Title of the clinical research project:

Dietary supplement, gut microbiota and ADHD

Short title: **Gut microbiota and ADHD**

Sponsor of the clinical research project: Hôpital Erasme – Cliniques universitaires de Bruxelles – Department of Infant, Child, Adolescent and Young Adult Psychiatry – H.U.B – Université Libre de Bruxelles

Investigators: Barbara Luyens; Dr François Felgueroso-Bueno; Prof. Dr Isabelle Massat

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Type of research project: Prospective, interventional, single-centre

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PROTOCOL

Introduction

1. ADHD

Attention-Deficit/Hyperactivity Disorder (ADHD) is the most prevalent neurodevelopmental disorder in child and adolescent psychiatry (7.2% of the world population) (APA, 2013; Thomas et al., 2015) and constitutes an undeniable public health priority. Its aetiopathogenesis is considered to be multifactorial; however, it must be acknowledged that, to date, decades of research have proved disappointing, since neither genetic factors nor specific environmental factors have been formally identified (Akutagava-Martins et al., 2016; Bélanger et al., 2018).

2. The gut-brain axis

The digestive system, also referred to as the "second brain", comprises 100 million neurons distributed along its length, forming part of the "autonomic nervous system". It maintains bidirectional relationships with the central nervous system and plays a role in the control of functions such as the regulation of digestive motility, nutrient absorption and the control of the intestinal barrier, which protects against external pathogens (Cryan & Mazmanian, 2022; Cussotto et al., 2019). Recent research on the gut microbiota (GM) has led to rapid progress in the understanding of the highly complex balance between the micro-organisms that compose it and of its close mutualistic relationships with the host organism. In addition to its role in general health, the GM is involved in the modulation of the gut-brain axis (Borre et al., 2014; Cryan & Dinan, 2015; Cryan & Mazmanian, 2022). For example, it acts on the intestinal mucosa, which secretes neurotransmitters involved in emotional regulation, such as serotonin, or dopamine precursors, which are involved in concentration and in ADHD (Aarts et al., 2017).

The GM may be impaired (or, conversely, rebalanced) by external factors (diet, medication, stress, age, environment, etc.) that influence bacterial abundance, richness and diversity. This is referred to as intestinal dysbiosis.

To our knowledge, only 7 studies have investigated the gut-brain axis in ADHD (Soltysova et al., 2022). These studies nevertheless present too many biases and methodological variations for a consensus to emerge regarding the association between a possible "dysbiosis" and ADHD. It should nonetheless be noted that, in a recent review, the authors identified 49 bacterial taxa that differed significantly between individuals with ADHD and typically developing subjects (mainly including Bifidobacterium, Dialister and Faecalibacterium), which constitutes a sufficiently promising lead to warrant further investigation (Szopinska-Tokov et al., 2020).

This could have clinical implications, on the assumption that rebalancing the GM could improve clinical symptoms in patients (Soltysova et al., 2022). To date, no study has measured the associations between GM composition and, more specifically, cognitive performance objectively assessed by standardised tests, either in subjects with ADHD or in typically developing subjects.

3. Pycnogenol

Pycnogenol is not considered a medicinal product, since it acts physiologically on the organism, in the same way that food and dietary supplements potentially have the same effect.

No therapeutic claim:

- As a dietary supplement, Pycnogenol cannot claim to treat, prevent or cure a disease such as ADHD.
- Pycnogenol is not authorised to be presented as a treatment for a specific disease, even though studies exist.
- Pycnogenol has no marketing authorisation (MA) demonstrating its efficacy and safety.

- Pycnogenol is not prescribed as a medicinal product. Its use remains preventive or supportive, within the framework of well-being.

Pycnogenol is a dietary supplement, a natural extract of ground maritime pine bark (Pinus pinaster; extraction process patented by Horphag Research). It is essentially composed of polyphenols (flavonoids, phenolic acids and procyanidins) recognised for their antioxidant, immunomodulatory and anti-inflammatory properties (Dvořáková et al., 2006; Lee et al., 2014). Its constituents are present in the diet, but their concentration and stability are lower than those of Pycnogenol, and they vary according to the diet (more or less rich in fibre), body mass index, etc. Pycnogenol meets the quality and safety requirements established by the competent regulatory authority in France (A. A. J. Verlaet et al., 2019). Its exact mode of action remains unknown but results, after absorption, from its biotransformation by microbial enzymes present in the colon, generating molecules capable of crossing the intestinal barrier. There is therefore an interaction between the GM and Pycnogenol (Trebatická & Ďuračková, 2015).

