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STUDY PROTOCOL

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Official Study Title:

**DEVELOPING MICROBIOME AND DIETARY STRATEGIES BASED ON
CHRONO-NUTRITION FOR TARGETING ADHD (CRONONUTRIBIOME)**

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1. INTRODUCTION

Attention Deficit Hyperactivity Disorder (ADHD) is the most prevalent neurodevelopmental and neuropsychiatric disorder in childhood (Polanczyk, G. et al., 2007). Although this disorder is primarily diagnosed in childhood, it is estimated that symptoms persist into adulthood in approximately 40% of cases (Childress, A.C. & Sallee, F.R., 2014). This disorder, which carries a major economic, social, and healthcare burden, is characterized by significant cognitive and behavioral difficulties that negatively impact an individual's cognitive and/or behavioral development and, consequently, their academic, occupational, and/or social life (Garcia, C.R. et al., 2012).

Moreover, ADHD is frequently associated with other mental disorders (Agarwal, R. et al., 2022), such as anxiety, depression, sleep disorders (Valsecchi, P. et al., 2022), and obsessive-compulsive disorders, as well as common metabolic disorders including

obesity and type II diabetes (Cortese, S. et al., 2016), all of which strongly affect patients' quality of life.

Currently, ADHD is considered a complex neurobiological disorder of multifactorial origin (Cenit M.C. et al., 2017). Despite recent advances in understanding its etiology, the pathogenic processes leading to its development and manifestation remain largely unknown. Additionally, ADHD is clinically very heterogeneous in terms of symptomatology, therapeutic response (about 30–40% of children treated with psychotropic drugs do not respond to treatment), and psychiatric comorbidities (Dias, T.G.C. et al., 2013).

It is important to note that the factors responsible for this clinical heterogeneity have not yet been elucidated, though identifying them could help define subgroups of patients who may benefit from different intervention strategies (personalized medicine/nutrition) (Ana Larroya et al., 2021). Specifically, scientific evidence has shown that this disorder is associated with alterations in specific brain regions—such as the prefrontal cortex—leading to imbalances in neurotransmitters like dopamine, GABA, norepinephrine, and serotonin, which regulate key cognitive and behavioral functions such as attention and impulsivity (Cenit M.C. et al., 2017; Del Campo, N. et al., 2011).

ADHD diagnosis is still based on clinical symptoms, as no biomarkers currently exist to support diagnosis or predict prognosis. Furthermore, current therapeutic efficacy is limited (approximately 30–40% non-response to psychopharmacological treatment) (Dias, T.G.C. et al., 2013), and such treatments may cause significant side effects (Martinez-Raga et al., 2013). Therefore, there is a clear need for new strategies to elucidate ADHD mechanisms, identify biomarkers to aid diagnosis, and improve clinical management of this challenging disorder.

ADHD is currently understood as a multifactorial disorder that develops in genetically predisposed individuals upon exposure to certain environmental factors. Many of these factors—particularly dietary components or patterns—have been shown to affect gut microbiota development and composition (Cenit M.C. et al., 2017). Factors such as mode of delivery, maternal health during pregnancy, and early infections are known to modulate the gut microbiota and may influence ADHD risk.

Dietary patterns are a key determinant of gut microbiota composition and may modulate ADHD symptomatology. Certain dietary components, such as preservatives and food additives like colorants, have been associated with worsening ADHD symptoms (Traxel W.L. et al., 1982), while others—such as omega-3 fatty acids and specific polyphenols—may improve symptoms, at least in some groups of ADHD patients. Several studies have also found low levels of copper, iron, zinc, magnesium, and omega-3 in children with ADHD (Cenit M.C. et al., 2017; Ana Larroya et al., 2021).

Recent studies have demonstrated the key role of the gut microbiota in brain development, maturation, and function. The so-called **gut-brain axis**, largely regulated by intestinal microbiota, consists of neural, hormonal, immunological, and metabolic pathways mediating bidirectional communication between both organs (Collins et al., 2012). Evidence shows that gut microbiota influence brain circuit development and that imbalances in microbiota composition can lead to immunological and neurological

dysregulation, increasing the risk of neurodevelopmental disorders affecting social, cognitive, behavioral, and emotional functions (Ihekweazu & Versalovic, 2018).

In 2013, psychiatrist Ted Dinan (University College Cork) coined the term *psychobiotic*, referring to bacteria that, when ingested in adequate amounts, confer mental health benefits (Dinan T.G. et al., 2013). However, clinical evidence remains limited, and more research is needed to establish causal relationships between microbiota composition changes and cognitive or behavioral outcomes (Martin & Mayer, 2017).

In recent years, the role of gut microbiota in ADHD risk and manifestation has been strongly suggested (Cenit et al., 2017). Thus, preventive and therapeutic interventions based on microbiome modulation could open new, safer avenues for managing this condition. Indeed, studies have shown microbiota alterations associated with ADHD (Bundgaard-Nielsen, C. et al., 2020), and preclinical research has demonstrated causality, with ADHD-like phenotypes transferable via fecal transplant (Tengeler, A.C. et al., 2020). However, it remains unknown which changes precede disorder onset or which may contribute to prevention.

Nutritional psychiatry is a new field of research that provides scientific evidence on how our dietary habits and the quality of food intake can modulate our mental health (Sarris et al., 2015; Adan R.A. et al., 2019). Studies conducted in this field aim to determine whether certain dietary patterns or different components of the diet, specific foods, or specific components of these foods could help prevent or treat mental health problems. In this regard, both nutritionists and psychiatrists around the world acknowledge that an unhealthy diet can worsen mental health, especially in individuals already suffering from neuropsychiatric diseases or disorders. Conversely, numerous studies have indicated that different dietary patterns or various components of the diet could help prevent or treat mood-related disorders, such as depression, or neurodevelopmental disorders such as attention-deficit/hyperactivity disorder (ADHD) (Adan R.A. et al., 2019; Firth et al., 2020).

Scientific evidence demonstrates that the diets which appear to improve aspects related to mental health are those that prioritize whole or natural foods over ultra-processed foods and generally reduce the inflammatory state (Adan R.A.H. et al., 2019). However, there are still many studies to be carried out in the field of nutritional psychiatry (Loughman et al., 2021). In fact, we do not yet know the exact mechanisms through which certain dietary patterns may influence mental health. Moreover, it is necessary to identify which specific foods and which precise components of these foods exert beneficial effects.

We know that a healthy diet, such as the Mediterranean diet, is capable of benefiting our mental health status, and although the exact mechanisms through which this diet could be influencing improvements in mental health are not fully understood, we know that the gut microbiota, through various mechanisms, could be involved (Marx et al., 2021). In any case, the microbiota appears to be a possible external factor—highly modifiable by lifestyle-related factors such as diet, medication use, and exercise, among the most important—implicated in mental health and in the aforementioned neurobiological or neurodevelopmental pathologies (Pelsser et al., 2011).

A multitude of studies have focused on investigating the importance of diet quality in mental health, but few have yet explored the importance of the timing of food intake and

its regularity for mental health. Lastly, although diet appears to play an important role in ADHD and different dietary strategies seem to improve the disorder, at least in certain groups of individuals with the condition (Pelsser, L.M., 2017), the best intervention strategies based on the modulation of diet and the gut microbiota that could assist in the clinical management of ADHD are still unknown (Larroya A. et al., 2021).

In this regard, several recent studies have indicated that not only the quality of the diet appears to impact our mental health, but also the timing at which food is consumed seems to matter for our cognitive, emotional, and/or behavioral state (Tahara, Y. et al., 2021; Currenti, W. et al., 2021; Qian, J. et al., 2022; Feillet, C.A. et al., 2006). **Chrononutrition** is an emerging field of research within nutritional psychiatry, based on the key relationship between temporal eating patterns, circadian rhythms, and health (Franzago, M. et al., 2023).

With respect to the gut microbiota, chrononutrition, and their impact on health, scientific evidence has recently demonstrated that the gut microbiota exhibits diurnal rhythmicity, largely determined by meal timing and the feeding window, and that this circadian rhythmicity of the microbiota substantially regulates the host's circadian rhythms (Wang, H. et al., 2022). Recently, it has also been shown that meal timing in humans affects the diurnal rhythms of the microbial profile present in saliva, which is closely related to the intestinal microbial profile; thus, a late intake of the main meal has been associated with an increase in pro-inflammatory microbial taxa present in saliva (Collado, M.C. et al., 2018).

On the other hand, some studies have reported that circadian disruption may alter fecal microbiota composition in a sex-dependent manner (Liang, X. et al., 2015). In relation to this, diurnal oscillations of the gut microbiota appear to be more pronounced in women than in men (Liang, X. et al., 2015), suggesting the importance of considering sex stratification in studies conducted on microbiota and circadian health. Furthermore, evidence indicates that the microbiome is required for the various sex-specific circadian rhythms and, therefore, essential for sexual development (Weger, B.D. et al., 2019).

Several studies have indicated that circadian health and the diurnal rhythmicity of the gut microbiota—factors that are strongly and bidirectionally interconnected (Voigt, R. et al., 2016)—are key determinants of cognitive status, behavior, and mood (Teichman, E.M. et al., 2020). This established connection between the gut microbiota, circadian rhythms, and mental health supports the suggested idea that modulation of the gut microbiota could help prevent circadian misalignment and/or assist in the resynchronization of altered circadian rhythms in psychiatric disorders (Codoñer-Franch, P. et al., 2023).

Alterations in circadian rhythms, such as irregular eating patterns and sleep disturbances, are commonly observed among individuals with psychiatric disorders (Codoñer-Franch, P. et al., 2023). In fact, a common hypothesis regarding the recognized increase in the incidence of mental illnesses lies in current lifestyle factors, such as unhealthy dietary patterns, inadequate physical activity and sleep habits, and the “stressful” environment associated with modern life in Western societies (Rajaratnam, S.M. et al., 2001; Logan et al., 2015a, 2015b). In this sense, the circadian rhythm misalignment associated with modern lifestyle has been suggested to play a significant role in the worsening of the population's mental health. Conversely, a favorable lifestyle associated with proper

circadian health has been linked to a substantial reduction in the risk of onset and better prognosis of mental illnesses (Codoñer-Franch, P. et al., 2023; Ng, C.M. et al., 2022).

Regarding ADHD, several studies have provided biological and clinical data that have strongly linked the disorder to various alterations in circadian rhythms (Coogan, A.N. et al., 2017). Reductions in sleep quality, delays in circadian phase, as well as an evening chronotype, are consistently associated with ADHD in both children and adults (Coogan, A.N. et al., 2017; Van Veen, M.M. et al., 2010), and these alterations, in turn, have been suggested to correlate with the severity of ADHD symptomatology. A recently published study described specific changes in the gut microbiota of children with autism comorbid with sleep disorders, as well as correlations between these gut microbiota changes and melatonin and serotonin levels (Xueying Hua et al., 2020). It is highly likely that alterations also exist in the diurnal oscillations of the gut microbiota associated with these sleep disturbances linked to neurodevelopmental disorders. If these alterations in gut microbiota rhythmicity underlying sleep disturbances were to be fully elucidated, such disturbances could potentially be reduced through gut microbiota modulation. However, further studies are needed to determine the best microbiome-targeted strategies for improving sleep.

Furthermore, the evening chronotype, associated with circadian misalignment, is considered a risk factor for severe psychiatric symptoms and psychiatric metabolic comorbidities. In this regard, patients with ADHD have a high prevalence of obesity, and it has been proposed that the evening chronotype—an expression of circadian misalignment linked to ADHD and associated with unhealthy eating habits and obesity—could contribute as an important mechanism underlying the strong connection between ADHD and the obese phenotype (Türkoğlu, S. et al., 2019). On the other hand, the high efficacy of chronotherapeutic approaches in improving ADHD (Van Andel, E. et al., 2021) suggests the circadian system as an interesting therapeutic target to address in ADHD management.

Taking all of the above into account, the evidence indicates that resynchronization of circadian rhythms through basic lifestyle changes related to diet could improve ADHD-related symptomatology or even prevent the onset of the disorder in susceptible individuals. Moreover, data support the idea that resynchronization of circadian rhythms could also reduce the risk of developing metabolic disorders that are highly prevalent among individuals with ADHD and are strongly associated with increased severity and poorer quality of life in patients with the disorder (Codoñer-Franch, P. et al., 2023).

Circadian rhythms are regulated by the interaction between genetic and environmental factors. Among the environmental factors, lifestyle-related elements such as diet—strongly linked to the composition of the gut microbiota—have been suggested as major determinants of human circadian health (Wang, H. et al., 2022). Additionally, irregular and mistimed eating patterns have recently been described as disruptive factors for mental health (Tahara, Y. et al., 2021; Currenti, W. et al., 2021; Qian, J. et al., 2022). In this sense, conversely, scientific evidence suggests that the circadian temporal regulation of food intake represents a promising chronotherapeutic strategy to prevent and/or assist in the treatment of mental illnesses, largely through modulation of the gut microbiota (Codoñer-Franch, P. et al., 2023).

2. JUSTIFICATION OF THE STUDY

Despite the extensive scientific evidence suggesting possible alterations in the circadian rhythmicity of the gut microbiota associated with ADHD, as well as the potential beneficial effect of circadian fasting for improving the disorder, we currently lack human intervention studies that have evaluated possible alterations in the diurnal oscillations of the gut microbiota and the therapeutic effect of circadian temporal regulation of food intake as a potential chronotherapeutic strategy for improving its clinical management.

Confirmation of the beneficial effect of regular eating aligned with circadian rhythms (circadian fasting) on ADHD-related alterations, and of the role of gut microbiota changes mediating the intervention effect, would allow the design of new intervention strategies based on more specific microbiota modulation. Furthermore, should modulatory factors of the intervention response be identified, such strategies could be directed toward specific subgroups of individuals with ADHD (precision nutrition) to be evaluated in future studies.

Therefore, if our working hypothesis is confirmed, the results of this project could, in the near future, enable the use of new intervention strategies that may improve ADHD in patients refractory to conventional therapies, as well as reduce the use of pharmacological treatments for ADHD management by proposing adjunctive therapeutic approaches.

3. HYPOTHESIS

This project is based on the hypothesis that the gut microbiota exhibits diurnal oscillations in its composition and function, which are highly modifiable by food intake timing and strongly impact the host's circadian health and brain function. Evidence suggests that, due to irregular and mistimed eating patterns linked to the disorder, these oscillations may be altered in patients with ADHD, and that the regular shortening of the eating window and its alignment with circadian rhythms could help improve ADHD through the positive modulation of the gut microbiota's diurnal rhythmicity, at least in certain patients, such as those with alterations in circadian health or individuals with immunometabolic disturbances.

Thus, the modulation of the gut microbiota through changes in the eating window, promoting a healthy lifestyle via regular temporal restriction of food intake aligned with circadian rhythms, could represent a strategy—at least for specific patient subgroups—for improving the cognitive, behavioral, and emotional alterations associated with ADHD.

