11,12] The bioavailability of plant-based melatonin from nuts, particularly walnuts (*Juglans regia* L.), has been demonstrated in humans, as evidenced by increased blood concentrations of melatonin and its metabolite, 6-sulphatoxymelatonin (6-SMT), after consumption.[11] However, evidence on the potential impact of walnut consumption on both urinary 6-SMT levels and sleep quality parameters is still needed.

Furthermore, a recent study showed that the melatonin content of walnuts can range from 1.2 to 3.3 ng $g^{-1}$ as a function of cultivar, harvesting season, and fruit maturity,[13] making further studies necessary. Concerning the effectiveness of other melatonin-rich foods, such as tart cherries[7] or beefsteak tomato,[14] studies have provided evidence supporting their role in promoting sleep quality.[7] This is particularly relevant given the ongoing demand for natural strategies to promote better sleep quality.[2] This demand is underscored by the fact that ~80% of individuals using pharmacological sleep aids report experiencing grogginess and difficulty concentrating during the day after medication use.[2]

Considering all of the above, we designed a randomized crossover trial (RCT) to investigate whether regular walnut consumption could improve sleep quality. Walnuts were selected for the intervention due to their unique nutritional profile among nuts, particularly their content of tryptophan and plant-based melatonin, which we hypothesized could influence sleep quality.[6,15,16] Our primary objective was to investigate whether daily walnut consumption could positively impact sleep quality parameters and modulate 6-SMT production, as measured by its urinary levels in young adults. In addition, we aimed to analyze the tryptophan content of walnuts, its ratio relative to other CAAs, and their melatonin content.

## Materials and methods

### Quantification of tryptophan, large neutral amino acids, and melatonin content in walnuts

Walnut samples (California Walnut Commission, California, USA) were ground to pass through a 0.5 mm sieve and analyzed, as described below, to quantify their content of tryptophan, large neutral amino acids (*e.g.*, isoleucine, leucine, phenylalanine, tyrosine, valine, histidine, methionine and threonine), and melatonin. All analyses were performed in duplicate.

Amino acid determination was performed according to the official methods for food and feed described in the Commission Regulation (EC) No. 152/2009.[17] Specifically, for tryptophan determination, samples were previously hydrolyzed under alkaline conditions with a saturated barium hydroxide solution (heated to 110 °C for 20 h) and analyzed by high-performance liquid chromatography (HPLC) with fluorescence detection (excitation: 280 nm, emission: 356 nm). For all other amino acids, samples were hydrolyzed with hydrochloric acid for 23 h and subsequently adjusted to pH 2.20 with sodium hydroxide solution. In the case of methionine, determination in oxidized samples was required. For this purpose, samples were exceptionally oxidized to methionine sulphone at 0 °C with a mixture of performic acid and phenol prior to hydrolysis. All pH-adjusted hydrolysates were subjected to an amino acid analyzer, consisting of a cation exchange HPLC coupled to post-column derivatization with ninhydrin and photometric detection at 570 nm.

Determination of melatonin was carried out according to Verde *et al.*[13] and consisted of sample extraction with a mixture of hexane : methanol : water (3 : 3 : 1 v/v) to effectively separate the analyte from the lipid content in walnuts. This was followed by an evaporation step combined with two-fold solid-phase extraction. The resulting extracts were then analysed by HPLC coupled with fluorescence detection (excitation: 285 nm, emission: 345 nm).

### Subjects

Young adults (aged 20–35 years) were recruited to participate in an 18-week, open label RCT under free-living conditions. Recruitment was conducted between February 2021 and February 2022 using various strategies, such as distributing flyers, conducting informative talks, and leveraging word of mouth to reach potential volunteers. Those expressing interest received comprehensive details about the study, and upon confirming their willingness to participate, underwent screening to assess their eligibility. Inclusion criteria were: having a body mass index (BMI) between 19 and 26 kg $m^{-2}$, maintaining a moderate level of exercise, and exhibiting no substantial weight change exceeding 2.3 kg in the last 3 months. Exclusion criteria were: nut allergies, acute or chronic illnesses, smoking habits, medications or supplements, adherence to specific dietary restrictions or diets, being shift workers, or inability to provide written informed consent. According to these criteria, out of the initial 171 volunteers recruited, 98 individuals met the eligibility requirements and were enrolled in the study. As shown in Fig. 1, of the 98 participants initially enrolled in the RCT, 5 withdrew due to personal reasons, leaving 93 individuals who began the trial. Over the course of the study, 13 participants also withdrew, again for personal reasons, resulting in an analytical sample of 80 participants. Then, upon data inspection, 4 participants were deemed ineligible and thus excluded from the analysis, resulting in a final analytical sample of 76 individuals who completed both arms of the RCT.

### Ethical aspects

All participants signed the informed consent form before being included in the study. The study was carried out under the guidelines of Good Clinical Practice in Research and the Declaration of Helsinki, and was also approved by the Ethics Committee of the University of Barcelona (IRB00003099). After approval, the study protocol was registered at ClinicalTrials.gov (NCT04799821).

 

