The gut-brain-axis in adult externalizing behavior

How brain functioning, diet and the gut-microbiome may influence adult ADHD and reactive aggression

Babette Jakobi

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Author: Babette Jakobi
Title: The gut-brain-axis in adult externalizing behavior. How brain functioning, diet and the gut-microbiome may influence adult ADHD and reactive aggression.

Radboud Dissertations Series
ISSN: 2950-2772 (Online); 2950-2780 (Print)

Published by RADBOUD UNIVERSITY PRESS
Postbus 9100, 6500 HA Nijmegen, The Netherlands
www.radbouduniversitypress.nl

Design: Proefschrift AIO | Katarzyna Kozak
Cover: Proefschrift AIO | Guntra Laivacuma
Printing: DPN Rikken/Pumbo

ISBN: 9789493296565
DOI: 10.54195/9789493296565
Free download at: www.boekenbestellen.nl/radboud-university-press/dissertations

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The gut-brain-axis in adult externalizing behavior

How brain functioning, diet, and the gut-microbiome may influence adult ADHD and reactive aggression

Proefschrift ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. dr. J.M. Sanders,
volgens besluit van het college voor promoties
in het openbaar te verdedigen op

vrijdag 21 februari 2025
om 12.30 uur precies

door

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Dissertation to obtain the degree of doctor
from Radboud University Nijmegen
on the authority of the Rector Magnificus prof. dr. J.M. Sanders,
according to the decision of the Doctorate Board
to be defended in public on

Friday, February 21, 2025
at 12.30 pm

by

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Chapter 1

General Introduction

The aim of this thesis was to gain a better understanding of adult externalizing behaviors such as attention-deficit/hyperactivity (ADHD) and reactive aggression by investigating the gut-microbiome-brain axis. Understanding the contribution of diet and the gut microbiota to brain development and functioning may open up avenues to develop targeted interventions and reduce adverse outcomes. This thesis describes the role of 1) altered brain functioning during emotion processing, 2) robust alterations in the gut-microbiome composition, and 3) the complex interplay of diet and the gut-microbiota for adult ADHD and reactive aggression. The general introduction provides an overview of ADHD and reactive aggression, the gut-microbiome-brain axis, and diet, and it provides an outline of the following chapters of this thesis.

WHAT IS ADHD?

Attention-deficit/hyperactivity disorder (ADHD) is a common neurodevelopmental condition with a worldwide prevalence of around 5.9% in children and 2.5% in adults [3]. It is characterized by developmentally inappropriate symptoms of inattention (e.g. failure to complete tasks, difficulty concentrating, distractibility) and hyperactivity/impulsivity (e.g. impatience, restlessness, fidgeting, excessive talking) [2]. The symptoms observed in ADHD are present on a continuum within the general population, and can impact the daily functioning of affected individuals. Experiencing excessive ADHD symptoms and impairment can result in a clinical diagnosis, see **Box 1** 'ADHD diagnosis and treatment' [4]. The clinical presentation of ADHD is heterogeneous; impairment, hyperactivity/impulsivity, and inattention symptoms can vary greatly between individuals. While ADHD symptoms often occur during childhood or early adolescence, they can change across the individual's lifespan, be more or less present in different phases, or gradually diminish over time. However, in more than half of the children with ADHD, symptoms and impairment persist into adulthood [5]. Risk associated with ADHD, including lower educational attainment, unemployment, social problems, and even premature death, causes an annual global economic burden of hundreds of billions of dollars, making ADHD a long-term strain on affected individuals, society and healthcare systems [3, 4].

Diagnosis and Treatment of adult ADHD

ADHD is diagnosed in a clinical interview (e.g. Diagnostic Interview for ADHD in adults [1]). Current and past symptoms of inattention and hyperactivity/impulsivity are assessed and evaluated according to DSM-5 criteria [2]:

- > 5 symptoms in at least one symptom domain in adults (> 6 in children)
- Present before the age of 12 and across settings (e.g. school, home, ...)
- Impairment in at least one of five domains of life including occupation, social functioning and self-image

Treatment of ADHD typically follows a multi-modal approach including:

- Psychoeducation
- Cognitive Behavioral Therapy
- Pharmacotherapy (e.g. targeting dopamine or noradrenaline reuptake)
- Lifestyle modifications: regular exercise, healthy diet, sufficient sleep, structured routines [3]

Box 1. ADHD Diagnosis and Treatment [1, 2].

The etiology of ADHD is not yet fully understood, but genetic predisposition as well as environmental influences play a role. ADHD is highly heritable, with a suggested complex and polygenic genetic make-up [3]. Findings of genetic studies converge on common biological pathways with a role in the development of the nervous system (neurodevelopment), the communication between neurons using messenger molecules (neurotransmission, e.g. dopamine and serotonin) [6], and immune system regulation [7]. Known environmental risk factors for the development of ADHD include pre- and perinatal toxin exposure (e.g. smoking or alcohol), maternal stress, and malnutrition; these factors can directly affect the developing nervous system or influence gene-expression (e.g. through epigenetic mechanisms) [8, 9]. Also later in life, environmental changes, e.g. in diet and lifestyle, have been shown to modulate ADHD symptoms, presenting potential non-invasive targets for treatment support [10, 11]. ADHD frequently co-occurs with non-mental conditions concerning the metabolism (e.g. obesity) and immune reactions (e.g. asthma and eczema), but also neurodevelopmental and psychiatric comorbidities such as autism spectrum disorder, substance use disorders, anxiety and depression, as well as behavioral problems are frequent [4, 12]. One of the most frequently co-occurring behavioral issues, particularly in adults, is emotion dysregulation. Up to 70% of adults with ADHD are affected by problems with emotion regulation [13]. While emotional symptoms are not part of the core diagnostic criteria, they have recently been recognized as a characteristic feature of ADHD [2, 14]. They can range from emotional lability or irritability up to more severe and impairing forms of emotion dysregulation, like reactive aggression [13, 15].

WHAT IS REACTIVE AGGRESSION?

Reactive aggression describes aggressive behavior in response to an emotional stimulus, such as a perceived social threat, provocation, or frustration [16]. Situation-appropriate reactive aggression serves evolutionary purposes like defending boundaries. Elevated reactive aggression, however, as observed in the context of ADHD, is often maladaptive and has negative consequences for the individuals and their environment [17]. In theory, reactive aggression can result from disturbances during emotion generating and emotion regulating stages of emotion processing: Events that are perceived relevant for an individual's wellbeing or resources are intrinsically salient and can elicit an intrinsic emotional response (e.g. arousal). Situation-appropriate responses require the appropriate perception and recognition of the event (e.g. considering the context, including social and self-referential processes), as well as an adequate regulation of the emotional response (e.g. impulse control, re-appraisal) [18]. In reactive aggression, inappropriate assignment of attention, salience, or meaning towards an event can result in an exaggerated intrinsic response (hyperreactivity). Similarly, a lack of reappraisal or impulse control can result in aggressive reactions [17]. Imagine being pushed in a busy shopping street. This situation might be perceived as an insult, resulting in feelings of anger and irritation, and, if not regulated properly, in reactive aggression.

Reactive aggression can be investigated by using questionnaires, in which examples of reactive aggressive tendencies and behaviors are scored, according to how much a person identifies with them, e.g. the *reactive and proactive aggression* questionnaire (RPQ [19]). Individuals with high levels of aggression and ADHD have considerably worse social and occupational prognoses than those with only either aggression or ADHD [20-22]. In addition to these adverse effects, reactive aggression is associated with suicidal thoughts and attempts, which might be aggravated by symptoms of hyperactivity and impulsivity [23]. Individuals presenting ADHD with comorbid reactive aggression often suffer from peer rejection during childhood and adolescence, unstable relationships with friends and family, poor school performance, have problems securing and/or maintaining employment, and are at risk of engaging in criminal activities [23-27]. Yet, treatment strategies for ADHD often do not target and sufficiently ameliorate aggressive symptoms [28, 29]. Despite the large proportion of adults affected by ADHD and emotion dysregulation such as reactive aggression, and the previously described clinical and societal relevance of these emotional problems, little is known about the etiological basis of their co-occurrence. Genetic findings on reactive aggression suggest alterations of endocrine systems, e.g. influencing the hypothalamic-pituitary-adrenal (HPA)-axis related to stress-responses, and sex hormonal influences; this may partly explain the observed sex-differences in aggressive behavior [30, 31]. Similar to ADHD, altered neurodevelopment and monoamine neurotransmission, resulting in altered brain functioning, have been suggested for the etiology of emotional dysregulation [32]. This overlap, and the high genetic correlations observed between ADHD and aggression and antisocial behaviors [31], point towards higher genetic susceptibility of individuals with ADHD to develop aggressive behaviors and shared biological pathways. One mechanism that might be shared between ADHD and reactive aggression is related to brain development and brain functioning.

Reactive and proactive aggression are often studied in contrast to each other, e.g. with the Reactive-Proactive Aggression Questionnaire [15]. The two types of aggressive behavior differ in their underlying motives and triggers as well as partly separate brain circuits involved. Reactive aggression is impulsive, emotionally-driven, triggered by a perceived threat or provocation. Proactive aggression, on the other hand, is a more premeditated and goal-directed type of aggression that is motivated by a desire to achieve some specific goal, such as gaining status, power, or resources.

THE BRAIN

For decades, psychological research has focused on the brain as the anatomical center of the human mind, emotion, and cognition [33]. The brain processes and saves our experiences and determines our behavior.

Neurodevelopment in ADHD

For ADHD and its onset early in life, altered neurodevelopment is thought to play a causal role. Large-scale neuroimaging studies have shown subtle structural differences between children with and without ADHD: Subcortical structures, such as the nucleus accumbens, amygdala, caudate nucleus, hippocampus, and putamen were smaller in children with ADHD [34]. Additionally, cortical thickness and surface area were reduced in children with ADHD, mainly in (pre-)frontal, cingulate, and temporal regions [35]. While some structural differences were less pronounced, reduced overall subcortical volumes, and overall and regional cortical surface area (e.g. in the middle and inferior temporal gyri (MTG, ITG), pre- and postcentral gyri, anterior and posterior cingulate cortex (PCC, ACC), and the cuneus) were also reported in large population-based samples of children and adolescents [36, 37]. The notably small effect sizes might reflect the phenotypical heterogeneity observed in ADHD: Similar to the presentation and severity of symptoms, brain structural alterations may not be present in all children and vary between individuals or subgroups with different (neuro-)developmental trajectories [38]. More pronounced symptoms in case-control studies compared to population-based studies may contribute to different results. The subtle morphological differences observed in childhood and early adolescence further seem to diminish or normalize over time, suggesting altered patterns of brain maturation in ADHD. Indeed, the pattern of structural alterations in adult ADHD is less clear; Several large-scale studies found no brain structural differences in adulthood, despite continued behavioral ADHD [34, 35]. Brain structural and genetic studies, although well-powered, only explain small fractions of phenotypic variance of externalizing behaviors like ADHD [31, 39]. Investigations of brain function and interactions with the environment can complement these models and bear the potential for the development of treatment targets.

Brain functioning in ADHD

In contrast to brain structural alterations, altered brain functioning is often reported across developmental periods in ADHD and aggression research. Functional studies therefore are particularly suitable to study persistent /adult phenotypes. Fluctuations in the activity and connectivity of particular brain regions can be measured in functional magnetic resonance imaging (*fMRI*). Resting-state fMRI measures the intrinsic functional organization of brain networks during rest, while task-based fMRI measures brain activity or connectivity between regions relevant for a particular cognitive domain or task (e.g. working memory or emotion processing). The most prominent findings across fMRI studies in ADHD report lower connectivity of frontoparietal and ventral attention networks, relevant for response inhibition and the redirection of attention, in combination with a hyperactive or disconnected default mode network, which is associated with self-referential processes and social/emotional evaluation [40]. Task-based fMRI studies report differences in cingulate and (pre-)frontal cortex (PFC) activity, and differences in its connectivity to subcortical regions (e.g. basal ganglia, striatum) [36, 40-43]. While most studies and meta-analyses focus on working memory, attention, and response inhibition, a smaller number of individual studies have investigated brain functioning during emotion processing in ADHD. Altered activity profiles of pre- and orbitofrontal, temporo-parietal, insular, occipital and cingulate cortices as well as subcortical regions like the striatum, hippocampus, and amygdala have been reported in children with ADHD [42, 44, 45]. Altered amygdala reactivity and less connectivity with prefrontal areas have been reported in children and adolescents with ADHD when presented with emotional faces [44, 46, 47]. These patterns have also been associated with emotional lability and severe mood dysregulation in ADHD [48, 49], and they might play a role in reactive aggressive behaviors.

Brain functioning in reactive aggression

Disruptions at different stages of emotion processing relevant for reactive aggression may be represented by different sets of brain regions. As an example, activity in subcortical structures like the amygdala are associated with assessment of salience and risk (does an event require attention, is it threatening/rewarding?) and the generation of an intrinsic emotional response [50]. Exposed to threat or frustration, amygdala activity increases [50]. Hyperreactivity of the amygdala, as part of the limbic system, initiates the so-called fight or flight response. Resulting physiological arousal can provoke defensive or aggressive behavior [50]. Regions like the prefrontal or anterior cingulate cortex can modulate these responses: they are relevant for emotional learning, cognitive control, response inhibition, and behavioral adaptation [50]. Accordingly, the working model of reactive aggression summarizes relevant brain regions according to their function in 1) emotion-generating processes related to threat sensitivity and frustration (amygdala, caudate nucleus, hypothalamus, ventral striatum, and periaqueductal gray) and 2) emotion regulation through response inhibition and social and self-referential processes (ACC, PFC, and OFC, but also inferior frontal gyrus, insula, and inferior parietal regions) [50]. In children with ADHD, high aggression was associated with less cognitive control (reflected in the ACC), and less influence of social cognition, reflected in lower temporo-parietal activity and deviating middle temporal, insular, and striatal activity [51]. Some brain regions reported to be functionally relevant for both ADHD and reactive aggression are illustrated in **Figure 1**.

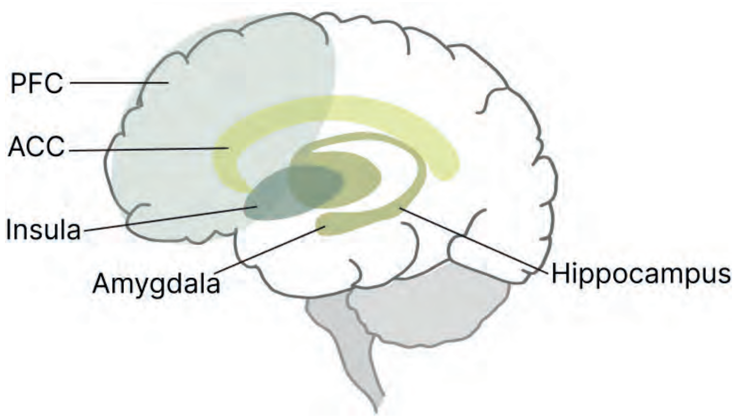

Figure 1. Illustration of brain regions functionally implicated in ADHD and reactive aggression, including the prefrontal cortex (PFC), the anterior cingulate cortex (ACC), the insula, amygdala, and the hippocampus.

Notably, the reports on brain structural and functional alterations are often inconsistent, and the localization and direction of effects vary across studies and tasks [42, 43]. Results, in particular citing the amygdala or striatum, might be biased by region of interest (ROI) approaches. Neuroimaging studies of emotion processing in adults with ADHD and emotionally dysregulated behavior are entirely lacking [42]. It is still unclear if alterations of brain functioning during emotion processing diminish in adulthood similar to the morphological brain alterations, and how altered brain functioning in adult ADHD is related to reactive aggression. While brain functioning is assumed to generate and modulate behaviors like aggression, our behavior and environment also have strong influences on brain functioning and neurodevelopment. One way in which environment and lifestyle can have an effect on brain functioning and behavior is through the gut-microbiome.