4. OBJECTIVES

Primary

1. To evaluate the feasibility of shortening the eating window and aligning it with circadian rhythms (circadian fasting) in adults with ADHD and circadian rhythm alterations (patients with sleep disturbances and a habitual eating window longer than 14 hours, with their last food intake occurring after 8:00 p.m.).

2. To evaluate the therapeutic potential of circadian fasting on cognitive and behavioral alterations in adults with ADHD.
3. To identify the beneficial effects of circadian fasting on sleep disturbances and quality of life in patients with ADHD.
4. To evaluate possible alterations in the circadian rhythms of the salivary microbiota associated with ADHD.
5. To evaluate the microbiota changes associated with circadian fasting in the context of ADHD.
6. To determine possible correlations between microbiota changes associated with circadian fasting and the beneficial effects of circadian fasting observed in patients with ADHD.

Secondary

7. To evaluate the influence of baseline microbiota profiles on the effects of circadian fasting.
8. To analyze lifestyle factors and/or anthropometric or immunometabolic variables related to better or worse response to circadian fasting intervention, and the possible association of these factors with baseline microbiota profiles.
9. To analyze whether participants' lifestyle—at a multidimensional level or within specific lifestyle domains—may influence the degree of adherence to the circadian fasting protocol and/or its effect on ADHD improvement and/or participants' quality of life.
10. To identify possible microbiota profiles associated with difficulty in adhering to the circadian fasting intervention.

5. MATERIALS AND METHODS

5.1. DESIGN

Longitudinal 12-week intervention study. Factorial 2x2 protocol, including non-ADHD and ADHD adult populations and non-fasting and fasting intervention.

5.2. POPULATION

The study will include adult participants (>18 years of age) diagnosed with ADHD according to DSM-5 criteria. The study will also include participants without an ADHD diagnosis and with no family history of the disorder. This control group will be recruited at IATA-CSIC, outside the hospital setting, and matched by age and sex with the ADHD participants.

Both ADHD participants and individuals without an ADHD diagnosis or family history will be divided into two groups:

- A control group for the disorder that will follow an unmodified diet (ADHD non-intervention group).

- An intervention group (ADHD fasting group) in which the feeding window will be modified to align with circadian rhythms.

Each study group will include 45 participants of both sexes. Participant allocation to the different groups (non-intervention and fasting) will first be stratified by sex (male/female), followed by block randomization within each sex. This type of randomization will ensure periodic balance in the number of participants assigned to each intervention group and equal sex representation across groups.

Participants will be recruited from the Psychiatry Department at Hospital Doctor Peset (Valencia) under the supervision of Head of Service Dr. José Martínez Raga. The intervention period will last three months, during which follow-up assessments will be conducted, including the collection of biological samples (saliva, urine, feces, and blood) at the beginning and end of the study. Participants will also complete the specified questionnaires to obtain the necessary data at three time points: baseline (start of the study), mid-intervention (six weeks), and post-intervention (end of the study).

All individuals included in the study will participate voluntarily and sign an informed consent form prior to participation.

5.3. STUDY SITE

Psychiatry Department, Hospital Doctor Peset, Valencia (Spain).

5.4. SELECTION CRITERIA

5.4.1. Inclusion Criteria

- Adults of both sexes with a DSM-5 diagnosis of ADHD, with or without comorbid depression and/or anxiety.
- Participants must have maintained a normal, stable diet without major changes during the past month.
- They must have been on a stable pharmacological treatment for at least the last three months.
- At study entry, participants with ADHD must have a habitual food intake window longer than 14 hours, including late-night eating (after 9:00 p.m.).
- Participants must present regular intestinal transit and, in the case of women, a regular menstrual cycle.

5.4.2. Exclusion Criteria

Participants who do not meet the inclusion criteria described above will be excluded. Additional exclusion criteria include:

- Treatment with antibiotics and/or prebiotic, probiotic, or symbiotic supplements during the previous month.
- Presence of intestinal diseases during that period.
- Initiation of a new medication during the past three months that could affect circadian rhythms and/or metabolism.
- Diagnosis of any type of eating disorder (ED) or risk of developing one due to risky eating behaviors.

Before entering the study, individuals expressing interest in participation will complete the **Risky Eating Behavior Questionnaire (CAR)** (Annex I), consisting of 10 items. Those scoring **greater than 6** on the CAR questionnaire—indicating a risk of developing an eating disorder—will be excluded.

The CAR questionnaire includes four response options: *never or almost never*, *sometimes*, *frequently* (twice a week), and *very frequently* (more than twice a week), scored from 0 to 3. Higher scores indicate greater eating behavior abnormalities. Participants are classified according to their CAR scores into three levels:

1. **No risk** (0–6 points)
2. **Moderate risk** (7–10 points)
3. **High risk** (>10 points)

Throughout the study, a **weekly phone call** will be made to each participant to evaluate the risk of developing an eating disorder using the CAR questionnaire. If at any point a participant's CAR score exceeds **6**, they will be instructed to **discontinue the fasting recommendations** and withdraw from the intervention phase of the study.

5.5. VARIABLES

5.5.1. RESPONSE VARIABLES

The following parameters will be evaluated:

1. **Indicators of Cognitive, Behavioral, and Emotional Status**
 - **Cognitive and behavioral alterations** associated with ADHD will be assessed using the **Conners' Adult ADHD Rating Scale**, the **ADHD Rating Scale-IV (18 items)**, and the **Clinical Global Impression (CGI) Scale**.
 - **Impulsive behavior** will be evaluated through analysis of the three main dimensions of impulsivity:
 - *Cognitive impulsivity* (lack of task focus),
 - *Motor impulsivity* (acting without thinking), and
 - *Non-planning impulsivity* (present-oriented rather than future-oriented behavior),using the **Barratt Impulsiveness Scale (BIS-11)** self-report instrument.
 - **Subjective changes in sleep quality** will be assessed through variations in the **Pittsburgh Sleep Quality Index (PSQI)** and through self-reported data obtained from an **actigraphy wristband** worn by each participant to record sleep–wake cycle parameters, efficiency, and quality.
 - **Changes in quality of life** will be evaluated using the **AAQoL (Adult ADHD Quality of Life)** questionnaire (29 items).
 - **Changes in stress, depression, and anxiety symptoms** will be assessed using the **Perceived Stress Scale (PSS)**, the **Beck Depression Inventory (BDI-II)**, and the **Beck Anxiety Inventory (BAI)**, respectively.
 - **Chronotype** will be determined using the **Munich Chronotype Questionnaire (MCTQ)** to identify diurnal preferences.
 - **Clinical evaluation of biological rhythm alterations / global circadian health** will be conducted using the **Biological Rhythms Interview of Assessment in**

Neuropsychiatry (BRIAN), consisting of 18 items assessing five major domains of daily functioning.

- **Lifestyle** will be assessed multidimensionally across seven major domains (diet and nutrition, substance use, physical activity, stress management, restorative sleep, social support, and environmental exposures) using the **SMILE self-administered questionnaire** (43 items).

5.6. TIMELINE

- The recruitment period for participants in the intervention study is estimated to last approximately 18–24 months, until voluntary participation reaches 170 subjects. This estimate accounts for potential study dropouts and incomplete data provided by participants during the study, ensuring a final participation of 60 subjects diagnosed with ADHD (30 per group) and 60 controls (subjects without ADHD or a family history of the disorder). Considering this, the initial number of participants recruited per group will be 45 for the ADHD groups and 40 for the control groups (a lower dropout rate is expected among the control participants).
- First, all individuals will be informed in detail about the study (they will be given a participant information sheet included in Annex XVI), as well as about the questionnaires to be completed, the clinical examination, and the procedures for biological sample collection required for subsequent analysis. Based on this information, individuals will decide whether they wish to participate voluntarily, understanding that they may withdraw from the study at any time without penalty. If they agree to participate, they will be asked to sign the informed consent form (Annex XVII) and to complete the Risky Eating Behavior Questionnaire (CAR, Annex I), which will determine their eligibility for inclusion in the study. Participants who do not obtain a CAR score greater than 6 will be included in the study.
- During the first visit, participants will be asked to collect samples of urine, saliva, and feces. They will receive detailed written and verbal instructions on how to properly collect these samples, along with all necessary materials. The samples will be delivered at the second scheduled visit. Participants will also be instructed on how to complete the questionnaires as thoroughly as possible, and a personal interview will be conducted using a general questionnaire to record sociocultural, sociodemographic, anthropometric, bowel habit, family history, and health risk factor information (Annex II).
- In addition, qualified laboratory personnel from the Psychiatry Unit at Hospital Doctor Peset (Valencia) will perform a blood extraction (5 mL) to obtain serum. This blood sample will be collected from each participant both at the beginning and at the end of the study.
- Participants will also be provided with the following: the 72-hour dietary record questionnaire, the food frequency questionnaire, the eating behavior and dietary habits questionnaire, the Mediterranean diet adherence questionnaire, the International Physical Activity Questionnaire (IPAQ), the SMILE lifestyle questionnaire, and the sleep habits and quality questionnaire. Furthermore, they will complete the Munich Chronotype Questionnaire, which will assess chronotype and the possible presence of social jet lag (i.e., different sleep schedules between weekdays and weekends) associated with metabolic and neuropsychiatric risks. They will also receive the BDI-II and BAI inventories and the BRIAN scale. Instructions on how to complete all questionnaires and scales

will be provided, and participants will be informed that these must be submitted during the second visit.

- During the second visit, in addition to collecting the questionnaires and the samples of feces, saliva, urine, and blood, the clinical investigator will assess the participant's social, behavioral, and cognitive performance using the selected clinical scales (the Conners Adult ADHD Rating Scale, the 18-item ADHD Rating Scale-IV, the Clinical Global Impression [CGI] scale, and the Barratt Impulsiveness Scale [BIS-11]).
- At this stage, participants—both those with ADHD and controls—will be provided with a wrist actigraph. They will be instructed to record the actigraph data over four consecutive days following this visit and again during the four days preceding the end of the study (participants will receive a telephone reminder at that time). These records will provide information to the investigator on sleep–wake cycle and activity parameters.
- During the second visit, participants in the intervention groups (ADHD fasting group and Control fasting group) will be instructed that, four days after recording the actigraph data, they must shorten their eating window for the duration of the three-month study to a total of 10 hours, ensuring that the last meal does not occur later than 8:00 p.m. Adherence to this intervention will be monitored weekly for the fasting group, and compliance with the unchanged eating window will be verified for the non-intervention group.
- In all four participant groups (ADHD non-intervention, ADHD fasting, Control non-intervention, and Control fasting), adherence to the Mediterranean diet and absence of eating disorder (ED) risk will be assessed weekly using the PREDIMED and CAR questionnaires via telephone interview. During these calls, participants in the ADHD and Control intervention groups with CAR scores below 6 will be encouraged to maintain adherence to the time-restricted eating recommendation and circadian alignment. As previously indicated, if a participant's CAR score exceeds 6 (indicating risk of developing an ED), they will be instructed to discontinue participation in the study.
- A mid-study telephone assessment (approximately six weeks after study initiation) will be conducted to identify potential changes in dietary habits using the 72-hour dietary record, the food frequency questionnaire, the eating behavior and dietary habits questionnaire, and the PREDIMED questionnaire, as well as changes in physical activity, chronotype, perceived stress, and quality of life through the IPAQ, Munich, SSP, and ADHDQoL questionnaires.
- At the end of the study, follow-up assessments will include new biological sample collection and administration of the following questionnaires: PREDIMED, APALQ, SMILE, perceived stress, chronotype, BDI-II, BAI, ADHDQoL, and the 72-hour dietary record, as well as evaluation of sleep habits. Finally, at the end of the study, the clinician will once again assess cognitive and behavioral alterations related to ADHD using the same scales administered at baseline (Conners Adult ADHD Rating Scale, 18-item ADHD Rating Scale-IV, CGI scale, and BIS-11 scale).
- After the collection of all biological samples at each study time point (saliva, feces, urine, and blood), all proposed analyses will be performed, and the results will be correlated with the data obtained from questionnaires and with baseline and final clinical evaluations to determine possible associations between time-restricted feeding, observed clinical and quality-of-life improvements, and changes in microbiota composition—both intestinal and salivary.

- Upon completion of the study, fasting recommendations will cease for the intervention group. However, four weeks after the end of the study, all participants will be asked to complete once again all questionnaires administered at the beginning and end of the study, to evaluate any lifestyle changes that persist one month after completion—particularly those related to the adoption of new eating habits in the intervention group.

5.7. STATISTICAL ANALYSIS

The analysis of the sequences of the amplified variable regions of the 16S rRNA gene will be carried out using the publicly accessible QIIME software, which will allow us to identify and quantify the different taxonomic groups present in the microbial community. Specifically, the sequences obtained through this program will be clustered according to their similarity into different Operational Taxonomic Units (OTUs) and annotated at various taxonomic levels by comparison with different public reference databases.

The proportion of the different species or taxonomic groups identified will be compared among the study groups using the LDA algorithm or the Linear Discriminant Analysis Effect Size (LEfSe) algorithm implemented in the bioinformatics tool Lefse (<https://huttenhower.sph.harvard.edu/galaxy>).

In addition, by using the previously described JTK algorithm, we will determine and compare the diurnal oscillations of the microbiota present in the saliva of each ADHD participant both the day before the start of the study and at the end of the intervention. Likewise, the microbiota oscillations observed in the ADHD group will be compared with those observed in the control group (participants without an ADHD diagnosis matched to the ADHD group by age and sex).

Diet assessment and evaluation will be conducted using the DietPro software based on a 72-hour dietary record. Using this software, we will calculate the mean and standard deviation of daily energy intake (Kcal), protein (g), carbohydrates (g), saturated fats (g), unsaturated fats (g), cholesterol (mg), and fiber (g). Subsequently, the means of the different groups for all these quantitative variables, as well as for the remaining clinical and sociodemographic quantitative variables, will be compared using the Student's t-test or the Mann-Whitney U test when normal distribution assumptions are not met. Other qualitative variables will be compared among groups using the Chi-square test or Fisher's exact test.

Significant changes between pre- and post-intervention variable values will be determined using the parametric paired Student's t-test (if both continuous variables follow a normal distribution) or the Wilcoxon test (if the normality assumption is not met).

Participants' adherence to the Mediterranean diet will be determined through the analysis and interpretation of results from the MeDiet-PREDIMED questionnaire (14 items), validated in the PREDIMED (Prevención con Dieta Mediterránea) study—a well-known clinical trial focused on the primary prevention of chronic diseases through the Mediterranean diet. Based on the questionnaire validation study and participants' scores, participants will be divided into four categories: high adherence to the Mediterranean diet (12–14 points); medium adherence (8–11.99 points); and low adherence (0–7.99 points).

BMI will be determined and analyzed using the World Health Organization's age- and sex-specific thresholds, establishing four categories: underweight (BMI <18.5 kg/m²); normal weight (BMI = 18.5–24.9 kg/m²); overweight (BMI = 25.0–29.9 kg/m²); and obesity (BMI ≥ 30.0 kg/m²). Additionally, subjects will be grouped into two categories: those with excess weight (overweight + obesity) and those without (normal weight + underweight).

