Nutritional Modulation of Sleep Latency, Duration, and Efficiency: A Randomised, Repeated-Measures, Double-Blind Deception Study

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ABSTRACT

Purpose: To test the hypothesis that a novel nutritional blend comprised of tryptophan, glycine, magnesium, tart cherry powder and L-theanine, enhances subjective and objective measures of sleep during free living conditions. **Methods:** In a randomised, repeated measures crossover and double blind deception design, participants (n = 9 male and 7 female; age: 24 ± 3 years; body mass: 69.8 ± 11.6 kg; stature: 170.8 ± 9.1 cm) completed a 3 day familiarisation period, followed by 3 day intervention and placebo trials. Subjective Pittsburgh Quality Sleep Index, Core Consensus Sleep Diary and Karolinska Sleepiness Scale survey tools, alongside objective actigraphy measures of sleep were assessed, with daily nutritional intake, activity and light exposure standardised between trials. Participants provided daily urine samples for assessment of targeted and untargeted metabolomes. **Results:** The intervention trial reduced sleep onset latency (-24 ± 25 mins; p = 0.002), increased total sleep time (22 ± 32 mins; p = 0.01) and sleep efficiency (2.4 ± 3.9 %; p = 0.03), whilst also reducing morning sleepiness (p = 0.02). Throughout the study, 75 % of participants remained blinded to sleep assessment as a primary outcome measure, with 56 % subjectively indicating improved sleep during the intervention trial. Metabolomic analysis highlighted several significantly altered metabolomes related to sleep regulation between trials, inclusive of 6-sulfatoxymelatonin, D-serine and L-glutamic acid. **Conclusions:** Data demonstrate that employing the proposed blend of novel nutritional ingredients during free living conditions reduced sleep onset latency, increased total sleep duration and increased sleep efficiency, leading to reduced perceptions of morning sleepiness. These effects may be mediated by the upregulation of key metabolites involved in the neurophysiological modulation of the sleep/wake cycle. **Key Words:** ACTIGRAPHY, NUTRITION, ERGOGENIC DIETARY SUPPLEMENTS, METABOLOMICS

INTRODUCTION

Sleep is an essential process in aiding the recovery of several psycho-physiological functions, including learning and memory, the central nervous and metabolic cost of daytime activities and the restoration of the immune and endocrine systems (1). In order to maintain optimal health and daytime functioning, it is recommended that adults achieve at least 7 hours of total sleep time with ≥85 % sleep efficiency (2, 3). However, a large scale global survey of participants aged 15 to 65 years, has highlighted that 56 % of respondents from the United States of America, alongside 31 % in Western Europe and 23 % in Japan have experienced some form of sleep disturbance, such as poor sleep induction (55-69 %), maintenance (78-63 %) and overall sleep quality (52-31 %) (4). The impact of these sleep disruptions often results in deleterious effects on daytime functioning, therefore causing negative repercussions to both personal and professional activities. Indeed, there is accumulating evidence demonstrating how sleep disturbance may cause reductions in both athletic performance and physical activity levels (5, 6). Moreover, it has been reported that sleep disturbance is a well understood factor in all cause morbidity and mortality across a range of populations (7). To that end, changes in lifestyle factors, improved sleep hygiene and nutrition have all been proposed as potential modulators for sleep disturbances in order to offset these issues (8, 9).

The nutritional regulation of sleep has been of consistent research interest for many years, most notably in athletic populations, as has been recently addressed in a number of narrative reviews and an expert consensus statement (1, 10, 11). Several studies have examined various nutritional ingredients with proposed sleep enhancing qualities across a range of participant groups with and without sleep disorders, inclusive of high glycaemic index carbohydrates (12, 13), tryptophan (14), tart cherries (15, 16), glycine (17), magnesium (18) and L-theanine (19).

Within these investigations there have been numerous suggested regulatory mechanisms, with metabolomic interactions of specific neurotransmitters involved in the sleep/wake cycle, purported to enhance regulation of the circadian rhythm of sleep (20). However, despite promising outcomes in the capacity to improve sleep, many of these studies have been conducted with predominantly male participants and with limited samples sizes in order to make definitive conclusions (21). Furthermore, these ingredients have principally been studied in isolation, with only one investigation to date examining their combined efficacy on various sleep outcome measures (22). On this basis, there is considerable scope to further investigate the combined benefit of these sleep enhancing agents, particularly given their reported individual effectiveness.

A recent critical review (23) has suggested that to sufficiently investigate and determine the true efficacy of any intervention treatment on sleep mechanisms, participants should be appropriately blinded to their sleeping patterns being monitored. To examine sleep as a primary variable there are several available methods, with the criterion standard of polysomnography affording an in-depth view across numerous outcome measures, inclusive of sleep stages (24). However, despite the validity of this technique, measurement needs to be conducted within a laboratory setting, leading to indirect disturbances in participants normal sleeping patterns and reducing ecological validity as assessments cannot be conducted during free living conditions (23). Consequently, given the complexity of configuration prior to and during polysomnography utilisation, this makes the capacity to deceive participants difficult, thereby not fulfilling the potential to truly blind experiments. Despite being limited in the capability to assess sleep stages, the use of actigraphy has been proposed as a method that is less invasive than polysomnography and has been suggested as less likely to interrupt participants regular sleeping patterns (11, 25). As such, given actigraphy monitors can be easily administered and also utilised to measure

daytime activities, this facilitates the opportunity to blind participants and provide a more accurate overview of sleep assessment in free living conditions (26).

With this in mind, the aim of the present study was to test the hypothesis that a novel nutritional blend comprised of tryptophan, glycine, magnesium, tart cherry powder and L-theanine, would improve subjective and objective measures of sleep in male and female participants during free living conditions. To this end, we adopted an experimental design inclusive of an initial familiarisation period for the assessment of habitual sleeping patterns, light exposures, physical activity levels and daily energy intakes, prior to participating in a randomised crossover trial whereby participants ingested either an intervention or placebo treatment. Importantly, the intervention was administered double blind, with participants being deceived as to the true aim of the study.

METHODS

Participants

The study was approved by the Liverpool John Moores University Research Ethics Committee (Protocol code: 21/SPS/003) and all procedures were conducted according to the manual of the Declaration of Helsinki 1964 and its later amendments. Sample size was determined a priori according to an effect size of $d = 1.00$, where a sample size of 16 would provide an α-value of 0.05 and a power of 0.96 (G*Power, version 3.1.9.6). Sixteen participants (9 males and 7 females; age: 24 ± 3 years; body mass: 69.8 ± 11.6 kg; stature: 170.8 ± 9.1 cm) were recruited for the study and prior to commencement, were provided with an information sheet and gave written informed consent. Inclusion criteria stipulated participants must be a.) aged between 18-40 years, b.) non-smokers, c.) not experiencing any medically diagnosed sleep

disorders, environmental sleep disturbances and/or taking sleep aid medications, d.) had not travelled across different time zones in the month prior to study, e.) have no history of neurological, cardiovascular, metabolic and/or psychiatric illnesses or diseases and f.) not be taking any medications or supplements, which may contraindicate the study.