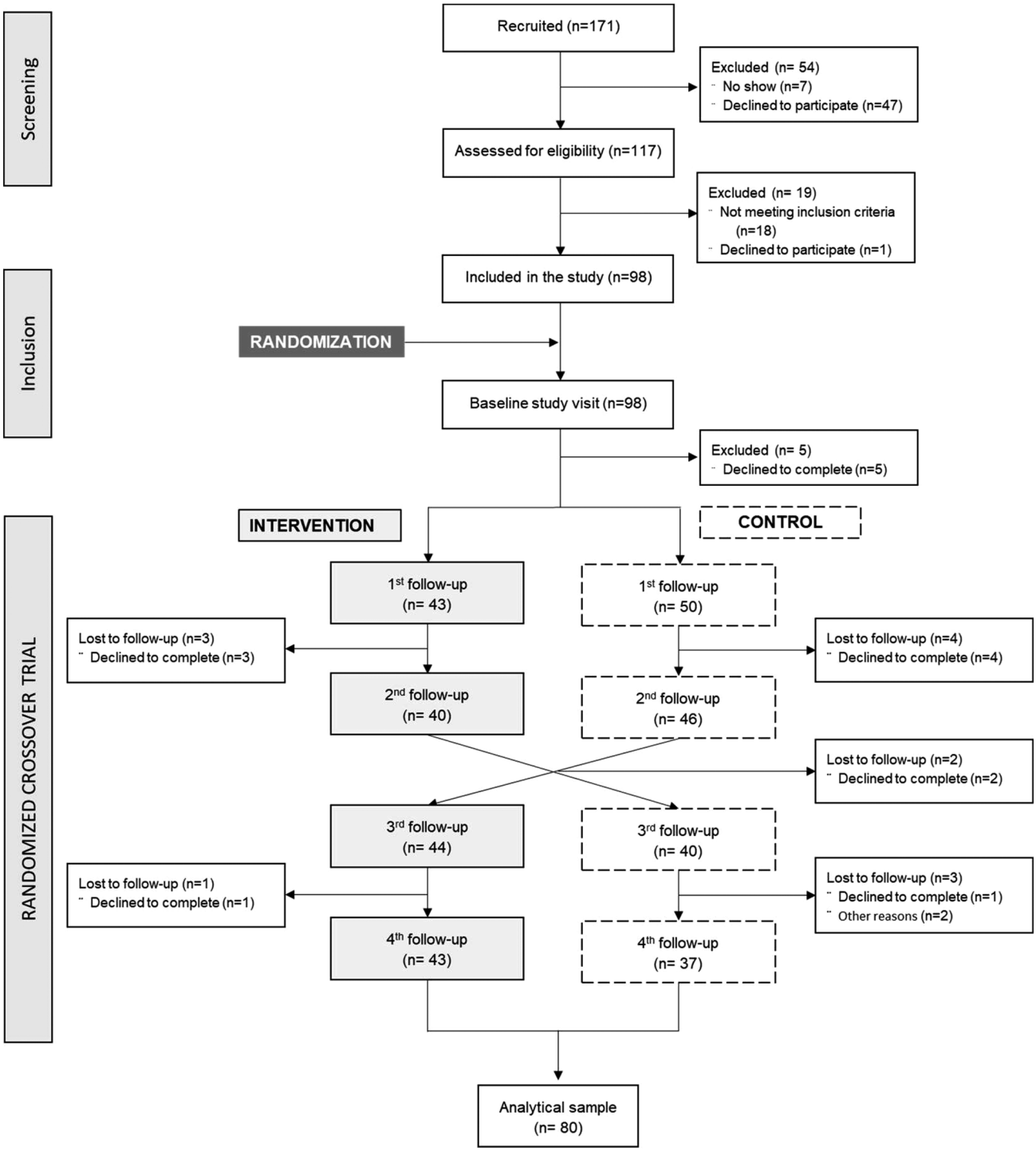


**Fig. 1** CONSORT diagram of participant flow.

### Study protocol

As shown in Fig. 2, we conducted an open label, 18-week RCT, in which participants were initially assigned to either the intervention or control conditions for an 8-week period. Simple randomization was performed at the moment of enrollment to allocate participants to one of the two arms of the trial:

i. Intervention: Participants were instructed to consume 40 g of walnuts per day along with dinner for 8 weeks. Each participant received individual walnut packages provided by the California Walnut Commission (California, USA). During this phase, participants were instructed to abstain from consuming other types of nuts and their derivatives due to their nutritional similarities to walnuts.

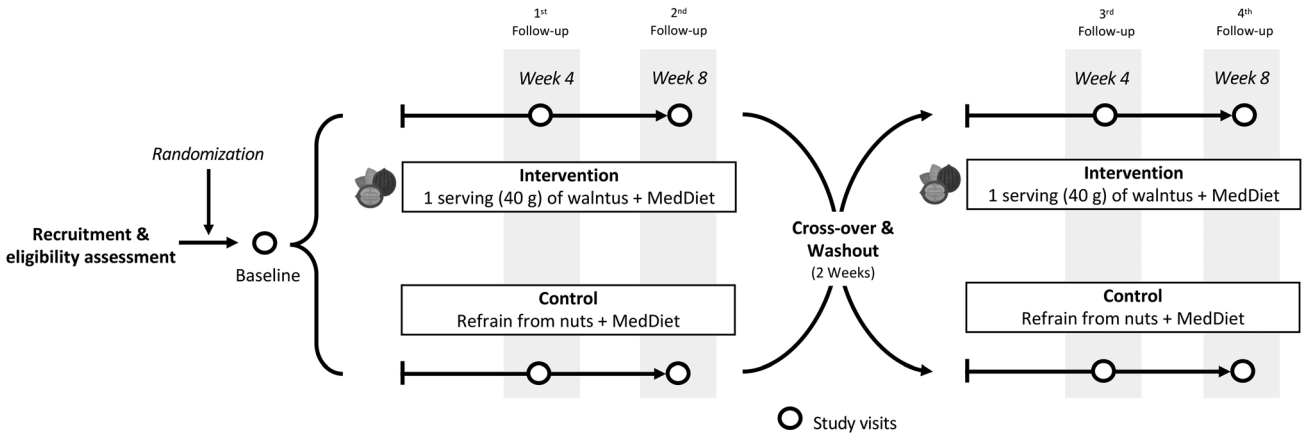


**Fig. 2** Study design.

ii. Control: Participants were instructed to refrain from consuming other types of nuts and their derivatives for 8 weeks.

Then, following a two-week washout period, participants were reassigned to the alternate arm of the trial (Fig. 2). Throughout both arms of the study, participants received guidance from a nutritionist to follow a Mediterranean-style diet. In addition, they were provided with sleep hygiene recommendations, which included refraining from consuming alcoholic beverages and/or caffeinated beverages (such as tea, coffee, cola, or energy drinks) in the afternoon or evening, to avoid performing physical exercise 2 h before bedtime, and minimizing exposure to electronic devices prior to bedtime. Participants were also advised to maintain regular eating and sleeping times and to have dinner preferably 2 h before their habitual bedtime. As shown in Fig. 2, throughout the study, all participants attended a total of 5 study visits, including the baseline assessment and 4 follow-up visits, which are explained below.

### Study visits

At the baseline study visit, we collected information on participants' sociodemographic variables (age, gender, and socioeconomic status).[18] Furthermore, during all study visits, we collected data related to anthropometric parameters, dietary intake, and physical activity. Additionally, at each study visit, participants were equipped with an ambulatory actigraphy monitor (ActTrust®, Condor Instruments, Sao Paulo, Brazil), worn on the non-dominant wrist for 7 consecutive days, except while showering or swimming. This device captured data on motor activity (accelerometer), skin temperature (°C), and light intensity (lux) at one-minute intervals. These data were recorded and stored in the actigraphy monitor memory for subsequent analysis. During each visit, participants were also provided with the necessary materials to collect two urine samples the night before returning the actigraphy monitor. They were instructed to collect urine samples in two separate containers during two consecutive periods: evening (from 20:00 to 23:00) and nighttime (from 23:00 to 07:00). Notably, these timeframes were established based on the methodology proposed by Mahlberg *et al.*,[19] considering that melatonin concentrations typically begin to rise 2–3 h before usual bedtime and remain elevated during nighttime sleep.