The frequency (days), duration (minutes per day), and intensity (moderate, vigorous, walking) of physical activity will be evaluated using the results from the IPAQ questionnaire. Physical activity will be described in terms of METs, a physiological measure representing the metabolic cost of everyday activities. MET-minutes per week are obtained by multiplying the number of METs by the minutes of activity per day and by the number of days per week (MET × min/day × days/week).

Physical activity levels will be categorized as high, moderate, or low, according to the intensity of aerobic physical activity, classified as follows: light (1.1–2.9 METs), moderate (3–5.9 METs), and vigorous (≥6 METs). Accordingly:

- **Low level:** no reported physical activity or insufficient activity to meet moderate or high criteria.
- **Moderate level:** meeting any of the following three criteria: (1) ≥3 days of vigorous activity ≥20 min/day; (2) ≥5 days of moderate/vigorous activity or walking ≥30 min/day; or (3) ≥5 days of any combination of walking, moderate, or vigorous activity totaling ≥600 MET-min/week.
- **High level:** meeting any of the following two criteria: (1) vigorous activity ≥3 days/week totaling ≥1,500 MET-min/week; or (2) ≥7 days of any combination of walking, moderate, or vigorous activity totaling ≥3,000 MET-min/week.

The degree of correlation between variables will be determined using Pearson's correlation coefficient. All statistical analyses will be conducted using SPSS version 17.0 for Windows (SPSS Inc., 2006) and the publicly available software R (<http://www.r-project.org>). Results will be considered statistically significant for *p* values < 0.05, accounting for multiple comparisons. The MaAsLin software will be used to adjust results for possible modifying and/or confounding effects of variables differing between study groups.

5.8. SAMPLE SIZE CALCULATION

This is a pilot study including a minimum of 30 subjects per experimental group. To date, no published or known study has evaluated the potential effect of modifying the feeding window on ADHD; therefore, it is not possible to estimate *a priori* the minimum sample size required to observe the potential effect of the evaluated intervention.

Indeed, data obtained from our study may serve as a basis for future power and sample size calculations. Moreover, this pilot study, by evaluating individual factors associated with better adherence to the intervention, will provide insights into factors to consider for properly implementing feeding window modulation in individuals with ADHD.

Considering our goal of including 30 participants per group and accounting for potential dropouts due to non-adherence or incomplete data, the initial recruitment target will be

45 participants per ADHD group and 40 per control group (as a lower dropout rate is expected in controls).

6. APPLICABILITY

ADHD symptoms are diverse and adversely affect various areas of social, professional, and family life, leading to significant academic or occupational dysfunction and social adaptation difficulties, with serious risks of social exclusion. ADHD is also associated with multiple comorbidities that negatively impact overall health. Affected individuals often experience higher levels of stress and anxiety, reduced social life, increased separation rates, feelings of loneliness and abandonment, and frequent depressive symptoms.

The most effective treatment approach currently combines psychological therapy and pharmacological treatment (stimulants such as methylphenidate and lisdexamfetamine, and non-stimulants such as atomoxetine and guanfacine). However, treatment outcomes are not always satisfactory and are often associated with side effects.

Therefore, this project aims to identify new intervention strategies based on modulating the feeding window to help improve ADHD-related symptoms and elucidate the precise mechanisms through which shortening and aligning the feeding window with circadian rhythms may exert beneficial effects in individuals with ADHD—particularly those with circadian health alterations.

Understanding these mechanisms could, in the future, allow us to reproduce their beneficial effects through more specific and targeted interventions without strict adherence to complex lifestyle modifications. Confirmation of the beneficial effect of feeding window modulation on ADHD-related alterations and the role of gut microbiota in mediating these effects could enable the design of new, microbiota-based interventions using specific probiotics, prebiotics, or synbiotics to be evaluated in future studies.

Finally, if our working hypothesis is confirmed, the project's results may support the use of microbiota modulation strategies as adjunctive interventions for improving ADHD in patients refractory to conventional therapies, potentially reducing pharmacological treatment reliance.

7. ETHICAL ASPECTS

7.1. APPROVAL BY ETHICS COMMITTEES

This study will comply with the ethical principles and standards originating from the current revised version (Seoul, 2008) of the Declaration of Helsinki adopted by the World Medical Association, the Oviedo Convention, and current Spanish regulations (Law 14/2007 on Biomedical Research and RD 1716/2011).

The study will be conducted in accordance with this protocol, after approval by the Clinical Research Ethics Committee (CEIC), and after obtaining informed consent from each participant. The rights, safety, and well-being of the study participants will take precedence over scientific and societal interests.

Study personnel will be qualified by education, training, and experience to perform their assigned tasks.

Each participant will provide informed consent. The participant information sheet/informed consent form will describe the study's nature, objectives, methods, expected benefits, potential risks, and possible discomforts in plain language. The consent form must be signed and dated prior to participant inclusion, and a copy will be kept in the participant's medical records.

7.2. SUMMARY OF ETHICAL APPROVAL REFERENCES

7.2.1. Ethics Committee – Spanish National Research Council (CSIC)

Committee: CSIC Ethics Committee

Institution: Spanish National Research Council (CSIC), Madrid, Spain

Reference Code: PID2022-142373OA-I00

Internal Code: 001/2024

Principal Investigators:

- María Carmen Cenit Laguna

- Marta Arroyo Calatayud

Committee President: Francisco José (Txetxu) Ausín Díez

Date of Approval: March 12, 2024

Evaluation Outcome: Favorable

7.2.1. Clinical Research Ethics Committee with Medicines

Committee: Clinical Research Ethics Committee with Medicines (CEIm)

Institution: Hospital Universitario Doctor Peset, Valencia, Spain

CEIm Code: 133/23

Principal Investigators:

- María Carmen Cenit Laguna

- Marta Arroyo Calatayud

- José Martínez Raga

Committee President: Dr. José Luis Rodrigo Pérez

Date of Approval: December 15, 2023

Evaluation Outcome: Favorable

7.2. DATA CONFIDENTIALITY

The processing, communication, and transfer of participants' personal data will comply with Spanish Organic Law 3/2018 (December 5) on Personal Data Protection and the EU Regulation 2016/679 (GDPR).

Both the Center and the Sponsor are respectively responsible for data processing and undertake to comply with applicable data protection regulations.

The Investigator and Sponsor will retain collected study data for at least five years after study completion. Afterwards, personal data will only be retained by the Center for healthcare purposes and by the Sponsor for further scientific research, when permitted by law and ethical requirements.

If coded data are transferred outside the European Union, participant data will be protected through safeguards such as contracts or other mechanisms approved by data protection authorities.

In accordance with the purposes of public health and biomedical research, and as stated in section 2.d) of the seventeenth additional provision of Organic Law 3/2018, personal data will be pseudonymized. To this end:

1. A technical and functional separation will be established between the research team and those responsible for pseudonymization and reidentification key storage.
2. Pseudonymized data will only be accessible to the research team when:
 - There is an explicit confidentiality commitment and no reidentification activities are performed.
 - Specific security measures are in place to prevent reidentification and unauthorized third-party access.

Reidentification may occur only when pseudonymized data indicate a real and specific danger to the safety or health of an individual or group, or when necessary to ensure adequate healthcare.

To ensure technical and functional separation, two groups will be established: one handling participant identification and pseudonymization, and another analyzing results.

8. PRACTICAL CONSIDERATIONS

8.1. CONFLICT OF INTEREST

The study will be publicly funded, and none of the participating researchers have any conflicts of interest regarding the proposed topic.

9. BUDGET

Drs. María Carmen Cenit Laguna and Marta Calatayud, senior scientists at IATA-CSIC, have secured funding for this study through the project PID2022-142373OA-I00 titled *DEVELOPING MICROBIOME AND DIETARY STRATEGIES BASED ON CHRONO-NUTRITION FOR TARGETING ADHD*, funded by the Spanish Ministry of Science and Innovation under the 2021–2023 National Research, Development, and Innovation Program (final resolution attached).

11. STUDY PERSONNEL

Members of the research team associated with project PID2022-142373OA-I00 belong to IATA-CSIC (Marta Arroyo Calatayud), EEZ-CSIC (María Carmen Cenit Laguna) and Doctor Peset University Hospital of Valencia (Pablo Cervera, Nuria López, and Alberto Real Dasi), under the supervision of José Martínez Raga, Head of the Psychiatry Department.

12. ALTERNATIVE METHODS CONSIDERED

There are no alternative techniques to the proposed procedures capable of achieving the study's objectives.

The evaluation of the impact of circadian fasting in a genetic animal model of ADHD could be considered a complementary, though not alternative, approach. However, due to high inter-individual variability in microbiota composition and lifestyle factors among humans, such an animal model would not allow assessment of lifestyle, anthropometric, or immunometabolic variables associated with differential responses to circadian fasting or their relationship with baseline microbiota profiles.

THERAPEUTIC EFFECT OF CIRCADIAN FASTING FOR THE IMPROVEMENT OF ATTENTION DEFICIT AND/OR HYPERACTIVITY DISORDER (ADHD) IN ADULTS AND THE ROLE OF THE INTESTINAL MICROBIOTA AS A MEDIATOR AND/OR MODULATOR OF THE EFFECT

Principal Investigators: Marta Arroyo Calatayud and María Carmen Cenit Laguna
Sponsor: Agencia Estatal de Investigación (AEI), Spanish National Research Council (Consejo Superior de Investigaciones Científicas, CSIC)

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1. INTRODUCTION

Attention Deficit Hyperactivity Disorder (ADHD) is the most prevalent neurodevelopmental and neuropsychiatric disorder in childhood (Polanczyk, G. et al., 2007). Although this disorder is primarily diagnosed in childhood, it is estimated that symptoms persist into adulthood in approximately 40% of cases (Childress, A.C. & Sallee, F.R., 2014). This disorder, which carries a major economic, social, and healthcare burden, is characterized by significant cognitive and behavioral difficulties that negatively impact an individual's cognitive and/or behavioral development and, consequently, their academic, occupational, and/or social life (Garcia, C.R. et al., 2012).

Moreover, ADHD is frequently associated with other mental disorders (Agarwal, R. et al., 2022), such as anxiety, depression, sleep disorders (Valsecchi, P. et al., 2022), and obsessive-compulsive disorders, as well as common metabolic disorders including obesity and type II diabetes (Cortese, S. et al., 2016), all of which strongly affect patients' quality of life.

Currently, ADHD is considered a complex neurobiological disorder of multifactorial origin (Cenit M.C. et al., 2017). Despite recent advances in understanding its etiology, the pathogenic processes leading to its development and manifestation remain largely unknown. Additionally, ADHD is clinically very heterogeneous in terms of symptomatology, therapeutic response (about 30–40% of children treated with psychotropic drugs do not respond to treatment), and psychiatric comorbidities (Dias, T.G.C. et al., 2013).

It is important to note that the factors responsible for this clinical heterogeneity have not yet been elucidated, though identifying them could help define subgroups of patients who may benefit from different intervention strategies (personalized medicine/nutrition) (Ana Larroya et al., 2021). Specifically, scientific evidence has shown that this disorder is associated with alterations in specific brain regions—such as the prefrontal cortex—leading to imbalances in neurotransmitters like dopamine, GABA, norepinephrine, and serotonin, which regulate key cognitive and behavioral functions such as attention and impulsivity (Cenit M.C. et al., 2017; Del Campo, N. et al., 2011).

ADHD diagnosis is still based on clinical symptoms, as no biomarkers currently exist to support diagnosis or predict prognosis. Furthermore, current therapeutic efficacy is limited (approximately 30–40% non-response to psychopharmacological treatment) (Dias, T.G.C. et al., 2013), and such treatments may cause significant side effects (Martinez-Raga et al., 2013). Therefore, there is a clear need for new strategies to elucidate ADHD mechanisms, identify biomarkers to aid diagnosis, and improve clinical management of this challenging disorder.

ADHD is currently understood as a multifactorial disorder that develops in genetically predisposed individuals upon exposure to certain environmental factors. Many of these factors—particularly dietary components or patterns—have been shown to affect gut microbiota development and composition (Cenit M.C. et al., 2017). Factors such as mode of delivery, maternal health during pregnancy, and early infections are known to modulate the gut microbiota and may influence ADHD risk.

Dietary patterns are a key determinant of gut microbiota composition and may modulate ADHD symptomatology. Certain dietary components, such as preservatives and food additives like colorants, have been associated with worsening ADHD symptoms (Traxel W.L. et al., 1982), while others—such as omega-3 fatty acids and specific polyphenols—may improve symptoms, at least in some groups of ADHD patients. Several studies have also found low levels of copper, iron, zinc, magnesium, and omega-3 in children with ADHD (Cenit M.C. et al., 2017; Ana Larroya et al., 2021).

Recent studies have demonstrated the key role of the gut microbiota in brain development, maturation, and function. The so-called **gut-brain axis**, largely regulated by intestinal microbiota, consists of neural, hormonal, immunological, and metabolic pathways mediating bidirectional communication between both organs (Collins et al., 2012). Evidence shows that gut microbiota influence brain circuit development and that imbalances in microbiota composition can lead to immunological and neurological dysregulation, increasing the risk of neurodevelopmental disorders affecting social, cognitive, behavioral, and emotional functions (Ihekweazu & Versalovic, 2018).

In 2013, psychiatrist Ted Dinan (University College Cork) coined the term *psychobiotic*, referring to bacteria that, when ingested in adequate amounts, confer mental health benefits (Dinan T.G. et al., 2013). However, clinical evidence remains limited, and more research is needed to establish

causal relationships between microbiota composition changes and cognitive or behavioral outcomes (Martin & Mayer, 2017).

In recent years, the role of gut microbiota in ADHD risk and manifestation has been strongly suggested (Cenit et al., 2017). Thus, preventive and therapeutic interventions based on microbiome modulation could open new, safer avenues for managing this condition. Indeed, studies have shown microbiota alterations associated with ADHD (Bundgaard-Nielsen, C. et al., 2020), and preclinical research has demonstrated causality, with ADHD-like phenotypes transferable via fecal transplant (Tengeler, A.C. et al., 2020). However, it remains unknown which changes precede disorder onset or which may contribute to prevention.

Nutritional psychiatry is a new field of research that provides scientific evidence on how our dietary habits and the quality of food intake can modulate our mental health (Sarris et al., 2015; Adan R.A. et al., 2019). Studies conducted in this field aim to determine whether certain dietary patterns or different components of the diet, specific foods, or specific components of these foods could help prevent or treat mental health problems. In this regard, both nutritionists and psychiatrists around the world acknowledge that an unhealthy diet can worsen mental health, especially in individuals already suffering from neuropsychiatric diseases or disorders. Conversely, numerous studies have indicated that different dietary patterns or various components of the diet could help prevent or treat mood-related disorders, such as depression, or neurodevelopmental disorders such as attention-deficit/hyperactivity disorder (ADHD) (Adan R.A. et al., 2019; Firth et al., 2020).