Study Design

Initially, participants were familiarised to the study measures and procedures, whilst also establishing a baseline for nocturnal sleeping patterns, daily energy/macronutrient intakes and activity profiles, which enabled duplication during the main intervention and placebo trials (see Figure 1a & b). In a randomised, repeated measures crossover and double blind deception design, participants were then assigned to either the intervention or placebo trial determined by an online randomisation system (See Figure 1c & d). Both the familiarisation and the main trial assessments were 3 days in length, all of which commenced on the same days of the week for all participants and with a 4 day period between conditions to minimise any crossover effects. The intervention treatment comprised of 1000 mg of tryptophan, 3000 mg of glycine, 300 mg of magnesium, 220 mg of tart cherry powder and 200 mg of L-theanine, with the placebo treatment containing 4720 mg of cellulose, both of which were administered across all 3 nights of each respective main trial in opaque pill format. Additionally, the participants were also informed that the study aim was to assess the effect of two supplemental treatments on resting fat oxidation measured on the final day of main trial periods (see Figure 1c) and with all subsequent assessments being administered for standardisation between conditions. The intention of this deception blinding, was to make participants unaware of the true aim of the study (27).

Study Measures

Body Mass and Stature: Body mass was measured to the nearest 0.01 kg on a calibrated digital scale, with measures of stature established to the nearest 0.1 cm using a free standing stadiometer (Seca 702 & 123; Seca GmbH, Hamburg, Germany).

Subjective Sleep Assessments: To examine subjective assessments of nocturnal sleep patterns, alongside evening and morning alertness and sleepiness, participants recorded responses within electronic versions of the Pittsburgh Quality Sleep Index (PSQI) (28), Core Consensus Sleep Diary (CSD) (29) and Karolinska Sleepiness Scale (KSS) (30). The PSQI is a 9-unit questionnaire, which retrospectively examines sleep across a one month period and is rated on 7 components of sleep quality, latency, duration, efficiency, disturbance, medication and day time dysfunction, to generate a global score. The core CSD is a 10-unit survey, assessing a range of information related to sleep patterns, whereas the KSS is a 9-point Likert scale utilised to examine levels of alertness or sleepiness, both prospectively captured at the time of response. Upon bed time, each participant recorded the clock time for the first and second units within the core CSD and the following morning upon getting out of bed, recorded the information for the remainder of the units.

Objective Sleep, Activity and Light Assessments: To objectively assess nocturnal sleep patterns, daytime activity profiles and light luminous intensity (lux) exposure, each participant was equipped with a lightweight, waterproof, wrist worn actigraphy monitor (MotionWatch 8, CamNtech Ltd, Cambridgeshire, United Kingdom). The tri-axial accelerometer within the monitor detects acceleration ranging from 0.01 to 8 *G* in magnitude, with a frequency of 3-

11 Hz. Additionally, the monitor contains a digital human eye response optimized light sensor, capable of capturing 0 to 64,000 lux at a resolution of 0.25 to 16.0 lux in one sample per second. For sleep and activity measurement, the monitor has been assessed for validity and reliability of its algorithms against both polysomnography and indirect calorimetry across a range of demographics and is effective when set above medium sensitivity and epoch lengths of 30 seconds (31, 32). Each monitor was configured in these modes and participants were requested to press the marker button during specific morning and night time periods (see *Study Procedures*). The recorded markers from the monitor and the collated information from the core CSD, were then used to determine bed time, sleep onset, sleep offset and get up time, so that nocturnal sleep behaviours could be automatically calculated using the appropriate software (MotionWare version 1.2.5, CamNtech Ltd, Cambridgeshire, United Kingdom). From the analysis, the following sleep characteristics were established: sleep onset latency (SOL), wake after sleep onset (WASO), total sleep time (TST), time in bed (TIB), and sleep efficiency (SE). After excluding sleep measurement periods, day time activity profiles were automatically calculated by the software into vigorous, moderate, low and sedentary phases, with light exposure averaged across each of the night and day time periods.

Daily Energy and Macronutrient Intakes: Within the familiarisation period, all *ad libitum* food and fluid intake were prospectively recorded by participants using both weighed food inventory and the remote food photography method (RFPM) (33). In brief, participants were guided on how to measure all food and fluids on an electronic digital scale (AccuWeight 201, Nanlgood Network Technology Co. Ltd., Shenzhen, China) and record the details within the MyFitnessPal application (Francisco Partners, San Francisco, California, USA). Additionally, participants were

also instructed to capture photographs at 45 and 90° angles before and after each eating and/or drinking occasion, which was recorded and timestamped via the WhatsApp application (WhatsApp Inc, Mountain View, California, USA) as previously described (34). These data were then assessed by two accredited sport nutrition practitioners utilising dietary analysis software (Nutritics V5. Nutritics Ltd., Swords, Co. Dublin, Ireland) to establish respective daily energy and macronutrient values (see Table 1), with systematic bias of measurements via independent *t*-tests highlighting no differences between analyses ($p < 0.05$). Within both main intervention and placebo trials, participants were instructed to repeat the eating and drinking patterns at the same times of day as in the familiarisation period, with reminders inclusive of previous recorded images, descriptions and MyFitnessPal data sent by members of the research team via the WhatsApp application. The participants were also instructed to follow the same procedures of weighed food inventory and RFPM as described above, for confirmation of adherence. Compliance to these procedures was 100% across all participants, which ensured daily energy and macronutrient values were duplicated across main trials.

Urine Collection, Storage and Metabolomic Analysis: Urinary voids were collected by participants into sterilised measuring cylinders and immediately stored at -20 °C within a home freezer unit. The samples were then returned to the research team, thawed at room temperature, centrifuged at 12,000 *G* for 1 min and subsequently transferred to a 1.5 mL Eppendorf tubes for immediate storage at −80 °C. A random sub group of $n = 8$ participants samples were then examined for targeted and untargeted metabolomic profiles. For sample preparation, 500 µL of urine was desalted using Zeba^{TM} spin columns (Thermo Scientific, Hemel Hempstead, United Kingdom), flash frozen in liquid nitrogen and lyophilized overnight. The samples were then

reconstituted in 100 µL (90/10 v/v) LC/MS grade water acetonitrile and spin filtered with Costar® Spin-X® 0·22 micron cellulose filters (Corning, Glendale, Arizona, USA) at 2000 *g* for 5 mins. The resulting concentrated urine samples were then transferred to 1.5 mL amber vials with 150 µL micro inserts. For the targeted analysis of 6-Sulfatoxymelatonin, conventional standard addition methodology was applied, whereby 1.0 mg of chemical standard (Biosynth® Carbosynth Ltd, Berkshire, United Kingdom) was reconstituted in 100 µL of LC/MS grade water to generate a 30 mM stock and then serial diluted down to a 10 µM working solution. The RT and accurate mass were confirmed using hydrophilic liquid interaction chromatography (HILIC) and the mass spectrometry signal acquisition was performed and quantified using a single ion monitoring mode with 10 ppm mass channel tolerance. The HILIC profiling of the samples was performed on a Vanquish™ Ultra High Pressure Liquid Chromatography (UHPLC) separation system connected to an IDX high-resolution mass spectrometer system (Thermo Scientific, Hemel Hempstead, United Kingdom).

Mass spectrometry data were captured using the AcquieX acquisition workflow (data dependent analysis methodology) and orbitrap detector operating parameters were MS1 mass resolution 60K, for MS2 30K collisional stepped energy (HCD) 20, 35, 50 in step collision mode. The mass scan range was 100-1000 m/z, RF lens was 35%, AGC target mode custom and normalised AGC target was set to 25 % (100 % = 3e6) with a maximum injection time of 50 ms. The intensity threshold was set to 2e4, with all data acquired in profile mode. A corresponding extraction blank was used to created background exclusion list and a pooled QC were used to create the inclusion list.