MRI or magnetic resonance imaging is a technique implemented to produce detailed images of internal structures of the body. MRI scanners use the magnetic field to align the protons in the body's atoms and disturb this alignment temporarily with radio waves. Different tissues of the body realign differently, which can be picked up and is translated to contrasts. In functional MRI, changes in blood flow, transporting oxygen, and nutrients to active neurons are assumed to reflect more brain activity. The differential magnetic properties of deoxygenated blood are then used to capture blood flow fluctuations in the brain during different tasks across time.

THE GUT-MICROBIOME

Humans are in constant interaction with microorganisms, like bacteria, fungi, and viruses. On average, 39 trillion of these so-called *microbiota* live in and on the human body [52]. The community of genetic material of those microorganisms in the digestive tract is called the *gut-microbiome*, a complex ecosystem of genomes, highly specific and uniquely adapted to each individual. This community changes across time based on natural aging, host genetics, environmental influences, such as changes in lifestyle factors, including diet and medication use [53].

As an essential part of the digestive tract, the gut-microbiota evidently play an important role in digestion. They can metabolize food that the human body cannot digest otherwise, such as complex carbohydrates and fiber. Thus, the gut-microbiome modulates the bioavailability of important nutrients and the energy household, but also key-signaling molecules such as neurotransmitters (e.g. dopamine and serotonin) or amino-acids (e.g. tryptophan) are produced by gut-microbiota [54]. As previously mentioned, these neurotransmitters are integral parts of healthy brain functioning and have been linked to ADHD [6] and aggressive behaviors [32].

The influence of the gut-microbiome on brain development, functioning, and behavior is often summarized as the so-called gut-brain axis [55]. The gut-brain axis describes the complex bidirectional communication between the brain and the gut-microbiome through a variety of functions, see **Figure 2** for an illustration. Microbial metabolites can interfere, for example, with the endocrine system (in particular the hypothalamic-pituitary-adrenal (HPA) axis, impacting stress responses), the enteric nervous system, and the immune system. From early in life, the gut-microbiome unfolds important functions for the human immune system: With colonization at birth it imprints and trains the developing immune system [56]. Also across the lifespan, gut-microbiota can regulate immune responses via e.g. immune cell activation, by stimulating the release of pro-inflammatory cytokines, and through the production of anti-inflammatory short-chain fatty acids (SCFAs). Through SCFA synthesis, which can regulate so-called tight junctions in the epithelium, and through mucosal layer maintenance, the gut-microbiome can influence the gut-barrier permeability. The gut-barrier prevents potential pathogens from entering the blood stream, having a substantial impact on infections and inflammation [56]. Similar effects of microbial metabolites like SCFAs on tight junctions are observed on the blood-brain barrier [57]. Among other effects, the modulation of the blood-brain-barrier integrity can lead to the activation of microglia, which can result in neuroinflammation, neuronal dysfunction, and cell death in the brain [57]. The gut-microbiome has been reported to impact neurodevelopment, where influences on gene-expression and epigenetic modification or the modulation of proteins like the brain-derived neurotrophic factor (BDNF) might play a role [58, 59]. Altered brain morphology across brain regions such as amygdala, hippocampus, and/or PFC related to gene- and protein-expression has been found in germ-free compared to microbiota-colonized mice [60]. Together, these functional pathways point towards a crucial role of the gut-microbiome in human health and wellbeing. In fact, alterations of gut-microbiome composition and diversity have been observed in psychiatric disorders, such as anxiety, depression, and autism spectrum disorder [61]; they seem to be related to commonly co-morbid gastro-intestinal complaints, metabolic, and immunological conditions [62].

A proxy of the gut-microbiota community can be obtained by sequencing genetic material from fecal samples. Research on the gut-microbiome often focuses particularly on the role of bacteria rather than fungi or viruses. For the purpose of this thesis, we will refer to bacteria as microbiota. Different strategies can be applied to investigate the gut-microbial community. In marker gene sequencing studies, variable regions of the 16S ribosomal RNA gene are used to identify individual bacterial *taxa* in the gut, while metagenomic sequencing identifies the entire genetic content in a sample. Most studies comparing microbial communities apply

16S sequencing, which is financially and computationally cost-effective and reliably identifies bacterial sequences up to the **genus** level [63]. While *metagenomic sequencing, metabolomics and bacterial culturing* studies can add information on prevalent functional pathways and functional profiles of individual species or strains, most measures of bacterial community characterization are consistent across both methods [64, 65]. The most commonly used measures to describe and investigate gut-microbial perturbations based on 16S sequencing are diversity and composition. *Alpha diversity* measures the number and distribution of different organisms within a sample; *beta diversity* describes differences in the distributions between samples, see glossary for more information. Compositional measures compare particular taxa and identify those that are more or less abundant in a particular group of people [64].

Gut-microbiota in ADHD and reactive aggression

Gut-microbiota studies have implicated alterations of gut-microbiome composition in ADHD [66, 67]. However, the few studies published to date report alterations in different taxa [67]. This lack of overlap can be related to limitations in the design and methodological approaches of the studies, of which I'm highlighting a few here: The sample sizes of most studies are too small to reliably detect expectedly small effects, considering the high inter- and intraindividual variability of gut-microbiome composition. Statistical tests of hundreds of taxa inflate false discovery rates. Inconsistent use of sequencing and statistical approaches produce different results [68], and the comparison of findings from different developmental stages (from infants to adults) and ethnic backgrounds (Caucasian, African, Asian, etc.) neglects natural compositional variation. Together, these factors have hampered the identification of robust gut-microbiome alterations in ADHD.

While the gut-microbiome has not been studied in relation to aggressive behavior in humans, animal studies point towards diet- and gut-microbiome alterations relevant for aggression [69, 70].

Such studies investigate gut-microbiota associations with behavior. The assumption that gut-microbiota alterations may cause the investigated behavioral changes (e.g. through influences on immune activation, the endocrine system and neurotransmitters) suggest potential therapeutic angles of gut-microbiota research. Notably, most pathways of the gut-brain axis are bi-directional: The gut-microbiome is highly susceptible to changes in these pathways, moderated by environmental and lifestyle influences. Not only may indirect cause changes in the gut-microbiota, and are important to control for; factors influencing gut-microbiota may also provide treatment targets. The most pronounced environmental factor influencing gut-microbiome diversity and composition, is diet [71].

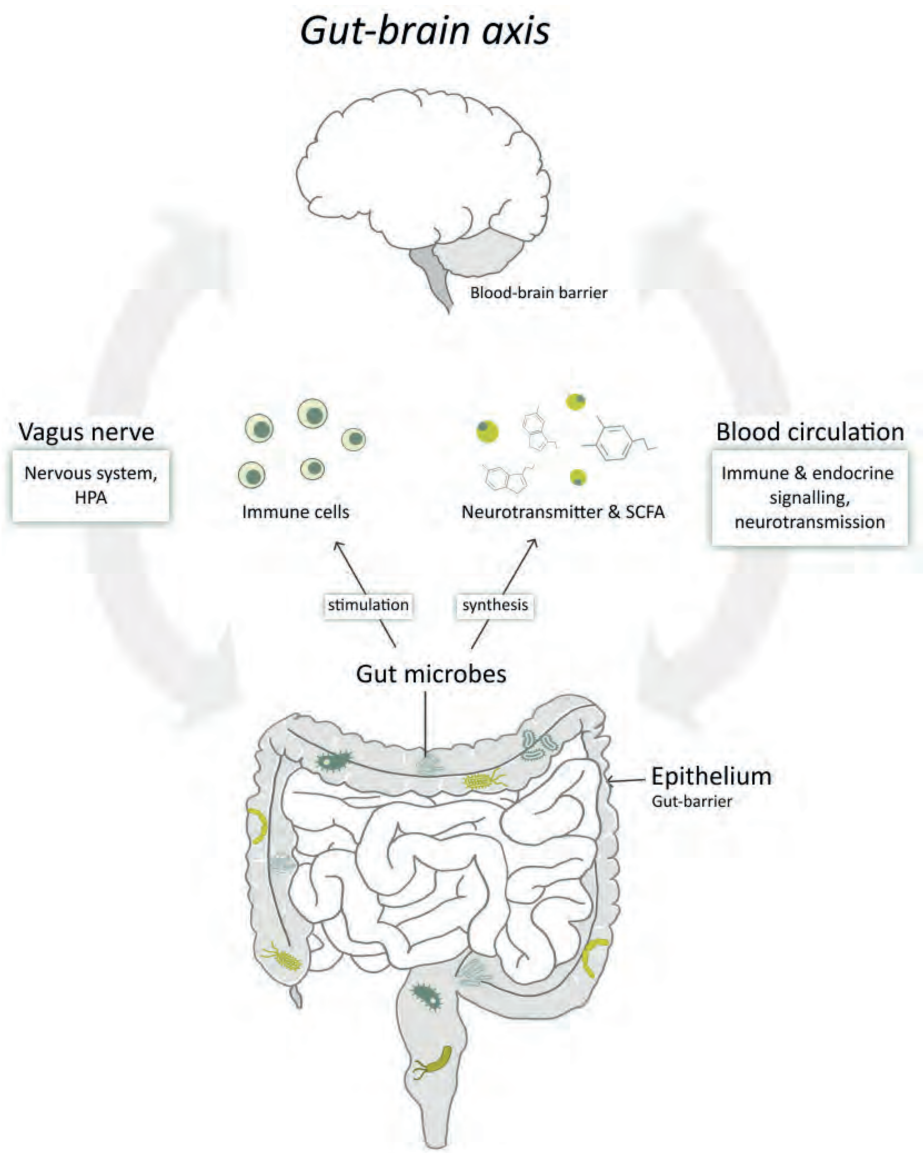

Figure 2. Illustration of the gut-microbiome-brain axis. Explanation of the figure can be found in the main text.

Alpha diversity is a measure of the diversity of microbial species within a particular sample or individual. Alpha diversity indices typically refer to 1) the richness of the microbiome, describing the number of different microbial taxa present in a sample (e.g. observed taxa), 2) the evenness or relative distribution of those taxa (e.g. Shannon's index), and 3) their phylogenic relationship, namely how closely related the individual taxa are with each other (e.g. Faith's phylogenic diversity or PD), see **Figure 4** for an illustration. Low diversity is often associated with a higher risk for certain diseases, such as inflammatory bowel disease (IBD) and obesity. Additionally, changes in alpha diversity may be used as a marker of response to interventions, such as dietary changes or probiotic supplementation.

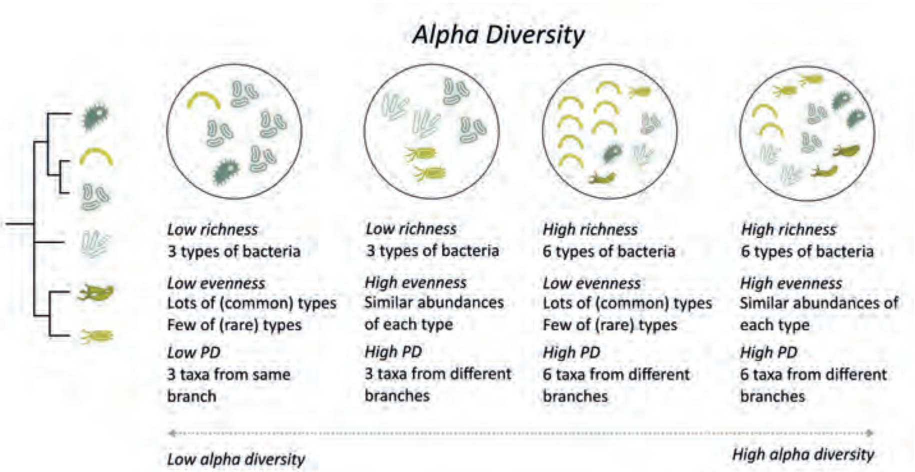

Figure 3. Alpha diversity explained as richness, evenness and phylogenic relatedness of individual taxa within a sample.

Beta diversity is a measure of the differences in microbial community composition (often also described as dissimilarity) between different samples or individuals, see **Figure 5** for an illustration. Beta diversity can be assessed, among others, using principal coordinates analysis, taking the abundance and presence/absence of microbial taxa in each sample into account and visualizing the distances between microbial community composition of different samples in a multidimensional space. One example of the distance between two samples, where all relative abundances are transformed (center-log-ratio transformation) and transferred to the Euclidean space, is Aitchison's Distance.

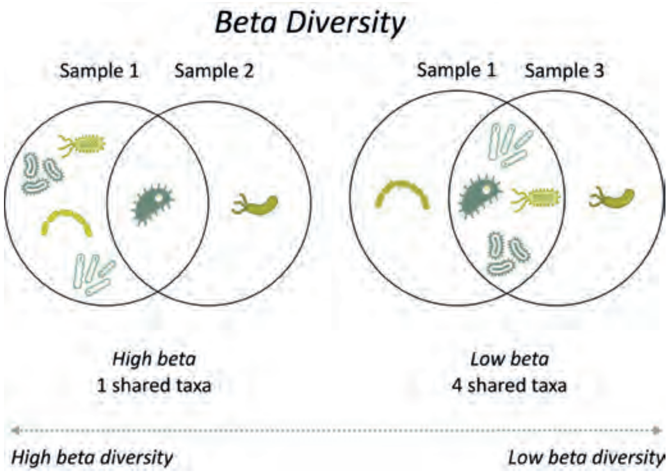

Figure 4. Beta diversity explained as the (dis)similarity or overlap between two samples. Low beta diversity identifies a high number of common and a low number of unique taxa, high beta diversity identifies a high number of unique and low number of common taxa between two samples..

Metagenomic sequencing of all the DNA in a sample can identify the types of microorganisms with higher precision compared to 16S and enables clustering by potential functional pathways the products formed from these sequences are involved in. ***Metabolomics*** uses advanced analytical techniques, such as mass spectrometry, to quantify metabolites and identify metabolic pathways, which can provide insights into the underlying biological processes. Bacterial ***culturing studies*** aim to grow and study bacterial strains and their function in the laboratory environment. While this is useful to detect functional properties, not all bacteria can be cultured and studied this way.

Microbiome / microbiota are often used interchangeably, but they actually refer to different aspects of the microbial communities that live in and on our bodies. Microbiota refers to the actual microorganisms that inhabit a particular environment, while microbiome refers to the collective genetic material of those microorganisms.

Taxon (plural taxa) refers to an individual organism or a group of organisms that are classified in a hierarchical system (see **Figure 3**), following their characteristics and phylogenic relationships. Each taxonomic group represents a different level of biological organization (domain, kingdom, phylum, class, order, family, ***genus***, and species), with species being the most specific and domain being the most general level.

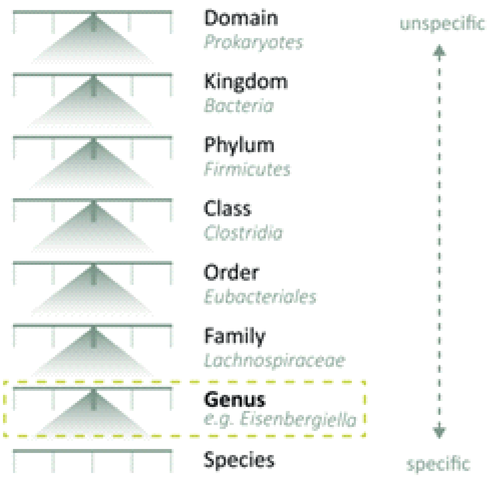

Figure 5. Levels of taxonomic classification on the example of the genus Eisenbergiella.