Scientific evidence demonstrates that the diets which appear to improve aspects related to mental health are those that prioritize whole or natural foods over ultra-processed foods and generally reduce the inflammatory state (Adan R.A.H. et al., 2019). However, there are still many studies to be carried out in the field of nutritional psychiatry (Loughman et al., 2021). In fact, we do not yet know the exact mechanisms through which certain dietary patterns may influence mental health. Moreover, it is necessary to identify which specific foods and which precise components of these foods exert beneficial effects.

We know that a healthy diet, such as the Mediterranean diet, is capable of benefiting our mental health status, and although the exact mechanisms through which this diet could be influencing improvements in mental health are not fully understood, we know that the gut microbiota, through various mechanisms, could be involved (Marx et al., 2021). In any case, the microbiota appears to be a possible external factor—highly modifiable by lifestyle-related factors such as diet, medication use, and exercise, among the most important—implicated in mental health and in the aforementioned neurobiological or neurodevelopmental pathologies (Pelsser et al., 2011).

A multitude of studies have focused on investigating the importance of diet quality in mental health, but few have yet explored the importance of the timing of food intake and its regularity for mental health. Lastly, although diet appears to play an important role in ADHD and different dietary strategies seem to improve the disorder, at least in certain groups of individuals with the condition (Pelsser, L.M., 2017), the best intervention strategies based on the modulation of diet and the gut microbiota that could assist in the clinical management of ADHD are still unknown (Larroya A. et al., 2021).

In this regard, several recent studies have indicated that not only the quality of the diet appears to impact our mental health, but also the timing at which food is consumed seems to matter for our cognitive, emotional, and/or behavioral state (Tahara, Y. et al., 2021; Currenti, W. et al., 2021; Qian, J. et al., 2022; Feillet, C.A. et al., 2006). **Chrononutrition** is an emerging field of research within nutritional psychiatry, based on the key relationship between temporal eating patterns, circadian rhythms, and health (Franzago, M. et al., 2023).

With respect to the gut microbiota, chrononutrition, and their impact on health, scientific evidence has recently demonstrated that the gut microbiota exhibits diurnal rhythmicity, largely determined by meal timing and the feeding window, and that this circadian rhythmicity of the microbiota substantially regulates the host's circadian rhythms (Wang, H. et al., 2022). Recently, it has also been shown that meal timing in humans affects the diurnal rhythms of the microbial profile present in saliva, which is closely related to the intestinal microbial profile; thus, a late intake of the main meal has been associated with an increase in pro-inflammatory microbial taxa present in saliva (Collado, M.C. et al., 2018).

On the other hand, some studies have reported that circadian disruption may alter fecal microbiota composition in a sex-dependent manner (Liang, X. et al., 2015). In relation to this, diurnal oscillations of the gut microbiota appear to be more pronounced in women than in men (Liang, X. et al., 2015), suggesting the importance of considering sex stratification in studies conducted on microbiota and circadian health. Furthermore, evidence indicates that the microbiome is required for the various sex-specific circadian rhythms and, therefore, essential for sexual development (Weger, B.D. et al., 2019).

Several studies have indicated that circadian health and the diurnal rhythmicity of the gut microbiota—factors that are strongly and bidirectionally interconnected (Voigt, R. et al., 2016)—are key determinants of cognitive status, behavior, and mood (Teichman, E.M. et al., 2020). This established connection between the gut microbiota, circadian rhythms, and mental health supports the suggested idea that modulation of the gut microbiota could help prevent circadian misalignment and/or assist in the resynchronization of altered circadian rhythms in psychiatric disorders (Codoñer-Franch, P. et al., 2023).

Alterations in circadian rhythms, such as irregular eating patterns and sleep disturbances, are commonly observed among individuals with psychiatric disorders (Codoñer-Franch, P. et al., 2023). In fact, a common hypothesis regarding the recognized increase in the incidence of mental illnesses lies in current lifestyle factors, such as unhealthy dietary patterns, inadequate physical activity and sleep habits, and the “stressful” environment associated with modern life in Western societies (Rajaratnam, S.M. et al., 2001; Logan et al., 2015a, 2015b). In this sense, the circadian rhythm misalignment associated with modern lifestyle has been suggested to play a significant role in the worsening of the population's mental health. Conversely, a favorable lifestyle associated with proper circadian health has been linked to a substantial reduction in the risk of onset and better prognosis of mental illnesses (Codoñer-Franch, P. et al., 2023; Ng, C.M. et al., 2022).

Regarding ADHD, several studies have provided biological and clinical data that have strongly linked the disorder to various alterations in circadian rhythms (Coogan, A.N. et al., 2017). Reductions in sleep quality, delays in circadian phase, as well as an evening chronotype, are consistently associated with ADHD in both children and adults (Coogan, A.N. et al., 2017; Van Veen, M.M. et al., 2010), and these alterations, in turn, have been suggested to correlate with the severity of ADHD symptomatology. A recently published study described specific changes in the gut microbiota of children with autism comorbid with sleep disorders, as well as correlations between these gut microbiota changes and melatonin and serotonin levels (Xueying Hua et al., 2020). It is highly likely that alterations also exist in the diurnal oscillations of the gut microbiota associated with these sleep disturbances linked to neurodevelopmental disorders. If these alterations in gut microbiota rhythmicity underlying sleep disturbances were to be fully elucidated, such disturbances could potentially be reduced through gut microbiota modulation. However, further studies are needed to determine the best microbiome-targeted strategies for improving sleep.

Furthermore, the evening chronotype, associated with circadian misalignment, is considered a risk factor for severe psychiatric symptoms and psychiatric metabolic comorbidities. In this regard, patients with ADHD have a high prevalence of obesity, and it has been proposed that the

evening chronotype—an expression of circadian misalignment linked to ADHD and associated with unhealthy eating habits and obesity—could contribute as an important mechanism underlying the strong connection between ADHD and the obese phenotype (Türkoğlu, S. et al., 2019). On the other hand, the high efficacy of chronotherapeutic approaches in improving ADHD (Van Andel, E. et al., 2021) suggests the circadian system as an interesting therapeutic target to address in ADHD management.

Taking all of the above into account, the evidence indicates that resynchronization of circadian rhythms through basic lifestyle changes related to diet could improve ADHD-related symptomatology or even prevent the onset of the disorder in susceptible individuals. Moreover, data support the idea that resynchronization of circadian rhythms could also reduce the risk of developing metabolic disorders that are highly prevalent among individuals with ADHD and are strongly associated with increased severity and poorer quality of life in patients with the disorder (Codoñer-Franch, P. et al., 2023).

Circadian rhythms are regulated by the interaction between genetic and environmental factors. Among the environmental factors, lifestyle-related elements such as diet—strongly linked to the composition of the gut microbiota—have been suggested as major determinants of human circadian health (Wang, H. et al., 2022). Additionally, irregular and mistimed eating patterns have recently been described as disruptive factors for mental health (Tahara, Y. et al., 2021; Currenti, W. et al., 2021; Qian, J. et al., 2022). In this sense, conversely, scientific evidence suggests that the circadian temporal regulation of food intake represents a promising chronotherapeutic strategy to prevent and/or assist in the treatment of mental illnesses, largely through modulation of the gut microbiota (Codoñer-Franch, P. et al., 2023).

2. JUSTIFICATION OF THE STUDY

Despite the extensive scientific evidence suggesting possible alterations in the circadian rhythmicity of the gut microbiota associated with ADHD, as well as the potential beneficial effect of circadian fasting for improving the disorder, we currently lack human intervention studies that have evaluated possible alterations in the diurnal oscillations of the gut microbiota and the therapeutic effect of circadian temporal regulation of food intake as a potential chronotherapeutic strategy for improving its clinical management.

Confirmation of the beneficial effect of regular eating aligned with circadian rhythms (circadian fasting) on ADHD-related alterations, and of the role of gut microbiota changes mediating the intervention effect, would allow the design of new intervention strategies based on more specific microbiota modulation. Furthermore, should modulatory factors of the intervention response be identified, such strategies could be directed toward specific subgroups of individuals with ADHD (precision nutrition) to be evaluated in future studies.

Therefore, if our working hypothesis is confirmed, the results of this project could, in the near future, enable the use of new intervention strategies that may improve ADHD in patients refractory to conventional therapies, as well as reduce the use of pharmacological treatments for ADHD management by proposing adjunctive therapeutic approaches.

3. HYPOTHESIS

This project is based on the hypothesis that the gut microbiota exhibits diurnal oscillations in its composition and function, which are highly modifiable by food intake timing and strongly impact the host's circadian health and brain function. Evidence suggests that, due to irregular and mistimed eating patterns linked to the disorder, these oscillations may be altered in patients with ADHD, and that the regular shortening of the eating window and its alignment with circadian rhythms could help improve ADHD through the positive modulation of the gut microbiota's

diurnal rhythmicity, at least in certain patients, such as those with alterations in circadian health or individuals with immunometabolic disturbances.

Thus, the modulation of the gut microbiota through changes in the eating window, promoting a healthy lifestyle via regular temporal restriction of food intake aligned with circadian rhythms, could represent a strategy—at least for specific patient subgroups—for improving the cognitive, behavioral, and emotional alterations associated with ADHD.

4. OBJECTIVES

Primary

7. To evaluate the feasibility of shortening the eating window and aligning it with circadian rhythms (circadian fasting) in adults with ADHD and circadian rhythm alterations (patients with sleep disturbances and a habitual eating window longer than 14 hours, with their last food intake occurring after 8:00 p.m.).
8. To evaluate the therapeutic potential of circadian fasting on cognitive and behavioral alterations in adults with ADHD.
9. To identify the beneficial effects of circadian fasting on sleep disturbances and quality of life in patients with ADHD.
10. To evaluate possible alterations in the circadian rhythms of the salivary microbiota associated with ADHD.
11. To evaluate the microbiota changes associated with circadian fasting in the context of ADHD.
12. To determine possible correlations between microbiota changes associated with circadian fasting and the beneficial effects of circadian fasting observed in patients with ADHD.

Secondary

11. To evaluate the influence of baseline microbiota profiles on the effects of circadian fasting.
12. To analyze lifestyle factors and/or anthropometric or immunometabolic variables related to better or worse response to circadian fasting intervention, and the possible association of these factors with baseline microbiota profiles.
13. To analyze whether participants' lifestyle—at a multidimensional level or within specific lifestyle domains—may influence the degree of adherence to the circadian fasting protocol and/or its effect on ADHD improvement and/or participants' quality of life.
14. To identify possible microbiota profiles associated with difficulty in adhering to the circadian fasting intervention.

5. MATERIALS AND METHODS

5.1. DESIGN

Longitudinal 12-week intervention study. Factorial 2x2 protocol, including non-ADHD and ADHD adult populations and non-fasting and fasting intervention.

5.2. POPULATION

The study will include adult participants (>18 years of age) diagnosed with ADHD according to DSM-5 criteria. The study will also include participants without an ADHD diagnosis and with no family history of the disorder. This control group will be recruited at IATA-CSIC, outside the hospital setting, and matched by age and sex with the ADHD participants.

Both ADHD participants and individuals without an ADHD diagnosis or family history will be divided into two groups:

- A control group for the disorder that will follow an unmodified diet (ADHD non-intervention group).
- An intervention group (ADHD fasting group) in which the feeding window will be modified to align with circadian rhythms.

Each study group will include 45 participants of both sexes. Participant allocation to the different groups (non-intervention and fasting) will first be stratified by sex (male/female), followed by block randomization within each sex. This type of randomization will ensure periodic balance in the number of participants assigned to each intervention group and equal sex representation across groups.

Participants will be recruited from the Psychiatry Department at Hospital Doctor Peset (Valencia) under the supervision of Head of Service Dr. José Martínez Raga. The intervention period will last three months, during which follow-up assessments will be conducted, including the collection of biological samples (saliva, urine, feces, and blood) at the beginning and end of the study. Participants will also complete the specified questionnaires to obtain the necessary data at three time points: baseline (start of the study), mid-intervention (six weeks), and post-intervention (end of the study).

All individuals included in the study will participate voluntarily and sign an informed consent form prior to participation.

5.3. STUDY SITE

Psychiatry Department, Hospital Doctor Peset, Valencia (Spain).

5.4. SELECTION CRITERIA

5.4.1. Inclusion Criteria

- Adults of both sexes with a DSM-5 diagnosis of ADHD, with or without comorbid depression and/or anxiety.
- Participants must have maintained a normal, stable diet without major changes during the past month.
- They must have been on a stable pharmacological treatment for at least the last three months.
- At study entry, participants with ADHD must have a habitual food intake window longer than 14 hours, including late-night eating (after 9:00 p.m.).
- Participants must present regular intestinal transit and, in the case of women, a regular menstrual cycle.

5.4.2. Exclusion Criteria

Participants who do not meet the inclusion criteria described above will be excluded. Additional exclusion criteria include:

- Treatment with antibiotics and/or prebiotic, probiotic, or symbiotic supplements during the previous month.
- Presence of intestinal diseases during that period.
- Initiation of a new medication during the past three months that could affect circadian rhythms and/or metabolism.
- Diagnosis of any type of eating disorder (ED) or risk of developing one due to risky eating behaviors.

Before entering the study, individuals expressing interest in participation will complete the **Risky Eating Behavior Questionnaire (CAR)** (Annex I), consisting of 10 items. Those scoring **greater than 6** on the CAR questionnaire—indicating a risk of developing an eating disorder—will be excluded.

The CAR questionnaire includes four response options: *never or almost never*, *sometimes*, *frequently* (twice a week), and *very frequently* (more than twice a week), scored from 0 to 3. Higher scores indicate greater eating behavior abnormalities. Participants are classified according to their CAR scores into three levels:

4. **No risk** (0–6 points)
5. **Moderate risk** (7–10 points)
6. **High risk** (>10 points)

Throughout the study, a **weekly phone call** will be made to each participant to evaluate the risk of developing an eating disorder using the CAR questionnaire. If at any point a participant's CAR score exceeds **6**, they will be instructed to **discontinue the fasting recommendations** and withdraw from the intervention phase of the study.

5.5. VARIABLES

5.5.1. RESPONSE VARIABLES

The following parameters will be evaluated:

2. Indicators of Cognitive, Behavioral, and Emotional Status

- **Cognitive and behavioral alterations** associated with ADHD will be assessed using the **Conners' Adult ADHD Rating Scale**, the **ADHD Rating Scale-IV (18 items)**, and the **Clinical Global Impression (CGI) Scale**.
- **Impulsive behavior** will be evaluated through analysis of the three main dimensions of impulsivity:
 - *Cognitive impulsivity* (lack of task focus),
 - *Motor impulsivity* (acting without thinking), and
 - *Non-planning impulsivity* (present-oriented rather than future-oriented behavior), using the **Barratt Impulsiveness Scale (BIS-11)** self-report instrument.
- **Subjective changes in sleep quality** will be assessed through variations in the **Pittsburgh Sleep Quality Index (PSQI)** and through self-reported data obtained from an **actigraphy wristband** worn by each participant to record sleep–wake cycle parameters, efficiency, and quality.
- **Changes in quality of life** will be evaluated using the **AAQoL (Adult ADHD Quality of Life)** questionnaire (29 items).
- **Changes in stress, depression, and anxiety symptoms** will be assessed using the **Perceived Stress Scale (PSS)**, the **Beck Depression Inventory (BDI-II)**, and the **Beck Anxiety Inventory (BAI)**, respectively.
- **Chronotype** will be determined using the **Munich Chronotype Questionnaire (MCTQ)** to identify diurnal preferences.