HILIC separation was achieved using a Waters™ Acquity UPLC BEH amide column (2.1 x 150mm with particle size of 1.7 µm), operating at 65°C with a flow rate of 200 $\mu L \cdot min^{-1}$

(Waters Ltd., Wilmslow, UK). The LC gradient consisted of a binary buffer system, of which buffer A was 95/5 % (LC/MS grade water/ACN) and Buffer B was 90/10 % (ACN/water), with both containing 10 mM ammonium formate additives. Independent buffer systems were used for positive and negative modes, respectively. For positive modes, the pH of buffers was adjusted using 0.1 % formic acid and for negative modes 0.1 % ammonia solution. The LC gradient were the same for both polarities, with 95 % B at T0 hold for 2 min and a linearly decrease to 50 % B at 11 min hold for 4.5 min and return to the starting condition and hold for further 4.5 min (column stabilization). The total run was approximately 21.5 min per injection, with each volume and applied voltage being 3 μL/3.5 kV and 5 μL/2.5 kV for positive and negative modes, respectively. The HESI conditions for 200 $\mu L \cdot min^{-1}$ were sheath gas 35, aux gas 7 and sweep gas of 0, with an ion transfer tube temperature of 300°C and vaporizer temperature of 275°C. Positive and negative data sets were processed via Thermo Scientific Compound Discoverer 3.2 with untargeted metabolomic workflow, alignment model adaptive curve, minimum intensity $1e^6$, S/N threshold 3:1, compound consolidation, mass tolerance 10 ppm and retention time tolerance 0.3 min settings. Database matching was performed using Thermo Scientific m/z Cloud^{TM} spectral library for targets with a similar index of >70% MS2 spectra. Those metabolites that could be matched (n = 134) and had a relative standard deviation of 30% or less within the quality controls were retained for analysis.

Deception Blinding Protocol: Given the status of COVID-19 at the time of the study assessment period and with governmental restrictions on certain testing procedures in place, the research team were unable to conduct valid assessments of resting fat oxidation via indirect calorimetry. To compensate, a *mock* fat oxidation assessment was conceived, whereby participants were

provided with an exetainer collection vial equipped with a bespoke breathing tube. Following the final assessment day of both intervention and placebo main trials and upon waking, participants were instructed to blow into the breathing tube for a period of 60 seconds, so their subsequent breath sample could be assessed for endogenous fat utilisation utilising indirect calorimetry. However once samples were collected, they were discarded, given they were not required for analysis within the primary examinations of the deception design.

Study Procedures

Familiarisation: On the day prior to the beginning of the familiarisation assessment period, participants were measured for body mass and stature, completed the PSQI and then provided with the subjective and objective sleep measurement apparatus and urine cylinders as described in the *Study Measures*. Participants were then subsequently categorised based on their global PSQI score, with < 5 regarded as *good sleep quality* and > 5 as *poor sleep quality* types (28). During this assessment period, all communication was recorded and timestamped via the WhatsApp application. On the evening prior to the first day of assessment, participants were instructed to wear the actigraphy monitor on their non-dominant wrist, with recording programmed to begin at 6.00am on the following morning. Participants were requested to continuously wear the monitor at all times, other than if there was a need to remove it for safety reasons i.e., contact exercise. On the morning of each assessment day, upon sleep offset and getting out of bed, participants were instructed to press the marker button of the monitor, provide an initial urine sample and then complete the electronic core CSD and KSS survey tools. Throughout the remainder of each assessment day, participants provided weighed food inventory and RFPM for each subsequent individual food and fluid intake occasion (see *Study Measures*),

279 whilst also indicating the details of any exercise bouts, inclusive of modality and load (i.e.,
280 duration, distance, sets, repetitions etc.). A further two urine samples were collected at regular
281 spaced intervals, based on the estimated get up and bed times derived from individual participant
282 PSQI responses. Finally, participants were directed to complete the relevant section within the
283 core CSD and KSS, press the wristwatch actigraphy monitor marker button when getting into
284 bed and again when they intended to go to sleep. The monitor and urine samples were then
285 returned to the research team on the day after the final assessment day (see Figure 1a & b).
286
287 *Main Trials:* Following the familiarisation period, sleep measures were analysed from the
288 actigraphy monitor and if SE was ≥ 85 % and TST ≥ 7 hours, then participants were further
289 classified as *normal* and below both of these values as *deficient* sleep types (2, 3). During main
290 trials, participants continued to be contacted via the WhatsApp online application, with
291 collection of measures, morning procedures, energy/macronutrient/fluid intakes, exercise
292 occasions and urine sampling repeated in the same manner and time of day as established in the
293 familiarisation period. The placebo and intervention treatments were administered two hours
294 prior to each participant's respective bed time, which was replicated at the same time within the
295 familiarisation period. The sleep offset and get up times of participants were not standardised, to
296 examine if any of the conditions resulted in an extended time in bed (see Figure 1c & d). Upon
297 completion of the study, all participants were interviewed to assess the efficacy of the deception
298 blinding procedures. Initially, participants were queried as to if they could detect any differences
299 between the two main trials. Participants were then informed that the deception was not the true
300 aim of the study and asked if they could speculate as to the real aim. Finally, participants were

informed of the study aim and asked if they could identify the intervention and placebo conditions.

Statistical Analyses

Descriptive statistics inclusive of mean ± *SD*, 95 % confidence intervals (95 % CI) and frequency are provided for all data where appropriate, with analyses performed using SPSS version 26 (PASW, Chicago, Illinois, USA) and the α-level set at $p<0.05$. Residuals of the data were explored for normality utilising histograms and Shapiro-Wilk tests, with box plots examined for outliers. All data are presented as the mean of each 3 day period for the intervention and placebo trials; therefore, comparisons were assessed via parametric paired samples *t*-tests or non-parametric Wilcoxon tests for normally and non-normally distributed data, respectively. Additionally, effect sizes (ES) were calculated utilising Hedges *g* with the following quantitative criteria to explain the practical significance of the findings: trivial <0.2, small 0.2 – 0.59, moderate 0.6 – 1.19, large 1.20 – 1.99, and very large >2.0 (35). Given the ordinal nature of the KSS data, Pearson's Chi Squared test was employed to cross tabulate frequency percentages between participant responses. Metabolomics data was auto scaled and cube root transformed using MetaboAnalyst 5.0 software (36) before performing detailed partial least squares discriminant analysis (PLS-DA) between intervention and placebo trial conditions. The relative abundance of the metabolites from the intervention with variable importance in projection (VIP) factor >1 was then compared with the placebo trial.

RESULTS

Sleep Classifications and Bed Times

Prior to starting the familiarisation assessment period, *n* = 8 participants were subjectively classified as having *poor sleep quality* (50 %) with the remaining *n* = 8 classified as having *good sleep quality* (50 %) based on global PSQI scores. Following the familiarisation period, *n* = 14 participants were objectively classified as *deficient* (87.5 %) and *n* = 2 classified as *normal* (12.5 %) sleep types based on actigraphy monitoring. Mean bed time during the familiarisation assessment period was 11.47 pm ± 26 mins, which was consistently replicated at 11.48 pm ± 26 mins and 11.50 pm ± 26 mins in both the intervention and placebo trials, respectively.