In addition to the beneficial effects observed in certain conditions (asthma, diabetes) (Liu et al., 2004; Shin et al., 2013), recent studies have shown significant effects on the improvement of cognitive symptoms (memory, planning, attention, etc.) in populations of young students and of elderly people (Belcaro et al., 2014, 2015; Hosoi et al., 2018; Luzzi et al., 2011; Paarmann et al., 2019).

Of particular interest for our study are the effects of Pycnogenol observed in populations of children and adolescents with ADHD.

It has been shown that the administration of Pycnogenol for 1 month significantly reduced ADHD symptoms (improvement in attention and visual-motor coordination, and reduction in hyperactivity/impulsivity symptoms) (Hsu et al., 2021; Trebatická et al., 2006; A. Verlaet et al., 2018; Weyns et al., 2022). Moreover, no side effects were reported (23). A recent study (2022) demonstrated an improvement in hyperactivity and impulsivity symptoms after 10 weeks of administration, which constitutes a very promising result (Weyns et al., 2022).

Nevertheless, no study has evaluated the efficacy of Pycnogenol on the cognitive performance associated with ADHD, as objectively assessed by standardised tests, in order to better dissect the cognitive components that are improved and thus better delineate the processes probably involved in the gut-brain axis.

Furthermore, across 70 clinical studies conducted in healthy subjects (children and adults) and in patients (5,723 subjects), the overall frequency of adverse effects of Pycnogenol is very low (1.8%); these effects are very mild/minor (gastrointestinal discomfort, which can be attenuated if Pycnogenol is taken during or after meals) and unrelated to the dose or duration of treatment (Storebø et al., 2015; A. Verlaet et al., 2018). Consequently, the administration of Pycnogenol as a dietary supplement in children and adolescents could constitute a promising, effective and safe form of support.

4. Hypothesis

We hypothesise that Pycnogenol modulates the GM, thereby regulating the gut-brain axis, leading to an improvement in the clinical symptoms of ADHD and in its associated cognitive components.

This study opens up a field of research that could help to elucidate certain processes involved in the aetiology of ADHD and lead to highly promising preventive and therapeutic support strategies.

5. Objectives

The aim of our research is (1) to identify a possible bacterial signature (dysbiosis) that could be linked to ADHD symptoms and/or to the associated cognitive functions, by comparing the GM of children with ADHD with that of typically developing children, and (2) to explore whether the presumed efficacy of Pycnogenol on the clinical symptoms of ADHD and on cognitive functions is associated with a rebalancing of the GM.

Summary:

- On the one hand, we will explore whether the composition of the GM differs in children with ADHD (6–12 years) compared with typically developing children, in order to identify a possible dysbiosis in subjects with ADHD.
- We will administer Pycnogenol to children with ADHD in order to evaluate its efficacy/supportive effect both on clinical symptoms and on cognitive functions assessed by standardised tests.

We will explore the extent to which the expected improvement in the clinical symptoms of ADHD and in the cognitive functions studied can be correlated with changes in the GM (rebalancing).

Expected results

- The first expected result is the replication of recent findings suggesting a different bacterial composition of the gut microbiota in subjects with ADHD.
- The second expected result is to confirm that Pycnogenol, as a dietary supplement, exerts a beneficial (supportive) effect on the improvement of ADHD.
- The third expected result is to identify which cognitive indices are the most sensitive to the beneficial effect of Pycnogenol (an aspect that has never yet been demonstrated).
- The fourth expected result is to demonstrate that Pycnogenol modulates the gut microbiota in patients with ADHD, by establishing a link between the rebalancing of the microbiota and the improvement in ADHD and in the cognitive indices highlighted in our study.
- The regulation of the microbiota-gut-brain axis by Pycnogenol constitutes the main originality of our research.

6. Method

This is a prospective interventional study including 2 groups of participants, namely children with ADHD (to whom Pycnogenol will be administered over a total period of 10 weeks) and typically developing children (without administration of Pycnogenol). Stool samples will be collected at several time points during follow-up in order to analyse the GM.

6.1 Participants

Children (6–12 years) with ADHD, combined presentation (n = 50), and typically developing children of the same age (n = 50). Patients with ADHD will be recruited at the Child and Adolescent Psychiatry Outpatient Clinic of the Hôpital Universitaire de Bruxelles (H.U.B), Erasme Medical Center site, under the supervision of Prof. Dr Massat. Typically developing subjects will be recruited through advertisements and the investigators' acquaintances.