- **Clinical evaluation of biological rhythm alterations / global circadian health** will be conducted using the **Biological Rhythms Interview of Assessment in Neuropsychiatry (BRIAN)**, consisting of 18 items assessing five major domains of daily functioning.
- **Lifestyle** will be assessed multidimensionally across seven major domains (diet and nutrition, substance use, physical activity, stress management, restorative sleep, social support, and environmental exposures) using the **SMILE self-administered questionnaire** (43 items).

5.6. TIMELINE

- The recruitment period for participants in the intervention study is estimated to last approximately 18–24 months, until voluntary participation reaches 170 subjects. This estimate accounts for potential study dropouts and incomplete data provided by participants during the study, ensuring a final participation of 60 subjects diagnosed with ADHD (30 per group) and 60 controls (subjects without ADHD or a family history of the disorder). Considering this, the initial number of participants recruited per group will be 45 for the ADHD groups and 40 for the control groups (a lower dropout rate is expected among the control participants).
- First, all individuals will be informed in detail about the study (they will be given a participant information sheet included in Annex XVI), as well as about the questionnaires to be completed, the clinical examination, and the procedures for biological sample collection required for subsequent analysis. Based on this information, individuals will decide whether they wish to participate voluntarily, understanding that they may withdraw from the study at any time without penalty. If they agree to participate, they will be asked to sign the informed consent form (Annex XVII) and to complete the Risky Eating Behavior Questionnaire (CAR, Annex I), which will determine their eligibility for inclusion in the study. Participants who do not obtain a CAR score greater than 6 will be included in the study.
- During the first visit, participants will be asked to collect samples of urine, saliva, and feces. They will receive detailed written and verbal instructions on how to properly collect these samples, along with all necessary materials. The samples will be delivered at the second scheduled visit. Participants will also be instructed on how to complete the questionnaires as thoroughly as possible, and a personal interview will be conducted using a general questionnaire to record sociocultural, sociodemographic, anthropometric, bowel habit, family history, and health risk factor information (Annex II).
- In addition, qualified laboratory personnel from the Psychiatry Unit at Hospital Doctor Peset (Valencia) will perform a blood extraction (5 mL) to obtain serum. This blood sample will be collected from each participant both at the beginning and at the end of the study.
- Participants will also be provided with the following: the 72-hour dietary record questionnaire, the food frequency questionnaire, the eating behavior and dietary habits questionnaire, the Mediterranean diet adherence questionnaire, the International Physical Activity Questionnaire (IPAQ), the SMILE lifestyle questionnaire, and the sleep habits and quality questionnaire. Furthermore, they will complete the Munich Chronotype Questionnaire, which will assess chronotype and the possible presence of social jet lag (i.e., different sleep schedules between weekdays and weekends) associated with metabolic and neuropsychiatric risks. They will also receive the BDI-II and BAI inventories and the BRIAN scale. Instructions on how to complete all questionnaires and scales will be provided, and participants will be informed that these must be submitted during the second visit.
- During the second visit, in addition to collecting the questionnaires and the samples of feces, saliva, urine, and blood, the clinical investigator will assess the participant's social, behavioral, and cognitive performance using the selected clinical scales (the Conners Adult ADHD Rating Scale, the 18-item ADHD Rating Scale-IV, the Clinical Global Impression [CGI] scale, and the Barratt Impulsiveness Scale [BIS-11]).

- At this stage, participants—both those with ADHD and controls—will be provided with a wrist actigraph. They will be instructed to record the actigraph data over four consecutive days following this visit and again during the four days preceding the end of the study (participants will receive a telephone reminder at that time). These records will provide information to the investigator on sleep–wake cycle and activity parameters.
- During the second visit, participants in the intervention groups (ADHD fasting group and Control fasting group) will be instructed that, four days after recording the actigraph data, they must shorten their eating window for the duration of the three-month study to a total of 10 hours, ensuring that the last meal does not occur later than 8:00 p.m. Adherence to this intervention will be monitored weekly for the fasting group, and compliance with the unchanged eating window will be verified for the non-intervention group.
- In all four participant groups (ADHD non-intervention, ADHD fasting, Control non-intervention, and Control fasting), adherence to the Mediterranean diet and absence of eating disorder (ED) risk will be assessed weekly using the PREDIMED and CAR questionnaires via telephone interview. During these calls, participants in the ADHD and Control intervention groups with CAR scores below 6 will be encouraged to maintain adherence to the time-restricted eating recommendation and circadian alignment. As previously indicated, if a participant’s CAR score exceeds 6 (indicating risk of developing an ED), they will be instructed to discontinue participation in the study.
- A mid-study telephone assessment (approximately six weeks after study initiation) will be conducted to identify potential changes in dietary habits using the 72-hour dietary record, the food frequency questionnaire, the eating behavior and dietary habits questionnaire, and the PREDIMED questionnaire, as well as changes in physical activity, chronotype, perceived stress, and quality of life through the IPAQ, Munich, SSP, and ADHDQoL questionnaires.
- At the end of the study, follow-up assessments will include new biological sample collection and administration of the following questionnaires: PREDIMED, APALQ, SMILE, perceived stress, chronotype, BDI-II, BAI, ADHDQoL, and the 72-hour dietary record, as well as evaluation of sleep habits. Finally, at the end of the study, the clinician will once again assess cognitive and behavioral alterations related to ADHD using the same scales administered at baseline (Conners Adult ADHD Rating Scale, 18-item ADHD Rating Scale-IV, CGI scale, and BIS-11 scale).
- After the collection of all biological samples at each study time point (saliva, feces, urine, and blood), all proposed analyses will be performed, and the results will be correlated with the data obtained from questionnaires and with baseline and final clinical evaluations to determine possible associations between time-restricted feeding, observed clinical and quality-of-life improvements, and changes in microbiota composition—both intestinal and salivary.
- Upon completion of the study, fasting recommendations will cease for the intervention group. However, four weeks after the end of the study, all participants will be asked to complete once again all questionnaires administered at the beginning and end of the study, to evaluate any lifestyle changes that persist one month after completion—particularly those related to the adoption of new eating habits in the intervention group.

5.7. STATISTICAL ANALYSIS

The analysis of the sequences of the amplified variable regions of the 16S rRNA gene will be carried out using the publicly accessible QIIME software, which will allow us to identify and quantify the different taxonomic groups present in the microbial community. Specifically, the sequences obtained through this program will be clustered according to their similarity into different Operational Taxonomic Units (OTUs) and annotated at various taxonomic levels by comparison with different public reference databases.

The proportion of the different species or taxonomic groups identified will be compared among the study groups using the LDA algorithm or the Linear Discriminant Analysis Effect Size

(LEfSe) algorithm implemented in the bioinformatics tool Lefse (<https://huttenhower.sph.harvard.edu/galaxy>).

In addition, by using the previously described JTK algorithm, we will determine and compare the diurnal oscillations of the microbiota present in the saliva of each ADHD participant both the day before the start of the study and at the end of the intervention. Likewise, the microbiota oscillations observed in the ADHD group will be compared with those observed in the control group (participants without an ADHD diagnosis matched to the ADHD group by age and sex).

Diet assessment and evaluation will be conducted using the DietPro software based on a 72-hour dietary record. Using this software, we will calculate the mean and standard deviation of daily energy intake (Kcal), protein (g), carbohydrates (g), saturated fats (g), unsaturated fats (g), cholesterol (mg), and fiber (g). Subsequently, the means of the different groups for all these quantitative variables, as well as for the remaining clinical and sociodemographic quantitative variables, will be compared using the Student's t-test or the Mann-Whitney U test when normal distribution assumptions are not met. Other qualitative variables will be compared among groups using the Chi-square test or Fisher's exact test.

Significant changes between pre- and post-intervention variable values will be determined using the parametric paired Student's t-test (if both continuous variables follow a normal distribution) or the Wilcoxon test (if the normality assumption is not met).

Participants' adherence to the Mediterranean diet will be determined through the analysis and interpretation of results from the MeDiet-PREDIMED questionnaire (14 items), validated in the PREDIMED (Prevención con Dieta Mediterránea) study—a well-known clinical trial focused on the primary prevention of chronic diseases through the Mediterranean diet. Based on the questionnaire validation study and participants' scores, participants will be divided into four categories: high adherence to the Mediterranean diet (12–14 points); medium adherence (8–11.99 points); and low adherence (0–7.99 points).

BMI will be determined and analyzed using the World Health Organization's age- and sex-specific thresholds, establishing four categories: underweight (BMI <18.5 kg/m²); normal weight (BMI = 18.5–24.9 kg/m²); overweight (BMI = 25.0–29.9 kg/m²); and obesity (BMI ≥ 30.0 kg/m²). Additionally, subjects will be grouped into two categories: those with excess weight (overweight + obesity) and those without (normal weight + underweight).

The frequency (days), duration (minutes per day), and intensity (moderate, vigorous, walking) of physical activity will be evaluated using the results from the IPAQ questionnaire. Physical activity will be described in terms of METs, a physiological measure representing the metabolic cost of everyday activities. MET-minutes per week are obtained by multiplying the number of METs by the minutes of activity per day and by the number of days per week (MET × min/day × days/week).

Physical activity levels will be categorized as high, moderate, or low, according to the intensity of aerobic physical activity, classified as follows: light (1.1–2.9 METs), moderate (3–5.9 METs), and vigorous (≥6 METs). Accordingly:

- **Low level:** no reported physical activity or insufficient activity to meet moderate or high criteria.
- **Moderate level:** meeting any of the following three criteria: (1) ≥3 days of vigorous activity ≥20 min/day; (2) ≥5 days of moderate/vigorous activity or walking ≥30 min/day; or (3) ≥5 days of any combination of walking, moderate, or vigorous activity totaling ≥600 MET-min/week.

- **High level:** meeting any of the following two criteria: (1) vigorous activity ≥ 3 days/week totaling $\geq 1,500$ MET-min/week; or (2) ≥ 7 days of any combination of walking, moderate, or vigorous activity totaling $\geq 3,000$ MET-min/week.

The degree of correlation between variables will be determined using Pearson's correlation coefficient. All statistical analyses will be conducted using SPSS version 17.0 for Windows (SPSS Inc., 2006) and the publicly available software R (<http://www.r-project.org>). Results will be considered statistically significant for p values < 0.05 , accounting for multiple comparisons. The MaAsLin software will be used to adjust results for possible modifying and/or confounding effects of variables differing between study groups.

5.8. SAMPLE SIZE CALCULATION

This is a pilot study including a minimum of 30 subjects per experimental group. To date, no published or known study has evaluated the potential effect of modifying the feeding window on ADHD; therefore, it is not possible to estimate *a priori* the minimum sample size required to observe the potential effect of the evaluated intervention.

Indeed, data obtained from our study may serve as a basis for future power and sample size calculations. Moreover, this pilot study, by evaluating individual factors associated with better adherence to the intervention, will provide insights into factors to consider for properly implementing feeding window modulation in individuals with ADHD.

Considering our goal of including 30 participants per group and accounting for potential dropouts due to non-adherence or incomplete data, the initial recruitment target will be 45 participants per ADHD group and 40 per control group (as a lower dropout rate is expected in controls).

6. APPLICABILITY

ADHD symptoms are diverse and adversely affect various areas of social, professional, and family life, leading to significant academic or occupational dysfunction and social adaptation difficulties, with serious risks of social exclusion. ADHD is also associated with multiple comorbidities that negatively impact overall health. Affected individuals often experience higher levels of stress and anxiety, reduced social life, increased separation rates, feelings of loneliness and abandonment, and frequent depressive symptoms.

The most effective treatment approach currently combines psychological therapy and pharmacological treatment (stimulants such as methylphenidate and lisdexamfetamine, and non-stimulants such as atomoxetine and guanfacine). However, treatment outcomes are not always satisfactory and are often associated with side effects.

Therefore, this project aims to identify new intervention strategies based on modulating the feeding window to help improve ADHD-related symptoms and elucidate the precise mechanisms through which shortening and aligning the feeding window with circadian rhythms may exert beneficial effects in individuals with ADHD—particularly those with circadian health alterations.

Understanding these mechanisms could, in the future, allow us to reproduce their beneficial effects through more specific and targeted interventions without strict adherence to complex lifestyle modifications. Confirmation of the beneficial effect of feeding window modulation on

ADHD-related alterations and the role of gut microbiota in mediating these effects could enable the design of new, microbiota-based interventions using specific probiotics, prebiotics, or synbiotics to be evaluated in future studies.

Finally, if our working hypothesis is confirmed, the project's results may support the use of microbiota modulation strategies as adjunctive interventions for improving ADHD in patients refractory to conventional therapies, potentially reducing pharmacological treatment reliance.

7. ETHICAL ASPECTS

7.1. APPROVAL BY ETHICS COMMITTEES

This study will comply with the ethical principles and standards originating from the current revised version (Seoul, 2008) of the Declaration of Helsinki adopted by the World Medical Association, the Oviedo Convention, and current Spanish regulations (Law 14/2007 on Biomedical Research and RD 1716/2011).

The study will be conducted in accordance with this protocol, after approval by the Clinical Research Ethics Committee (CEIC), and after obtaining informed consent from each participant. The rights, safety, and well-being of the study participants will take precedence over scientific and societal interests.

Study personnel will be qualified by education, training, and experience to perform their assigned tasks.

Each participant will provide informed consent. The participant information sheet/informed consent form will describe the study's nature, objectives, methods, expected benefits, potential risks, and possible discomforts in plain language. The consent form must be signed and dated prior to participant inclusion, and a copy will be kept in the participant's medical records.

7.2. DATA CONFIDENTIALITY

The processing, communication, and transfer of participants' personal data will comply with Spanish Organic Law 3/2018 (December 5) on Personal Data Protection and the EU Regulation 2016/679 (GDPR).

Both the Center and the Sponsor are respectively responsible for data processing and undertake to comply with applicable data protection regulations.

The Investigator and Sponsor will retain collected study data for at least five years after study completion. Afterwards, personal data will only be retained by the Center for healthcare purposes and by the Sponsor for further scientific research, when permitted by law and ethical requirements.

If coded data are transferred outside the European Union, participant data will be protected through safeguards such as contracts or other mechanisms approved by data protection authorities.

In accordance with the purposes of public health and biomedical research, and as stated in section 2.d) of the seventeenth additional provision of Organic Law 3/2018, personal data will be pseudonymized. To this end:

3. A technical and functional separation will be established between the research team and those responsible for pseudonymization and reidentification key storage.
4. Pseudonymized data will only be accessible to the research team when:

- There is an explicit confidentiality commitment and no reidentification activities are performed.
- Specific security measures are in place to prevent reidentification and unauthorized third-party access.