After 7 days, participants returned the actigraphy monitor and urine samples to the laboratory. Actigraphy data were then downloaded using ActStudio Alpha software v1.0.21.1 (Condor Instruments, Sao Paulo, Brazil), while urine samples were aliquoted and stored at −80 °C until analysis.

### Outcome variables

**Sleep quality.** Sleep quality was assessed using an actigraphy monitor, a validated, non-invasive technique for assessing sleep quality under ambulatory conditions.[20] Participants wore the actigraphy monitor continuously for 7 days. Then, using ActStudio Alpha v1.0.21.1 software, we identified the main sleep period of each day using the Cole–Kripke algorithm.[21] However, considering that actigraphy is known to overestimate sleep and underestimate wake time,[22] these data were manually validated by a blinded expert (TC), who verified that the main sleep periods aligned with a significant decrease in activity, a consequent increase in wrist temperature, and lights being out.[20]

From these data, various sleep parameters including bed time (hh:mm), get up time (hh:mm), total time in bed (min), and total sleep time (min) were obtained. Additionally, we estimated the following sleep quality parameters:

- Latency: The length of time, in min, it takes to transition from wake to sleep onset.
- Efficiency: The percentage of time in bed spent asleep.
- Wake after sleep onset (WASO): The amount of time, in min, spent awake after sleep onset and before final awakening.
- Awakenings: Periods of 1 min or more in which the individual wakes up during the sleep period.

Then, a scoring system to evaluate global sleep quality was designed, incorporating the four sleep continuity measures that could be measured with an actigraphy monitor. According to the National Sleep Foundation (NSF)[23] criteria,

 

optimal sleep quality could be defined as: short sleep latencies (≤15 min), higher sleep efficiencies (≥85%), fewer awakenings (<4), and reduced wake after sleep onset (WASO <51 min).[23] Therefore, in our global sleep quality score, each sleep quality parameter (latency, efficiency, WASO and awakenings) was scored separately. Participants received a score of 0 if they did not meet the NSF criteria for a given parameter and a score of 1 if they did. The scores from each of the four parameters were then summed to obtain the global sleep quality score, which ranged from 0 (if none of the sleep quality criteria were met) to 4 (if all sleep quality criteria were met).

**Chronobiological analyses.** Actigraphy-derived data were also analyzed using "El Temps" (v293) (Diez-Noguera, University of Barcelona, Spain, **https://www.el-temps.com**), an integrated package for chronobiological analysis. Cosinor analysis[24,25] was first used to calculate the following variables:

- Mesor: Defined as the mean 24 h value of the parameter fitted to a cosine function.
- Acrophase: Peak time referenced to local midnight of the cosinusoidal curve.
- Amplitude: Defined as the difference between the maximum value of the cosine function and the mesor.
- Rayleigh test: Defined as the rhythm's phase stability. This analysis provides an *r* vector with its origin at the center of a circumference of radius 1. The *r* vector ranges between 0 and 1, where the higher values indicate the stability of the acrophase over successive days.

Second, using a non-parametric analysis,[26] we calculated the following circadian variables:

- Relative amplitude: Defined as the amplitude related to the daily mean value and an indicator of the robustness of the rhythm.
- Interdaily stability: Defined as the variance of data (in percentage) explained by the rhythm. Higher values indicate a more stable rhythm.
- Intradaily variability: Defined as the fragmentation of the rhythm. This value oscillates, ranging from 0 (when the wave is perfectly sinusoidal) to 2 (Gaussian noise).
- M10: Defined as the average value of the 10 h of maximum activity.
- L5: Defined as the average of the 5 h of least activity.

**Urinary 6-sulfatoxymelatonin (6-SMT) levels.** The concentration of 6-SMT in the urine samples collected throughout the study was quantified using the Melatonin-Sulfate Urine ELISA kit (RE54031, IBL International, Germany). Analyses were conducted in duplicate. Concentrations of 6-SMT were corrected for creatinine (Cre) concentration using the reaction kinetics of the Jaffe method (modified by Larsen) (7). The values are expressed as ng 6-SMT per mg Cre.

**Daytime sleepiness.** This variable was assessed for all participants at the baseline and at the 2nd follow-up and 4th follow-up study visits (Fig. 2) using the Spanish version of the Epworth Sleepiness Scale (ESS).[27] The ESS is a self-administered questionnaire consisting of 8 items, used to measure the probability of experiencing daytime sleepiness and the propensity to doze off in different situations. The scale items are scored on a range from 0 (no possibility of becoming drowsy) to 3 (high possibility of becoming drowsy), with total scores ranging from 0 to 24. Higher scores indicate a greater level of daytime sleepiness. According to the questionnaire instructions, scores exceeding 10 points are indicative of daytime sleepiness.

### Covariates

**Anthropometric parameters.** Weight (in kg) and body fat (in percentage) were measured using a body composition analyzer (InBody 120, Biospace, Seoul, Korea), with participants wearing light clothes and without shoes. Height was determined using a fixed wall stadiometer (Seca 217, Seca, Hamburg, Germany) to the nearest 0.1 cm. BMI was calculated as weight (kg) divided by squared height ($m^2$).

**Chronotype.** The chronotype was estimated using the phase (local time) of the midpoint between bed and wakeup timing (midpoint of sleep) on free days (MSF), based on the instructions accompanying the Munich Chronotype Questionnaire.[28]

**Dietary intake.** Dietary intake was assessed in each study visit using 7-day food registers, completed on consecutive days that coincided with the same days that sleep quality was evaluated. Participants were instructed by a nutritionist on how to register the type of food, including brand name where possible, portion sizes, as well as the time and the location of each meal (*i.e.* home or restaurant). This allowed us to evaluate the time in which each food or beverage was consumed. From these data, energy and nutrient intake were calculated using PCN Pro 1.0 Software.[29] Adherence to the intervention was evaluated using the 14-item Mediterranean Diet Assessment Tool, validated in the Spanish population.[30] This questionnaire includes the question "How many times do you consume nuts per week?". Scores ranged from 0 to 14, with higher scores reflecting greater adherence to the Mediterranean diet.

**Physical activity.** The level of physical activity was measured in Metabolic Equivalents of Task (METs) using the short version of the International Physical Activity Questionnaire (IPAQ), validated for the Spanish population.[31] Higher scores indicate more intense levels of physical activity. Additionally, physical activity was objectively measured using an actigraphy device, and the values were expressed as "mesor" (as described in the Chronobiological analyses section).

**Socioeconomic variables.** The educational level and work status were evaluated through the questions: 'What is your education level?', with options 'primary studies' or 'more than primary studies' and 'what is your current employment status?', with options 'employed', 'student' or 'unemployed'.