The 6–12 year age range was chosen for the following reasons: it is the most frequent age for the diagnosis of ADHD; tolerability problems related to methylphenidate treatment; more reliable assessment of diet by the parents; good adherence (Pycnogenol administered/supervised by the parents).

6.2 Sample size calculation

The sample size is consistent with the existing literature in this field of research (Chovanová et al., 2006; Dvořáková et al., 2006; Trebatická et al., 2006; Verlaet et al., 2017; Weyns et al., 2022).

6.3 Inclusion criteria

French-speaking girls and boys, children with ADHD-combined presentation (n = 50) and neurotypical children (n = 50) aged 6 to 12 years inclusive. Consent of the children and of their legal representatives.

6.4 Exclusion criteria

Comorbid psychiatric disorders; anorexia nervosa, bulimia or significant eating disorders; lactose intolerance or cow's milk protein allergy; gluten intolerance; prematurity; known intellectual disability (IQ < 75); any serious chronic disease (inflammatory bowel disease, gastrointestinal disorders deemed incompatible with the conduct of the study, obesity, diabetes, joint disease, cancer, ongoing hepatic or

biliary disorders, or other chronic respiratory disorder, etc.); vitamin supplementation; consumption of dietary supplements (omega-3 fatty acids, probiotics, prebiotics, plant stanols/sterols) in the 4 weeks preceding the study; use of psychotropic treatments (e.g. methylphenidate or others), proton pump inhibitors, or antibiotic therapy within the previous 3 months. These exclusion criteria will be verified by history-taking.

6.5 Clinical assessments, behavioural rating scales and neuropsychological assessment

T0: inclusion

T1: week 1

T2: week 5

T3: week 10

6.5.1 Medical data

DSM-5 criteria for ADHD and child psychiatric examination confirming ADHD according to standardised international criteria, with exploration of clinical comorbidities (in order to exclude them) at T0 (inclusion), T2 (week 5) and T3 (week 10).

6.5.2 Questionnaires

- ADHD-RS IV (DuPaul et al., 1998) (a scale measuring the frequency of occurrence and the severity of ADHD symptoms), completed by the physician during the consultation (T0, T2, T3) in subjects with ADHD and at T1 in neurotypical subjects.
- The Physical Complaints Questionnaire (PCQ) (Pelsser & Buitelaar, 2002) assesses physical complaints and sleep disturbances in order to evaluate possible side effects; it will be administered at each visit, T2 and T3, in subjects with ADHD only.
- The Food Frequency Questionnaire (FFQ) (De Keyzer et al., 2013), intended to document the participants' dietary habits (a non-negligible factor in the study of the GM), will be administered at each visit (T1, T2, T3) in subjects with ADHD and at T1 in neurotypical subjects.
- Bristol Stool Scale (Heaton) (stool quality) (T1, T2, T3) in subjects with ADHD and at T1 in neurotypical subjects.

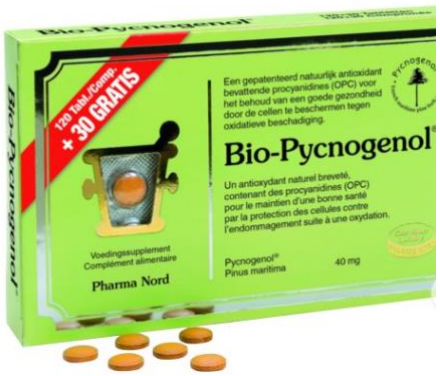
6.5.3 Cognitive assessment

Performed at T1 and T3 in subjects with ADHD and at T1 in typically developing subjects:

- The intellectual functioning of all participants will be assessed using the WISC-V (Wechsler Intelligence Scale for Children; Wechsler, 2003). An abbreviated IQ, referred to as the "estimated IQ", will be calculated (Crawford et al., 2010).
- Attentional and executive functions: the TAP attention test battery (Test of Attentional Performance; Zimmermann & Fimm, 2010) allows the computerised assessment of various cognitive parameters. In light of the existing scientific literature on cognitive profiles in ADHD (Khaledi et al., 2019; Pievsky & McGrath, 2018), the following subtests were selected: visual and auditory selective attention, divided attention, vigilance, flexibility, inhibition (Go/NoGo) and working memory.
- The Maudsley Index of Delay Aversion (MIDA; Kuntsi et al., 2001) will be used to assess delay aversion. In order to counter possible test-retest effects, all the neuropsychological tests mentioned above will be administered only twice (T0 and T3). Only the Go/NoGo and vigilance tasks, which have been shown to be relatively insensitive to test-retest effects, will also be administered during the study (T2) in children with ADHD.