Reidentification may occur only when pseudonymized data indicate a real and specific danger to the safety or health of an individual or group, or when necessary to ensure adequate healthcare.

To ensure technical and functional separation, two groups will be established: one handling participant identification and pseudonymization, and another analyzing results.

8. PRACTICAL CONSIDERATIONS

8.1. CONFLICT OF INTEREST

The study will be publicly funded, and none of the participating researchers have any conflicts of interest regarding the proposed topic.

9. BUDGET

Drs. María Carmen Cenit Laguna and Marta Calatayud, senior scientists at IATA-CSIC, have secured funding for this study through the project PID2022-142373OA-I00 titled *DEVELOPING MICROBIOME AND DIETARY STRATEGIES BASED ON CHRONO-NUTRITION FOR TARGETING ADHD*, funded by the Spanish Ministry of Science and Innovation under the 2021–2023 National Research, Development, and Innovation Program (final resolution attached).

11. STUDY PERSONNEL

Members of the research team associated with project PID2022-142373OA-I00 belong to IATA-CSIC (Marta Arroyo Calatayud), EEZ-CSIC (María Carmen Cenit Laguna) and Doctor Peset University Hospital of Valencia (Pablo Cervera, Nuria López, and Alberto Real Dasi), under the supervision of José Martínez Raga, Head of the Psychiatry Department.

12. ALTERNATIVE METHODS CONSIDERED

There are no alternative techniques to the proposed procedures capable of achieving the study's objectives.

The evaluation of the impact of circadian fasting in a genetic animal model of ADHD could be considered a complementary, though not alternative, approach. However, due to high inter-individual variability in microbiota composition and lifestyle factors among humans, such an animal model would not allow assessment of lifestyle, anthropometric, or immunometabolic variables associated with differential responses to circadian fasting or their relationship with baseline microbiota profiles.

ANNEX I

RISK EATING BEHAVIOR QUESTIONNAIRE (CAR)

Item	Never or almost never	Sometimes	Frequently (twice a week)	Very frequently (more than twice a week)
1. At times I have eaten too much or binged on food.				
2. I have lost control over what I eat (I feel like I can't stop eating).				
3. I have vomited after eating in an attempt to lose weight.				
4. I have fasted (gone without eating for 12 hours or more) to try to lose weight.				
5. I have used diet pills to try to lose weight.				
6. I have taken diuretics (substances to lose water) to try to lose weight.				
7. I have taken laxatives (substances to facilitate bowel movements) to try to lose weight.				
8. I have been worried about gaining weight.				
9. I have followed diets to try to lose weight.				
10. I have exercised to try to lose weight.				

ANNEX II

Clinical and Sociodemographic Questionnaire

Record of Sociodemographic and Clinical Variables:

- Age:
- Weight:
- Height:
- BMI:
- Abdominal circumference / Waist-hip ratio:
- Sex:
- Education level:
- Marital status: (Single, widowed, or partnered)
- Employment status: Indicate if unemployed, employed (salaried), or self-employed.
- Smoker (Yes or No): If Yes, indicate number of cigarettes per day and age at onset.
- Former smoker (Yes or No):
- Never smoked:
- Alcohol units per week:
- Alcohol abuse (Yes or No):
- Past alcohol abuse (Yes or No):
- Other drug abuse (Yes or No):
- Past drug abuse (Yes or No):
- Family member with alcohol use disorder:

Resilience

1. When you are in a difficult situation, can you usually find a way out? (Yes or No)
2. Do you see failure as an opportunity for success in the future?
3. Are you capable of working for months or years to achieve a goal?
4. Do you experience sleep disturbances? (Yes or No; type — e.g., sleepiness or difficulty sleeping — and since when)
5. Do you currently experience loss or increase of appetite? (Yes or No; since when)
6. Do you or any family member have or have had any cardiovascular disease? (Yes or No; specify disease)
7. Diagnosed anxiety disorder (Yes or No):
8. Beck Anxiety Inventory score:
9. Diagnosed depressive disorder (Yes or No):
10. Family member diagnosed with an anxiety disorder (Yes or No; specify relationship):
11. During the past week, how many hours per day did you spend sitting?
12. During the past week, how many hours per day did you spend lying down or reclining but awake?
13. Physical Activity (IPAQ Scale):
14. Diagnosed diabetes (Yes or No):
15. Are you receiving treatment for diabetes and/or hypertension? If yes, indicate medication:
16. Arterial hypertension treated with medication (Yes/No):
17. Triglyceride levels:
18. Fasting glucose levels:
19. Cholesterol levels:
20. Blood pressure: systolic and diastolic (both in mmHg):
21. Beck Depression Inventory (BDI-II) score:
22. Perceived Stress Scale (PSS) score:
23. Quality of life (AAQoL 29-item questionnaire):
24. Family member diagnosed with depression (Yes or No; specify who):
25. Current treatments: specify whether, in addition to medication, the patient is receiving psychotherapy. For medication, indicate active ingredient, daily dose, and duration of treatment (months).

ANNEX III

Bristol Stool Scale – Intestinal Habits

INTESTINAL HABITS

How many times do you have a bowel movement per day?

▫ 0–1 ▫ 2–3 ▫ More than 3

How many times do you have a bowel movement per week?

▫ 0–3 ▫ 4–7 ▫ 8–14 ▫ More than 14

According to the Bristol Stool Scale, indicate the general form of your stools:

Type 1	Type 2	Type 3	Type 4	Type 5	Type 6	Type 7
Type 1			Separate hard lumps, like nuts (hard to pass)			
Type 2			Sausage-shaped but lumpy			
Type 3			Like a sausage, but with cracks on the surface			
Type 4			Like a sausage or snake, smooth and soft			
Type 5			Soft blobs with clear-cut edges (passed easily)			
Type 6			Fluffy pieces with ragged edges, a mushy stool			
Type 7			Watery, no solid pieces (entirely liquid)			

ANNEX IV

FOOD FREQUENCY QUESTIONNAIRE

Food Item	Portion	How often do you consume it?	
		Per week	Per month
1. CEREALS, PASTA, RICE, AND POTATOES			
Breakfast cereals (cornflakes, puffed cereals, etc.)	Bowl		
“María”-type biscuits	1–3 units		
Industrial pastries (muffins, donuts, croissants, etc.)	1–3 units		
Homemade pastries (cakes, cookies, pies, etc.)	1–3 units		
Potatoes (baked, fried, or boiled)	1 large or 2 small potatoes		
White rice, paella, etc.	1 plate		
Pasta (macaroni, spaghetti, etc.)	1 plate		
Soups and creams	1 bowl/deep plate		
Bread (sandwiches, at meals, etc.)	1 slice (4–6 fingers width)		
Pizza, empanadas	Medium serving (2–4 portions)		
2. LEGUMES, VEGETABLES, NUTS, AND OIL			
Salads (lettuce, endive, tomato, onion, etc.)	1 plate		
Green beans, chard, or spinach	1 plate		
Side vegetables (eggplant, zucchini, peppers, mushrooms, etc.)	Small portion (half a plate)		
Legumes (lentils, beans, chickpeas, etc.)	1 plate		
Nuts (peanuts, hazelnuts, almonds, etc.)	Handful		
Extra virgin olive oil	1 tablespoon		
3. MEAT, EGGS AND FISH			
Eggs	1–2 units		
Chicken or turkey	1 individual fillet		
Ground meat (sausages, hamburgers)	—		
Beef, pork, or lamb (steak, breaded, grilled, etc.)	1 individual fillet		
White fish (hake, cod, sole, trout, sea bass, etc.)	1 individual fillet		
Oily fish (salmon, mackerel, tuna, sardine, anchovy, bonito)	1 individual fillet		
Shellfish (mussels, shrimp, prawns, squid, etc.)	1 serving (1 can / 6–7 pieces)		
Cured ham, cooked ham, and cold cuts	2–3 slices/pieces		
4. MILK AND DAIRY PRODUCTS (YOGURT, CHEESE, DAIRY DESSERTS, ETC.)			
Milk	1 glass		
Yogurt	2 yogurts		

Food Item	Portion	How often do you consume it?	
		Per week	Per month
Croquettes	3–4 units		
Fresh or white cheese	1 container		
Semi-cured, cured, or creamy cheeses	2–3 portions		
Dairy desserts (ricotta, flan, custard)	1 unit		
5. SUGARS			
Dark chocolate / cocoa	1 glass or 1–2 squares		
Sugar	1 spoon (small)		
Chocolate bars (“Kit-Kat”, “Mars”, etc.)	1–3 ounces of chocolate / 1 unit		
Cream or chocolate pastries	1 unit		
Savory snacks (“Cheetos”, “Fritos”, etc.)	1 bag		
Candies / sweets	—		
Ice cream	—		
Cookies with chocolate or cream	1–3 units		
6. FRUITS AND JUICES			
Citrus fruits (orange, mandarin, kiwi)	1 medium piece		
Other fruits (apple, pear, peach, banana, etc.)	1 medium piece		
Canned fruits (in syrup, etc.)	1 unit		
Natural fruit juices	1 glass		
Commercial / packaged juices	1 unit		
7. BEVERAGES			
Sugary drinks (“Coca-Cola”, “Fanta”, etc.)	1 can		
Low-calorie drinks (“Coca-Cola Light”, etc.)	1 can		
Wine, sangria	1 glass		
Beer	1 glass / 1 bottle (330 ml)		
Distilled drinks (whisky, gin, cognac, etc.)	1 shot / glass		

ANNEX V

72-HOUR DIETARY RECORD

Days of recording:

- Day 1: _____
- Day 2: _____
- Day 3: _____

INSTRUCTIONS FOR COMPLETION

First of all, we would like to thank you for your collaboration. In this questionnaire, we will collect information about your diet, one of the most important aspects that can affect your health status.

You must record **all foods and beverages consumed during three consecutive days** (two weekdays and one weekend day).

The questionnaire consists of **three sheets per day**:

- **First sheet:** from when you wake up until midday (before lunch).
- **Second sheet:** lunch and everything consumed until dinner.
- **Third sheet:** dinner and everything consumed afterwards until bedtime.

To avoid forgetting any item, **record foods immediately after consuming them**.

Do not forget to include foods and beverages consumed between meals (chocolate, candies, sweets, coffee, beer, etc.), or foods you taste while cooking or waiting for meals.

Please also include **all meals eaten outside the home**.

HOW TO FILL IN THE QUESTIONNAIRE

1. **Specify the menu and location** where each meal is consumed.
 - Example:
Lunch: at home
 - Macaroni with tomato and cheese
 - Beef steak with peppers
 - Bread and orange
2. **Clearly describe the foods and beverages consumed:**
 - Type of food or beverage (for example, whole or skimmed milk, white or whole-grain bread).
 - Type of meat (lean or fatty), type of oil or fat used, type of cheese, etc.
 - Whenever possible, **indicate the commercial brand**.
Note: The commercial brand is nutritionally relevant (regardless of its cost), since the same type of product may vary in composition and weight depending on the manufacturer, which in turn affects its nutrient composition.
Example: a cookie may weigh 6, 8, 12, or 20 grams depending on the brand.
3. **When describing a dish**, indicate the **cooking method** used.
 - For example: fried potatoes or boiled potatoes, stewed meat or grilled meat.
 - It is also important to indicate whether a food is **precooked** (ready-to-eat).
 - If so, please **cut out and attach the product label** (brand and composition) to the notebook.
4. **Quantify the food:**
 - If possible, use an electronic scale to record exact weights.
 - If a scale is not available, use **household measures** (cups, spoons, etc.).
 - Record the **raw weight** (as purchased).
 - For pasta, rice, or legumes, you may record the cooked weight, but please specify this.
Example:
 - Macaroni (brand name): 80 g raw or 1 small cup (raw).
 - 250 g cooked or 2 ladles of pasta (cooked).
5. **Record only your own portion size.**
 - If quantities refer to a dish for several people, indicate the total and the number of portions similar to yours.
Example:
 - Lentil stew with vegetables: 320 g lentils (raw) for 4 people;
200 g pepper and onion for 4 people.
6. **Do not forget to record foods that are often omitted**, such as:
 - Sugar added to coffee or other drinks,
 - Bread,
 - Oil,
 - Beverages.

LEFTOVERS

If you leave any uneaten food (excluding inedible parts like peels), indicate **as precisely as possible** the quantity discarded or eaten by someone else.

If leftovers are saved for later consumption, you do **not** need to rewrite the ingredients—just note the meal and the amount saved.

FINAL NOTES

It is highly recommended to write down the menu and foods **immediately after each meal** to avoid omissions that could negatively affect the study results.

Please fill out this questionnaire carefully over the three days, as the **quality of the final report will depend on the accuracy of the data provided by each participant.**

[illegible]

AFTER LUNCH UNTIL DINNER	

DINNER	
MENU prepared and LOCATION	
FOODS CONSUMED (including beverages) (Specify type and/or brand)	AMOUNT CONSUMED (in g or units)
AFTER DINNER UNTIL BEDTIME:	

ANNEX VI

Eating Behavior in the Adult Population and Eating Habits

No.	Statement	Response Options: <i>Never – Rarely – Sometimes – Often – Always</i>
1.	I frequently feel hungry	
2.	I love food	
3.	I enjoy a wide variety of foods	
4.	I often leave food on my plate when I finish eating	
5.	I am usually the last to finish eating	
6.	I often know I don't like a food before trying it	
7.	I often get so hungry that I must eat something immediately	
8.	I often feel full before finishing a meal	
9.	I enjoy trying new foods	
10.	I often finish eating quickly	
11.	My stomach often growls because I'm hungry	
12.	I am almost always thinking about food	
13.	I like to try new foods and dishes	
14.	I often feel hungry when I'm with someone who is eating	
15.	I like to eat	
16.	I eat less when I am anxious	
17.	As the meal progresses, I notice that I eat more slowly	
18.	I eat less when I am worried	
19.	I look forward to mealtimes	
20.	I eat more when I am irritated	
21.	At first, I reject foods that I do not know	
22.	I eat more when I am worried	
23.	I eat slowly	
24.	I eat less when I am upset	
25.	I get full easily	
26.	I eat less when I am angry	
27.	If a meal is delayed, I get dizzy	
28.	When I smell or see food that I like, I feel like eating	
29.	I eat more when I am upset	
30.	I eat more when I am angry	
31.	If I could, I would be eating most of the time	
32.	If I miss a meal, I easily get irritated	
33.	I eat more when I am anxious	
34.	I cannot finish a meal if I have eaten something beforehand	
35.	I eat less when I am irritated	

EATING HABITS

1. Most days, do you experience loss of appetite in the morning?
☐ Yes ☐ No
2. How often do you usually have breakfast?
☐ Always ☐ Sometimes ☐ Almost never ☐ Never
3. How many meals do you have per day?
☐ Fewer than 2 ☐ 2–5 ☐ More than 5
4. Mark with an “X” the meals you usually have and indicate the times:
5. Are your meals eaten at regular intervals within 24 hours, or do you have different schedules and a different number of meals depending on the day?