**Statistical analyses.** Normality of distribution was assessed by histograms and Q–Q plots. Descriptive characteristics were presented for all participants, including mean ± standard deviation for continuous normally distributed variables, and median and interquartile range for melatonin concentrations, which were found to be non-normally distributed variables. Proportions were used for categorical variables. Differences in outcome variables between the baseline and study conditions

(baseline *vs.* control [4 weeks], baseline *vs.* intervention [4 weeks], control [4 weeks] *vs.* intervention [4 weeks], baseline *vs.* control [8 weeks], baseline *vs.* intervention [8 weeks], control [8 weeks] *vs.* intervention [8 weeks]) were tested using Wilcoxon signed-ranked tests for non-normally distributed variables or paired *t*-tests for normally distributed variables.

We then used linear mixed models to study whether significant differences in outcome variables were independent of potential confounders, accounting for the cross-over design of the study. Mixed models were performed including study conditions (baseline, control and intervention) and follow-up time as fixed effects, while participants were modeled as a random effect to account for repeated measures within individuals. All models were adjusted for age, gender, socioeconomic status, chronotype, physical activity, and energy intake.

Furthermore, considering that the main differences in outcome variables were found between the baseline and intervention conditions, we conducted a secondary analysis to explore the potential associations between evening and nighttime 6-SMT urinary concentrations with sleep quality parameters and global sleep quality score, all assessed on the same study night. The latter were tested using partial correlations controlled for age, gender, chronotype, diet quality, and physical activity. Note that, we applied a logarithmic transformation to variables with a non-parametric distribution (such as 6-SMT urinary concentrations, latency, efficiency and WASO) before performing the statistical analysis. We also examined correlations between evening and nighttime 6-SMT urinary concentrations and circadian variables between the baseline and intervention conditions using partial correlations controlled for age, gender, chronotype, diet quality, and physical activity.

Finally, we tested the associations between the global sleep quality score and circadian-related variables using partial correlations controlled for age, gender, chronotype, and BMI. These analyses were conducted using the data from all study visits ($n$ = 380 observations) to verify that the score obtained was actually compatible with better sleep quality and healthier circadian function.

For all analyses, statistical significance was inferred when $p$-value was <0.05. All statistical analyses were conducted using SPSS v27 (IBM, Chicago, IL, USA).

# Results

### Tryptophan, large neutral amino acids, and melatonin content of the walnuts used to conduct the RCT

The mean contents of tryptophan, other large neutral amino acids, and melatonin in the walnuts used for the RCT are shown in Table 1. A single 40 g serving of walnuts provided an average of 84.6 mg of tryptophan and 118.0 ng of melatonin (Table 1). The tryptophan/CAA ratio in walnuts was 0.058.

**Table 1** Tryptophan, amino acid profile, and melatonin content in the walnuts used to conduct the clinical trial

| | Mean (SD) | Amount per serving (40 g) |
|---|---|---|
| **Large neutral amino acids** | | |
| Tryptophan, g per 100 g | 0.21 (0.02) | 84.6 mg |
| Isoleucine[a], g per 100 g | 0.59 (0.04) | 237.8 mg |
| Leucine[a], g per 100 g | 1.13 (0.08) | 452.0 mg |
| Phenylalanine[a], g per 100 g | 0.70 (0.05) | 281.0 mg |
| Tyrosine[a], g per 100 g | 0.52 (0.06) | 207.4 mg |
| Valine[a], g per 100 g | 0.72 (0.03) | 289.0 mg |
| Histidine, g per 100 g | 0.39 (0.05) | 157.6 mg |
| Methionine, g per 100 g | 0.23 (0.02) | 90.4 mg |
| Threonine, g per 100 g | 0.56 (0.08) | 225.6 mg |
| **Tryptophan/CAA ratio**, A.U. | 0.058 (0.00) | — |
| **Melatonin**, ng $g^{-1}$ | 2.95 (0.21) | 118.0 ng |

A.U., arbitrary units; SD, standard deviation. [a] Competing amino acids (CAAs): isoleucine, leucine, phenylalanine, tyrosine and valine – these are the five large neutral amino acids typically included in the tryptophan/CAA ratio (10).

### Characteristics of the population studied

A summary of the general characteristics of the study population is shown in Table S1.† Briefly, participants had a mean age of 24.1 ± 3.4 years and were mostly women (85.5%). At the baseline, all participants were classified as having a normal weight (BMI: 21.3 ± 2.1 kg $m^{-2}$). Further details regarding body fat percentage, dietary intake, physical activity levels, bedtime, and chronotype at the baseline are given in Table S1.† As for sleep quality parameters, most participants met the criteria for optimal sleep latency (97.4%), efficiency (94.7%), and WASO (85.5%) at the baseline. However, only 7.9% of the individuals met the NSF criteria for the number of awakenings, resulting in a mean global sleep quality score of 2.8 ± 0.6 points. As for daytime sleepiness (Table S1†), the average EES score was 7.9 ± 3.8 points, with 22.4% of the participants experiencing daytime sleepiness at the baseline. Furthermore, mean urinary 6-SMT concentrations in the evening and nighttime periods were 5.0 ± 4.34 ng 6-SMT per mg Cre and 32.4 ± 21.1 ng 6-SMT per mg Cre, respectively.

### Effect of the 8-week walnut intervention on the levels of urinary 6-SMT

The walnut intervention led to a significant increase in urinary 6-SMT concentrations during the evening period (from 20:00 to 23:00), a trend observed consistently throughout the intervention (Fig. 3a). Specifically, after 4 weeks of intervention, evening urinary 6-SMT concentrations significantly increased compared to the baseline ($p$ = 0.039). This increase remained significant after 8 weeks of the walnut intervention ($p$ = 0.004) compared to the baseline. Results from the mixed-effects models confirmed that, compared to the baseline, the intervention resulted in higher urinary 6-SMT concentrations at both 4 and 8 weeks (1.98 ng 6-SMT per mg Cre [95% CI: 0.47, 3.50; $p$ = 0.011] and 1.64 ng 6-SMT per mg Cre [95% CI: 0.17, 3.11; $p$ = 0.029], respectively).

Regarding urinary 6-SMT concentrations during the nighttime period (from 23:00–7:00), we did not observe significant changes (Fig. 3b).

