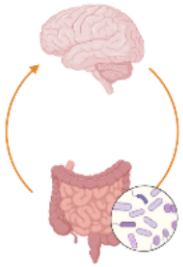
Hôpital Erasme – Hôpital Universitaire de Bruxelles (HUB)
Department of Infant, Child, Adolescent and Young Adult Psychiatry
Clinic for Neurodevelopmental and Learning Disorders



Role of the gut-brain axis in ADHD: Exploring the links between gut microbiota, cognition and symptomatology

Clinical research protocol

Prospective, interventional, single-centre study

English version – translated from the French original signed on 20/12/2024

Review date: 11 April 2025

Principal Investigator	Prof. Dr Isabelle Massat – Child Psychiatrist, Senior Research Associate (FRS-FNRS); Director of the Clinic for Neurodevelopmental and Learning Disorders, HUB
Investigators	Barbara Luyens – Neuropsychologist, PhD candidate Dr François Felgueroso-Bueno – General practitioner, PhD candidate Prof. Dr Véronique Delvenne – Head of the Department of Infant, Child, Adolescent and Young Adult Psychiatry, HUB
Sponsor	Hôpital Erasme – Hôpital Universitaire de Bruxelles (HUB)
Ethics Committee approval	CCB B4062025000026 – Ethics Committee, Hôpital Erasme, Brussels

Title of the clinical research project:

Role of the gut-brain axis in ADHD: Exploring the links between gut microbiota, cognition and symptomatology

Promoter/sponsor of the clinical research project: Hôpital Erasme – Hôpital Universitaire de Bruxelles (HUB) – Department of Infant, Child, Adolescent and Young Adult Psychiatry

Investigators:

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Type of research project:

Prospective, interventional, single-centre

Research project number:

PROTOCOL

1. Introduction

1.1. ADHD

Attention-Deficit/Hyperactivity Disorder (ADHD) is the most prevalent neurodevelopmental disorder in child and adolescent psychiatry (8% of the world population) (Akmatov et al., 2021; Polanczyk et al., 2014), making it an unquestionable public health priority. Characterised by developmentally inappropriate levels of inattention, hyperactivity and impulsivity (APA, 2013), ADHD is also associated with executive and attentional cognitive deficits (Khaledi et al., 2019; Kofler et al., 2013; Pievsky & McGrath, 2018; Sjöwall et al., 2013; Willcutt et al., 2005). These deficits lie at the heart of the various theoretical models of ADHD developed over the past 25 years, which converge on the predominant involvement of executive and attentional functions in the symptomatology of the disorder (Barkley, 1997; Pennington & Ozonoff, 1996; Sonuga-Barke, 2002). Its aetiopathogenesis is considered multifactorial with complex determinism, but it must be acknowledged that, to date, decades of research have been disappointing, since neither the specific genetic nor the specific environmental factors have been formally identified (Akutagava-Martins et al., 2016; Faraone et al., 2015).

Exploring a possible imbalance in the composition of the gut microbiota, induced by diet or other environmental factors, constitutes a new and original research approach to better understand the aetiopathophysiology of ADHD (Cenit et al., 2017; Ceylan et al., 2012) and to identify very promising avenues for the development of new prevention and treatment strategies.

1.2. The gut-brain axis

The digestive system, also known as the “second brain”, contains 100 million neurons distributed along its length, forming part of the “autonomic nervous system”. It maintains bidirectional relationships with the central nervous system, playing a role in the control of functions such as the regulation of digestive motility, nutrient absorption and the control of the intestinal barrier that protects against external pathogens (Cryan & Mazmanian, 2022; Cussotto et al., 2019). Recent research on the gut microbiota (GM) has brought about a dramatic advance in our understanding of the highly complex balance between the micro-organisms that compose it and its close mutualistic relationships with our body. In addition to its role in general health, it is involved in modulating 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 managing stress or emotions, such as serotonin, or dopamine precursors, which are involved in concentration and in ADHD (Aarts et al., 2017).