7. Administration of Pycnogenol to subjects with ADHD

Visit T1. The dose will be adjusted to the weight of the child with ADHD according to current recommendations: 30 mg/day if < 30 kg, 40 mg/day if ≥ 30 kg and 50 mg/day if > 50 kg (20 mg/day during the first two weeks) (A. A. J. Verlaet et al., 2017; Weyns et al., 2022). Each subject with ADHD will receive the Pycnogenol tablets in a bottle. One tablet is to be taken per day in the middle of the evening meal (under parental supervision). Pycnogenol will be provided to patients free of charge (purchased by the study team from a parapharmacy) https://www.pycnogenol.com/company/



Summary of assessments

	T0 (inclusion)	T1 (week 1)	T2 (week 5)	T3 (week 10)
ADHD group	Confirmation of ADHD / DSM-5 criteria ADHD-RS	Cognitive assessment Stool sample Pycnogenol (start) FFQ Bristol Stool Scale	Rating scales DSM-5 criteria ADHD-RS PCQ FFQ Bristol Stool Scale Stool sample	Cognitive assessment Stool sample DSM-5 criteria ADHD-RS PCQ FFQ Bristol Stool Scale
Typically developing group	Exclusion of ADHD criteria ADHD-RS	Cognitive assessment Stool sample FFQ Bristol Stool Scale	—	—

8. Recruitment start and end dates

Between 01/08/2025 and 31/12/2028

9. Procedure, data collection for the GM (stool samples) and analysis

9.1 Stool collection

Stool samples for GM analysis will be collected at T1, T2 and T3 in subjects with ADHD and at T1 and T3 in typically developing children. Sample collection will be carried out "at home", in a container provided for this purpose.



https://www.dnagenotek.com/US/products/collection-microbiome/omnigene-gut-kit/OMR-200.100.html

The sample (which may be kept unfrozen for 60 days at room temperature in a stabiliser) will be brought to the consultation to be stored in a −80 °C freezer. Once all the samples have been collected, the analysis will be performed in a laboratory within the framework of an academic collaboration (see point 9.2).

Biobank

Biothèque of the Université catholique de Louvain, located at Avenue Emmanuel Mounier 50, 1200 Brussels, represented by Professor Etienne MARBAIX.

The stool sample will be destroyed after analysis of the gut microbiota, which makes its use in any other study impossible. Should additional analyses be required, they would be submitted to the Ethics Committee for approval.

9.2 Sequencing and analysis of the gut microbiota (GM)

The laboratory analyses of the GM and the statistical analyses will be performed within the framework of an academic collaboration with Prof. Dr Amandine Everard, PhD, Research Associate FRS-FNRS, WELBIO (Walloon Excellence in Life Sciences and Biotechnology), Louvain Drug Research Institute, Metabolism and Nutrition, UCL/LDRI/MNut, Av. E. Mounier 73, box B1.73.11, B-1200 Brussels, BELGIUM. Metagenomic DNA is extracted from faeces using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, with modifications. The V1–V3 region of the 16S rRNA gene is amplified using the following universal eubacterial primers: 27Fmod (5'-AGRGTTTGATCMTGGCTCAG-3') and 519Rmodbio (5'-GTNTTACNGCGGCKGCTG-3'). The purified amplicons are sequenced on an Illumina MiSeq. Sequence reads are demultiplexed and processed using the QIIME2 pipeline. Biostatistical analyses will be performed within this laboratory by bioengineers with expertise in this specific field.

9.3 Data collection, processing and protection

Data will be collected using the in-house REDCap software in the form of an eCRF, in which each participant's identification code will be requested. Variables containing personally identifiable information will be coded as "identifiers" and will not be exportable for data analysis. Data collection will be strictly limited to the data necessary for the research, and will be organised in such a way that re-identification of the patient by combining data is impossible.

Each participant's personal data will be processed by anonymisation and replaced by a code linking the subject's anonymised identification in the research to the patient's medical record. The code will contain no clue allowing the patient to be recognised. Only the principal investigator, Dr Isabelle Massat, will keep this database with the codes and correspondences, in the form of a password-protected Excel file kept on the Erasme site. For confidentiality reasons, the file will be destroyed as soon as the results are published.

Code construction

Subjects will be coded as follows:

A database under the exclusive responsibility of the PI (Isabelle Massat) containing the patients' surname, first name, date of birth and medical record number.