 

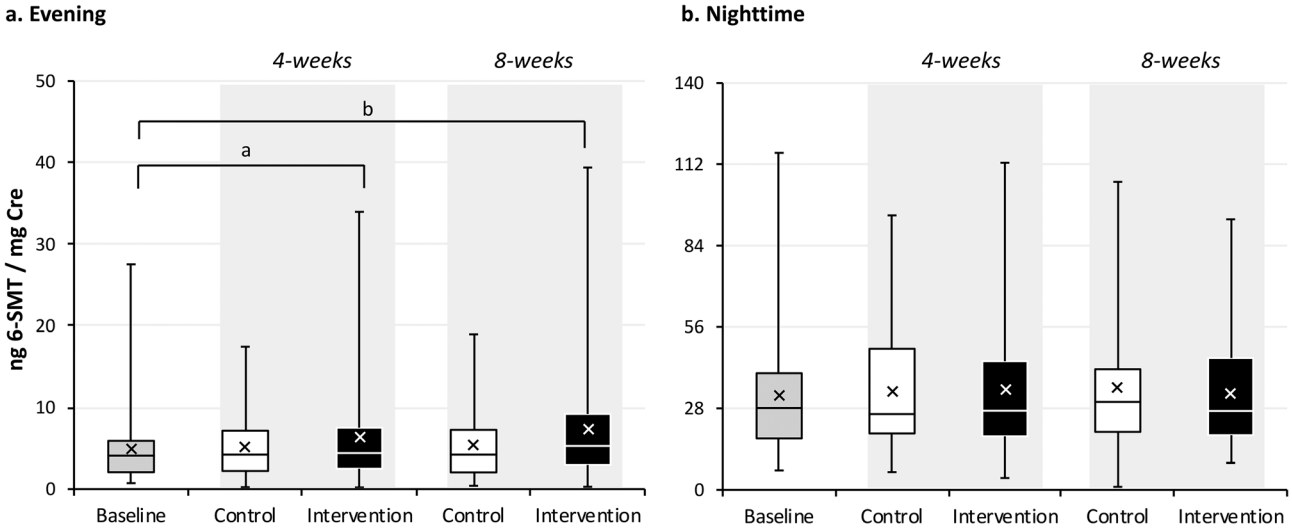


**Fig. 3** Comparison of urinary 6-sulfatoxymelatonin (6-SMT) between the baseline, intervention (weeks 4 and 8), and control (weeks 4 and 8) conditions. Data are expressed as median and interquartile range, while X represents the mean value. Wilcoxon signed-ranked tests were used to compare the 6-SMT values between the study visits. Superscripts represent significant differences: [a] Baseline *vs.* intervention (4 weeks) and [b] baseline *vs.* intervention (8 weeks).

### Effect of the 8-week walnut intervention on sleep quality parameters and on the daily rhythm of motor activity

Our results revealed significant improvements in sleep latency following the walnut intervention (Fig. 4). Compared to the baseline, sleep latency decreased after the first 4 weeks of the intervention ($p$ = 0.009). This reduction remained significant throughout the intervention period, with sleep latency still significantly lower after 8 weeks of the walnut intervention ($p$ = 0.004) compared to the baseline. Furthermore, results from the mixed-effects models confirmed that compared to the baseline, the intervention resulted in lower sleep latency at both 4 and 8 weeks (−1.21 min [95% CI: −2.03, −0.37; $p$ = 0.005] and −1.29 min [95% CI: −2.07, −0.51; $p$ = 0.001], respectively).

No significant differences were observed in other sleep quality parameters (Fig. 4). In addition, differences between the intervention and control conditions were studied. In which case, we observed that after 8 weeks of walnut consumption, sleep efficiency increased by 0.70% (95% CI: 0.10, 1.29; $p$ = 0.022) (Fig. 4). Results from the mixed-effects models confirmed that compared to the control, the intervention resulted in higher sleep efficiency at 8 weeks (0.72% [95% CI: 0.07, 1.38], $p$ = 0.030).

The global sleep quality score significantly improved following the 8-week walnut intervention compared to the baseline ($p$ = 0.033). Daytime sleepiness significantly decreased after 8 weeks of walnut intervention ($p$ = 0.004), while no significant differences were found under the control conditions. Notably, results from the mixed-effects models confirmed a significant improvement in sleep quality (0.30 points [95% CI: 0.11, 0.49], $p$ = 0.002) and a reduction in daytime sleepiness (−1.37 [95% CI: −2.21; −0.52], $p$ = 0.002) after 8 weeks of walnut intervention.

The comparison of other sleep variables as well as of the daily rhythms of motor activity throughout the study is shown in Table S2.† On average, participants slept more than 7 h per day across the entire trial period. No significant differences were observed between the baseline and the intervention conditions. We only observed that during the 1st follow up of the control (4-weeks), participants went to bed ∼13 minutes later, spent ∼0.2 h less time in bed and slept 0.2 h less compared to the baseline. Meanwhile, no significant differences were observed in circadian-related variables between the baseline and the intervention conditions, nor between the intervention and control conditions.

### Association between the evening and nighttime urinary 6-SMT concentrations with sleep quality parameters and circadian variables

Partial correlations analyses (Table 2) showed that during the intervention, higher evening urinary 6-SMT concentrations were significantly associated with greater sleep efficiency ($r$ = 0196; $p$ = 0.026) and a better global sleep quality score ($r$ = 0.232, $p$ = 0.006). Similarly, higher nighttime urinary 6-SMT concentrations during the intervention were significantly associated with greater sleep efficiency ($r$ = 0.200, $p$ = 0.023), lower WASO ($r$ = −0.207, $p$ = 0.019), fewer awakenings ($r$ = −0.188, $p$ = 0.033), and reduced daytime sleepiness ($r$ = −0.206, $p$ = 0.016). These associations were not observed at the baseline.

Regarding circadian-related variables (Table 2), significant associations were found between urinary 6-SMT concentrations and intradaily variability. At the baseline, higher urinary 6-SMT evening values were significantly associated with lower intradaily variability ($r$ = −0.295, $p$ = 0.026). During the intervention, both evening and nighttime urinary 6-SMT