The GM can be damaged (or, conversely, rebalanced) by external factors (diet, medication, stress, age, environment, etc.) that influence bacterial quantity, richness, diversity and composition. Alterations of the gut microbiota and the emergence of bacterial signatures have been consistently reported in the literature across various neurological, neurodevelopmental, neurodegenerative and neuropsychiatric disorders (Caputi et al., 2024; Lacorte et al., 2019; Mathee et al., 2020; You et al., 2024; Zimmermann-Rösner & Prehn-Kristensen, 2024). With regard to ADHD, microbiota research remains limited (N. Wang et al., 2022). The fact that individuals with ADHD suffer from gastrointestinal dysfunction, notably digestive difficulties during childhood and low-grade

inflammation, as well as constipation, suggests a potential role of the gut microbiome in this disorder (Cickovski et al., 2023; Duel et al., 2003; Ming et al., 2018). Moreover, dietary interventions are attracting growing interest for their potential to alleviate ADHD symptoms, possibly by reducing subclinical allergic reactions or inflammation, since children with ADHD show increased susceptibility to allergies (Bull-Larsen & Mohajeri, 2019; Turiaco et al., 2024). These observations thus suggest an involvement of the gut microbiota in ADHD and its associated clinical manifestations (Mathee et al., 2020).

To date, around ten studies have compared the gut microbiota of individuals with ADHD with control groups, i.e. typically developing individuals (Aarts et al., 2017; Jiang et al., 2018; Lee et al., 2022; Panpetch et al., 2024; Prehn-Kristensen et al., 2018; Richarte et al., 2021; Steckler et al., 2024; Szopinska-Tokov et al., 2020; Wan et al., 2020; L.-J. Wang et al., 2020; N. Wang et al., 2024; Zhou et al., 2021). Taken together, these studies reveal innovative findings on the GM in ADHD, highlighting significant variations in bacterial composition, diversity and richness of the GM between individuals with ADHD and control groups. These differences open promising new perspectives, suggesting that the GM could play a decisive role in understanding, and perhaps even treating, ADHD. A recent review by Sukmajaya et al. (2021) identified 49 bacterial taxa showing significant differences between individuals with ADHD and typically developing individuals, including genera such as *Bifidobacterium*, *Dialister* and *Faecalibacterium*. Two meta-analyses (Payen et al., 2022; N. Wang et al., 2022) have also reported significant differences in microbial diversity in people with ADHD. These data constitute a solid and encouraging lead, justifying further research to better understand the involvement of the GM in the pathophysiology of ADHD.

However, methodological discrepancies and biases in these studies still prevent a consensus from being reached on the existence of a definitive association between GM imbalance and ADHD, as well as on the identification of a possible specific bacterial signature (Sukmajaya et al., 2021).

In addition, several studies have reported links between the severity of ADHD symptoms and variations in the gut microbiota (Jiang et al., 2018; Panpetch et al., 2024; Prehn-Kristensen et al., 2018; Szopinska-Tokov et al., 2020; L.-J. Wang et al., 2020). These studies identified certain bacterial species and genera associated with the intensity of inattention and/or hyperactivity/impulsivity symptoms, as reported by others (parents and/or teachers) or by the individuals themselves. For example, Szopinska-Tokov et al. (2020) observed a correlation between the abundance of the genus *Ruminococcus* and the severity of inattention symptoms, while L.-J. Wang et al. (2020) found an association between the abundance of two other bacterial genera and the intensity of hyperactivity and impulsivity symptoms.

Although results vary across studies and symptom categories, these observations have important clinical implications. They suggest that the gut microbiota may play a role in the manifestation of ADHD and that symptomatology could be favourably influenced by rebalancing the microbiota, or even by therapies targeting specific bacteria (Soltysova et al., 2022).

The studies mentioned above explored the gut-brain axis mainly on the basis of clinical questionnaires, which are subjective tools liable to be influenced by biases related to the respondent and to the observation conditions (Power et al., 1998; Tripp et al., 2006). It is likely that assessing the bidirectional interactions between ADHD and the GM using simple behavioural scales does not allow the nature of these links to be captured rigorously and with sufficient specificity.

Indeed, cognitive deficits, particularly in the executive and attentional domains in ADHD (Pievsky & McGrath, 2018), are not taken into account in these approaches. To date, no study has investigated the links between GM composition and cognitive performance assessed by standardised neuropsychological tests in subjects with ADHD compared with typically developing individuals.

Although these initial investigations are very promising, the field of research on interactions between the GM and ADHD is still in its infancy and requires more in-depth studies with more methodologically rigorous approaches (Jurek et al., 2021; N. Wang et al., 2022). The current challenge is to better understand the links between the microbiota and cognitive functions, particularly those identified as impaired in ADHD and considered central to its symptomatology: divided and selective attention, inhibition, cognitive flexibility, working memory, vigilance, delay aversion, and reaction time variability (Khaledi et al., 2019; Kofler et al., 2013; Pievsky & McGrath, 2018; Sjöwall et al., 2013; Willcutt et al., 2005). It is precisely in this respect that our study stands out and finds its originality, by seeking to explore the interactions between the GM and cognitive functioning in ADHD.