A CRF database containing only the code and the data collected.

Only the PI can link the two databases via the code number, should it be necessary to trace the patient.

10. Risks and benefits of the clinical research project

No risk is incurred, except possibly gastrointestinal signs such as cramps or diarrhoea, or a risk of allergy, as with any dietary supplement.

There can be no incidental findings. Microbiota analyses are group-level analyses and we will not need to examine cases individually; therefore no incidental anomalies will be brought to light. Furthermore, the global, non-targeted analysis of the microbiota is not of a kind to reveal a particular pathology (except Clostridium difficile, but this is unlikely given its characteristic clinical signs). There is no microbial signature that could be identified as an incidental finding of pathology.

11. Potential benefits for participants

- Advancement of knowledge on the GM in ADHD and in typically developing subjects.
- Pycnogenol, a dietary supplement provided to the patient free of charge. Demonstration of additional support for the improvement of ADHD that carries no drug-related risk.
- Advancement of knowledge on attentional and executive alterations in ADHD in relation to the GM.
- No procedure or examination will be charged to the patient beyond usual clinical practice (clinical follow-up consultations).
- The results of the cognitive tests (which form part of the standard multidisciplinary work-up for ADHD) will be provided to the family through the physician who usually coordinates the patient's care, or by the neuropsychologist who carried out the assessment, as in clinical practice.

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Sponsor and investigators

Sponsor: Hôpital Erasme / H.U.B

Name and affiliation of principal investigator (address and phone number)

Prof. Dr Isabelle Massat, Child and Adolescent Psychiatrist

- Research Associate (Chercheure Qualifiée) of the FRS-FNRS, Laboratory of Experimental Neurology, ULB
- Director of the Neurodevelopmental Disorders Clinic within the Department of Infant, Child, Adolescent and Young Adult Psychiatry, H.U.B, headed by Prof. Véronique Delvenne

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Other investigators

- Dr François Felgueroso-Bueno, general practitioner — francisco.felgueroso-bueno@ulb.be
- Barbara Luyens, neuropsychologist — barbaraluyens@gmail.com

Investigator's statement

I agree to conduct this clinical study in accordance with the design and specific provisions of this protocol and will only make changes in the protocol after notifying the sponsor.

I understand that I may terminate or suspend enrolment of the study at any time if it becomes necessary to protect the best interests of the study subjects. This study may be terminated by CUB – Hôpital Erasme, with or without cause.

I agree to personally conduct or supervise this investigation and to ensure that all associates, colleagues, and employees assisting in the conduct of this study are informed about their obligations in meeting these commitments.

I will conduct the study in accordance with Good Clinical Practice, the Declaration of Helsinki, and the moral, ethical and scientific principles that justify medical research. The study will be conducted in accordance with all relevant laws and regulations relating to clinical studies and the protection of patients.

I will ensure that the requirements relating to Ethics Committee review and approval are met. I will provide CUB – Hôpital Erasme with any material which is provided to the EC for Ethical approval.

I agree to maintain adequate and accurate records and to make those records available for audit and inspection in accordance with relevant regulatory requirements.

I agree to promptly report to the EC any changes in the research activity and all unanticipated problems involving risks to human subjects or others. Additionally, I will not make any changes in the research without EC approval, except where necessary to ensure the safety of study participants.

Isabelle Massat	(signed in the original)	**22/08/2025**
Name	Signature	Date

Budget

- DNA Genotek kits (EUR 4,600 for 300 sample kits) (estimated by quotation)
- Gut microbiota analyses in the laboratory: DNA extraction and 16S sequencing (150 × 250 = EUR 37,500) (EUR 150 being a preferential price per sample, thanks to our scientific collaboration with the laboratory; corresponds to half price)
- Meeting registrations and travel costs for participation in an international congress (once a year) and national meetings
- 4 international congresses (registration is usually around EUR 500, plus travel and accommodation): EUR 6,000
- 1 computer + 1 additional screen + printer + printer ink cartridges and small office equipment: EUR 2,500
- Publication costs (4 manuscripts × 3,500) = EUR 16,000
- Pycnogenol (boxes of 120 tablets at EUR 60; 50 × 60 = EUR 3,000)

Total: EUR 69,600

- + 15% administrative costs (EUR 10,440)

Total: EUR 80,040

Funding obtained: Fund Claude & Marie-Hélène Lelièvre-Meurée, for an amount of EUR 82,915 for this project.