DIET

The ancient truism "Let food be thy medicine", often ascribed to Hippocrates over 2,000 years ago, already appreciated the importance of diet for health and wellbeing. Diet describes the type and quantity of food and drinks a person consumes on a regular basis. It is the primary source of nutrients, vitamins, and minerals, and it determines energy sources (protein, fat, carbohydrates) and energy homeostasis (caloric intake in relation to expenditure). Diet provides the essential building blocks for nearly all bodily functions, impacts overall health, (chronic) disease risk, and mental wellbeing [72]. As a naturally occurring behavior that is relatively easy to control, food intake has become target for interventions to promote physical and mental health. The assessment of diet in human intervention and observational studies is often realized with food-frequency questionnaires, in which participants report the frequency (and sometimes quantities) of certain food items or groups they have consumed over a period of time [73]. Overall, health-promoting eating patterns are described by 1) high nutritional value, e.g. through consumption of vegetables, fruits, seeds/nuts, and fish, by 2) a well-balanced energy consumption, avoiding added sugars, foods high in saturated fats, and high caloric foods, and by 3) minimally processed ingredients [74]. One example of a diet often described as healthy is the Mediterranean diet, characterized by vegetables, unsaturated fats, and seafood [75]. In contrast, the so-called Western diet (processed, high-caloric food, rich in sugars/carbohydrates and saturated fat, low in vegetables, fruits or food with high nutritional value) is described as unhealthy due to associations with adverse health-outcomes such as obesity, diabetes mellitus, and chronic heart diseases [76].

Differences in food intake between children and adolescents with and without ADHD have been observed in some studies. Eating habits associated with ADHD were mostly characterized to resemble aspects of the Western diet; high in processed foods with high caloric value (e.g. high consumption of sugars/carbohydrates and saturated fats) and low nutritional value (e.g. low consumption of fruit, vegetables and polyunsaturated fatty acids (*PUFAs*)) [77, 78]. However, the findings are not conclusive, and studies in adults are scarce [79]. Cause and consequence are also hard to disentangle: while ADHD symptoms such as inattention and impulsivity might affect food choices and hinder adherence to healthy eating patterns, diet might also affect ADHD symptoms. Dietary intervention studies in ADHD often focus on either the restriction of particular food groups, like sugar or allergens, or the supplementation with minerals, vitamins, or PUFAs. While restriction-based interventions seem to be promising, a direct comparison between a restriction-based, a "healthy diet" (following Dutch governmental guidelines), and *care as usual* showed that the healthy diet was more effective than the restriction-based diet; the healthy diet ameliorated ADHD symptoms to a comparable extent as care as usual [80]. Interestingly, this healthy diet amended not only inattention and hyperactivity/impulsivity but also emotion regulation problems. Indeed, similar patterns (more consumption of sweet foods and lower consumption of vegetables) have been associated with negative affect and low emotional self-regulation. Supplementation with PUFAs, minerals, and vitamins reduced reported incidents and aggressive behavior in imprisoned adults [69]. However, observational studies investigating associations of diet with reactive aggression and adult ADHD are lacking.

PUFAs or polyunsaturated fatty acids are building blocks of fats with a specific chemical structure (unsaturated structures have double bonds instead of side groups, poly-unsaturated fatty acids have more than one of these double bonds). PUFAs are essential for human health. They cannot be produced by the human body and must be taken up in the diet, in particular omega-3 (and omega-6 in moderation). They are often ascribed anti-inflammatory properties and associated with the maintenance of brain structure and functioning, as they are important components of the brain cell's membrane.

Care as usual in ADHD refers to the standard treatment typically provided to individuals with ADHD. While treatment is strongly dependent on the individual situation, a combination of psychoeducation, cognitive behavioral therapy, and medication (typically stimulant or amphetamine treatment) are often applied.

DATA AVAILABILITY

To investigate the potential role of the gut-brain axis for ADHD and the co-occurrence of reactive aggression with ADHD - especially in adults, where the is a scarcity of published studies - data sources are needed that combine information about: 1) brain functioning, preferably during emotion processing/relevant for reactive aggression, 2) gut-microbiota, 3) relevant confounders and influential factors like diet, 4) phenotyping of ADHD and reactive aggression.

Some large-scale neuroimaging studies investigating ADHD include functional scans like resting state, or tasks studying e.g. executive functioning [36]. However, recent systematic reviews show that functional imaging studies of adult ADHD are still rare and often lack relevant phenotypic information about emotion dysregulation or reactive aggression, or brain functioning measures of emotion processing, which are particularly relevant for reactive aggression research in the context of ADHD [41, 43, 81]. Another limiting factor is the availability of gut-microbiota sequencing data. The available resources for individual microbiota data or emotion processing-related neuroimaging data often have small sample sizes, are biased towards young (childhood or adolescence) or (partially) remitted study populations, and are missing relevant information, e.g. measures of reactive aggression and lifestyle factors such as diet [67, 82, 83]. Few study populations have been published combining gut-microbial signatures together with measures of brain-functioning; none of them in ADHD [84]. Due to the limited availability of datasets with complete data for research on the role of the gut-brain axis in adult ADHD and aggression, I collected data from adults with and without ADHD, including brain functioning during emotion processing, 16S gut-microbiota sequencing data, and relevant phenotypic and dietary information (IMpACT2-NL cohort). Other study populations with relevant data for this thesis are parts of the MIND-Set and NeuroIMAGE cohorts, as well as the Mental-Cat cohort, described below.

IMpACT2-NL is a case-control cohort including approximately 170 participants with and without ADHD. IMpACT2-NL is part of the Dutch site of the International Multi-centre persistent ADHD CollaboraTion (IMpACT), a consortium of eight countries from Europe and North- and South-America, aimed at studying ADHD across the lifespan. The IMpACT2-NL cohort includes relevant neuroimaging measures, such as task-based and resting state functional MRI and structural imaging, fecal microbiome sequencing data, blood-samples, as well as a battery of neuropsychological tests and questionnaires, collected at the Radboudumc in Nijmegen, The Netherlands. ADHD symptoms in this cohort were assessed using the DIVA structured diagnostic interview for adults [1]; a proportion of participants additionally filled in the adult ADHD self-report [85].

MIND-Set stands for Measuring Integrated Novel Dimensions in Neurodevelopmental and Stress-Related Mental Disorders. The MIND-Set study is a cross-sectional cohort investigating shared and specific mechanisms of psychiatric disorders including ADHD, ASD, bipolar disorder, major depressive disorder, and anxiety [86]. In chapter 2, we include approximately 200 participants with either an ADHD diagnosis or no psychiatric diagnosis from MIND-Set. The ADHD sample is highly comorbid with other psychiatric disorders. ADHD symptoms were assessed using a short version of the Conners Adult ADHD Rating Scale (CAARS [87]).

NeuroIMAGE is a cohort collected in continuation of the International Multisite ADHD Genetics (IMAGE) study. It is a longitudinal study investigating brain-structure and functioning from childhood to adult ADHD [88]. NeuroIMAGE2 was a follow-up study, where participants additionally provided fecal samples for gut-microbiome analyses. A total of 70 of those participants were adults; they were included in the meta-analysis in chapter 2.

Mental-Cat is a cohort collected at the Vall d'Hebron Research Institute in Barcelona (VHIR) focusing on medication-naïve adults with and without ADHD. The 200 participants provided fecal samples, and ADHD symptoms were assessed with the ADHD-Rating scale, an adult self-report instrument [89].

THESIS OUTLINE

With this thesis, I aimed to investigate the role of the gut-microbiome-brain-axis for adult ADHD and co-occurring reactive aggression. To this end, I first collected data from adults with and without ADHD, including brain functional scans, gut-microbiota fecal samples, and phenotypic and dietary information (IMpACT2-NL cohort). In three scientific chapters, I subsequently explored this topic from different perspectives:

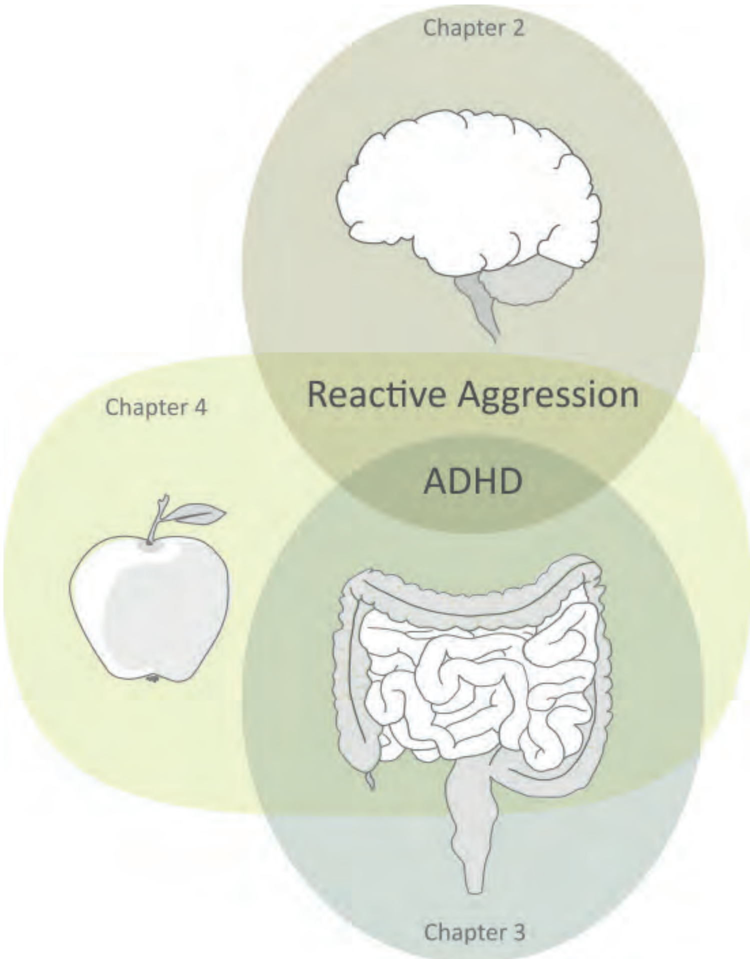

Figure 6. Overview of the scientific chapters.

In **Chapter 2** of my thesis, I focused on brain functioning related to emotion processing. Understanding the relationship between emotion processing, brain functioning, and emotionally dysregulated behavior like reactive aggression in adult ADHD might help developing interventions and treatment strategies that target e.g. reactive aggression in individuals with ADHD. Altered activity in particular brain regions might point towards sub-processes during emotion regulation (higher intrinsic emotional response or less down-regulation) that underlie the co-occurrence of reactive aggression with adult ADHD. I therefore studied whole-brain activity during emotion processing using an fMRI task in the *IMpACT2-NL* cohort. I used the reactive aggression subscale of the Reactive-Proactive Aggression Questionnaire (RPQ [15]), to identify which brain regions change activity in the fMRI task in association with higher reactive aggression scores in adults with and without ADHD.

In **Chapter 3** of this thesis, I focused on the gut-microbiome. Alterations in the gut-microbiome might point towards biological/environmental pathways linked to co-occurring ADHD and reactive aggression. Compositional alterations of the gut-microbiome are particularly interesting targets for future functional observational studies as well as intervention studies to improve social and occupational outcomes for participants. The detection of robust signals from gut-microbiome sequencing data is an absolute necessity for this endeavor. In this chapter, I aimed to tackle limitations of small individual studies and identify robust signals in the gut-microbiome by refining and harmonizing the preprocessing and statistical analysis of four European cohorts including adults with and without ADHD (*IMpACT2-NL*, *MIND-Set*, *Mental-Cat*, and *NeuroIMAGE*, N=617). I summarized the findings across cohorts and statistical tools in meta-analyses. I further investigated relationships of gut-microbiota with the number of ADHD symptoms across affected and unaffected individuals.

Since the gut-microbiome is highly susceptible to age and lifestyle changes, such as food intake, dietary interventions are promising targets to alleviate emotion dysregulation and ADHD symptoms, **Chapter 4** of this thesis is dedicated to the role of diet and the gut-microbiome in reactive aggression in the context of adult ADHD. I applied exploratory factor analysis, using a semi-quantitative food questionnaire, to identify patterns of eating habits and investigate associations with reactive aggression and ADHD in the IMpACT2-NL cohort. I further investigated associations of the gut-microbiome with reactive aggression and explored the potential mediating role of selected gut-microbiota in the relationships of diet with reactive aggression and ADHD.

In the final **Chapter 5**, the General Discussion, implications of the results and the conclusions of previous chapters are discussed in the context of the existing literature and knowledge as well as the setting of this work within a medical faculty. Limitations and future directions for the research field of the gut-microbiome-brain-axis for reactive aggression and ADHD are presented.

REFERENCES

1. Kooij, J. and M. Francken, *DIVA 2.0.* Diagnostic Interview Voor ADHD in Adults bij volwassenen [DIVA 2 0 Diagnostic Interview ADHD in Adults]. DIVA Foundation (http://www. divacenter. eu/ DIVA. aspx), 2010.
2. American Psychiatric Association, *Diagnostic and Statistical Manual of Mental Disorders*. 5th ed. 2013, Washington, DC.
3. Faraone, S.V., et al., *The world federation of ADHD international consensus statement: 208 evidence-based conclusions about the disorder.* Neuroscience & Biobehavioral Reviews, 2021. **128**: p. 789-818.
4. Franke, B., et al., *Live fast, die young? A review on the developmental trajectories of ADHD across the lifespan.* European Neuropsychopharmacology, 2018. **28**(10): p. 1059-1088.
5. Faraone, S.V., J. Biederman, and E. Mick, *The age-dependent decline of attention deficit hyperactivity disorder: a meta-analysis of follow-up studies.* Psychological medicine, 2006. **36**(2): p. 159-165.
6. Gizer, I.R., C. Ficks, and I.D. Waldman, *Candidate gene studies of ADHD: a meta-analytic review.* Human genetics, 2009. **126**: p. 51-90.
7. Cabana-Domínguez, J., et al., *Comprehensive analysis of omics data identifies relevant gene networks for Attention-Deficit/Hyperactivity Disorder (ADHD).* Translational Psychiatry, 2022. **12**(1): p. 409.
8. Franke, B. and J.K. Buitelaar, *Gene-environment interactions.* Oxford Textbook of Attention Deficit Hyperactivity Disorder, 2018: p. 35.
9. Sonuga-Barke, E.J., et al., *Annual Research Review: Perspectives on progress in ADHD science–from characterization to cause.* Journal of Child Psychology and Psychiatry, 2023. **64**(4): p. 506-532.
10. Uldall Torp, N.M. and P.H. Thomsen, *The use of diet interventions to treat symptoms of ADHD in children and adolescents–a systematic review of randomized controlled trials.* Nordic Journal of Psychiatry, 2020. **74**(8): p. 558-568.
11. Fotoglou, A., et al., *Nutritious Diet, Physical Activity and Mobiles. The Game Changers of ADHD.* Technium BioChemMed, 2022. **3**(2): p. 87-106.
12. Kittel-Schneider, S., et al., *Non-mental diseases associated with ADHD across the lifespan: Fidgety Philipp and Pippi Longstocking at risk of multimorbidity?* Neuroscience & Biobehavioral Reviews, 2022. **132**: p. 1157-1180.
13. Shaw, P., et al., *Emotion dysregulation in attention deficit hyperactivity disorder.* American Journal of Psychiatry, 2014. **171**(3): p. 276-293.
14. Nigg, J.T., et al., *Toward a revised nosology for attention-deficit/hyperactivity disorder heterogeneity.* Biological Psychiatry: Cognitive Neuroscience and Neuroimaging, 2020. **5**(8): p. 726-737.
15. Christiansen, H., et al., *Attention-deficit/hyperactivity disorder (ADHD) and emotion regulation over the life span.* Current psychiatry reports, 2019. **21**: p. 1-11.
16. Blair, R., *Traits of empathy and anger: implications for psychopathy and other disorders associated with aggression.* Philosophical Transactions of the Royal Society B: Biological Sciences, 2018. **373**(1744): p. 20170155.
17. Vitaro, F. and M. Brendgen, *Proactive and Reactive Aggression: A Developmental Perspective.* 2005.
18. Gross, J.J., *Emotion regulation: Current status and future prospects.* Psychological inquiry, 2015. **26**(1): p. 1-26.
19. Raine, A., et al., *The reactive–proactive aggression questionnaire: Differential correlates of reactive and proactive aggression in adolescent boys.* Aggressive Behavior: Official Journal of the International Society for Research on Aggression, 2006. **32**(2): p. 159-171.