6. Do you usually repeat servings?
☐ Yes ☐ No
 If yes, for which meal(s)? ☐ Breakfast ☐ Mid-morning ☐ Lunch ☐ Snack ☐ Dinner
☐ Others
7. How often do you usually have dinner?
☐ Always ☐ Sometimes ☐ Almost never ☐ Never
8. Most days, do you feel a strong need to eat between dinner and going to bed and/or during the night?
☐ No ☐ Yes
9. Do you experience nighttime binge eating episodes?
☐ No ☐ Yes
10. Do you use the consumption of sweets, candy, or snack foods (such as potato chips) as a reward?
☐ No ☐ Yes
11. Do you usually snack between meals on “unhealthy” foods (candy, industrial pastries, packaged chips, etc.)?
☐ No ☐ Yes

Meal	Do you have it?	Time
Breakfast		
Mid-morning snack		
Lunch		
Afternoon snack		
Dinner		
Supper (late evening snack)		
Others		

ANNEX VII

MEDITERRANEAN DIET ADHERENCE QUESTIONNAIRE

Date: _____

Participant Name: _____

1. Do you use **olive oil as the main fat for cooking**?
☐ Yes ☐ No
2. **How much olive oil do you consume in total per day** (including oil used for frying, meals outside the home, salads, etc.)?
☐ Two or more tablespoons ☐ Less than two tablespoons
3. **How many servings of vegetables or greens do you consume per day?** (Side dishes count as ½ serving)
☐ Two or more per day (at least one raw or in salads) ☐ Less than two servings
4. **How many pieces of fruit (including natural juice) do you consume per day?**
☐ Three or more per day ☐ Less than three
5. **How many servings of red meat, hamburgers, sausages, or cold cuts do you consume per day?** (One serving = 100–150 g)
☐ Less than one per day ☐ More than one per day
6. **How many servings of butter, margarine, or cream do you consume per day?** (One individual portion = 12 g)
☐ Less than one per day ☐ More than one per day
7. **How many carbonated and/or sugary drinks (soft drinks, colas, tonics, bitter sodas) do you consume per day?**
☐ Less than one per day ☐ More than one per day
8. **Do you drink wine? How much do you consume per week?**
☐ Three or more glasses per week ☐ Less than three per week
9. **How many servings of legumes do you consume per week?** (One serving or plate = 150 g)
☐ Three or more per week ☐ Less than three per week
10. **How many servings of fish or seafood do you consume per week?** (One serving = 100–150 g fish or 4–5 pieces of seafood)
☐ Three or more per week ☐ Less than three per week
11. **How many times per week do you consume commercial baked goods** (not homemade) such as cookies, flans, sweets, or pastries?
☐ Less than three per week ☐ Less than three per week
12. **How many times per week do you consume nuts?** (One serving = 30 g)
☐ Once or more per week ☐ Less than once per week
13. **Do you preferably consume chicken, turkey, or rabbit instead of beef, pork, hamburgers, or sausages?** (Chicken serving = 100–150 g)
☐ Yes ☐ No
14. **How many times per week do you consume cooked vegetables, pasta, rice, or other dishes seasoned with a tomato, garlic, onion, or leek sauce prepared slowly with olive oil (sofrito)?**
☐ Two or more per week ☐ Less than two per week

ANNEX VIII

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE (IPAQ)

We are interested in understanding the type of physical activity you perform in your daily life. The questions refer to the time you spent being active over the last 7 days. Please note that this questionnaire is completely anonymous.

1. During the last 7 days, on how many days did you perform vigorous physical activities such as lifting heavy weights, digging, aerobic exercises, or fast cycling?

Days per week (indicate number)

No vigorous physical activity (skip to question 3)

2. On those days, how much total time did you usually spend on vigorous physical activity?

Indicate hours per day

Indicate minutes per day

Don't know/Not sure

3. During the last 7 days, on how many days did you perform moderate physical activities such as carrying light loads or cycling at a regular pace? Do not include walking.

Days per week (indicate number)

No moderate physical activity (skip to question 5)

4. On those days, how much total time did you usually spend on moderate physical activity?

Indicate hours per day

Indicate minutes per day

5. During the last 7 days, on how many days did you walk for at least 10 minutes continuously?

Days per week (indicate number)

No walking (skip to question 7)

6. On those days, how much total time did you usually spend walking?

Indicate hours per day

Indicate minutes per day

Don't know/Not sure

7. During the last 7 days, how much time did you spend sitting on a weekday?

Indicate hours per day

Indicate minutes per day

Don't know/Not sure

TEST SCORING:

1. Walking: $3.3 \text{ MET} \times \text{minutes of walking} \times \text{days per week}$ (e.g., $3.3 \times 30 \text{ minutes} \times 5 \text{ days} = 495 \text{ MET}$)
2. Moderate Physical Activity: $4 \text{ MET} \times \text{minutes} \times \text{days per week}$
3. Vigorous Physical Activity: $8 \text{ MET} \times \text{minutes} \times \text{days per week}$

Next, sum the three values obtained:

Total = walking + moderate physical activity + vigorous physical activity

CLASSIFICATION CRITERIA:

- **Moderate Physical Activity:**
 1. 3 or more days of vigorous physical activity of at least 20 minutes per day
 2. 5 or more days of moderate physical activity and/or walking of at least 30 minutes per day
 3. 5 or more days of any combination of walking, moderate or vigorous physical activity achieving at least 600 MET
- **Vigorous Physical Activity:**
 1. Vigorous physical activity at least 3 days per week achieving a total of at least 1500 MET
 2. 7 days of any combination of walking, moderate and/or vigorous physical activity achieving a total of at least 3000 MET

RESULT - ACTIVITY LEVEL

HIGH LEVEL

MODERATE LEVEL

LOW OR INACTIVE

ANEXO IX

Name: _____

Date: _____

Pittsburgh Sleep Quality Index (PSQI)

Instructions: The following questions relate to your usual sleep habits during the past month only. Your answers should indicate the most accurate reply for the majority of days and nights in the past month. **Please answer all questions.**

1. During the past month, what time have you usually gone to bed at night? _____
2. During the past month, how long (in minutes) has it usually taken you to fall asleep each night? _____
3. During the past month, what time have you usually gotten up in the morning? _____
4. During the past month, how many hours of actual sleep did you get at night? (This may be different than the number of hours you spent in bed.) _____

5. During the <u>past month</u> , how often have you had trouble sleeping because you...	Not during the past month	Less than once a week	Once or twice a week	Three or more times a week
a. Cannot get to sleep within 30 minutes				
b. Wake up in the middle of the night or early morning				
c. Have to get up to use the bathroom				
d. Cannot breathe comfortably				
e. Cough or snore loudly				
f. Feel too cold				
g. Feel too hot				
h. Have bad dreams				
i. Have pain				
j. Other reason(s), please describe:				
6. During the past month, how often have you taken medicine to help you sleep (prescribed or "over the counter")?				
7. During the past month, how often have you had trouble staying awake while driving, eating meals, or engaging in social activity?	No problem at all	Only a very slight problem	Somewhat of a problem	A very big problem
8. During the past month, how much of a problem has it been for you to keep up enough enthusiasm to get things done?	No problem at all	Only a very slight problem	Somewhat of a problem	A very big problem
9. During the past month, how would you rate your sleep quality overall?	Very good	Fairly good	Fairly bad	Very bad
10. Do you have a bed partner or room mate?	No bed partner or room mate	Partner/room mate in other room	Partner in same room but not same bed	Partner in same bed
If you have a room mate or bed partner, ask him/her how often in the past month you have had:	Not during the past month	Less than once a week	Once or twice a week	Three or more times a week
a. Loud snoring				
b. Long pauses between breaths while asleep				
c. Legs twitching or jerking while you sleep				
d. Episodes of disorientation or confusion during sleep				
e. Other restlessness while you sleep, please describe:				

ANNEX X

MUNICH CHRONOTYPE QUESTIONNAIRE (MCTQ)

WORKDAYS

1. I go to bed at hours
2. *Please note that some people stay awake for a while when they are in bed!*
3. I am actually ready to fall asleep at hours
4. I need ... minutes to fall asleep
5. I wake up at hours
 - a. With an alarm clock
 - b. Without an alarm clock
6. I get up after ... minutes

NON-WORKDAYS

1. I go to bed at hours
2. *Please note that some people stay awake for a while when they are in bed!*
3. I am actually ready to fall asleep at hours
4. I need ... minutes to fall asleep
5. I wake up at hours
 - a. With an alarm clock
 - b. Without an alarm clock
6. I get up after ... minutes

ANNEX XI

Barratt Impulsiveness Scale (BIS-11) – Items

Answer each item as:

Rarely or never Occasionally Often Always or almost always

0 1 3 4

1. I plan what I have to do
2. I do things without thinking
3. I make decisions quickly
4. I am a carefree person
5. I do not pay attention to things
6. My thoughts go too fast
7. I plan my free time
8. I am a self-controlled person
9. I can concentrate easily
10. I am thrifty
11. I cannot sit still in the cinema or at school
12. I like to think and mull things over
13. I plan my future
14. I say things without thinking
15. I like to think about complicated problems
16. I change my mind about what I want to do when I grow up
17. I act on impulse
18. I get bored easily when trying to solve mental problems
19. I act according to the moment
20. I think things through quite well
21. I change friends
22. I buy things on impulse
23. I can focus on only one problem at a time
24. I change hobbies and sports
25. I spend more than I should
26. When I think about something, other thoughts crowd my mind
27. I am more interested in the present than the future
28. I am restless in cinemas and classes
29. I like board games such as chess, checkers, or Parcheesi
30. I think about the future

ANNEX XII

PERCEIVED STRESS SCALE (PSS)

The questions in this scale refer to your feelings and thoughts during the last month. For each item, please indicate with an X how you have felt or thought in each situation.

Item Never Almost never Sometimes Often Very often

- | | 0 | 1 | 2 | 3 | 4 |
|-----|---|---|---|---|---|
| 1. | | | | | |
| 2. | | | | | |
| 3. | | | | | |
| 4. | | | | | |
| 5. | | | | | |
| 6. | | | | | |
| 7. | | | | | |
| 8. | | | | | |
| 9. | | | | | |
| 10. | | | | | |
| 11. | | | | | |
| 12. | | | | | |
| 13. | | | | | |
| 14. | | | | | |

Scoring Instructions:

This self-report scale assesses perceived stress over the past month. It consists of 14 items with a 5-point response format (0 = never, 1 = almost never, 2 = sometimes, 3 = often, 4 = very often).

To calculate the total PSS score:

- Reverse the scores of items 4, 5, 6, 7, 8, 9, 10, and 13 (reverse scoring: 0 = 4, 1 = 3, 2 = 2, 3 = 1, 4 = 0).
- Then sum all 14 items.

A higher total score indicates a higher level of perceived stress.

ANNEX XIII

BECK ANXIETY INVENTORY (BAI)

Please answer this questionnaire considering your current difficulties. Use as reference the difficulties you have experienced during the past month.

The Beck Anxiety Inventory is a useful tool for assessing somatic symptoms of anxiety, both in anxiety disorders and depressive states.

The questionnaire consists of 21 questions, providing a total score range from 0 to 63. Suggested cut-off points for interpreting the total score are:

- 00–21 – Very low anxiety
- 22–35 – Moderate anxiety
- Over 36 – Severe anxiety

Each item is scored from 0 to 3, with the following meanings:

- 0 = Not at all
- 1 = Mildly, it does not bother me much
- 2 = Moderately, it was very unpleasant but I could stand it
- 3 = Severely, I could hardly bear it

The total score is the sum of all item scores. The symptoms refer to the past week and the current moment.

Read each item carefully and indicate how much it has affected you in the past week, including today.

#	Symptom	Not at all	Mild	Moderate	Severe
1	Clumsy or numb	-	-	-	-
2	Hot or flushed	-	-	-	-
3	Hands or legs trembling	-	-	-	-
4	Unable to relax	-	-	-	-
5	Fear of the worst happening	-	-	-	-
6	Dizzy or lightheaded	-	-	-	-
7	Heart pounding or racing	-	-	-	-
8	Unsteady	-	-	-	-
9	Terrified or afraid	-	-	-	-
10	Nervous	-	-	-	-
11	Feeling of choking	-	-	-	-
12	Hands trembling	-	-	-	-
13	Shaky or unsteady	-	-	-	-
14	Fear of losing control	-	-	-	-
15	Feeling of suffocation	-	-	-	-
16	Fear of dying	-	-	-	-
17	Afraid	-	-	-	-
18	Digestive problems	-	-	-	-
19	Fainting spells	-	-	-	-
20	Blushing or flushed face	-	-	-	-
21	Sweating, hot or cold	-	-	-	-

ANNEX XIV
Short Multidimensional Inventory Lifestyle Evaluation (SMILE)

Domain	In your daily routine during the past month, how often have you...?	Always	Frequently	Sometimes	Never
Diet and Nutrition	1. Consumed homemade food prepared by you or another family member.	4	3	2	1
	2. Checked labels while shopping, paying attention to ingredients such as salt content.	4	3	2	1
	3. Eaten processed foods, such as frozen pizzas, fries, industrial pastries, fried foods, or canned foods.	1	2	3	4
	4. Consumed junk food or high-calorie foods, sweets, or fats when feeling stressed or sad.	1	2	3	4
	5. Eaten healthy foods such as fresh fruits, fresh vegetables, whole grains, legumes, or nuts.	4	3	2	1
	6. Maintained a regular meal schedule.	4	3	2	1
	7. Shared main meals of the day with friends or family.	4	3	2	1
Substance Use	8. Consumed 5 or more drinks (if male) or 4 or more drinks (if female) in one occasion (less than 2 hours). <i>1 drink = 1 glass of beer, 1 glass of wine, or 1 shot of distilled spirits (rum, vodka, whisky, tequila, gin)</i>	1	2	3	4
	9. Smoked tobacco (cigarettes, e-cigarettes, cigars, pipe, smokeless tobacco).	1	2	3	4
	10. Smoked marijuana or hashish.	1	2	3	4
	11. Used other drugs (cocaine, crack, amphetamines, ecstasy, opioids without prescription, or other drugs).	1	2	3	4
Physical Activity	12. Engaged in at least 30 minutes of physical activity per day (or 150 minutes per week).	4	3	2	1
	13. Played team sports (soccer, volleyball, basketball, rugby...) at least 2 hours per week.	4	3	2	1
	14. Chose stairs over elevators and/or walked to perform daily tasks instead of using a car or public transport.	4	3	2	1
	15. Felt good after engaging in physical activity.	4	3	2	1
Stress Management	16. Dedicated time to relax.	4	3	2	1
	17. Used psychological strategies or support to manage stress (e.g., meditation, mindfulness, or psychotherapy).	4	3	2	1
	18. Used physical strategies to manage stress (e.g., yoga, tai-chi, physical exercise).	4	3	2	1
	19. Practiced a religion or spiritual belief.	4	3	2	1
	20. Felt that you have a good balance between work and personal life.	4	3	2	1

Domain	In your daily routine during the past month, how often have you...?	Always	Frequently	Sometimes	Never
Restorative Sleep	21. Felt that your work or tasks never end.	1	2	3	4
	22. Felt satisfied with the time it takes to get to work.	4	3	2	1
	23. Felt that your life has meaning.	4	3	2	1
	24. Felt grateful for the life you have.	4	3	2	1
	25. Slept between 7 and 9 hours each night.	4	3	2	1
	26. Felt rested with the number of hours you sleep.	4	3	2	1
	27. Rested (sleep or nap) after lunch.	4	3	2	1
	28. Maintained a regular sleep schedule.	4	3	2	1
Social Support	29. Took medication to sleep.	1	2	3	4
	30. Interacted with friends and/or family.	4	3	2	1
	31. Felt part of a group of friends, your community, or society.	4	3	2	1
	32. Had someone you trust to listen to your problems or concerns.	4	3	2	1
	33. Had someone to help with daily tasks, such as cooking, cleaning, or shopping.	4	3	2	1
	34. Had someone in your life to go out or have fun with when you wanted.	4	3	2	1
	35. Participated in celebrations or gatherings with family, friends, or coworkers.	4	3	2	1
	36. Enjoyed your free time.	4	3	2	1
Environmental Exposure (screen time / outdoor time)	37. Were available to help your loved ones.	4	3	2	1
	38. Were satisfied with your sex life.	4	3	2	1
	39. Felt loved.	4	3	2	1
	40. Spent more than 2 hours a day watching TV, playing computer/video games, or using the internet.	1	2	3	4
	41. Used a computer or mobile phone one hour before bedtime.	1	2	3	4
	42. Spent time in contact with nature, e.g., in parks, beach, countryside, or mountains.	4	3	2	1
	43. Felt that your relationship with nature, i.e., all living beings, is an important part of who you are.	4	3	2	1

ANNEX XV

ADULT ADHD QUALITY OF LIFE – 29 : (AAQoL-29)

The following questions are about how ADHD has impacted your life over the past 2 weeks. For each item, evaluate the degree or frequency with which you find each quality of life issue troublesome or problematic. There are no right or wrong answers.