Activity Profiles and Light Exposure

There were no differences in mean night time light exposure between intervention (1.06 ± 0.91 lux) and placebo (1.29 ± 0.95 lux) main trials, with a -0.24 ± 0.53 lux variance of a small effect between conditions (p = 0.10; 95 % CI = -0.53 to 0.55 lux; ES = 0.24). Additionally, there were also no differences in mean day time light exposure between intervention (351.66 ± 289.58 lux) and placebo (352.48 ± 361.74 lux) main trials, with a -0.80 ± 295.19 lux difference of a trivial effect between conditions (p = 0.99; 95 % CI = -158.10 to 156.50 lux; ES = 0.01). Measurements of mean participant activity are highlighted in Figure 2, demonstrating no differences in vigorous (Figure 2a, 9 ± 1 vs. 8 ± 11 mins·day^{-1}, p = 0.83; 95 % CI = -4 to 5 mins·day^{-1}; ES = 0.09), moderate (Figure 2b, 25 ± 23 vs. 35 ± 29 mins·day^{-1}, p = 0.22; 95 % CI = -25 to 6 mins·day^{-1}; ES = 0.37), low (Figure 2c, 473 ± 93 vs. 453 ± 80 mins·day^{-1}, p = 0.15; 95 % CI = -7 to 47 mins·day^{-1}; ES = 0.22) or sedentary (Figure 2d, 360 ± 117 vs. 378 ± 109

mins·day^{-1}, p = 0.13; 95 % CI = -40 to 5 mins·day^{-1}; ES = 0.16) profiles, resulting in trivial to small effects between the intervention and placebo main trials, respectively.

Actigraphy Sleep Assessments

For measures of SOL, there were differences of a large effect between the intervention (16 ± 6 mins) and placebo (40 ± 25 mins) main trials, with a mean reduction of -24 ± 25 mins (p = 0.002; 95 % CI = -38 to -10 mins; ES = 1.29) between conditions (see Figure 3a). There were no differences in WASO between the intervention (49 ± 15 mins) and placebo (51 ± 17 mins) main trials, resulting in a trivial effect based on the mean difference of -2 ± 11 mins (p = 0.36; 95 % CI = -8 to 3 mins; ES = 0.12) as highlighted in Figure 3b. For measures of TST there were differences of a small effect between the intervention (422 ± 34 mins) and placebo (400 ± 44 mins) main trials, due to a mean increase of 22 ± 32 mins (p = 0.01; 95 % CI = 6 to 39 mins; ES = 0.55) between conditions (see Figure 3c). There were no differences for overall TIB between the intervention (506 ± 38 mins) and placebo (495 ± 42 mins) main trials, with a small effect given the mean difference of 11 ± 26 mins (p = 0.13; 95 % CI = -4 to 25 mins; ES = 0.27) as shown in Figure 3d. Finally, there were differences of a small effect in measures of SE between the intervention (83.3 ± 3.5 %) and placebo (80.9 ± 5.9 %) main trials, based on an increase in efficiency of 2.4 ± 3.9 % (p = 0.03; 95 % CI = 0.3 to 4.5 %; ES = 0.48) between conditions (see Figure 3e).

Subjective Sleep Assessments & Deception Blinding

There were no differences evident in subjective scores of evening sleepiness between the intervention and placebo main trials (see Figure 4a). However, there were differences in morning

sleepiness (see Figure 4b), whereby in the intervention trial participants reported a lower level of some signs of sleepiness when compared to the placebo trial ($p = 0.02$). During post testing exit interviews, $n = 12$ (75 %) participants were unable to identify that the true outcome of the study was to assess sleep, with the remaining $n = 4$ (25 %) being aware. Of the $n = 16$ participants, $n = 9$ (56 %) identified the intervention trial as the assessment period where they perceived they had their best sleep, $n = 1$ (6 %) stated the placebo trial and $n = 6$ (38 %) were unable to identify any difference between conditions.

Targeted and Untargeted Urinary Metabolomic Analysis

Results of the PLS-DA visualization for both treatments is presented in Figure 5, demonstrating a distinct phenotypic change in the urinary metabolome between the intervention and placebo treatments. Twenty five database matched metabolites were highlighted to be statistically different between the intervention and placebo trials based on VIP score factor > 1. Figure 6 highlights those metabolites identified as proxy markers of sleep regulation inclusive of the targeted metabolite 6-sulfatoxymelatonin (a.), alongside untargeted amino acids D-serine (b.) and L-glutamic acid (c.), both of which were up and downregulated between the intervention and placebo trials, respectively.

DISCUSSION

In a randomised, repeated measures crossover and double blind deception design, the aim of the present study was to test the hypothesis that a novel nutritional blend comprised of tryptophan, glycine, magnesium, tart cherry extract and L-theanine, would improve subjective and objective measures of sleep in male and female participants during free living conditions.

The data from this study demonstrates that a blend of sleep enhancing agents, shortened time to fall asleep and extended time asleep, therefore resulting in greater sleep efficiency and leading to reduced feelings of morning sleepiness. Evaluation of the urinary metabolome also suggests that such alterations to sleeping patterns may be mediated by the upregulation of 6-sulfatoxymelatonin and glycine co-agonist D-serine, in parallel to a downregulation of the excitatory neurotransmitter L-glutamic acid, which are modulators of the sleep/wake cycle, respectively. Taken together, these results indicate the efficacy of this novel blend of nutritional ingredients to improve outcome measures of sleep during free living conditions, in both male and females without medically diagnosed sleep disorders.

A key strength of this investigation was in deception blinding participants to sleep assessment as the true aim of the study. On this basis, 75 % of participants were unaware that sleep was being assessed as a primary outcome measure and over 50 % identified they had a perceived sense of improved sleep during the intervention trial. Whilst this method has been utilised in other nutritional investigations (27), to the authors' knowledge this study represents the first application of this approach when examining the potential for nutritional modulation of subjective and objective sleep outcomes and therefore enhances the applicability of the findings (23). Additionally, participants were also exposed to an initial assessment period with the dual objective of familiarisation to outcome measures, whilst also establishing a baseline for standardisation of bed times, daily nutritional intake, activity profiles and light exposure within the main intervention and placebo trials. This has been previously highlighted as an important consideration in deciphering the translational potential of nutrition intervention studies (37) and serves to further establish the ecological validity of the outcome results.

To date, only one other study by Halson and colleagues (22) has examined the effect of a combined blend of sleep active ingredients, inclusive of high glycaemic carbohydrates, tart cherry, tryptophan (in the form of α-lactalbumin), adenosine-5-monophosphate, valerian and L-theanine in a double blind, placebo controlled crossover design. Whilst utilising polysomnography in contrast to the actigraphy measures within this study, Halson et al. highlighted a 9 minute reduction in SOL between the intervention and placebo treatments, yet contrary to our findings, found no other differences in subjective or objective measures of sleep outcomes between conditions. It should be noted that a limitation within the present study, is the inability of a reductionist approach to understand the individual or combined effects of the agents within the intervention treatment, on the capacity to improve the subjective and objective measures of sleep. However, given the strength of the study design, the nutritional blend did indeed impact some of these measures and to that end, the subsequent sections will serve to discuss the potential sleep enhancing mechanisms of each ingredient contained within the intervention treatment.