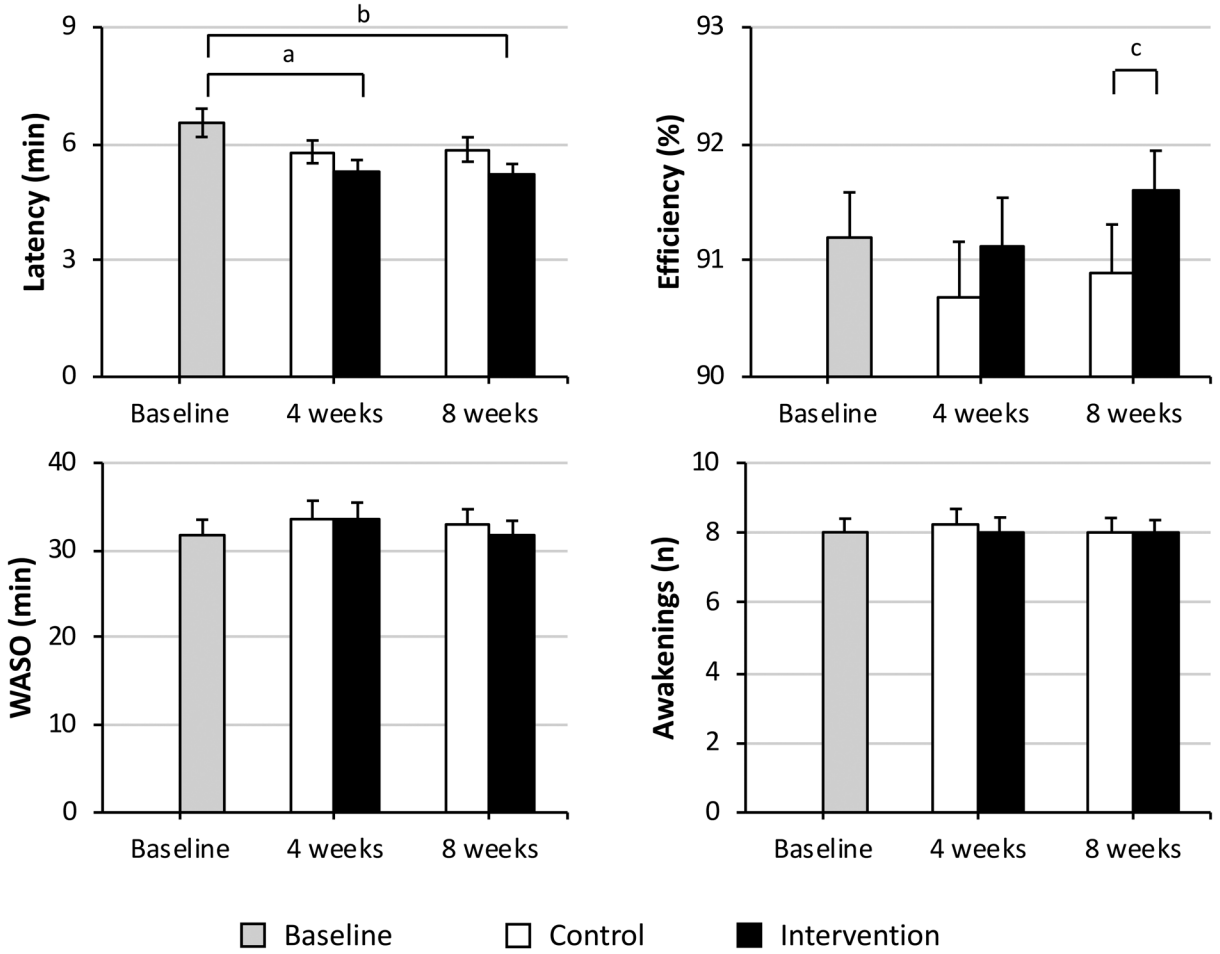


**Fig. 4** Comparison of sleep quality parameters. Data are expressed as mean and standard error measurements. Paired *t*-tests were used to compare the sleep quality parameters between the study visits. Superscripts represent significant differences: [a] Baseline *vs.* intervention (4 weeks), [b] baseline *vs.* intervention (8 weeks) and [c] control (8 weeks) *vs.* intervention (8 weeks).

concentrations were negatively correlated with intradaily variability ($r = -0.172$, $p = 0.043$ and $r = -0.227$, $p = 0.007$, respectively). Furthermore, greater evening 6-SMT concentrations were significantly related to the relative amplitude ($r = 0.175$, $p = 0.040$) and the maximum 10-hour of activity ($r = 0.178$, $p = 0.037$) during the intervention.

With regard to the global sleep quality score, which includes the four sleep continuity measures recommended by the NSF (latency, efficiency, WASO, and awakenings), it was significantly associated with a higher relative amplitude ($r = 0.137$, $p = 0.010$) and lower L5 ($r = -0.144$, $p = 0.007$).

### Effect of the 8-week walnut intervention on the study covariates throughout the RCT

Anthropometric parameters related to adiposity remained unchanged throughout the intervention (Table 3), with no significant differences in the BMI or body fat percentage across the study conditions. However, significant differences in dietary intake and diet quality were observed. On average, diet quality improved significantly under the intervention conditions compared to both the baseline and control conditions ($p < 0.001$, Table 3). In addition, we observed that following the walnut intervention, intake of energy, protein, fat, and fiber was significantly higher ($p < 0.05$). As for other covariates, physical activity levels and chronotype remained similar throughout the RCT ($p > 0.05$).

## Discussion

To our knowledge, this is the first RCT aimed at investigating the effect of daily walnut consumption as a sleep-promoting food in young adults under free-living conditions. Our findings showed that daily consumption of walnuts during dinner significantly increased evening urinary 6-SMT concentrations by approximately by 1.64 ng per mg Cre. Additionally, the 8-week walnut intervention modestly but significantly reduced sleep latency (by 1.29 minutes), improved global sleep quality (+0.30 points), and decreased daytime sleepiness by 1.37 points compared to the baseline.

**Table 2** Partial correlations between urinary 6-sulfatoxymelatonin (6-SMT) collected during the evening (20:00–23:00) and the nighttime (23:00–07:00) periods and the sleep-related variables measured on the same night

| | Baseline ($n$ = 76) | | Intervention ($n$ = 152) | |
|---|---|---|---|---|
| | 6-SMT (log) | | 6-SMT (log) | |
| | Evening $R$ | Nighttime $r$ | Evening $r$ | Nighttime $r$ |
| **Sleep quality parameters** | | | | |
| Latency, log | −0.041 | −0.264 | 0.040 | 0.027 |
| Efficiency, log | −0.044 | 0.108 | **0.196*** | **0.200*** |
| WASO, log | 0.093 | 0.002 | −0.148 | **−0.207*** |
| Awakenings, $n$ | 0.188 | −0.103 | −0.098 | **−0.188*** |
| Global sleep quality, score | 0.050 | −0.003 | **0.232**** | 0.124 |
| **Daytime sleepiness**, score | 0.087 | 0.098 | 0.012 | **−0.206*** |
| **Circadian variables** | | | | |
| Mesor, A.U. | −0.138 | 0.030 | 0.119 | −0.135 |
| Acrophase, hh:mm | −0.052 | −0.117 | −0.132 | −0.057 |
| Amplitude, ° | 0.171 | 0.104 | 0.157 | 0.050 |
| Relative amplitude, A.U. | 0.099 | 0.009 | **0.175*** | 0.099 |
| Intradaily variability, A.U. | **−0.295*** | −0.075 | **−0.172*** | **−0.227**** |
| Rayleigh test, A.U. | −0.001 | 0.029 | −0.050 | −0.066 |
| Interdaily stability, % | 0.088 | 0.088 | 0.134 | 0.084 |
| M10, A.U. | 0.166 | 0.092 | **0.178*** | 0.023 |
| L5, A.U. | −0.056 | 0.080 | −0.045 | −0.074 |

A.U., arbitrary units; L5, average 5 h of least activity; M10, average value of the 10 h of maximum activity. This table shows the Pearson correlation coefficient ($r$). Partial correlations controlled for age, gender, chronotype, diet quality, and physical activity were conducted to test the association between the baseline or the intervention condition with the outcome variables. Statistical significance: *$p < 0.05$ and **$p < 0.01$.