20. Brown, K., et al., *A revised teacher rating scale for reactive and proactive aggression.* Journal of abnormal child psychology, 1996. **24**: p. 473-480.
21. Day, D.M., L.A. Bream, and A. Pal, *Proactive and reactive aggression: An analysis of subtypes based on teacher perceptions.* Journal of Clinical Child and Adolescent Psychology, 1992. **21**(3): p. 210-217.
22. Jensen, P.S., et al., *Consensus report on impulsive aggression as a symptom across diagnostic categories in child psychiatry: implications for medication studies.* Journal of the American Academy of Child & Adolescent Psychiatry, 2007. **46**(3): p. 309-322.
23. Abel, M.R., et al., *Reactive aggression and suicidal behaviors in children receiving outpatient psychological services: the moderating role of hyperactivity and inattention.* Child Psychiatry & Human Development, 2020. **51**: p. 2-12.
24. Evans, S.C., et al., *The role of reactive aggression in the link between hyperactive–impulsive behaviors and peer rejection in adolescents.* Child Psychiatry & Human Development, 2015. **46**: p. 903-912.
25. King, S. and D.A. Waschbusch, *Aggression in children with attention-deficit/hyperactivity disorder.* Expert Review of Neurotherapeutics, 2010. **10**(10): p. 1581-1594.
26. Slaughter, K.E., et al., *Reactive and proactive aggression in children with and without ADHD and negative emotional lability.* Social Development, 2020. **29**(1): p. 320-338.
27. Martel, M.M., *Research review: A new perspective on attention-deficit/hyperactivity disorder: Emotion dysregulation and trait models.* Journal of Child Psychology and Psychiatry, 2009. **50**(9): p. 1042-1051.
28. Jensen, P.S., et al., *Management of psychiatric disorders in children and adolescents with atypical antipsychotics: a systematic review of published clinical trials.* European child & adolescent psychiatry, 2007. **16**: p. 104-120.
29. Aman, M.G., et al., *What does risperidone add to parent training and stimulant for severe aggression in child attention-deficit/hyperactivity disorder?* Journal of the American Academy of Child & Adolescent Psychiatry, 2014. **53**(1): p. 47-60. e1.
30. Pavlov, K.A., D.A. Chistiakov, and V.P. Chekhonin, *Genetic determinants of aggression and impulsivity in humans.* Journal of applied genetics, 2012. **53**: p. 61-82.
31. Demontis, D., et al., *Risk variants and polygenic architecture of disruptive behavior disorders in the context of attention-deficit/hyperactivity disorder.* Nature communications, 2021. **12**(1): p. 576.
32. Waltes, R., A.G. Chiocchetti, and C.M. Freitag, *The neurobiological basis of human aggression: a review on genetic and epigenetic mechanisms.* American Journal of Medical Genetics Part B: Neuropsychiatric Genetics, 2016. **171**(5): p. 650-675.
33. Carrier, M. and J. Mittelstrass, *Mind, brain, behavior: The mind-body problem and the philosophy of psychology*. 1991: Walter de Gruyter.
34. Hoogman, M., et al., *Subcortical brain volume differences in participants with attention deficit hyperactivity disorder in children and adults: a cross-sectional mega-analysis.* The Lancet Psychiatry, 2017. **4**(4): p. 310-319.
35. Hoogman, M., et al., *Brain imaging of the cortex in ADHD: a coordinated analysis of large-scale clinical and population-based samples.* American Journal of Psychiatry, 2019. **176**(7): p. 531-542.
36. Owens, M.M., et al., *Multimethod investigation of the neurobiological basis of ADHD symptomatology in children aged 9-10: baseline data from the ABCD study.* Translational psychiatry, 2021. **11**(1): p. 64.
37. Bernanke, J., et al., *Structural brain measures among children with and without ADHD in the Adolescent Brain and Cognitive Development Study cohort: a cross-sectional US population-based study.* The Lancet Psychiatry, 2022. **9**(3): p. 222-231.
38. Li, T., et al., *Characterizing neuroanatomic heterogeneity in people with and without ADHD based on subcortical brain volumes.* Journal of child psychology and psychiatry, 2021. **62**(9): p. 1140-1149.

39. Teeuw, J., et al., *Polygenic risk scores and brain structures both contribute to externalizing behavior in childhood-A study in the Adolescent Brain and Cognitive Development (ABCD) cohort.* Neuroscience Applied, 2023. **2**: p. 101128.

40. Cortese, S., et al., *Toward systems neuroscience of ADHD: a meta-analysis of 55 fMRI studies.* American Journal of Psychiatry, 2012. **169**(10): p. 1038-1055.

41. Yap, K.H., H.A. Manan, and S. Sharip, *Heterogeneity in brain functional changes of cognitive processing in ADHD across age: a systematic review of task-based fMRI studies.* Behavioural Brain Research, 2021. **397**: p. 112888.

42. Rubia, K., *Cognitive neuroscience of attention deficit hyperactivity disorder (ADHD) and its clinical translation.* Frontiers in human neuroscience, 2018. **12**: p. 100.

43. Cortese, S., et al., *Systematic review and meta-analysis: resting-state functional magnetic resonance imaging studies of attention-deficit/hyperactivity disorder.* Journal of the American Academy of Child & Adolescent Psychiatry, 2021. **60**(1): p. 61-75.

44. Viering, T., et al., *Amygdala reactivity and ventromedial prefrontal cortex coupling in the processing of emotional face stimuli in attention-deficit/hyperactivity disorder.* European Child & Adolescent Psychiatry, 2021: p. 1-13.

45. Musella, K.E. and L.L. Weyandt, *Attention-deficit hyperactivity disorder and youth's emotion dysregulation: A systematic review of fMRI studies.* Applied Neuropsychology: Child, 2022: p. 1-14.

46. Posner, J., et al., *Abnormal amygdalar activation and connectivity in adolescents with attention-deficit/hyperactivity disorder.* Journal of the American Academy of Child & Adolescent Psychiatry, 2011. **50**(8): p. 828-837. e3.

47. Bottelier, M.A., et al., *Age-dependent effects of acute methylphenidate on amygdala reactivity in stimulant treatment-naive patients with attention deficit/hyperactivity disorder.* Psychiatry Research: Neuroimaging, 2017. **269**: p. 36-42.

48. Brotman, M.A., et al., *Amygdala activation during emotion processing of neutral faces in children with severe mood dysregulation versus ADHD or bipolar disorder.* American Journal of Psychiatry, 2010. **167**(1): p. 61-69.

49. Hulvershorn, L.A., et al., *Abnormal amygdala functional connectivity associated with emotional lability in children with attention-deficit/hyperactivity disorder.* Journal of the American Academy of Child & Adolescent Psychiatry, 2014. **53**(3): p. 351-361. e1.

50. Bertsch, K., J. Florange, and S.C. Herpertz, *Understanding brain mechanisms of reactive aggression.* Current psychiatry reports, 2020. **22**: p. 1-16.

51. Bubenzer-Busch, S., et al., *Neural correlates of reactive aggression in children with attention-deficit/hyperactivity disorder and comorbid disruptive behaviour disorders.* Acta Psychiatrica Scandinavica, 2016. **133**(4): p. 310-323.

52. Sender, R., S. Fuchs, and R. Milo, *Revised estimates for the number of human and bacteria cells in the body.* PLoS biology, 2016. **14**(8): p. e1002533.

53. Baweja, R. and J.G. Waxmonsky, *Updates in pharmacologic strategies for emotional dysregulation in attention deficit hyperactivity disorder.* Child and Adolescent Psychiatric Clinics, 2022. **31**(3): p. 479-498.

54. Strandwitz, P., *Neurotransmitter modulation by the gut microbiota.* Brain research, 2018. **1693**: p. 128-133.

55. Morais, L.H., H.L. Schreiber IV, and S.K. Mazmanian, *The gut microbiota–brain axis in behaviour and brain disorders.* Nature Reviews Microbiology, 2021. **19**(4): p. 241-255.

56. Perez-Muñoz, M.E., et al., *A critical assessment of the "sterile womb" and "in utero colonization" hypotheses: implications for research on the pioneer infant microbiome.* microbiome, 2017. **5**(1): p. 1-19.

57. Parker, A., S. Fonseca, and S.R. Carding, *Gut microbes and metabolites as modulators of blood-brain barrier integrity and brain health.* Gut Microbes, 2020. **11**(2): p. 135-157.

58. Nichols, R.G. and E.R. Davenport, *The relationship between the gut microbiome and host gene expression: a review.* Human genetics, 2021. **140**(5): p. 747-760.

59. Bercik, P., et al., *The intestinal microbiota affect central levels of brain-derived neurotropic factor and behavior in mice.* Gastroenterology, 2011. **141**(2): p. 599-609. e3.

60. Warner, B.B., *The contribution of the gut microbiome to neurodevelopment and neuropsychiatric disorders.* Pediatric Research, 2019. **85**(2): p. 216-224.

61. Andrioaie, I.-M., et al., *The Role of the Gut Microbiome in Psychiatric Disorders.* Microorganisms, 2022. **10**(12): p. 2436.

62. Person, H. and L. Keefer, *Psychological comorbidity in gastrointestinal diseases: Update on the brain-gut-microbiome axis.* Progress in Neuro-Psychopharmacology and Biological Psychiatry, 2021. **107**: p. 110209.

63. Johnson, J.S., et al., *Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis.* Nature communications, 2019. **10**(1): p. 5029.

64. Knight, R., et al., *Best practices for analysing microbiomes.* Nature Reviews Microbiology, 2018. **16**(7): p. 410-422.

65. Rausch, P., et al., *Comparative analysis of amplicon and metagenomic sequencing methods reveals key features in the evolution of animal metaorganisms.* Microbiome, 2019. **7**(1): p. 1-19.

66. Gkougka, D., et al., *Gut microbiome and attention deficit/hyperactivity disorder: a systematic review.* Pediatric Research, 2022: p. 1-13.

67. Shirvani-Rad, S., et al., *The role of gut microbiota-brain axis in pathophysiology of ADHD: a systematic review.* Journal of Attention Disorders, 2022. **26**(13): p. 1698-1710.

68. Nearing, J.T., et al., *Microbiome differential abundance methods produce different results across 38 datasets.* Nature Communications, 2022. **13**(1): p. 342.

69. Tcherni-Buzzeo, M., *Dietary interventions, the gut microbiome, and aggressive behavior: Review of research evidence and potential next steps.* Aggressive behavior, 2023. **49**(1): p. 15-32.

70. Gulledge, L., D. Oyebode, and J.R. Donaldson, *The Influence of the Microbiome on Aggressive Behavior: an Insight into Age Related Aggression.* FEMS Microbiology Letters, 2023: p. fnac114.

71. Wilson, A.S., et al., *Diet and the human gut microbiome: an international review.* Digestive diseases and sciences, 2020. **65**: p. 723-740.

72. Organization, W.H., *Diet, nutrition, and the prevention of chronic diseases: report of a joint WHO/FAO expert consultation*. Vol. 916. 2003: World Health Organization.

73. Cade, J.E., *Measuring diet in the 21st century: use of new technologies.* Proceedings of the Nutrition Society, 2017. **76**(3): p. 276-282.

74. Herforth, A., et al., *A global review of food-based dietary guidelines.* Advances in Nutrition, 2019. **10**(4): p. 590-605.

75. Davis, C., et al., *Definition of the Mediterranean diet: a literature review.* Nutrients, 2015. **7**(11): p. 9139-9153.

76. Carrera-Bastos, P., et al., *The western diet and lifestyle and diseases of civilization.* Research Reports in Clinical Cardiology, 2011: p. 15-35.

77. Pinto, S., et al., *Eating Patterns and Dietary Interventions in ADHD: A Narrative Review.* Nutrients, 2022. **14**(20): p. 4332.

78. Del-Ponte, B., et al., *Dietary patterns and attention deficit/hyperactivity disorder (ADHD): a systematic review and meta-analysis.* Journal of affective disorders, 2019. **252**: p. 160-173.

79. Breda, V., et al., *Is there a place for dietetic interventions in adult ADHD?* Progress in Neuro-Psychopharmacology and Biological Psychiatry, 2022: p. 110613.
80. Bosch, A., et al., *A two arm randomized controlled trial comparing the short and long term effects of an elimination diet and a healthy diet in children with ADHD (TRACE study). Rationale, study design and methods.* BMC psychiatry, 2020. **20**(1): p. 1-16.
81. Sutcubasi, B., et al., *Resting-state network dysconnectivity in ADHD: A system-neuroscience-based meta-analysis.* The World Journal of Biological Psychiatry, 2020. **21**(9): p. 662-672.
82. Lindholm, P., et al., *Brain response to facial expressions in adults with adolescent ADHD.* Psychiatry Research: Neuroimaging, 2019. **292**: p. 54-61.
83. Schulz, K.P., et al., *Emotional bias of cognitive control in adults with childhood attention-deficit/hyperactivity disorder.* NeuroImage: Clinical, 2014. **5**: p. 1-9.
84. Mulder, D., et al., *A systematic review exploring the association between the human gut microbiota and brain connectivity in health and disease.* medRxiv, 2022: p. 2022.11. 29.22282879.
85. Kessler, R.C., et al., *The World Health Organization Adult ADHD Self-Report Scale (ASRS): a short screening scale for use in the general population.* Psychological medicine, 2005. **35**(2): p. 245-256.
86. van Eijndhoven, P., et al., *Measuring Integrated Novel Dimensions in Neurodevelopmental and Stress-Related Mental Disorders (MIND-SET): protocol for a cross-sectional comorbidity study from a Research Domain Criteria perspective.* JMIRx Med, 2022. **3**(1): p. e31269.
87. Conners, C., et al., *Self-ratings of ADHD symptoms in adults I: Factor structure and normative data.* Journal of Attention Disorders, 1999. **3**(3): p. 141-151.
88. von Rhein, D., et al., *The NeuroIMAGE study: a prospective phenotypic, cognitive, genetic and MRI study in children with attention-deficit/hyperactivity disorder. Design and descriptives.* European child & adolescent psychiatry, 2015. **24**: p. 265-281.
89. Richarte, V., et al., *Gut microbiota signature in treatment-naïve attention-deficit/hyperactivity disorder.* Translational psychiatry, 2021. **11**(1): p. 382.

Chapter 2

Neural Correlates of Reactive Aggression in Adult ADHD

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Published as:
(2022). Neural correlates of reactive aggression in adult attention-deficit/hyperactivity disorder. Frontiers in psychiatry, 13, 840095.Doi: https://doi.org/10.3389/fpsyt.2022.840095.

ABSTRACT

Despite not being part of the core diagnostic criteria for attention-deficit/hyperactivity disorder (ADHD), emotion dysregulation is a highly prevalent and clinically important component of (adult) ADHD. Emotionally dysregulated behaviors such as reactive aggression have a significant impact on the functional outcome in ADHD. However, little is known about the mechanisms underlying reactive aggression in ADHD. In this study, we aimed to identify the neural correlates of reactive aggression as a measure of emotionally dysregulated behavior in adults with persistent ADHD during implicit emotion regulation processes. We analyzed associations of magnetic resonance imaging-based whole-brain activity during a dynamic facial expression task with levels of reactive aggression in 78 adults with and 78 adults without ADHD, and also investigated relationships of reactive aggression with symptoms and impairments. While participants with ADHD had higher reactive aggression scores than controls, the neural activation patterns of both groups to processing of emotional faces were similar. However, investigating the brain activities associated with reactive aggression in individuals with and without ADHD showed an interaction of diagnosis and reactive aggression scores. We found high levels of activity in the right insula, the hippocampus, and middle and superior frontal areas to be particularly associated with high reactive aggression scores within the ADHD group. Furthermore, the limbic activity was associated with more hyperactivity/impulsivity symptoms. These results suggest a partly differential mechanism associated with reactive aggression in ADHD as compared to controls. Emotional hyper-reactivity in the salience network as well as more effortful top-down regulation from the self-regulation network might contribute to emotionally dysregulated behavior as measured by reactive aggression.