2. Hypotheses

We hypothesise that (1) children with ADHD will have a GM composition significantly different from that of typically developing (TD) children, in terms of diversity and composition; (2) children with ADHD will show significantly lower attentional and executive performance than TD children, and this performance will be associated with the participants' GM composition; (3) finally, the GM of children with ADHD will be associated with symptom severity as reported by clinical scales and, above all, by clinical medical observation.

3. Objectives

- ➢ First, we will explore whether GM composition differs in children with ADHD (6–12 years) compared with TD children, in order to identify a possible imbalance and/or bacterial signature in ADHD subjects.
- ➢ Second, we will compare the cognitive performance of the two groups (ADHD vs. TD) on attentional and executive tasks, and then examine their associations with GM composition.
- ➢ Third, we will also explore the correlations between the severity of inattention and hyperactivity/impulsivity symptoms and GM composition.

4. Method

This is a prospective, interventional case-control comparison study including 2 groups of participants: 70 children with ADHD and 70 typically developing children. The data collected are as follows: a stool sample for gut microbiota analysis; a neuropsychological assessment of attentional and executive functions; a clinical rating scale of ADHD symptom severity (completed by parents and the physician); a dietary habits scale; and a clinical assessment of symptoms and their severity.

4.1. Participants

Children (6–12 years) with ADHD, combined presentation (n = 70), and age-matched typically developing children (n = 70). ADHD patients will be recruited from the Child Psychiatry Outpatient Clinic of the Hôpital Universitaire de Bruxelles (HUB) at both sites (Erasme and HUDERF), under the supervision of Dr Massat. Typically developing subjects will be recruited through advertisements and the investigators' personal networks.

The 6–12 age range was chosen for the following reasons: it is the most common age for ADHD diagnosis, and parents can assess diet more reliably.

4.2. Sample size calculation

To determine the sample size required for our study, we performed an a priori power analysis to compare the mean between two independent groups using a two-tailed test. Input parameters included a medium effect size (d = 0.5), a significance level (α) of 0.05 and a statistical power of 0.80 (80%). The results of this analysis show that the total number of participants required is 128, divided equally between the two groups, with 64 participants per group. This sample size ensures a sufficient probability of detecting a statistically significant difference between the two groups, while controlling the risk of type I error at the defined threshold. As the drop-out rate is generally 5 to 10%, the total number of subjects needed is approximately 140 participants (i.e. 70 per group).

The sample size is consistent with the existing literature in this field of research (Aarts et al., 2017; Jiang et al., 2018; Lee et al., 2022; Panpetch et al., 2024; Prehn-Kristensen et al., 2018; Richarte et al., 2021; Steckler et al., 2024; Szopinska-Tokov et al., 2020; Wan et al., 2020; N. Wang et al., 2024; Zhou et al., 2021).

4.3. Inclusion criteria

French-speaking girls and boys: children with ADHD, combined type (n = 70), and typically developing children (n = 70), aged 6 to 12 years inclusive. Consent of the children and their legal representatives.

4.4. Exclusion criteria

Prematurity (< 37 weeks); known intellectual disability (IQ < 75); psychiatric disorders; presence of a serious chronic disease (e.g. cancer, HIV, renal failure, ongoing hepatic or biliary disease, chronic inflammatory bowel disease, inflammatory bowel disease, irritable bowel syndrome or other chronic respiratory disorder, etc.) or of gastrointestinal disorders deemed incompatible with the conduct of the study by the investigator (e.g. coeliac disease); use of dietary supplements (omega-3 fatty acids, probiotics, prebiotics, plant stanols/sterols) in the 4 weeks preceding the study; anorexia nervosa, bulimia or significant eating disorders according to the investigators; lactose intolerance or milk protein allergy; gluten intolerance; use of antibiotics in the 3 months preceding the study; proton pump inhibitors; any psychotropic treatment in the 3 months preceding the study (methylphenidate and others).

4.5. Clinical assessments, behavioural scales and neuropsychological assessment

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).

- **ADHD-RS IV** (DuPaul et al., 1998) (a scale measuring the frequency and severity of ADHD symptoms), completed by the physician during the consultation.
- The **Food Frequency Questionnaire (FFQ)** (De Keyzer et al., 2013), designed to document participants' dietary habits (a non-negligible factor in GM research), to be completed 1 week before the visit and on the day before the visit.