Although the exact mechanisms underlying these effects remain to be fully elucidated, we hypothesize that the tryptophan content of walnuts (0.20 ± 0.02 mg per 100 g) may have enhanced melatonin synthesis by the pineal gland during the evening, thereby improving sleep quality parameters.[2,7] The latter aligns with previous findings by Bravo *et al.*,[32] who demonstrated that a tryptophan-enriched cereal intervention for breakfast and dinner (60 mg of tryptophan per 30 g serving) significantly increased sleep efficiency, while reducing sleep fragmentation and latency in middle-aged adults. On the other hand, the tryptophan/CAA ratio plays a crucial role in regulating brain availability for melatonin synthesis.[32] In our study, walnuts showed a tryptophan/CAA ratio of 0.058, comparable to that of the tryptophan/CAA ratio of whole milk (0.081), which has been previously identified as a potential sleep-promoting food.[7]

It is also worth noting that, once in the brain, approximately 1–2% of dietary tryptophan is converted into serotonin, which is subsequently transformed to melatonin *via* two enzymatic reactions that require vitamins B5 and B6, as well as magnesium, as cofactors. Interestingly, walnuts are good sources of both vitamins B5 and B6 (0.57 mg per 100 g and 0.54 mg per 100 g, respectively),[33] and are among the plant-based foods with the highest magnesium content (158 mg per

**Table 3** Changes in the study covariates throughout the randomized controlled trial

| | | 4 weeks | | | | 8 weeks | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Baseline | Control | Intervention | $P$-value[a] | $P$-value[b] | $P$-value[c] | Control | Intervention | $P$-value[d] | $P$-value[e] | $P$-value[f] |
| **Anthropometric parameters** | | | | | | | | | | | |
| BMI, kg m$^{-2}$ | 21.3 (2.1) | 21.2 (2.0) | 21.3 (2.1) | 0.506 | 0.413 | 0.570 | 21.2 (2.0) | 21.3 (2.1) | 0.090 | 0.570 | 0.079 |
| Body fat, % | 25.0 (6.1) | 24.7 (6.2) | 24.7 (6.0) | 0.216 | 0.227 | 0.877 | 24.6 (6.2) | 24.8 (6.1) | 0.157 | 0.275 | 0.087 |
| **Dietary intake** | | | | | | | | | | | |
| Diet quality, score | 9.4 (2.2) | 9.2 (1.9) | 10.3 (1.6) | 0.212 | **<0.001** | **<0.001** | 9.4 (1.7) | 10.3 (1.9) | 0.952 | **<0.001** | **<0.001** |
| Energy intake, kcal day$^{-1}$ | 1756.8 (468.78) | 1639.1 (348.3) | 1843.6 (369.9) | **0.012** | **0.036** | **<0.001** | 1752.7 (430.1) | 1972.4 (441.7) | 0.980 | **<0.001** | **<0.001** |
| Fat, g day$^{-1}$ | 75.1 (19.5) | 68.7 (19.0) | 93.7 (19.4) | 0.008 | **<0.001** | **<0.001** | 79.0 (21.1) | 102.3 (22.5) | **0.150** | **<0.001** | **<0.001** |
| Protein, g day$^{-1}$ | 78.1 (22.2) | 77.2 (19.2) | 78.1 (19.6) | 0.598 | 0.841 | 0.232 | 78.9 (22.4) | 84.5 (22.9) | 0.404 | **0.003** | **0.012** |
| Carbohydrates, g day$^{-1}$ | 182.8 (54.2) | 170.6 (41.5) | 171.0 (41.9) | **0.024** | **0.018** | 0.807 | 173.8 (50.3) | 178.2 (50.5) | 0.135 | 0.551 | 0.316 |
| Dietary fiber, g day$^{-1}$ | 23.2 (8.4) | 22.6 (8.3) | 23.8 (7.9) | 0.399 | 0.545 | 0.107 | 22.5 (8.4) | 25.2 (8.14) | 0.288 | **0.016** | **0.001** |
| **Physical activity**, METS/min | 2205.5 (2145.6) | 1977.3 (1341.7) | 2054.9 (1855.5) | 0.249 | 0.312 | 0.742 | 2222.0 (2280.6) | 1865.2 (1686.7) | 0.590 | 0.151 | 0.160 |
| **Bedtime, hh:mm** | 00:03 (0:51) | 00:10 (0:52) | 00:05 (0:45) | 0.264 | 0.779 | 0.400 | 00:16 (0:53) | 00:06 (0:50) | 0.065 | 0.020 | 0.565 |
| **Chronotype, hh:mm** | 04:18 (1:35) | 04:26 (0:53) | 04:12 (1:33) | 0.432 | 0.626 | 0.176 | 4:28 (0:56) | 4:23 (0:58) | 0.417 | 0.847 | 0.428 |

BMI, body mass index; METS, metabolic equivalents of task. Paired $t$-tests were used to compare variables between the study visits. [a] Baseline *vs.* control (4 weeks). [b] Baseline *vs.* intervention (4 weeks). [c] Control (4 weeks) *vs.* intervention (4 weeks). [d] Baseline *vs.* control (8 weeks). [e] Baseline *vs.* intervention (8 weeks). [f] Control (8 weeks) *vs.* intervention (8 weeks). Paired $t$-tests were used to compare variables within subjects and between the study conditions.

100 g).[33,34] These nutrients could represent additional mechanisms contributing to the observed effects.