INTRODUCTION

Attention-deficit/hyperactivity disorder (ADHD) is a highly prevalent neuro-developmental disorder [1], characterized by core symptoms of inattention and/or hyperactivity and impulsivity [2]. Symptoms of ADHD persist into adulthood in up to 66% of affected individuals [1] and are commonly accompanied by emotion regulation problems [3]. Even though symptoms of emotion dysregulation (ED) are prevalent in people with ADHD (with 24-50% in children and up to 70% in adults) and are an important predictor of ADHD symptoms [4], they have long been disregarded in diagnostic and therapeutic context [3].

An important expression of severe ED in ADHD is reactive aggression [5-9]. Not only is aggressive behavior a frequent catalyst for diagnostic consultation [7], recent research reports that reactive aggression in ADHD remains significantly elevated after correction for comorbidities such as conduct disorder (CD) and oppositional defiant disorder (ODD) [9, 10]. Literature often distinguishes two types of aggressive behaviors. While proactive aggression links to instrumentalized and controlled aggressive behaviors, reactive aggression is a mirror of a dysregulated emotional response e.g. to fear or anger [11]. Reactive aggression can have a large impact on multiple dimensions of life. People with ADHD and co-occurring aggression show the most maladaptive strategies in emotion regulation and social decision making [12], often resulting in unstable dysfunctional relationships and families, peer rejection and victimization, functional impairments in school and later occupation, as well as an elevated risk of contact with criminality [5, 7, 10, 13]. Reactive aggression has also consistently been linked to suicidal behaviors and attempts [5, 14]. Abel and colleagues (2020) reported that this elevated risk of suicidal behavior in reactive aggression is modulated by hyperactivity and impulsivity symptoms, irrespective of comorbidities such as depression [5].

Altered structural or functional maturation of several brain areas might point towards a neurodevelopmental link of ADHD with reactive aggression. Among small morphological differences in several areas, the structural alterations implicated in ADHD involve reduced volumes within the limbic system, e.g. the amygdala and the hippocampus [15], as well as differential cortical thickness in frontal and parietotemporal brain regions [16]. Besides structural implications in ADHD, these regions also exhibit altered functional connectivity and altered activity profiles in ADHD [17]; they have been linked to altered emotional reactivity and memory (limbic system, orbito and ventromedial frontal cortex [18, 19] as well as executive functioning and attentional frontal and parietotemporal networks [20].

Emotional sub-processes are relevant for the emergence of reactive aggression and are frequently assessed using functional magnetic resonance imaging (fMRI) paradigms inducing an implicit or explicit emotional reaction to emotionally salient stimuli, e.g. the implicit processing of facial emotional expressions. FMRI studies in children and adolescents with ADHD have revealed patterns of elevated bottom-up emotional reactivity, reflected in altered activity in the amygdala, insula, ventral striatum, and the orbitofrontal cortex (OFC) [17, 21-24]. Additionally, differences in the top-down modulation of emotional responses were found in tasks involving active emotion regulation. Differential activation of the amygdala or insula and hypo-connectivity of those structures to prefrontal structures, such as the ventrolateral PFC (vlPFC), the anterior cingulate cortex (ACC) and the temporoparietal junction (TPJ) have been observed in ADHD [25-27]. Only few fMRI studies on face emotion processing were carried out in adults with ADHD, covering only small, remitted or partially remitted samples and focusing on response inhibition and attention. The authors reported (sub-threshold) activity- and connectivity differences in limbic and prefrontal circuit in an emotional go/no-go task [28] or hyperactivity in face-processing areas and differential connectivity to regions linked to attention in remitted adults in a dynamic facial expression task [29].

The above-mentioned brain regions altered in people with ADHD are overlapping broadly with the neural correlates of reactive aggression. While reactive aggressive behavior appears to be facilitated by activity of the limbic system and hypothalamus, prefrontal activity seems to indicate inhibition of such behaviors [30]. Alia-Klein et al. [31] summarize the emergence of anger as a basis of reactive aggression to 1) reactivity of the salience network (dACC, Insula and limbic structures), influenced by 2) social cognition and self-referential processes in the mentalizing network (TPJ, SFG, posteriorCC, dorsolateralPFC) and downregulated by 3) the self-regulation network (PFC, ACC, IFG). Hence, reactive aggression is associated with an imbalance of cognitive control (implemented in prefrontal areas) and hyper-reactivity to emotional stimuli of the limbic system and insula [9, 32, 33].

Despite the high impact on the quality of life, our understanding of the co-occurrence of ADHD with and reactive aggression and of the underlying mechanisms is limited. Neural circuits engaged in reactive aggression -as a severe form of ED - overlap with structurally and functionally implicated brain regions in ADHD and are linked together in functional neuroimaging studies on children and adolescents. However, research on the neural circuits or alterations during emotion processing underlying reactive aggression in adults with persistent ADHD is clearly underrepresented, does not cover implicit facial emotion processing nor integrate behavioral impairments such as ratings of reactive aggression.

This study aimed to identify the neural correlates of reactive aggression in adults with persistent ADHD during implicit emotion regulation processes. We acquired fMRI during a dynamic facial expression task [34, 35]. We investigated the neural correlates of reactive aggression in adults with and without ADHD and analyzed the covariance of whole-brain activity with levels of reactive aggression scores from a questionnaire. We expected reactive aggression to be associated with altered neural activation within the emotion regulation network. Moreover, we aimed to identify sub-processes relevant for reactive aggression. We hypothesized more emotional reactivity, as reflected in hyperactivity of the limbic system and anterior insula, and/or more effortful or less cognitive control processes, as reflected in differential prefrontal activity to be relevant for the occurrence of higher reactive aggression in ADHD. We also explored the association of ADHD diagnosis and clinically relevant variables with the reactive aggression scores and post-hoc correlations with the neural activity in areas implicated in reactive aggression in the fMRI analysis (insula, hippocampus, precentral gyrus, superior and middle frontal gyrus, middle temporal gyrus and lingual gyrus).

METHODS

Participants and Experimental Procedure

A total number of 83 adults with a confirmed diagnosis of ADHD and 79 healthy control subjects participated in this fMRI experiment. Participants were recruited via newspaper advertisements, patient organizations and local sports clubs in and around Nijmegen, The Netherlands. All of the participants provided written informed consent before participating in the study and received monetary compensation for their participation. The study was approved by the local medical ethical committee.

Participants were included in the ADHD group if they had been diagnosed with ADHD by a clinician. To confirm the diagnosis and assess previous and current symptoms in all participants, we conducted the Diagnostic Interview for Adult ADHD (DIVA 2.0; [36]). The DIVA includes nine subscales for the symptoms of inattentiveness and hyperactivity/impulsivity and further assesses subjective impairment over life domains such as occupation, family and relationships, social contacts, hobbies and self-image in childhood as well as adulthood. Participants were included in the control group when the following criteria were met: absence of previous diagnoses of ADHD, of current neurological or psychiatric disorders and of first-degree family members with ADHD. Exclusion criteria for all participants comprised (1) an age younger than 18 or older than 60 years, (2) neurological disorders, (3) psychosis or substance abuse in the last 6 months, (4) current major depression, (5) psycho-pharmaceutical therapy other than stimulants, (6) impairments of hearing, seeing and sensorimotor abilities as well as (7) problems with understanding Dutch (to ensure that all of the participants understood the study protocol and the task instructions). The 40 participants with ADHD that received regular pharmacological treatment with stimulants, were asked to pause their medication intake 24 hours prior to participation. Missing data of the reactive proactive aggression questionnaire of five participants from the ADHD and failed fMRI data preprocessing of one control subject resulted in a total sample of 78 participants with and 78 participants without ADHD. Both groups had comparable distributions of age, sex, IQ and educational background (see **Table 1** for a demographic description of the sample including the relevant questionnaire data).

Participation was structured in two parts. The first part included the diagnostic screening for ADHD using the DIVA [36] and a short screening for comorbid psychiatric disorders following the Structured Clinical Interview for DSM, SCID-5. Demographic information was collected and IQ testing was performed using block-design and vocabulary subtests of the Wechsler Adult Intelligence Scale (WAIS; [37]). In the second part, structural and functional MRI scans were acquired. After the visit, participants were asked to fill in questionnaires via an online platform, among others the Reactive-Proactive Aggression Questionnaire (RPQ; [11]). The RPQ is a 23-item self-report questionnaire inquiring 11 example sentences of reactive and 12 of proactive aggression that are scored by the participant in a scale of never, sometimes and often. As reactive, but not proactive aggression is implicated in ED as well as ADHD, only the reactive subscale was used in the subsequent analyses. In the **Supplementary Section** "Analysis of Proactive Aggression", **Supplementary Table 2**, we attached a linear regression on proactive aggression and ADHD.

Table 1. Demographic description of the sample

Measure	**Control group,** n=78	**ADHD group,** n=78	**Difference,** p-value
Sex, percentage male participants	48.7%	43.6%	0.596
Age in years (SD)	34.2 (13.1)	34.1 (10.54)	0.656
Education (SD)	4.6 (1.6)	4.1 (1.6)	0.019
IQ (SD)	106.1 (13.6)*	108.7 (13.8)	0.479
DIVA, mean number of symptoms			
Inattention Adult (SD)	0.79 (1.27)	7.32 (1.99)	p < 0.001
Inattention Child (SD)	0.49 (0.84)	7.23 (1.83)	p < 0.001
Hyperactivity/Impulsivity Adult (SD)	0.83 (1.37)	5.59 (2.24)	p < 0.001
Hyperactivity/Impulsivity Child (SD)	0.83 (1.36)	5.57 (2.65)	p < 0.001
DIVA, percentage of adults reporting impairment			
Occupation	0%	74.3%	p < 0.001
Relationship and Family	0%	65.4%	p < 0.001
Social Contacts	0%	39.7%	p < 0.001
Hobby	1.2%	53.8%	p < 0.001
Self-image	0%	64.1%	p < 0.001
RPQ, mean score			
Reactive Aggression Score (SD)	5.57 (3.31)	8.17 (4.05)	p < 0.001
Proactive Aggression Score (SD)	1.42 (2.43)	1.90 (2.61)	0.242

This table summarizes mean scores and standard deviations of age, highest achieved educational degree (measured on a scale of 1 to 8 in the Dutch education system),BMI, IQ score, number of present symptoms of inattention and hyperactivity/impulsivity in childhood and adulthood (or current) and the percentage of subjects reporting impairments in occupation, relationship and family, social contacts, hobbies and self-image from the diva. The bottom of the table shows the mean scores and standard deviation of the results from the RPQ, proactive aggression is excluded from further analysis. *the IQ estimate of one control subject was missing. Statistical testing was performed using the Mann Whitney test as well as the chi-squared test for distribution free comparisons of independent samples with a significance level of p = .001.

To investigate implicit emotion processing during MRI, an adapted dynamic facial expression task was applied, showing faces morphing from a neutral face to an angry, fearful or happy facial expression in short clips of four frames. This task has proven to elicit activity in structures reflecting emotion processing, such as the amygdala [38]. The stimuli were taken from a standardized set and consisted of 10 grey-scale clips per emotion, each represented by a different actor of male and female gender in equal distribution. During the experimental session, we presented 6 blocks per emotion with a duration of 22.5 seconds, which consisted of 50 trials, 5 repetitions for each of the 10 actors. The blocks were presented in counterbalanced order interleaved with 9 blocks showing a fixation cross. In one random trial of each block, a red dot was displayed on the forehead of the actor (see **Supplementary Figure 1** in the section **Supplementary Material**). Participants were asked to press the button on a response box fixated on their leg as soon as the red dot appeared on the actor's face, to sustain their attention while preserving passive processing of the emotional faces. The scanning time for this task was approximately 10 minutes.

Image Acquisition

MRI scans were conducted using a 32-channel coil and a 3 Tesla Siemens Magnetom Prisma scanner (Siemens Trio, Erlangen, Germany). A T1-weighted MPRAGE sequence (TI = 1100 ms, flip angle = 8°, TE = 3.03 ms, TR = 2300.0 ms, bandwidth = 130 Hz/Px) with 192 sagittal slices (slice thickness = 1.0 mm) was used for the structural scanning, providing whole-brain coverage. Functional blood oxygen level-dependent (BOLD) images were collected using a T2*-weighted echo-planar imaging (EPI) sequence (TR = 1000 ms, TE = 34.0, flip angle = 60°, FOV = 210 mm, voxel size = 2x2x2 mm^3, 66 slices, interleaved acquisition, slice thickness = 2.00 mm). Preprocessing was performed in FSL FEAT. The first 5 images were discarded from further analysis. Mean framewise displacement of all participants was below the cutoff of 0.5 mm for 10% of the frames. After grand mean scaling and boundary based registration to the structural image and realignment as motion correction, a Gaussian filter of 5 mm kernel was applied to the images. Motion correction was performed using a dedicated independent component analysis based selection algorithm (ICA-AROMA; [39]). Additionally, the average signal of white-matter and corticospinal fluid were subtracted from the data. For analyses on the group level, we normalized individual scans to Montreal Neurological Institute (MNI) 152 standard space, 2mm resolution.

Analysis

fMRI Task Activation

Single subject fMRI analysis were performed in FSL FEAT (version 6.0.3) using a general linear model (GLM) with three regressors of interest modeling the onsets of happy, angry and fearful face blocks with a duration of 22.5 seconds as well two regressors of no interest modeling the trials where the red dot indicated the attention control task and the timing of the response as event markers with a duration of 0 seconds. As the task distracted from emotion processing, related BOLD activity and behavioral results of the task were excluded. Results were corrected for age and sex. All events were convolved with the canonical hemodynamic response function (HRF).

Three group level contrasts were defined by contrasting each emotional condition against the implicit baseline of the fixation blocks resulting in Happy > Fixation, Angry > Fixation and Fear > Fixation images. We included the factors diagnosis in two groups and emotion in the three levels angry, fear and happy in a mixed factorial model and investigated the group effects for each emotion separately as well as for all conditions together (Emotion > Fixation). Results are reported at a cluster-level corrected significance threshold of $p < 0.05$.

We further investigated effects of sex on the brain activity during emotion processing.

fMRI Task Activation and Reactive Aggression

To analyze the relationship between reactive aggressive scores with implicit emotion regulation and emotional reactivity, we included the contrast of all emotions versus the implicit baseline (Emotion > Fixation) as one summary measure per subject as well as the individual reactive aggression scores as a mean centered continuous covariate in the second level GLM. We were interested in the group specific correlates of reactive aggressive behavior, more specifically which brain regions would be relevant for higher reactive aggression scores in ADHD. Therefore we first investigated the interaction of diagnosis with reactive aggression, to see if there were group differences dependent on reactive aggression scores. Based on this interaction, we further looked at the effect in each group individually to find clusters associated with the co-occurrence of higher reactive aggression within the ADHD group. To correct for multiple comparisons in this exploratory analysis, we applied a Monte-Carlo-simulation of 1000 iterations for cluster extent correction on the uncorrected t-maps at $p = .001$ [40, 41], this method is publicly accessible at https://drive.google.com/file/d/ 16HVUD-PZaEpwHoZE99YXDxhcuLawjW7O/, resulting in a cluster extent of 11 resampled voxels at a cluster extent threshold of $p = 0.05$ assuming a type 1 error of 0.01.

Post-hoc Associations of Clinical Measures

To investigate the association between ADHD and the reactive aggression score of the RPQ, we used a linear regression analysis, modeling the Reactive Aggression scores by the binomial factor ‘diagnosis’ (1 = ADHD group, 0 = control group). We included age and sex as covariates in the model. The analysis was conducted in R version 3.6.1.

To investigate the association of clinical outcome with aggression, we introduced DIVA subscales of adult symptoms (Hyperactivity/Impulsivity, Inattention) and impairments (Occupation, Relationships and family, Social contacts, Hobbies, Self-image) as compounds to a linear model of reactive aggression scores. To assess clinical implications of our results, we analyzed the relationship of ADHD specific neural correlates of reactive aggression with the clinical expression of ADHD. We therefore analyzed correlations between symptoms and the activity in the right limbic system, the right precentral, inferior middle temporal and lingual gyri as well as the middle and superior frontal clusters showing positive covariance with elevated reactive aggression. Results are reported as Spearman's ρ and Bonferroni-corrected for multiple correlations.