Dietary tryptophan (Trp) is a well-established sleep active ingredient, which has over four decades of research within this area (14). Trp crosses the blood brain barrier by active transport, therefore acting to release the monoamine neurotransmitter serotonin, a precursor of melatonin (38). However, given other large neutral amino acids (LNAAs) also compete to cross the blood brain active barrier system, it is the addition of an adequate dose of dietary Trp (1000-3000 mg), alongside an optimal Trp:LNAA ratio which increases brain bioavailability and can subsequently lead to enhanced sleep through the upregulation of melatonin (14, 39). In comparison to our study, Hartmann and Spinweber (40) found that a dose of 1000 mg of tryptophan resulted in significantly reducing subjective assessments of SOL in those with mild

insomnia (over 30 minutes SOL). Additionally, Chauffard-Alboucq and colleagues (41) also highlighted that a dose of 1000 mg of tryptophan was effective in quadrupling the Trp:LNAA ratio, whilst also leading to enhanced feelings of sleepiness after 90 minutes of ingestion. Based on these investigations, it could be postulated that the dosage of tryptophan included in the presented study increased brain Trp levels, thereby upregulating serotonin and melatonin pathways. Despite not directly assessing the Trp:LNAA ratio, this notion is supported by an enhanced metabolomic abundance of urinary 6-sulfatoxymelatonin (see Figure 6a), which could further explain the exhibited improvements in sleep during the intervention trial. In parallel to Trp, tart cherries are known to contain endogenous melatonin (42), which can also directly regulate sleep mechanisms via the aforementioned pathways. Howatson and colleagues (15) demonstrated that a 7 day administration of tart cherry juice resulted in significant improvements in sleep time and quality. Indeed, whilst the provision of the intervention treatment within the present study was for a 3 day period, this resulted in similar favourable increases in both TST (22 vs. 34 mins) and enhancements in SE (2.4 vs. 5.5 %). Furthermore, Howatson et al. also highlighted significant increases in urinary melatonin during tart cherry supplementation, also corresponding to the amplified metabolomic abundance of urinary 6-sulfatoxymelatonin within this study.

Glycine and L-theanine are amino acids, which are both endogenously synthesised/stored and can be ingested through diet. In a similar manner to Trp, glycine is also able to cross the blood brain barrier, which alongside co-agonist D-serine, targets the *N*-methyl-D-aspartate (NMDA) receptors within the suprachiasmatic nucleus, further acting to upregulate whole body vasodilation and therefore reduce core temperature leading to subsequent sleep enhancement (17). Additionally, L-theanine also crosses the blood brain barrier and acts on the ionotropic

glutamate receptors and partially on the NMDA receptors to upregulate both serotonin and glycine (19). A study by Yamadera and colleagues (43) examined the effects of glycine on subjective sleep parameters and objective sleep via polysomnography. Whilst utilising different measures compared to those within this study, a comparable dose of 3000 mg of glycine improved objective assessments of SOL and subjective assessments of sleep quality and daytime sleepiness. Although no assessments of core temperature were conducted within the present study, given the known effects of core temperature reduction on upregulating sleep (44), it is tempting to speculate that the glycine modulation of this mechanism may have also contributed to the improvements in sleep outcome measures, particularly given the increased metabolomic abundance of D-serine (see Figure 6b). However, to date this has only been conducted in animal model studies, with more conclusive evidence needed within human populations (45). In a similar manner to the present study, Ozeki and colleagues also examined the efficacy of L-theanine on sleep via both subjective sleep scores and objective measurements of actigraphy (19). Utilising a 200 mg dose and across a 3 day measurement period, the intervention significantly improved subjective assessments of sleep, alongside an actigraphy derived 2.8 % improvement in sleep efficiency when compared to a placebo condition, in direct parallel to the results of this study.

An additional interesting finding within this investigation was a downregulated metabolomic abundance of L-glutamic acid within the intervention compared to the placebo trials (see Figure 6c). L-glutamic acid has been highlighted as a potent excitatory neurotransmitter, resulting in stimulation of the orexinergic neurons, which in turn promotes arousal and inhibition of both non-rapid eye and rapid eye movement sleep stages (46). Conversely, both endogenous melatonin and D-serine have been demonstrated to attenuate the

excitatory neurotransmitter action of L-glutamic acid via inhibition of specific NMDA receptor binding sites (47, 48). To that end, it is tempting to speculate that the upregulated metabolomic abundance of urinary 6-sulfatoxymelatonin and D-serine in the intervention trial may have resulted in the downregulated metabolomic abundance of L-glutamic acid, therefore leading to greater subjective and objective sleep enhancement in comparison to the placebo trial. However, much of the former evidence in this area has principally been conducted in rodent models and more research is needed in human trials to substantiate this hypothesis.

Finally, magnesium is a key micronutrient, which also acts to regulate the conductivity of the NMDA receptors, whilst aiding the binding of monoamines such as serotonin to their respective sites (49), presenting the assumption that magnesium deficiency may lead to disturbances in sleep, which can be enhanced with exogenous supplementation (50). A recent systematic review and meta-analysis highlighted that administration of 320-729 $mg \cdot day^{-1}$ magnesium, resulted in improvements in subjective and objective sleep, that included 17 minute reductions in SOL and 16 minute increases in TST when compared to placebo conditions (50). However, it should be noted that these treatments were delivered across various dosing protocols, in predominantly elderly populations with magnesium deficiencies and across a range of sleep disturbances and disorders. Consequently, given magnesium status was not assessed in the present study participant cohort, it is difficult to consider the interaction of this ingredient within the intervention treatment and with more research on the efficacy of magnesium supplementation in diverse populations needed.

Finally, the present data may be considered of practical relevance for those individuals who partake in regular exercise and physical activities. Indeed, in athletic populations, it has been established that sleep disturbances can result in negative consequences on physical

performance, exercise recovery and exercise induced injuries/diseases, with these outcomes being mediated by an enhanced perception of exertion via increased psycho-physiological strain (5). Furthermore, reductions in sleep duration and quality are likely to reduce participation in physical activity, which contributes to a wide range of health related outcomes within general populations (6). Accordingly, improvements in sleep have been shown to enhance exercise performance and increase the likelihood of participation of physical activities in athletic and general populations, respectively (5, 6). On this basis, further investigation implementing the nutritional blend proposed within this study and the potential effects of ameliorated sleep outcomes on exercise performance and physical activity is certainly warranted.

CONCLUSIONS

In conclusion, this study highlights the efficacy of a novel blend of sleep enhancing nutritional agents on subjective and objective measurements of sleep in both male and female participants without medically diagnosed sleep disorders and during free living conditions. Additionally, a key strength of this investigation included 75 % of participants being unaware that sleep was being measured as a primary outcome, therefore enhancing the ecological validity and applicability of the findings. The combination of ingredients contained within the blend, led to significant decreases in sleep onset latency and increases in total sleep time and sleep efficiency, alongside subjective reductions in morning sleepiness, as has been demonstrated within the previous research literature. Furthermore, potential mechanisms for these effects may have been as the result of metabolomes related to the sleep/wake cycle. The replication of this study to examine potential efficacy across longer time periods and in those with medically diagnosed sleep disorders is also warranted.

527

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Conflicts of Interest

James P. Morton is a consultant for SiS plc. The other authors disclose no conflicts of interest. SiS plc had no role in the design, execution, interpretation, or writing of the study. The results of the study are presented clearly, honestly, without fabrication, falsification, or inappropriate data manipulation. The results of the present study do not constitute endorsement by the American College of Sports Medicine.