Answer option:

Not at all

A little

Somewhat

A lot

Extremely

Not applicable

During the past 2 weeks, how difficult has it been for you to:

1. Keep the house/apartment clean or uncluttered
2. Manage your finances, such as cashing checks, balancing your checkbook, paying bills on time
3. Remember important things
4. Get your shopping done (such as for food, clothes, or household items)
5. Pay attention when interacting with others

During the past 2 weeks, how often have you felt:

6. Overwhelmed
7. Anxious
8. Depressed
9. You have not been able to meet others' expectations of you (either at home or work)
10. You annoyed people
11. Getting things done requires too much effort
12. People are frustrated with you
13. You have overreacted in difficult or stressful situations
14. Your energy is well spent (has positive results)
15. Able to enjoy time spent with others
16. You can successfully manage your life
17. As productive as you would like to be
18. Tension in relationships
19. Not having quality time to spend with others
20. Feeling fatigued
21. Fluctuations (ups and downs) in your emotions

During the past 2 weeks, how much of a problem has it been for you to:

22. Complete projects or tasks (either at work or home)
23. Get started with tasks you don't find interesting
24. Balance multiple projects
25. Get things done on time
26. Keep track of important items (such as keys, wallet)

During the past 2 weeks, how often have you felt:

27. Good about yourself
28. People enjoy spending time with you
29. Your intimate relationship is going well emotionally

Each item is rated by patients on a five-point Likert scale, ranging from "Not at all/Never" (1) to "Extremely/Very often" (5). A total score is obtained (based on the 29 items) as well as scores for each of the four dimensions: vital productivity (11 items), psychological health (6 items), social relationships (5 items), and life perspective (7 items). The vital productivity dimension refers to the ability to organize, plan, and carry out everyday tasks, including work-related tasks. Issues assessed in this domain include disorganization, procrastination, problems initiating actions, and perseverance in completing tasks.

The psychological health dimension encompasses all aspects related to emotional well-being, such as feelings of being overwhelmed, loss of control, anxiety, frustration, depression, and low self-esteem. These issues are linked to difficulties in managing day-to-day life.

The life perspective dimension reflects positive aspects through which the individual can express satisfaction, for example: “I am able to manage my life satisfactorily.” This dimension relates to the degree to which a person feels satisfied with their life management.

Finally, the social relationships dimension includes items associated with the ability to establish and maintain interpersonal relationships, both in professional and personal contexts.

ANNEX XVI
PARTICIPANT INFORMATION SHEET
Title: DEVELOPING MICROBIOME AND DIETARY STRATEGIES BASED ON CHRONO-NUTRITION FOR TARGETING ADHD (CRONONUTRIBIOME)

Principal Investigators:

Marta Arroyo Calatayud and María Carmen Cenit Laguna

Center:

Institute of Agrochemistry and Food Technology (IATA-CSIC)

Sponsor:

Spanish National Research Council (CSIC)

Contact phone number:

963900022 extension 235249

+34614234168

1. INTRODUCTION

We are writing to inform you about a research study in which you are invited to participate. The study has been approved by the Research Ethics Committee with medicines (CEIm) of the Dr. Peset University Hospital of Valencia, in accordance with current legislation and the standards of good clinical practice. Our aim is to ensure that you receive clear and sufficient information so that you can decide whether or not to participate in this study. Please read this information sheet carefully, and we will be happy to clarify any questions you may have. You may also consult anyone you consider appropriate.

2. VOLUNTARY PARTICIPATION

You should know that your participation in this study is voluntary and that you may choose **not** to participate. If you decide to participate, you may change your decision and withdraw your consent at any time, without affecting your relationship with your doctor or your medical care in any way.

3. OBJECTIVE OF THE STUDY

To evaluate whether circadian fasting (the regular shortening of the eating window and alignment of food intake with circadian rhythms) may help improve ADHD symptoms and/or quality of life in adult patients with this disorder through modulation of the gut microbiota, as well as to assess whether intrinsic and/or extrinsic (lifestyle-related) factors in ADHD patients may influence the degree of response to circadian fasting.

4. DESCRIPTION OF THE STUDY

At the Dr. Peset University Hospital, we will conduct an interventional study in adults with ADHD in order to thoroughly evaluate the potential beneficial effect of circadian fasting on the cognitive and behavioral alterations associated with ADHD, as well as on other altered aspects such as sleep quality and emotional state. Confirming a beneficial effect of circadian fasting on ADHD-related alterations would enable the design of new intervention strategies to be evaluated in future studies aimed at improving the disorder.

If our working hypothesis is confirmed—that the intestinal microbiota may mediate the therapeutic effect of circadian fasting in improving ADHD—the results could, in the near future, support the use of new intervention strategies based on probiotics and/or prebiotics to improve ADHD in patients unresponsive to conventional therapies, as well as to reduce the use of pharmacological treatments for ADHD management.

The study will include adult participants over 18 years of age diagnosed with ADHD. It will also include participants without ADHD and without a family history of the disorder. A total of 45 participants will be recruited for each of the ADHD groups (ADHD Fasting Intervention Group and ADHD Non-Intervention Group) and 40 participants for each of the non-ADHD groups (Control Fasting Intervention Group and Control Non-Intervention Group).

5. STUDY ACTIVITIES

The study will last **12 weeks**. Each participant will undergo a clinical examination before starting the study, as well as complete questionnaires on lifestyle habits such as diet, exercise, and stress. This examination will be repeated monthly during the study.

If you are randomly assigned to the ADHD Fasting Intervention Group or the Control Fasting Intervention Group, you will be asked to reduce your daily eating window to 10 hours for the duration of the study, avoiding food intake after 8:00 p.m. This schedule will allow you to follow an intermittent fasting pattern known as *circadian fasting*.

Additionally, biological samples of stool, saliva, and urine will be collected both at the beginning and at the end of the study. These samples will be processed and analyzed at the Institute of Agrochemistry and Food Technology of the Spanish National Research Council (IATA-CSIC).

Please note that the biological samples will be used exclusively to determine the data or parameters scheduled and specified in this study as approved by the Ethics Committee.

6. POSSIBLE BENEFITS AND RISKS OR DISCOMFORTS OF PARTICIPATION

There are several potential benefits from participating in this study. Scientific evidence indicates that adhering to a regular eating schedule aligned with circadian rhythms may significantly benefit overall health. Improving a key lifestyle factor such as meal timing would generally be expected to improve quality of life, although in some cases, benefits may not be observed.

If the study hypothesis is confirmed, the results could serve as a basis for developing dietary intervention strategies aimed at restoring the composition and anti-inflammatory function of the intestinal microbiota, potentially improving quality of life in individuals with ADHD.

Importantly, there are no expected risks for participants, as most biological samples collected are considered waste products. Blood extraction will be performed at the beginning and at the end of the study by qualified personnel. Approximately 10 ml of blood will be drawn, posing no significant risk to participants. Given the study objectives and planned serum analyses, the collection of these samples is fully justified.

7. CONFIDENTIALITY

The processing, communication, and transfer of the personal data of all study participants will comply with **Organic Law 3/2018 of December 5** on the Protection of Personal Data and Guarantee of Digital Rights, and **Regulation (EU) 2016/679** of the European Parliament and of the Council of April 27, 2016 (General Data Protection Regulation).

Both the Center and the Sponsor are responsible for processing your data and commit to complying with applicable data protection regulations.

The Investigator and the Sponsor will retain the collected study data for at least five years after study completion. Thereafter, personal information will be retained only by the center for healthcare purposes and by the sponsor for other scientific research purposes, if permitted by law and ethical requirements. If coded data are transferred outside the European Union, participants' data will remain protected under safeguards such as contracts or other mechanisms recognized by data protection authorities.

In accordance with public health and biomedical research purposes, and as stipulated in section 2(d) of the seventeenth additional provision of Organic Law 3/2018, personal data used for research will be **pseudonymized**. To this end:

1. A technical and functional separation will be established between the research team and those responsible for pseudonymization and maintaining the information that allows re-identification.
2. Pseudonymized data will only be accessible to the research team when:
 - There is an explicit confidentiality commitment and an undertaking not to perform any re-identification activities.
 - Specific security measures are adopted to prevent re-identification and unauthorized third-party access.

Re-identification of the original data may occur only if, during a study using pseudonymized data, a real and specific danger to the safety or health of a person or group of people, or a serious threat to their rights, is identified, or if it is necessary to ensure adequate healthcare.

To ensure technical and functional separation, two groups will be established: one will receive identifiable participant information and perform pseudonymization, and the other will analyze the results.

8. FINANCIAL COMPENSATION

"No form of financial compensation is contemplated for study participants, the research team, or the center."

9. OTHER RELEVANT INFORMATION

You should know that you may be excluded from the study if the investigators deem it appropriate, either for safety reasons or due to non-compliance with study procedures. In all cases, you will receive an appropriate explanation of the reason for your withdrawal.

By signing the attached informed consent form, you agree to comply with the study procedures described to you.

10. CONTACT INFORMATION

If, during your participation, you have any questions or need further information, please contact:

Dr. María Carmen Cenit Laguna,

Research Scientist at the Spanish National Research Council (CSIC), Institute of Agrochemistry and Food Technology (IATA-CSIC)

Phone: +34617205498

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or

Dr. Marta Arroyo Calatayud,

Research Scientist at the Spanish National Research Council (CSIC), Institute of Agrochemistry and Food Technology (IATA-CSIC)

Phone: +34658608330

Email: **m.arroyo@iata.csic.es**

ANNEX XVII

INFORMED CONSENT

Title:

DEVELOPING MICROBIOME AND DIETARY STRATEGIES BASED ON CHRONO-NUTRITION FOR TARGETING ADHD (CRONONUTRIBIOME)

Principal Investigators:

Marta Arroyo Calatayud and María Carmen Cenit Laguna

Center:

Institute of Agrochemistry and Food Technology (IATA-CSIC)

Sponsor:

Spanish National Research Council (CSIC)

Contact phone number:

963900022 extension 235249

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Mr./Ms., ... years of age, holder of National ID No., hereby declares that he/she has been informed about the potential benefits that the analysis of the samples he/she provides may bring to the research entitled "*THERAPEUTIC EFFECT OF CIRCADIAN FASTING FOR THE IMPROVEMENT OF ADHD IN ADULTS AND THE ROLE OF INTESTINAL MICROBIOTA AS A MEDIATOR AND/OR MODULATOR OF THE EFFECT*", and that he/she receives a signed and dated copy of this informed consent document.

The study will include adult subjects over 18 years of age diagnosed with ADHD. Likewise, the study will also include participants not diagnosed with ADHD and without a family history of the disorder. The total number of participants will be 45 for each of the ADHD groups (ADHD Fasting Intervention Group and ADHD Non-Intervention Group) and 40 for each of the non-ADHD groups (Control Fasting Intervention Group and Control Non-Intervention Group).

If you are randomly assigned to the ADHD Fasting Intervention Group or to the Control Fasting Intervention Group, you will be asked to reduce your food intake window throughout the duration of the study to 10 hours, avoiding food intake after 8:00 p.m. This schedule will allow you to follow an intermittent fasting pattern known as *circadian fasting*.

In this document, I, Mr./Ms., confirm the following:

- ☐ I have read the information sheet provided to me about the study.
- ☐ I have been able to ask questions about the study.
- ☐ I have received sufficient information about the study and about my participation in it.
- ☐ I have had the opportunity to speak with Dr. María Carmen Cenit Laguna and/or Dr. Marta Arroyo Calatayud, the principal investigators of the study.
- ☐ I understand that my participation is voluntary.
- ☐ I understand that I may withdraw from the study:

- At any time.
- Without having to provide explanations.
- Without this affecting my medical care.

☐ I have been clearly, precisely, and adequately informed about how the personal data contained in this consent form and in the record or file created for this research will be processed:

- Such data will be handled and safeguarded with respect for my privacy and in compliance with current data protection regulations.
- I am entitled to exercise my rights of access, rectification, cancellation, and opposition by submitting a request to the principal investigator at the contact address provided in this document.

- I give my consent for the use of the samples and data generated from this study for further analysis (secondary use) in other projects during the next 20 years, provided they are treated for research purposes, in a strictly confidential manner, and in accordance with the applicable legal regulations.

I have also been informed that my personal data will be protected and included in a file subject to and guaranteed by Regulation (EU) 2016/679 of the European Parliament and of the Council of 27 April 2016 (General Data Protection Regulation, GDPR), and by Organic Law 3/2018 of 5 December on Personal Data Protection and the Guarantee of Digital Rights (LOPDGDD).

Taking all the above into consideration, I GIVE MY CONSENT to:

- Collection of data from my medical record
- Collection of stool samples
- Collection of urine samples
- Collection of saliva samples
- Collection of blood samples

And, taking into account that all of the above will be used solely to meet the objectives specified in the project, I freely give my consent to participate in the study.

In Valencia, on the day of, 20.....

Signed: Mr./Ms. _____

Participant's Signature: _____