We additionally performed a sensitivity analysis to investigate potential influences of medication on reactive aggression, the number of hyperactivity and impulsivity symptoms and impairments measured with the DIVA as well as the brain activity associated with reactive aggression in the ADHD group, see the section supplementary analysis.

RESULTS

fMRI Task Activation

For the whole-brain analysis of viewing all emotions compared to the implicit baseline, ADHD cases and controls did not show any differential activation patterns. Both diagnostic groups showed BOLD responses to emotional faces in broad clusters spanning several areas. These regions included temporoparietal, inferior, and superior frontal cortical areas as well as several subcortical areas including parts of the limbic system, see **Supplementary Table 1** and **Supplementary Figure 2**. No significant differences were found either when analyzing the emotions separately. Comparisons were made at the FWE-corrected threshold of $p = 0.05$.

We furthermore found two clusters in the left superior frontal as well as left orbitofrontal cortex associated with sex differences during emotion processing, see **Supplementary Table 3**.

Neural Correlates of Reactive Aggression

Interaction of Reactive Aggression and Diagnostic Group

We found a significant interaction between ADHD diagnosis and reactive aggression scores in the activation of clusters assigned to the right precentral and postcentral gyri, superior parietal, middle temporal areas, lingual gyrus, and the caudate nucleus at a significance level of $p = 0.05$. Activity was higher for high reactive aggression scores in the ADHD group and lower for low reactive aggression scores in healthy controls. **Figure 1** shows the cluster of significant interaction, for further information see **Table 2**. There were no clusters showing an inverse effect.

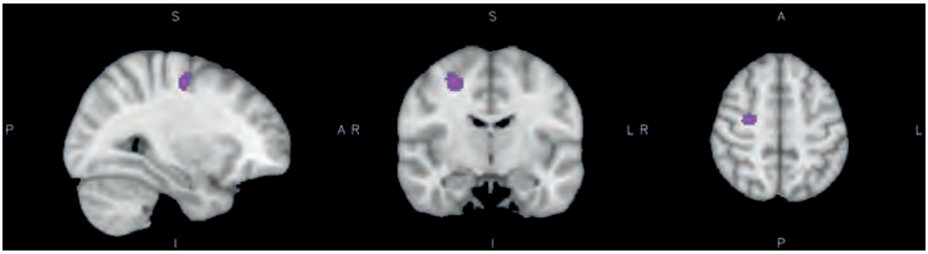


Figure 1. Interaction of diagnosis with reactive aggression. Results from the interaction of reactive aggression and diagnosis at the maximum of the cluster in the precentral gyrus (slices x= 30 left, y= -10 middle and z = 52 right), cluster extent corrected for p = .05.

Table 2. Interaction of diagnosis and reactive aggression

Cluster Label	Voxels	P	Z-MAX	X (mm)	Y (mm)	Z (mm)
R Precentral gyrus	127	<0.05	4.97	30	-10	52
R Lingual gyrus	63	<0.05	3.68	16	-54	-6
R Superior parietal lobe	39	<0.05	3.84	30	-56	58
R Inferior / middle temporal gyrus	24	<0.05	3.64	58	-36	-16
R Postcentral gyrus	22	<0.05	3.86	18	-42	58
R Occipital Pole	11	<0.05	3.34	4	-86	32
L Caudate	11	<0.05	3.54	-6	8	10

Results of the whole-brain analysis for the interaction of reactive aggression with diagnosis, cluster extent correction of 11 voxel for p =0 .05. R stands for right, L for left.

Reactive Aggression in the ADHD group

The analysis of reactive aggression within the ADHD group showed significantly elevated activation levels of clusters including the precentral gyrus, cortical frontal and temporal areas, as well as subcortical structures such as the hippocampus.

Furthermore two clusters within the right Amygdala ($p_{unc.} < 0.001$, xyz = (22,-8,-8) and $p_{unc.} < 0.001$ xyz = (28,-10,-1)) were activated, but did not exceed the threshold of 11 voxels each. The activity in all clusters was positively associated with higher reactive aggression scores. There was no significant effect of low reactive aggression, see **Figure 2** and **Table 3** for more information on the significant clusters.

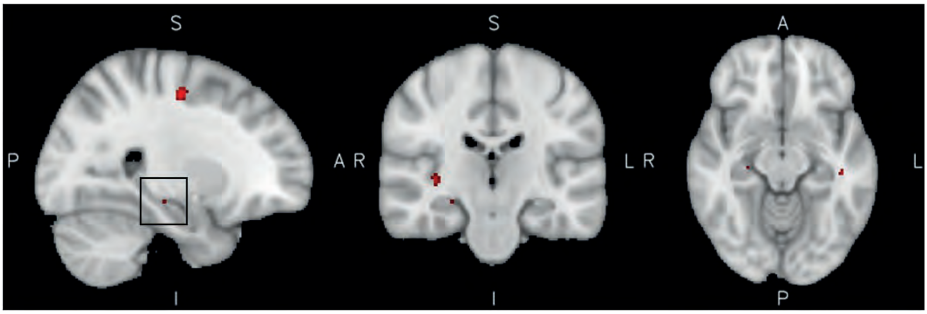


Figure 2. High reactive aggression in ADHD. Results from the reactive aggression analysis in the ADHD group at the maximum of the cluster of 14 voxel in the hippocampus, highlighted by a black square in the left figure (slices x= 26 left, y= -22 middle and z = -12 right), at a cluster extent correction of p = 0.05.

Reactive Aggression in the Control Group

No positive relationship of reactive aggression with brain activity was found in the control group. However, we additionally investigated a potential inverse effect, e.g. negative associations of reactive aggression scores with the brain activity of healthy controls. We found a negative relationship of reactive aggression with the activity in a cluster in the left middle temporal gyrus, right superior parietal lobule as well as the right precentral and lingual gyri (**Figure 3**, **Table 3**).

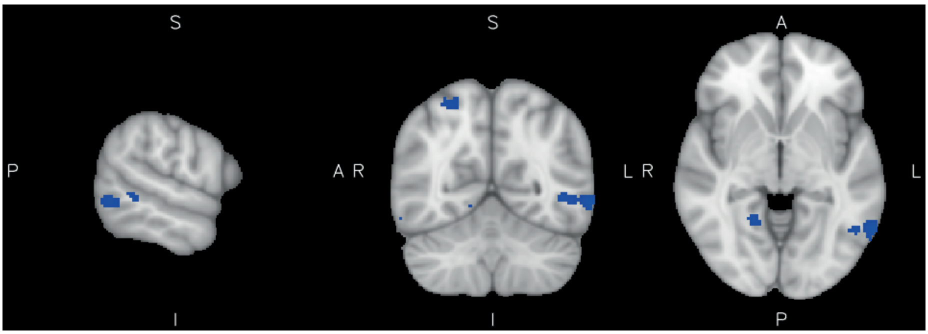


Figure 3. Low reactive aggression in the control group.Results from the reactive aggression analysis of the control group at the maximum of the cluster in the left middle temporal gyrus (slices x= -62 left, y= -60 middle and z = -4 right), at a cluster extent correction of p = 0.05.

Table 3. Neural correlates of reactive aggression

Cluster label	Voxels	P	Z-MAX	X (mm)	Y (mm)	Z (mm)
ADHD						
R Precentral gyrus	83	< 0.05	5.45	24	-12	50
L Middle frontal gyrus	42	<0.05	3.74	32	18	56
R Superior frontal gyrus	33	<0.05	3.78	6	32	62
R Inferior/middle temporal gyrus	33	<0.05	4.99	64	-32	-18
R Insula	20	<0.05	4.38	36	-22	2
R Lingual gyrus	18	<0.05	3.5	16	-70	-6
Not assigned	16	<0.05	4.27	-44	-26	-12
Not assigned	14	<0.05	3.82	30	2	28
R Hippocampus	14	<0.05	3.43	26	-22	-12
Control						
L Midde/inferior temporal gyrus	240	<0.05	4.37	-62	-60	-4
R Superior parietal lobule	88	<0.05	3.82	28	-56	58
R Lingual gyrus	60	<0.05	3.77	18	-52	-4
R Precentral gyrus	24	<0.05	3.78	32	-10	56
R Superior Parietal Lobule	13	<0.05	3.62	28	-42	58

Group results reactive aggression. Results of the whole-brain analysis for the analysis of reactive aggression within the ADHD group (top) and the control group (bottom) separately, cluster extent correction of 11 voxel for p = .05.

Post-hoc associations of clinical measures

The linear regression analysis of reactive aggression modeled by diagnostic group revealed a significant association of the factor diagnosis with reactive aggression scores (t=4.50; $p < 0.001$, $r_{standardized} = 0.34$). Age was not associated with reactive aggression, but male gender was (t=-3.72; $p = 0.004$, $r_{standardized} = -0.28$). **Table 4** summarizes the results of the regression analysis.

Table 4. Regression of Reactive Aggression

Regressors	Estimate	Standard Error	t-value	p-value
ADHD diagnosis (y/n)	0.33	0.57	4.50	<0.001
Age	0.03	0.02	0.40	0.69
Sex	-0.28	0.57	-3.72	<0.001

Summary of regression analysis of reactive aggressive behavior, showing regression coefficients, standard errors, t- and p-values.

We furthermore found associations between reactive aggression scores and the number of hyperactivity/impulsivity symptoms as well as self-reported impairment in the domains of "relationships and families" as well as "self-image" in adulthood from the DIVA in the regression analysis of the DIVA subscales, see **Table 5**.

Table 5. Association of Clinical Measures

Regressors	Estimate	Standard Error	t-value	p-value
Hyperactivity/Impulsivity	0.28	0.17	2.11	0.036
Inattention	0.09	0.14	0.71	0.479
Occupation	0.34	0.93	0.11	0.917
Relationship and family	6.41	0.89	2.07	0.041
Social contacts	2.03	0.81	0.71	0.479
Hobby	-2.17	0.79	-0.78	0.436
Self-Image	-6.59	0.93	-2.02	0.045

Associations of clinical measures with reactive aggression. Summary of regression analysis of reactive aggressive behavior, showing regression coefficients, standard errors, t- and p-values.

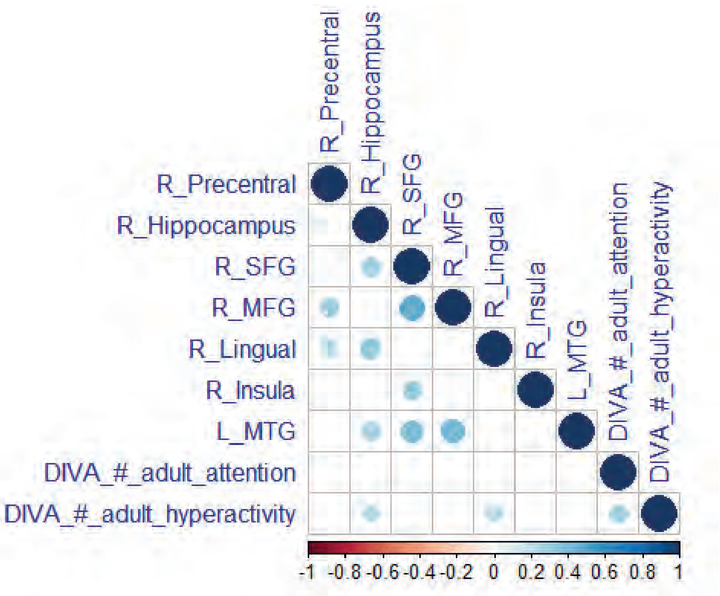


Figure 4. Correlation of neural activity with symptoms. Correlation matrix of mean estimates in the significant clusters from the reactive aggression whole-brain analysis with expressions of the core symptoms (attention and hyperactivity). We are reporting Spearman's correlation coefficient p (bottom scale) with a blue color for a positive and a red color for a negative correlation. Results were Bonferroni corrected.

Additionally, the number of hyperactivity/ impulsivity symptoms was correlated positively with the activity in two clusters associated with reactive aggression in the ADHD group; the more hyperactivity symptoms the adults with ADHD had, the more activation was seen in the right hippocampus (Spearman's $\rho = 0.29$, $p = 0.046$) and the lingual gyrus (Spearman's $\rho = 0.31$, $p = 0.041$). No correlations with neural activation in areas linked to reactive aggression were observed for inattention symptoms, see **Figure 4**.

The sensitivity analysis of medication effects yielded no significant medication effects, see the section **Supplementary Analysis**.

DISCUSSION

In this study, we aimed to identify neural correlates of reactive aggression, in adults with ADHD. To our knowledge, this is the first study to use a behavioral measure of reactive aggression as a marker of ED during implicit emotion regulation in adults with ADHD. We found areas of differential brain activity during emotion processing in the ADHD group in covariance with high reactive aggression to be localized in the limbic system and insula as well as in middle and superior frontal areas.

Reactive Aggression

In line with previous literature on reactive aggression in ADHD, our analysis of reactive aggression indicated significantly elevated levels of reactive aggression in adults with ADHD compared to the control group [32]. Higher scores of reactive aggression were also associated with male sex, in congruence with literature on male reactive aggression and externalizing behavior [6, 10]. Previous literature shows that increased reactive aggression often correlates with, or has predictive value for ADHD symptom severity [5], with closer developmental coupling with hyperactivity/ impulsivity symptoms [42]. Indeed, the association of reactive aggression with the subscales of the DIVA diagnostic instrument used in our study confirmed that symptoms of hyperactivity/impulsivity, but not inattention, were associated with reactive aggression. Interestingly, individuals reporting problems in relationships and/or family or with their self-image showed higher levels of reactive aggression, implying that impairments in the social life-domains could be particularly frequent in people with ADHD and high reactive aggression traits. This finding is in line with the literature on ED being an important predictor for the social, functional, and occupational outcome of ADHD [43, 44]. Especially for the persistent phenotype of ADHD, ED such as reactive aggression is considered a constitutional component [45], showing most pronounced expression and associated impairments in adulthood [46]. This clinical subgroup might thus be more vulnerable to social impairments and is particularly important to investigate further.

fMRI task activation

The fMRI whole-brain analysis of emotional faces revealed activation in the limbic system (associated with emotion processing and memory), the fusiform gyrus (associated with face processing), broad temporoparietal and frontal networks. These findings are in line with the original paradigm [38], which used this task to investigate manipulations of amygdala responsiveness, as well as a meta-analysis of implicit facial emotion processing [47]. Notably, the attention distraction task withdrew attention from the emotional stimulus towards the red dot, to measure implicit ED. This might skew the findings towards the study of emotion hyperreactivity and underrepresent the contribution of impaired executive functioning to the production of emotionally dysregulated behavior in ADHD. Studies on explicit emotion regulation, report more commonly associated areas such as the anterior cingulate and dorsal and ventromedial prefrontal cortex [25], which might be more relevant for tasks without distraction from the emotional stimulus. However, we find evidence, that even in this implicit emotion regulation scenario, middle and superior frontal areas are engaged, suggesting that executive functioning plays a role for implicit emotion regulation as well.

We found no neural activation differences between adults with and without ADHD, irrespective of emotional valence, suggesting that implicit emotional reactivity is not altered in people with ADHD. While several fMRI studies in children with ADHD reported group differences in the activity and connectivity of the amygdala, the ventral striatum, and the orbitofrontal cortex (OFC), these effects were often moderated by medical treatment and were not replicated in adult populations (for a review see [17]. In the current study, post-hoc analyses of medication effects on reactive aggression, symptoms, impairments and the neural correlates of reactive aggression revealed no significant associations (see section Supplementary Analyses).

However, sex seemed to influence emotion processing in the left orbitofrontral and superior frontal cortex. These areas are implicated in cognitive control processes, relevant for downregulation of emotional responses and might play a role for general sex differences in emotional reactions or aggression.

Neural Correlates of Reactive Aggression

While the neurocognitive architecture of emotion processing does not seem to be altered over the whole ADHD group compared to the controls, we expected the neurocognitive architecture to differ in adults with ADHD and co-occurring reactive aggression. Therefore, we investigated the association of reactive aggression with whole-brain activity in both diagnostic groups. We found a significant interaction between ADHD diagnosis and reactive aggression in areas such as the lingual gyrus, caudate, superior parietal, middle temporal, and premotor areas during processing of emotional faces, pointing towards differential neural correlates in adults with ADHD and reactive aggression compared to control subjects with reactive aggression.