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FIGURE LEGENDS

Figure 1 Overview of study design, inclusive of (a.) familiarisation assessment period, (b.) familiarisation assessment methods and procedures, (c.) intervention and placebo assessment periods and (d.) intervention and placebo assessment methods and procedures

Figure 2 Comparison of mean and individual responses for (a). vigorous, (b.) moderate, (c.) low and (d.) sedentary activity profiles, between the intervention (grey bars) and placebo (white bars) trials

Figure 3 Comparison of mean and individual responses for (a.) sleep onset latency, (b.) wake after sleep onset, (c.) total sleep time, (d.) time in bed and (E.) sleep efficiency outcomes, between the intervention (grey bars) and placebo (white bars) trials. *denotes significant difference between conditions ($p < 0.05$)

Figure 4 The frequency of subjective Karolinska Sleepiness Scale scores across (a.) evening and (b.) morning measurement periods between intervention and placebo trials. *denotes significant difference between equitable scoring measure ($p < 0.05$)

Figure 5 Partial least squares discriminant analysis of key identified urinary metabolomes between the intervention (red) and placebo (green) trials

682 **Figure 6** Original and normalised concentration of key urinary metabolites (a.) 6-
683 sulfatoxymelatonin, (b.) D-serine and (c.) L-glutamic acid between the intervention (red bars)
684 and placebo (green bars) trials

Table 1 Estimated daily energy and macronutrient intakes assessed during the familiarisation period, which were duplicated during main intervention and placebo trials

	Energy	**CHO**	**Protein**	**Fat**
INTAKE	**(kcal·day^{-1})**	**g·day^{-1}**	**g·day^{-1}**	**g·day^{-1}**
		(g·kg^{-1})	***(g·kg^{-1})***	***(g·kg^{-1})***
Day 1	2010 ± 567	257 ± 79	101 ± 38	62 ± 27
		(3.5 ± 1.0)	*(1.6 ± 0.5)*	*(0.9 ± 0.3)*
Day 2	2114 ± 769	273 ± 90	104 ± 49	65 ± 39
		(3.7 ± 1.1)	*(1.6 ± 0.8)*	*(1.0 ± 0.5)*
Day 3	2036 ± 782	264 ± 110	100 ± 50	63 ± 35
		(3.8 ± 1.2)	*(1.6 ± 0.7)*	*(0.9 ± 0.4)*
TOTAL	**2053 ± 640**	**265 ± 82**	**101 ± 39**	**63 ± 28**
		(3.7 ± 1.0)	***(1.6 ± 0.6)***	*(0.9 ± 0.3)*

Relative values are presented in italics within parenthesis. CHO = carbohydrate

Figure 1

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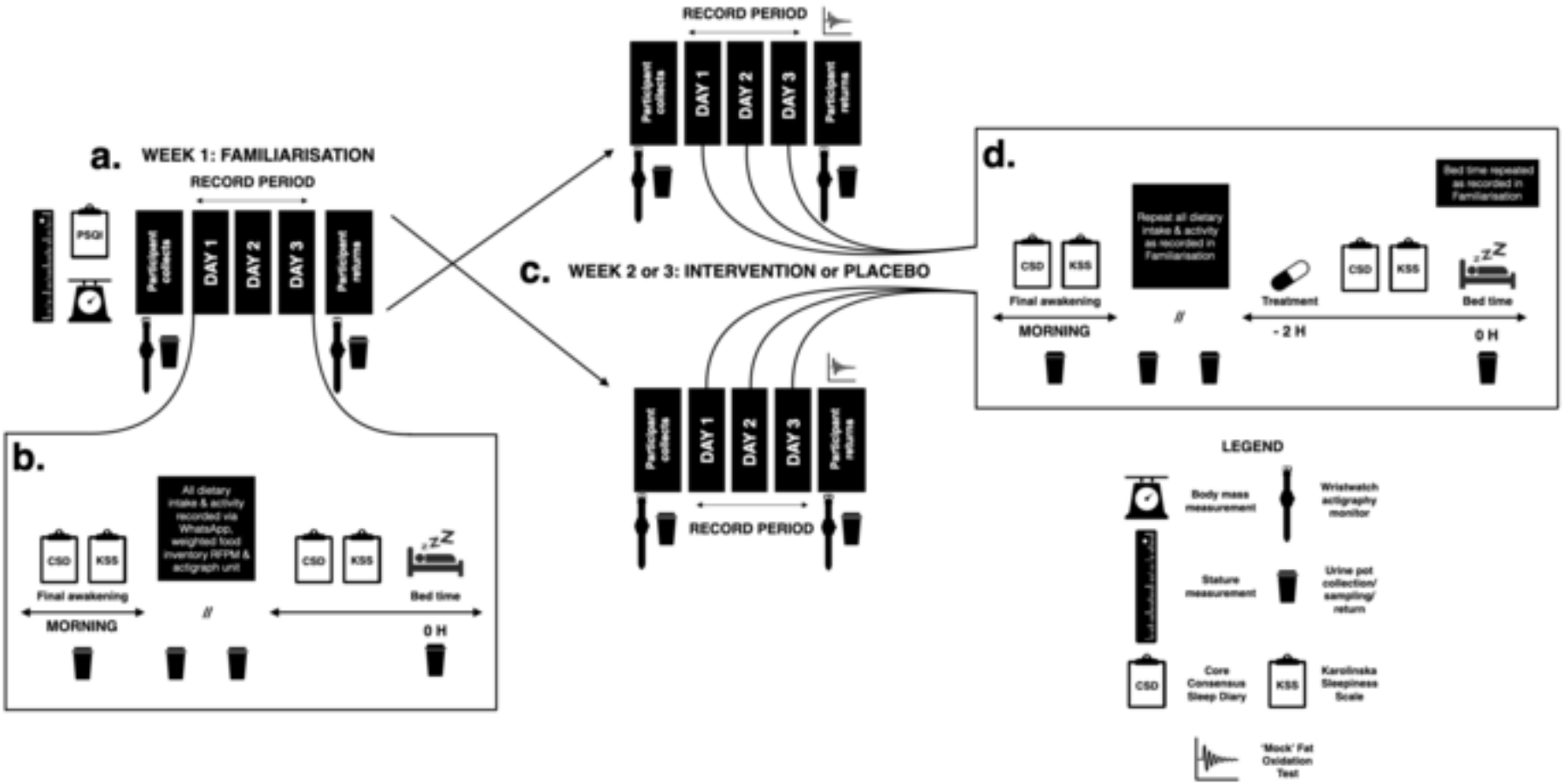