Cognitive assessment carried out with all participants:

- The intellectual functioning of all participants will be assessed using the **WISC-V** (Wechsler Intelligence Scale for Children; Wechsler, 2003). A short-form IQ, referred to as "estimated IQ", will be calculated (Crawford et al., 2010).
- Attentional and executive functions: the **TAP** attentional test battery (Test of Attentional Performance; Zimmermann & Fimm, 2010) allows 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 have been 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.

DuPaul, G. J., Power, T. J., Anastoupolous, A. D., & Reid, R. (1998). New York, NY: Guilford.

De Keyzer, W. et al. (2013). *European Journal of Public Health*, 23(5), 737–742.

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Kuntsi, J., Stevenson, J., Oosterlaan, J. and Sonuga-Barke, E. J. S. (2001). *British Journal of Developmental Psychology*, 19, 339–348.

Protocol – summary:

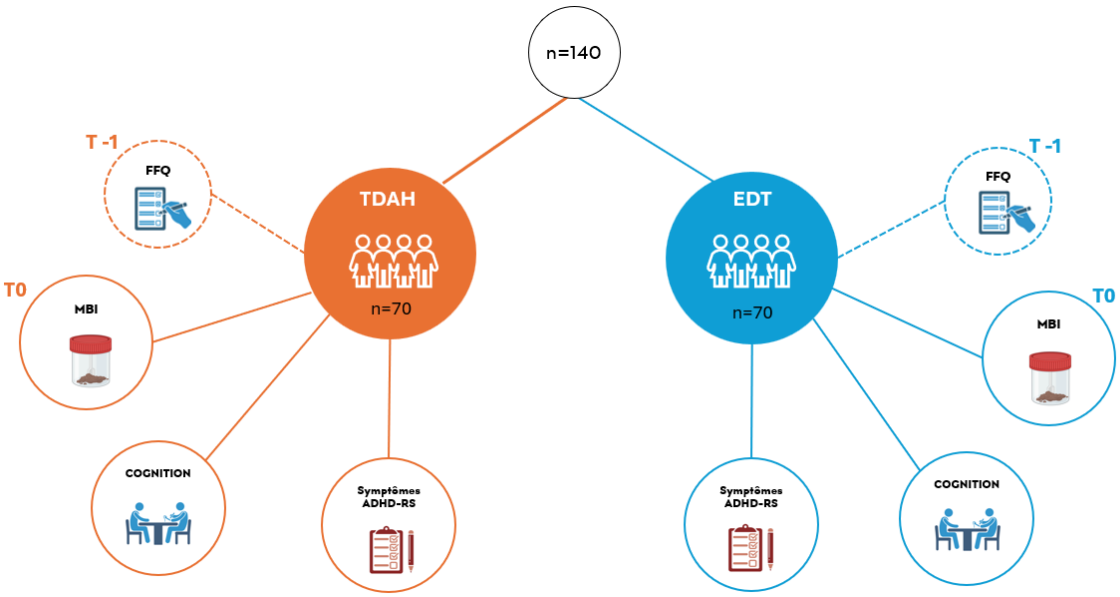


Figure 1. ***Protocol.*** ADHD-RS = ADHD Rating Scale IV. EDT (in figure) = TD, typically developing children. FFQ = Food Frequency Questionnaire. MBI (in figure) = GM, gut microbiota. TDAH (in figure) = ADHD. "Symptômes" = symptoms. T-1 = day before the visit; T0 = day of the visit.

4.6. GM data collection (stool)

Stool for gut microbiota analysis will be collected on the day of the visit (or the day before). The sample will be collected "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 can remain unfrozen for 60 days at room temperature in a stabiliser) will be brought to the consultation to be stored in a freezer at −80 °C. Once all samples have been collected, the analysis will be performed in a laboratory within the framework of an academic collaboration (see section 4.8).

4.7. Recruitment start/end date

Between 01/01/2025 and 31/12/2027.

4.8. Sequencing and analysis of the gut microbiota (GM)

Laboratory analyses of the GM and statistical analyses will be carried out 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 stool 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′). Purified amplicons are sequenced using 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.

5. 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 carried out in such a way that re-identification of the patient by linking data is impossible.

Each participant's personal data will be processed by anonymisation: they will be replaced by a code linking the subject's anonymous 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 stored on the Erasme site. For confidentiality reasons, the file will be destroyed as soon as the results are published.

6. Risks and benefits of the clinical research project

No risk incurred.

7. Potential benefits for the persons from whom the HBM is derived

Advancement of knowledge on the GM in ADHD and in typically developing subjects. Advancement of knowledge on attentional and executive impairments 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 (free of charge) will be given 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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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 on the original French version]*	20/12/2024
Name	Signature	Date