When focusing the analysis on the ADHD group, activity in the right precentral, right lingual and left middle temporal gyri was particularly increased in people with ADHD if they had higher reactive aggression scores. Furthermore, small clusters within the right insula, the right limbic system (hippocampus and sub-threshold parts of the amygdala) and middle and superior frontal areas were implicated in emotion processing in the ADHD group with high reactive aggression only.

The insula as well as the hippocampus and amygdala are associated with the reactivity and assignment of salience during emotion processing in ADHD [48]. Emotional reactivity in the amygdala might be influenced by the hippocampus, which controls emotional memory recalling and regulation, especially in positive contexts [49], and the insula, engaged in down-regulation and maintaining homeostasis during the experience of negative emotions [48, 50]. Knowing that measures of structure and volume of the brain in these areas show alterations in children with ADHD, one could hypothesize that an altered neurodevelopment of these structures could influence the vulnerability of individuals with ADHD to develop reactive aggressive behavior: differential maturation might lead to higher liability to develop a hyperreactivity of the limbic system, which might suggest a more intense emotional sensation and higher sensitivity to emotional stimuli in subjects with ADHD.

Our results on altered activity of limbic structures and the insula during emotion processing are in line with previous findings in medication-naive individuals with ADHD [23, 51]. These authors discuss medication might drive a normalization effect of differential amygdala response. Interestingly, in our sample medication was not associated with reactive aggression, brain activity or ADHD symptoms in adulthood, which suggests that for the persistent phenotype of ADHD in our sample, stimulant medication seemed to be inefficient to further improve symptoms significantly.

Interestingly, we observed higher activity in middle and superior frontal clusters in patients with high reactive aggression scores. This activation is associated with stronger cognitive control and might suggest more effortful top-down emotion regulation, a process frequently disturbed in ADHD [52], implying both sub-processes, emotional reactivity as well as top-down regulation are implied in reactive aggression in adult ADHD.

The activation of the left middle temporal gyrus (MTG) as well as premotor areas was observed specifically in the ADHD group. The left middle temporal gyrus or temporo-parietal junction is often implicated in theory of mind abilities [53]. This ability to understand other people's beliefs and intentions might represent a protective mechanism for emotion dysregulation. The activity in the MTG could reflect higher efforts to retrieve intentions from the facial expressions in the ADHD group. Higher premotor activity could be related to more allocation of attention. Both could be cause or consequence of higher estimation of salience in the ADHD group with higher reactive aggression scores (e.g. hippocampus), which could trigger a deeper processing of the seemingly important emotional stimuli.

The healthy control group did not show any clusters associated with high reactive aggression, in coherence with the notably low levels of reactive aggression in this group. Interestingly, decreased activity in people in the control group with particularly low reactive aggression scores in some clusters that are implicated in the ADHD group as well suggest a protective function of these areas to develop reactive aggression (in particular the left MTG, precentral and lingual gyri).

Associations of clinical measures

We found positive correlations between the expression of hyperactivity/impulsivity symptoms and the activity related to reactive aggression in the hippocampus as well as the lingual gyrus in individuals with ADHD. The more hyperactivity/impulsivity symptoms adults with ADHD showed, the more these areas were engaged during emotion processing in association with reactive aggression. This association of ADHD symptoms with brain activity related to reactive aggression could be an example of a deviant neurocognitive mechanism behind emotion dysregulation in ADHD where the brain mediates both, emotion dysregulation as well as ADHD symptoms. Inattentive symptoms or impairments were not significantly correlated with neural activities. These findings point towards a specific susceptibility of the hyperactive type of ADHD to exhibit emotion dysregulation such as reactive aggression, in line with findings on the close developmental coupling of hyperactive/impulsive symptoms with reactive aggression [42] and general association of the hyperactive subtype with emotion dysregulation and aggression [54].

Strengths and weaknesses

All results need to be viewed in the context of the strengths and limitations of this study. Our sample sizes exceed most previous fMRI studies related to this topic in ADHD and is demographically well balanced.

However, task activation maps across all subjects did not show some areas classically associated with emotion regulation, such as the anterior cingulate, orbitofrontal cortex, dlPFC and vmPFC, or the amygdala [55], the latter only showing up as small subthreshold clusters. This was likely due to the implicit nature of our task. While emotion regulation paradigms mostly direct the participant's attention towards the processing of an emotion or even ask for explicit emotion regulation (Ochsner et al., 2012), we employed an implicit paradigm in which the participant's attention was not focused on the processing of emotional content and they were not asked to regulate their emotions, potentially affecting effect sizes and activity in areas relevant for top-down control. Notably, the clustersize of the reported results of the covariance of reactive aggression with ADHD is overall small, all described results should be carefully discussed as true findings. The small clustersizes might be related to the small effect sizes elicited from implicit emotion processing task in combination with an indirect measure of trait-reactive aggression as well as the vast heterogeneity in the ADHD population. Future studies with sufficient statistical power to address for expected small effect sizes that investigate subgroups within the spectrum of ADHD could elucidate this matter further. Furthermore, contrasting the emotional conditions to a fixation cross (implicit baseline) instead of neutral faces, as we did here, expectedly captured not only emotion processing but also general social cognition processes. For the investigation of adult ADHD and the association to emotion dysregulation, broader social processes e.g. general face processing difficulties, might play a role as well. Notably, we included reactive aggression as a trait-behavioral measure of emotion dysregulation. The applied task is measuring emotion processing closer to everyday-life situations compared to explicit emotion regulation tasks. Importantly, the integration of trait behavioral reactive aggression as a reflection of very relevant real-life emotion dysregulation together with the activity during emotion processing is suitable to reveal which areas are relevant for emotionally dysregulated behavior and potentially highlight social cognition and emotional (sub-)processes that are implicated in emotion dysregulation. However, the implementation of a task to elicit an emotional response, without attention distraction during fMRI could result in bigger effect sizes and a more precise delineation of the network of emotion dysregulation in ADHD. We furthermore recommend the implementation of a longitudinal design to study relevant brain networks for emotion dysregulation in ADHD, due to the close coupling of developmental trajectory of hyperactivity/impulsivity with the expression of emotion dysregulation.

CONCLUSION

In conclusion, these findings convey evidence for a differential emotion processing mechanism in subjects with ADHD and reactive aggression, with a specific liability of individuals with hyperactivity/impulsivity symptoms to experience these alterations. The brain regions related to this mechanism suggest difficulties with both emotional hyper-reactivity (as reflected in the insula and amygdala), and more effortful regulation of emotional responses (implicated by hippocampus and frontal activity) in the ADHD group with higher reactive aggression. This differential mechanism appears to be related to an altered neurocognitive brain architecture of ADHD and supports the diagnostic and clinical view of emotionally dysregulated ADHD as a subgroup in the spectrum of the disorder, as discussed by [3]. In the light of these findings, future research may evaluate more targeted intervention for the emotionally dysregulated group, such as behavioral emotion regulation interventions and their effect on reactive aggressive behavior [56].

Funding and Acknowledgements

We acknowledge funding from the Netherlands Organization for Scientific Research (NWO), i.e. from the Veni Innovation Program (grant 016-196-115 to MH) and the Dutch National Science Agenda NeurolabNL project (grant 400-17-602). The work was also supported by funding from the European Community's Horizon 2020 Programme (H2020/2014 – 2020) under grant agreements n° 728018 (Eat2beNICE) and and n° 667302 (CoCA), and by the European College of Neuropsychopharmacology (ECNP) Network "ADHD Across the Lifespan".

REFERENCES

1. Faraone, S.V., *ADHD*. Nature Reviews Disease Primers, 2015. **15027**.
2. American Psychiatric Association. 5th ed. Diagnostic and Statistical Manual of Mental Disorders. 2013, Wahington, DC.
3. Shaw, P., et al., *Emotion dysregulation in attention deficit hyperactivity disorder.* American Journal of Psychiatry, 2014. **171**(3): p. 276-293.
4. Christiansen, H., et al., *Attention-deficit/hyperactivity disorder (ADHD) and emotion regulation over the life span.* Current psychiatry reports, 2019. **21**: p. 1-11.
5. Abel, M.R., et al., *Reactive aggression and suicidal behaviors in children receiving outpatient psychological services: the moderating role of hyperactivity and inattention.* Child Psychiatry & Human Development, 2020. **51**(1): p. 2-12.
6. Connor, D.F., et al., *Impulsive aggression in attention-deficit/hyperactivity disorder: symptom severity, co-morbidity, and attention-deficit/hyperactivity disorder subtype.* Journal of child and adolescent psychopharmacology, 2010. **20**(2): p. 119-126.
7. King, S. and D.A. Waschbusch, *Aggression in children with attention-deficit/hyperactivity disorder.* Expert Review of Neurotherapeutics, 2010. **10**(10): p. 1581-1594.
8. Saylor, K.E. and B.H. Amann, *Impulsive aggression as a comorbidity of attention-deficit/hyperactivity disorder in children and adolescents.* Journal of child and adolescent psychopharmacology, 2016. **26**(1): p. 19-25.
9. Lickley, R.A. and C.L. Sebastian, *The neural basis of reactive aggression and its development in adolescence.* Psychology, Crime & Law, 2018. **24**(3): p. 313-333.
10. Slaughter, K.E., et al., *Reactive and proactive aggression in children with and without ADHD and negative emotional lability.* Social Development, 2020. **29**(1): p. 320-338.
11. Raine, A., et al., *The reactive–proactive aggression questionnaire: Differential correlates of reactive and proactive aggression in adolescent boys.* Aggressive Behavior: Official Journal of the International Society for Research on Aggression, 2006. **32**(2): p. 159-171.
12. Martel, M.M., *Research Review: A new perspective on attention-deficit/hyperactivity disorder: emotion dysregulation and trait models.* Journal of Child Psychology and Psychiatry, 2009. **50**(9): p. 1042-1051.
13. Evans, S.C., et al., *The role of reactive aggression in the link between hyperactive–impulsive behaviors and peer rejection in adolescents.* Child Psychiatry & Human Development, 2015. **46**(6): p. 903-912.
14. Bridge, J.A., et al., *Impulsive aggression, delay discounting, and adolescent suicide attempts: effects of current psychotropic medication use and family history of suicidal behavior.* Journal of child and adolescent psychopharmacology, 2015. **25**(2): p. 114-123.
15. Hoogman, M., et al., *Subcortical brain volume differences in participants with attention deficit hyperactivity disorder in children and adults: a cross-sectional mega-analysis.* The Lancet Psychiatry, 2017. **4**(4): p. 310-319.
16. Hoogman, M., et al., *Brain imaging of the cortex in ADHD: a coordinated analysis of large-scale clinical and population-based samples.* American Journal of Psychiatry, 2019. **176**(7): p. 531-542.
17. Rubia, K., *Cognitive neuroscience of attention deficit hyperactivity disorder (ADHD) and its clinical translation.* Frontiers in human neuroscience, 2018. **12**: p. 100.
18. Phelps, E.A., *Human emotion and memory: interactions of the amygdala and hippocampal complex.* Current opinion in neurobiology, 2004. **14**(2): p. 198-202.

19. Sarkheil, P., et al., *Amygdala response and functional connectivity during cognitive emotion regulation of aversive image sequences.* European archives of psychiatry and clinical neuroscience, 2019. **269**(7): p. 803-811.
20. Makris, N., et al., *Cortical thinning of the attention and executive function networks in adults with attention-deficit/hyperactivity disorder.* Cerebral cortex, 2007. **17**(6): p. 1364-1375.
21. Brotman, M.A., et al., *Amygdala activation during emotion processing of neutral faces in children with severe mood dysregulation versus ADHD or bipolar disorder.* American Journal of Psychiatry, 2010. **167**(1): p. 61-69.
22. Hwang, S., et al., *Executive attention control and emotional responding in attention-deficit/hyperactivity disorder—A functional MRI study.* NeuroImage: Clinical, 2015. **9**: p. 545-554.
23. Posner, J., et al., *Abnormal amygdalar activation and connectivity in adolescents with attention-deficit/hyperactivity disorder.* Journal of the American Academy of Child & Adolescent Psychiatry, 2011. **50**(8): p. 828-837. e3.
24. Vetter, N.C., et al., *Anterior insula hyperactivation in ADHD when faced with distracting negative stimuli.* Human brain mapping, 2018. **39**(7): p. 2972-2986.
25. Materna, L., et al., *Adult patients with ADHD differ from healthy controls in implicit, but not explicit, emotion regulation.* Journal of psychiatry & neuroscience: JPN, 2019. **44**(5): p. 340.
26. Passarotti, A.M., J.A. Sweeney, and M.N. Pavuluri, *Emotion processing influences working memory circuits in pediatric bipolar disorder and attention-deficit/hyperactivity disorder.* Journal of the American Academy of Child & Adolescent Psychiatry, 2010. **49**(10): p. 1064-1080.
27. Posner, J., et al., *The attenuation of dysfunctional emotional processing with stimulant medication: an fMRI study of adolescents with ADHD.* Psychiatry Research: Neuroimaging, 2011. **193**(3): p. 151-160.
28. Schulz, K.P., et al., *Emotional bias of cognitive control in adults with childhood attention-deficit/hyperactivity disorder.* NeuroImage: Clinical, 2014. **5**: p. 1-9.
29. Lindholm, P., et al., *Brain response to facial expressions in adults with adolescent ADHD.* Psychiatry Research: Neuroimaging, 2019. **292**: p. 54-61.
30. Nelson, R.J. and B.C. Trainor, *Neural mechanisms of aggression.* Nature Reviews Neuroscience, 2007. **8**(7): p. 536-546.
31. Alia-Klein, N., et al., *The feeling of anger: From brain networks to linguistic expressions.* Neuroscience & Biobehavioral Reviews, 2020. **108**: p. 480-497.
32. Bubenzer-Busch, S., et al., *Neural correlates of reactive aggression in children with attention-deficit/hyperactivity disorder and comorbid disruptive behaviour disorders.* Acta Psychiatrica Scandinavica, 2016. **133**(4): p. 310-323.
33. Puiu, A.A., et al., *Impulsive aggression and response inhibition in attention-deficit/hyperactivity disorder and disruptive behavioral disorders: Findings from a systematic review.* Neuroscience & Biobehavioral Reviews, 2018. **90**: p. 231-246.
34. Hermans, E.J., P. Putman, and J. Van Honk, *Testosterone administration reduces empathetic behavior: A facial mimicry study.* Psychoneuroendocrinology, 2006. **31**(7): p. 859-866.
35. Sato, W., et al., *The amygdala processes the emotional significance of facial expressions: an fMRI investigation using the interaction between expression and face direction.* Neuroimage, 2004. **22**(2): p. 1006-1013.
36. Kooij, J. and M. Francken, *DIVA 2.0.* Diagnostic Interview Voor ADHD in Adults bij volwassenen [DIVA 2 0 Diagnostic Interview ADHD in Adults]. DIVA Foundation (http://www. divacenter. eu/DIVA. aspx), 2010.
37. Wechsler, D., *Wechsler adult intelligence scale.* 1955.

38. Van Marle, H.J., et al., *From specificity to sensitivity: how acute stress affects amygdala processing of biologically salient stimuli.* Biological psychiatry, 2009. **66**(7): p. 649-655.

39. Pruim, R.H., et al., *ICA-AROMA: A robust ICA-based strategy for removing motion artifacts from fMRI data.* Neuroimage, 2015. **112**: p. 267-277.

40. Slotnick, S.D., *Cluster success: fMRI inferences for spatial extent have acceptable false-positive rates.* Cognitive neuroscience, 2017. **8**(3): p. 150-155.

41. Slotnick, S.D., et al., *Distinct prefrontal cortex activity associated with item memory and source memory for visual shapes.* Cognitive Brain Research, 2003. **17**(1): p. 75-82.