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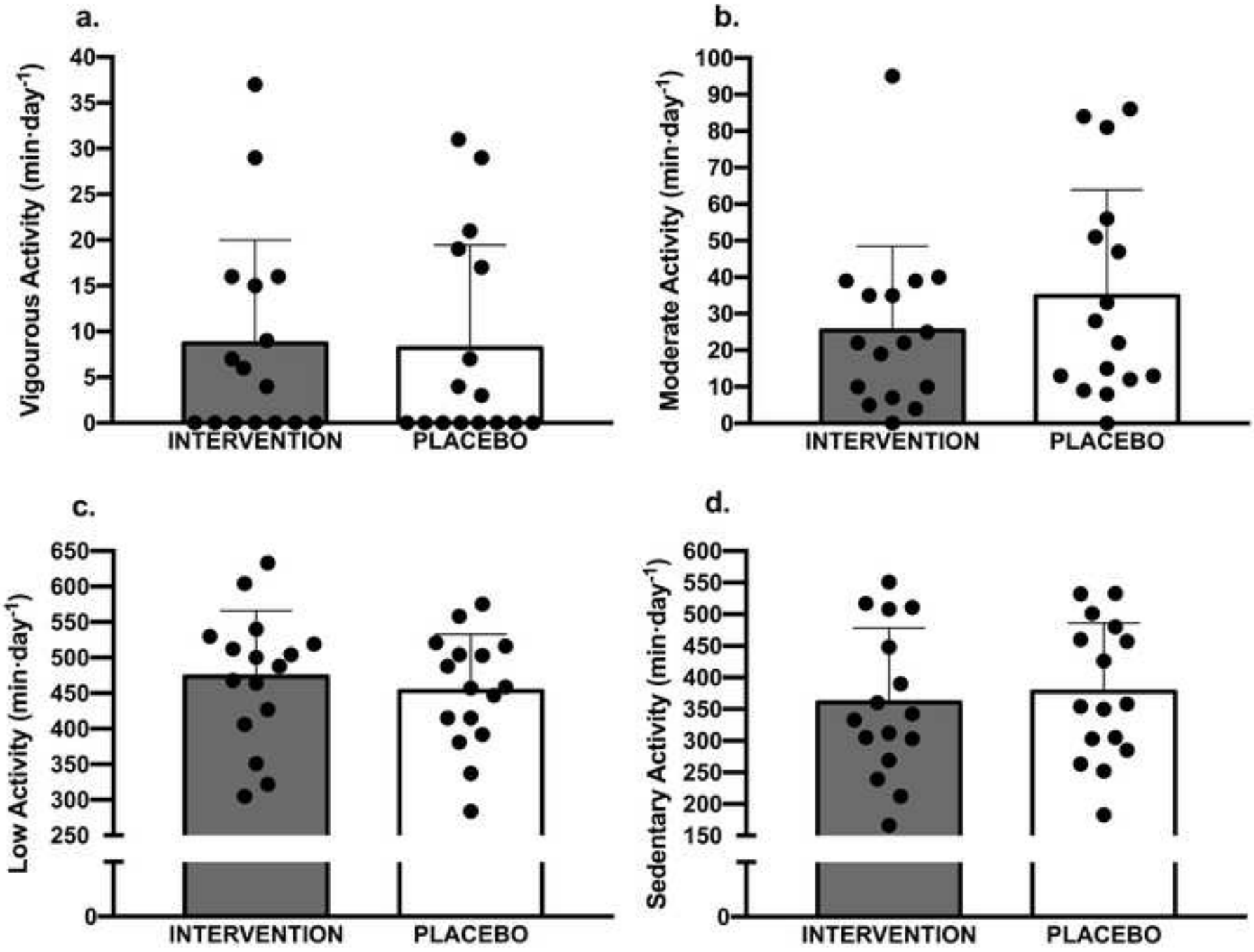

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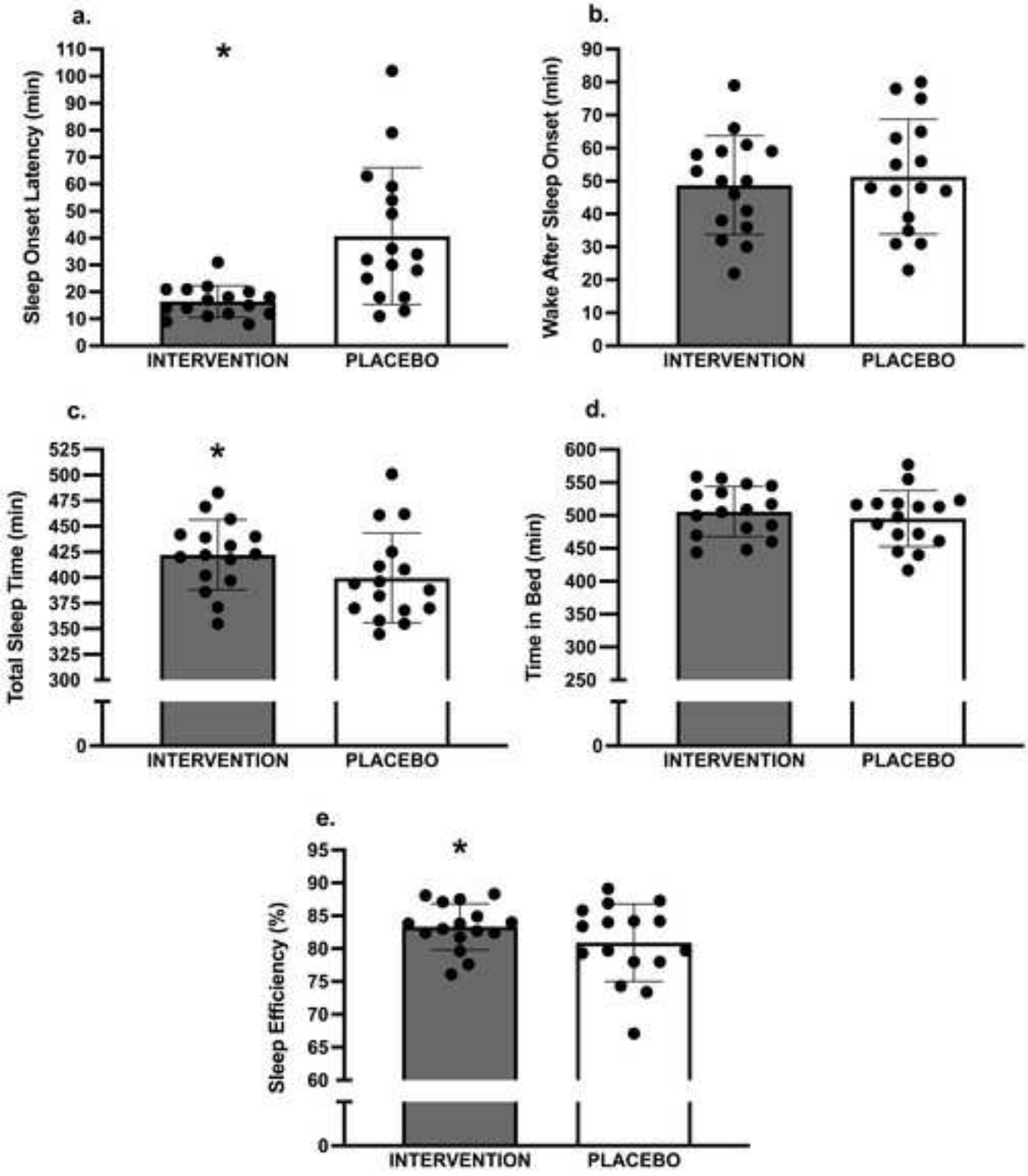