Among other potential mechanisms underlying our findings, walnuts provide a source of plant-based melatonin, quantified in this study at 118.0 ng per serving. This may further contribute to their sleep-promoting effect, supporting our findings. Consistent with our findings, an 8-week RCT in postmenopausal women with obesity showed that consuming 250 g of beefsteak tomato (containing 1274.5 ng of melatonin per serving) significantly improved urinary 6-SMT concentrations and subjective sleep quality, as measured by the Pittsburg Sleep Quality Index. This aligns with our findings, where higher evening 6-SMT concentrations were significantly associated with greater sleep efficiency and global sleep quality. Furthermore, the intervention was related to a significant decrease in daytime sleepiness, which is also another marker of good sleep quality. However, lower daytime sleepiness might not be directly associated with melatonin but rather with better sleep quality.[35]

Melatonin is associated with sleepiness and relaxation and thus plays a key role in sleep latency.[36,37] Consistently, our study and others have showed that nutritional interventions (either with walnuts, tart cherries,[38] or a cherry-based product[39]) increase urinary 6-SMT levels and improve sleep quality parameters such as latency, efficiency, number of awakenings, and/or total nocturnal activity, as measured *via* an actigraphy monitor.[40,41] However, unlike our study, previous interventions involving tart cherry consumption were more intense as they included the consumption of tart cherries or a cherry-based product twice a day for 3 days.

In addition, it is plausible that walnuts increased melatonin production and improved sleep latency through the gut–brain axis.[42–44] As a rich source of dietary fiber, walnuts may enhance the gut microbiota composition and promote the production of short-chain fatty acids, which have been shown to stimulate serotonin secretion from enterochromaffin cells.[43,44] Given that approximately 95% of the body's serotonin is synthetized in the gastrointestinal tract, its role in gut–brain communication is critical for sleep regulation.[44] In addition, the gut microbiota can produce and regulate the synthesis of melatonin and other sleep-regulating neurotransmitters, including GABA, which is known for reducing anxiety, stress, and balancing mood.[44] However, further research is warranted to elucidate these mechanisms in greater detail.

Another key finding of our study was that higher evening urinary 6-SMT levels were significantly associated with improved sleep efficiency and global sleep quality, as well as with a more robust and less fragmented circadian pattern of activity. Furthermore, higher evening urinary 6-SMT levels during the intervention were significantly associated with an increased daytime motor activity (expressed as M10). This is particularly noteworthy as daily walnut intake could provide a natural strategy to promote better sleep quality, without the side effects of pharmacological sleep aids. According to Wilson *et al.*,[2] approximately 80% of individuals using pharmacological sleep aids experience next-day grogginess and impaired concentration. Our findings indicate that walnut consumption may offer a dietary alternative for improving sleep quality, without these side effects.

Along these lines, partial correlation analyses revealed that nighttime urinary 6-SMT levels during the intervention were significantly associated with greater sleep efficiency and fewer WASO and awakenings. This aligns with a recent meta-analysis in young individuals, which showed that melatonin supplementation moderately improves sleep continuity parameters.[45] Furthermore, in line with our results, melatonin seems to act more as a chronobiotic than as a hypnotic, acting as an endogenous synchronizer of the body clock, inducing sleep and regulating circadian rhythms.[46,47]

Furthermore, our study and others included in the discussion suggest that age might influence the magnitude of dietary interventions on sleep quality parameters.[14,32,41] Garrido *et al.*[41] pointed out that the sleep quality-improving effects of dietary interventions were more pronounced with advancing age, possibly due to age-related declines in melatonin secretion[14,41,47] and sleep quality.[14,48] Therefore, elderly volunteers may be more responsive to these types of nutritional strategies aimed at enhancing sleep. Therefore, future studies should explore whether the sleep-promoting effect of walnut consumption is more pronounced among older populations.

In relation to the global sleep quality score, our study found that it was significantly associated with the robustness of the circadian rhythm of motor activity, as indicated by a greater relative amplitude and lower nighttime activity (L5). Both associations were compatible with better sleep quality and healthier functioning of the circadian system.[20,49] Therefore, we propose that the global sleep quality score could serve as an integrative marker for assessing sleep quality based on the NSF[23] criteria when measured objectively using actigraphy.

Our study has several strengths, including its RCT design and the fact that this is the first RCT evaluating the effect of a dietary intervention on urinary 6-SMT concentrations in two distinct time periods: evening (when melatonin secretion begins) and nighttime (when peak melatonin secretion occurs).[19] Additionally, we quantified the amino acid and melatonin contents of the walnuts used in the study, integrated actigraphy with skin temperature data to identify the main sleep periods, providing a reliable and more informative method to evaluate the sleep–wake cycles under ambulatory conditions,[20] and implemented a feasible dietary intervention with a daily serving of 40 g of walnuts, which likely improved adherence. Furthermore, a strength of this study is that it accounted for the timing of walnut intake, with participants consuming it at dinner.

However, this study has certain limitations that should be acknowledged, starting with the difficulty of blinding participants due to the nature of the nutritional intervention (walnuts), which constitute a methodological limitation. We also acknowledge the lack of dietary intake standardization as a limitation of the study. In addition, this study does not report dietary tryptophan and melatonin intake, which could limit the interpretation of our findings. Furthermore, the rela-

 

tively young age of the participants may limit the generalizability and clinical relevance of our results. Finally, although we ensured that no sleep measurements or urine samples were collected during the menstrual phase, we did not control for the menstrual phase in our analyses.

## Conclusion

Our findings highlight the potential of walnuts as sleep-promoting foods among young adults under free-living conditions. Specifically, we demonstrated that daily walnut consumption during dinner significantly reduces sleep latency and increased evening urinary 6-SMT concentrations, an effect that is observed from the 4th week of intervention. Additionally, the 8-week walnut intervention positively influenced global sleep quality and reduced daytime sleepiness. These results open a new framework for future intervention and experimental studies to investigate the role of walnut consumption as a sleep-promoting food across different populations. Further studies are warranted to elucidate the precise mechanisms underlying the diet–sleep relationship and to determine whether the observed benefits extend to older adults and individuals with sleep disorders.

## Author contributions

Maria Fernanda Zerón Rugerio: conceptualization, validation, investigation, formal analysis, data curation, visualization, writing – original draft, and project administration. Aradeisy Ibarra-Picón: investigation, formal analysis, visualization, and writing – original draft. María Diez-Hernández: investigation. Oriol Comas-Baste: investigation and writing – review & editing. Francisco José Pérez-Cano: validation, investigation, and writing – review & editing. Trinitat Cambras: investigation, formal analysis, and writing – review & editing. Maria Izquierdo-Pulido: conceptualization, validation, investigation, formal analysis, resources, writing – original draft, supervision, funding acquisition, and project administration. All authors have read and agreed to their individual contributions.

## Conflicts of interest

The authors declare no conflicts of interest.

## Data availability

The data supporting this article have been included as part of the ESI (Table S3†). The data collected from human participants are not available publicly for confidentiality reasons, but they will be made available by the corresponding author, Maria Izquierdo-Pulido (**maria_izquierdo@ub.edu**), upon reasonable request.

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