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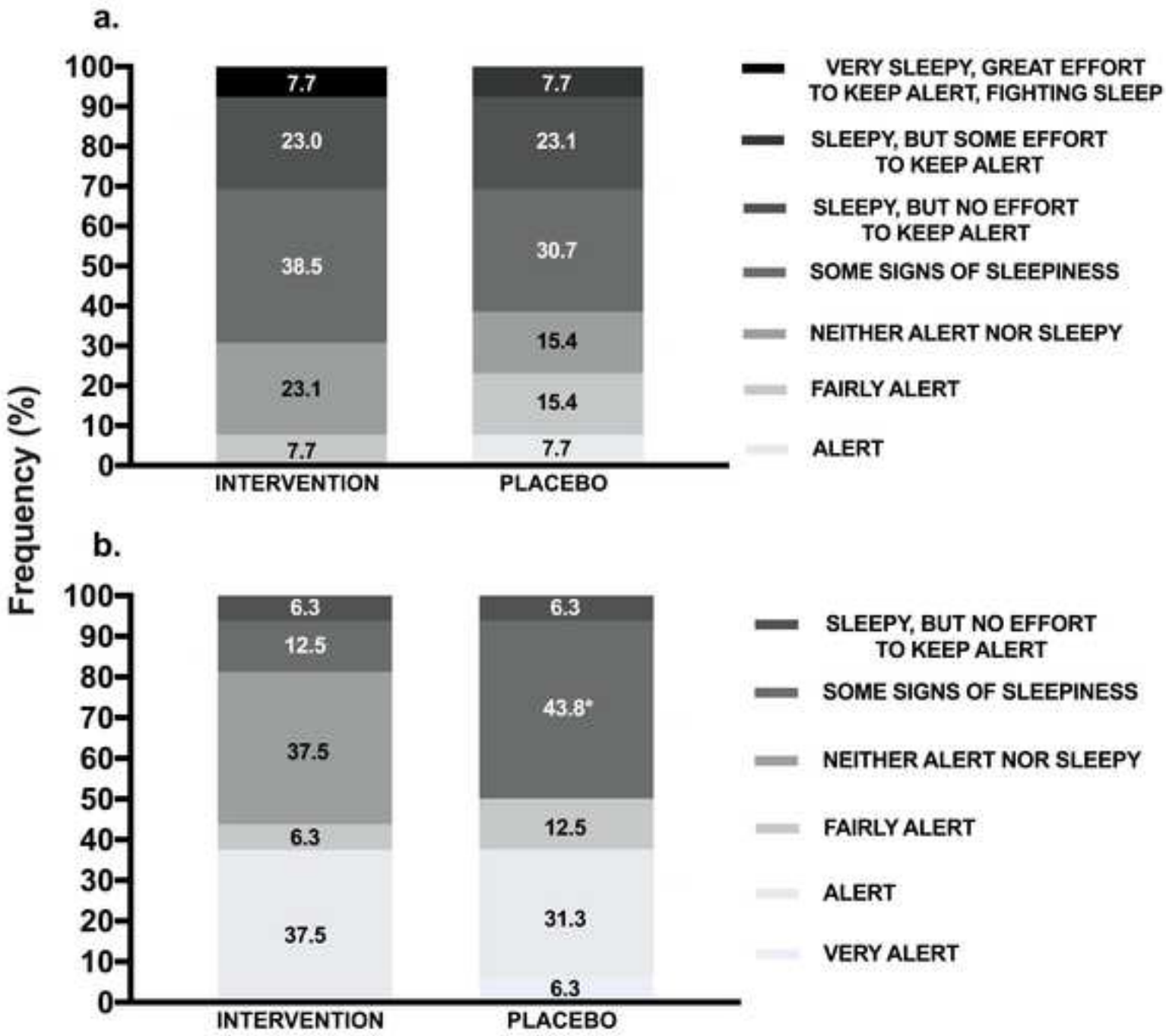

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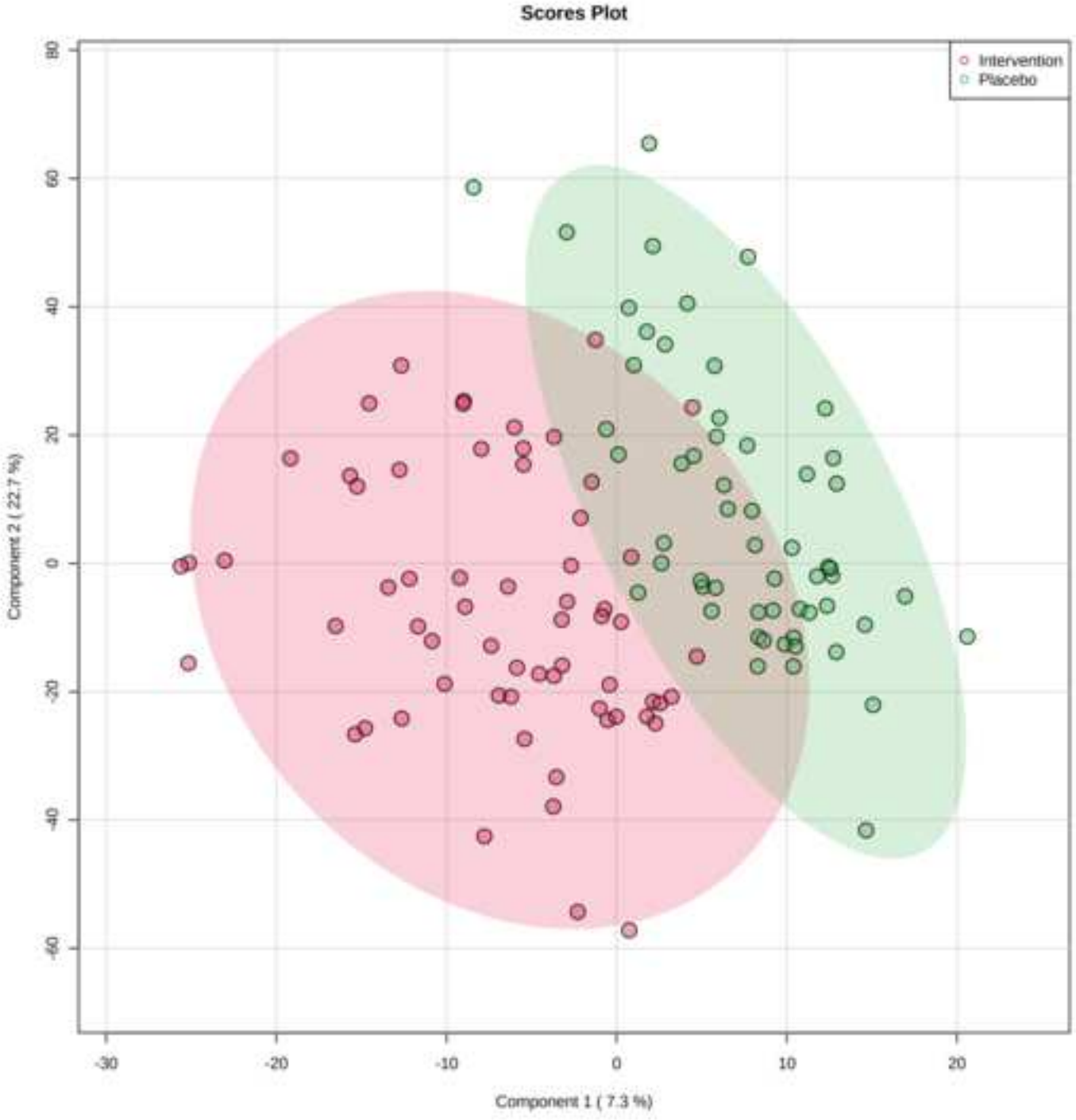

Figure 6

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