42. Murray, A.L., et al., *Developmental relations between ADHD symptoms and reactive versus proactive aggression across childhood and adolescence.* Journal of attention disorders, 2020. **24**(12): p. 1701-1710.

43. Bunford, N., S.W. Evans, and J.M. Langberg, *Emotion dysregulation is associated with social impairment among young adolescents with ADHD.* Journal of attention disorders, 2018. **22**(1): p. 66-82.

44. Bodalski, E.A., L.E. Knouse, and D. Kovalev, *Adult ADHD, emotion dysregulation, and functional outcomes: Examining the role of emotion regulation strategies.* Journal of Psychopathology and Behavioral Assessment, 2019. **41**: p. 81-92.

45. Beheshti, A., M.-L. Chavanon, and H. Christiansen, *Emotion dysregulation in adults with attention deficit hyperactivity disorder: a meta-analysis.* BMC psychiatry, 2020. **20**(1): p. 1-11.

46. Skirrow, C. and P. Asherson, *Emotional lability, comorbidity and impairment in adults with attention-deficit hyperactivity disorder.* Journal of affective disorders, 2013. **147**(1-3): p. 80-86.

47. Shi, H., X. Wang, and S. Yao, *Comparison of activation patterns between masking and inattention tasks: a coordinate-based meta-analysis of implicit emotional face processing.* Frontiers in human neuroscience, 2013. **7**: p. 459.

48. Zhu, Y., et al., *Emotion regulation of hippocampus using real-time fMRI neurofeedback in healthy human.* Frontiers in human neuroscience, 2019. **13**: p. 242.

49. Schumacher, A., et al., *Ventral hippocampal CA1 and CA3 differentially mediate learned approach-avoidance conflict processing.* Current Biology, 2018. **28**(8): p. 1318-1324. e4.

50. Steward, T., et al., *Emotion regulation and excess weight: impaired affective processing characterized by dysfunctional insula activation and connectivity.* PLoS One, 2016. **11**(3): p. e0152150.

51. Hulvershorn, L.A., et al., *Abnormal amygdala functional connectivity associated with emotional lability in children with attention-deficit/hyperactivity disorder.* Journal of the American Academy of Child & Adolescent Psychiatry, 2014. **53**(3): p. 351-361. e1.

52. Craig, F., et al., *A review of executive function deficits in autism spectrum disorder and attention-deficit/hyperactivity disorder.* Neuropsychiatric disease and treatment, 2016. **12**: p. 1191.

53. Samson, D., et al., *Left temporoparietal junction is necessary for representing someone else's belief.* Nature neuroscience, 2004. **7**(5): p. 499-500.

54. Wheeler Maedgen, J. and C.L. Carlson, *Social functioning and emotional regulation in the attention deficit hyperactivity disorder subtypes.* Journal of clinical child psychology, 2000. **29**(1): p. 30-42.

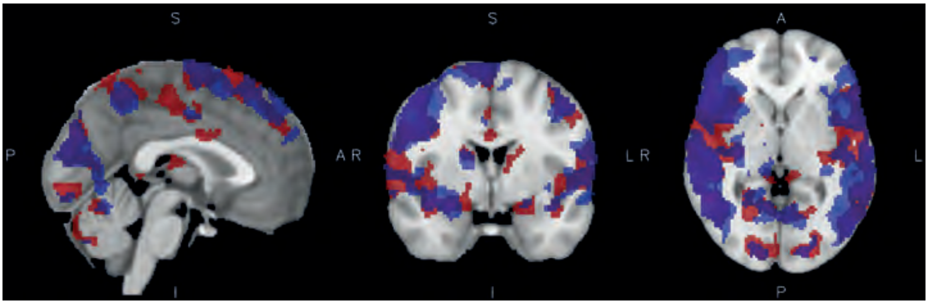
55. Ochsner, K.N., J.A. Silvers, and J.T. Buhle, *Functional imaging studies of emotion regulation: a synthetic review and evolving model of the cognitive control of emotion.* Annals of the new York Academy of Sciences, 2012. **1251**: p. E1.

56. Sánchez, M., et al., *Emotion regulation in participants diagnosed with attention deficit hyperactivity disorder, before and after an emotion regulation intervention.* Frontiers in psychology, 2019. **10**: p. 1092.

SUPPLEMENTARY MATERIAL

1 Supplementary Figures

Supplementary Figure 1. Example of the presented stimuli. Schematic example of a trials with a female actor in a happy face block and a male actor in a fear face block. Faces morphing from a neutral expression to the target expression in 450ms, described here in four example frames.Supplementary Figure 2. Overlap of emotion processing networks.



Supplementary Figure 2. Overlap of emotion processing networks. Results from the whole-brain contrast analysis Emotion<Fixation for the ADHD group in red overlapped with the control group in blue. Results are FWE-corrected at a significance level of $p<0.05$ at xyz = (0,0,0).

2 Supplementary Tables

Supplementary Table 1. Broad-scale emotion processing networks

Cluster index	Voxels	P	Z-MAX	X (mm)	Y (mm)	Z (mm)	Region of the Peak
ADHD							
5	51174	< 0.001	11,40	62	-24	18	Parietal Operculum
4	2263	< 0.001	9,83	16	-94	0	Occipital Pole
3	199	< 0.001	5,43	24	-78	-36	Cerebellum
2	194	< 0.001	5,53	-16	-10	20	Left Caudate
1	130	0,0385	5,16	-20	2	-16	Left Parahippocampal Gyrus / Left Amygdala
Control							
3	56835	< 0.001	10.6	52	38	8	Supramarginal Gyrus
2	572	< 0.001	5.57	-14	-92	-4	Occipital Pole
1	560	< 0.001	5.52	-22	54	16	Frontal Pole

Broad-scale emotion processing networks. Results of the whole-brain analysis for control subjects for the contrast Emotion < Fixation family-wise error corrected at p = 0.05. Cluster indices are reported for big cluster spanning several areas, the region of the peak according to the Harvard-Oxford cortical (and if relevant subcortical) atlases are indicated.

Supplementary Table 2. Regression of Proactive Aggression

Regressors	Estimate	Standard Error	t-value	p-value
ADHD diagnosis (y/n)	0.11	0.38	1.53	0.13
Age	-0.13	0.02	-1.76	0.08
Sex	-0.31	0.39	-4.03	<0.001

Regression of proactive aggression. Summary of regression analysis of proactive aggressive behavior, showing regression coefficients, standard errors, t- and p-values.

Supplementary Table 3. Effects of Sex on emotion processing

Cluster index	Voxels	P	Z-MAX	X (mm)	Y (mm)	Z (mm)	Region of the Peak
1	36	< 0.05	3,58	-12	16	62	Left SFG
2	33	< 0.05	3,64	-48	36	-14	Left Orbital Frontal Cortex

Effects of sex on emotion processing. Results of the whole-brain analysis for Sex effects during the fMRI paradigm, cluster extent correction of 11 voxel for p =0 .05.

3 Supplementary Analyses

Sensitivity Analysis

Our participants with ADHD were asked to withhold medication for at least 24 hours prior to participation, but long term stimulant treatment as well as therapeutic interventions could drive a generalized normalization effect of the limbic activity. In a post-hoc multivariate linear model we checked if ongoing treatment with stimulants was associated with I) reactive aggression scores, II) the number of inattentive symptoms, III) the number of hyperactive/impulsive symptoms, IV) impairments of the five life domains of the DIVA and V) the activity in the clusters from the reactive aggression analysis. We found no significant associations of medication with either variable.

Analysis of proactive aggression

While proactive aggression was not associated with ADHD diagnosis, it was significantly associated with male sex, which is in line with research reporting elevated levels of aggression in males.

Chapter 3

The gut-microbiome in adult Attention-deficit/hyperactivity disorder - A Meta-analysis

Jakobi, B., Vlaming, P., Mulder, D., Ribases, M., Richarte, V., Ramos-Quiroga, J. A., ... & Arias-Vasquez, A. (2024).

published as

The gut-microbiome in adult Attention-deficit/hyperactivity disorder-A Meta-analysis. European Neuropsychopharmacology, 88, 21-29.

ABSTRACT

Attention-deficit/hyperactivity disorder (ADHD) is a common neurodevelopmental condition that persists into adulthood in the majority of individuals. While the gut-microbiome seems to be relevant for ADHD, the few publications on gut-microbial alterations in ADHD are inconsistent, in the investigated phenotypes, sequencing method/region, preprocessing, statistical approaches, and findings. To identify gut-microbiome alterations in adult ADHD, robust across studies and statistical approaches, we harmonized bioinformatic pipelines and analyses of raw 16S rRNA sequencing data from four adult ADHD case-control studies (N_{ADHD}=312, N_{NoADHD}=305). We investigated diversity and differential abundance of selected genera (logistic regression and ANOVA-like Differential Expression tool), corrected for age and sex, and meta-analyzed the study results. Converging results were investigated for association with hyperactive/impulsive and inattentive symptoms across all participants. Beta diversity was associated with ADHD diagnosis but showed significant heterogeneity between cohorts, despite harmonized analyses. Several genera were robustly associated with adult ADHD; e.g., *Ruminococcus_torques_group* (LogOdds=0.17, p_{fdr}=4.42x10^{-2}), which was more abundant in adults with ADHD, and *Eubacterium_xylanophilum_group* (LogOdds= -0.12, p_{fdr}=6.9 x 10^{-3}), which was less abundant in ADHD. *Ruminococcus_torques_group* was further associated with hyperactivity/impulsivity symptoms and *Eisenbergiella* with inattention and hyperactivity/impulsivity (p_{fdr}<0.05). The literature points towards a role of these genera in inflammatory processes. Irreproducible results in the field of gut-microbiota research, due to between study heterogeneity and small sample sizes, stress the need for meta-analytic approaches and large sample sizes. While we robustly identified genera associated with adult ADHD, that might overall be considered beneficial or risk-conferring, functional studies are needed to shed light on these properties.

INTRODUCTION

Attention-deficit/hyperactivity disorder (ADHD) is a common neurodevelopmental condition [1], characterized by symptoms of inattention and hyperactivity/impulsivity (American Psychiatric Association 2013). The clinical presentation of ADHD is quite heterogeneous; symptoms and impairments persist into adulthood in the majority of affected individuals, and other psychiatric and somatic problems often accompany ADHD [2]. The etiology of ADHD is likely multifactorial, combining genetic and environmental risk-factors or protective influences [3]. Studies have suggested potential roles of e.g. immune- and inflammatory processes and alterations in dopaminergic and serotonergic neurotransmission, resulting in altered brain development and functioning, for the emergence of ADHD symptoms [4].

The gut-microbiome is involved in early brain-development as well as every-day brain functioning; it can modulate the bioavailability of key-signaling molecules (e.g. neurotransmitters or nutrients relevant for energy homeostasis) by influencing the metabolism and the integrity of intestinal- and-blood-brain barriers [5]. Through the regulation of the intestinal barrier, but also through the production of short-chain-fatty-acids (SCFA) and the release of cytokines, it further plays an important role in immune and inflammatory responses (for a review see [5]). These pathways might influence ADHD symptoms and pathophysiology [6, 7]. Studies showing associations of microbial diversity and composition with altered neurodevelopment (for a review, see [8]), psychiatric disorders (for a review, see [9]) and common metabolic comorbidities of ADHD [10, 11] have fueled hypotheses about the potential role of the gut-microbiome alterations for ADHD. The few published studies investigating gut-microbiome alterations in ADHD, however, report inconsistent results. Most authors reported no differences between individuals with and without ADHD or conflicting results for gut-microbiome diversity ([12-16]). Recent systematic reviews showed that differential abundance of some taxa between individuals with and without ADHD was reported in all published studies [13]. However, the results converged at the genus-level (marking the highest resolution of taxonomic assignment from 16S sequencing) in maximally two out of eight published studies in children and adolescents (see Supplementary Table 1 [17, 18]). The three studies published on adults so far showed no overlap in results, despite a substantial overlap in samples and wet-lab procedures between Aarts et al. (2017) and Szopinska-Tokov et al. (2020) [19-21]. The scarcity of consistent results renders biological interpretations of gut-microbiome alterations as well as consideration of the abundance of particular taxa as potential biomarkers or treatment targets for ADHD difficult.

Inconsistencies in reported results might be explained by a variety of factors; [22] provide an overview on this topic. Most relevant may be the expected small effect sizes of microbiome alterations due to high inter- and intrapersonal variability, in conjunction with the statistical testing of a high number of features and the small sample sizes of gut-microbiome studies published to date (mean $n_{ADHD} = 39$, mean $n_{noADHD} = 49$ [13]); those might lead to high false positive rates and at the same time low detection of true effects. Heterogeneity in terms of age (children, adolescents, adults), sex (some studies only focused on males), and ethnic origin (Asian or European) might render summarizing and comparing the results more difficult. Accounting for common confounders (e.g. age, sex, diet), focusing on one developmental stage, and meta-analysis across studies might help increase the robustness of findings. Importantly, methodological choices can strongly influence observed microbial diversity and composition, e.g. in the technical variation of wet-lab procedures and processing of microbiome data. While most studies use 16S rRNA sequencing, different sequencing methods (extraction, storing, platforms, protocols) and the choice of the regions of the 16S gene can have a substantial influence on the identified features (for a review, see [23]). The lack of consensus on preprocessing pipelines and statistical analysis tools or approaches further contributes to the scattered and irreproducible gut-microbiome associations with ADHD across the field. 16S microbiome data comes with particular properties, that should be accounted for: 1) Compositionality-bias (stemming from the fact that the count of a feature does not carry information about its absolute abundance) results from the sequencing and the restriction to the library size. Transformations such as the center-log-ratio (CLR) scale the counts to a reference and can successfully eliminate compositionality bias of between-sample comparisons [24]. 2) Zero-inflation and un-identifiable sources of zeros (e.g. sampling bias, sequencing bias, true zero) are particularly problematic for differential abundance analysis. Bias-related zeros increase the number of (uninformative) tests, and the resulting distribution has to be accounted for by the statistical approach. A recent paper [25] reviewed approaches to deal with these issues, showing that exclusion of features that are observed in less than 10% of the samples from further analysis (prevalence threshold) increased cross-method comparability and reduced the statistical testing burden, false discovery rates, and zero inflation bias while maintaining sufficient information content for downstream statistical analysis. Attempts to account for these biases in the statistical approaches, however, vary profoundly between research groups, resulting in incomparable results across studies. The comparison of results across tools is recommended, where converging results are most likely to reflect true findings. Next to logistic regression in case-control studies, ANOVA-like Differential Expression (ALDEx2 [26]) is a promising approach to analyze differential abundance; this method produced the most comparable results across statistical approaches and studies while preserving a low false discovery rate [25].

In this study, we aimed to investigate gut-microbiome alterations in adult ADHD and identify genera that might be risk-conferring for the condition. To assure robustness of the results, we applied four strategies: 1) We harmonized the bioinformatic pipelines for sequencing data of four case-control cohorts of adults with and without ADHD (N=617); 2) we focused on one developmental stage, i.e. adulthood; 3) we investigated diversity and differential abundance across tools and indices, correcting for common confounders per study; 4) we meta-analyzed the results across studies. Converging results across tools and studies were analyzed for associations with the clinical representations of ADHD such as hyperactive/impulsive and inattentive symptoms.

MATERIALS AND METHODS

Cohort description

We requested data from all ADHD case-control studies that included 16S fecal gut-microbiome samples, identified in two recent systematic reviews [13, 16], see Supplementary Section 1.1, Table 1 and Supplementary Figure 15. We received clinical information and raw sequencing data from four adult cohorts (comprising the three articles including adults published to date: the NeuroIMAGE cohort [20, 21] and the Mental-Cat cohort from the Vall d'Hebron Research Institute in Barcelona (VHIR) [19]; and unpublished data from the MIND-Set cohort [27] and our own cohort IMpACT2-NL [28].

The NeuroIMAGE cohort comprises adolescents and young adults with ADHD, their family members as well as unrelated healthy controls; only adult participants were included in this study [13, 29]. The Mental-Cat cohort consists of medication-naive adults with and without ADHD. The MIND-Set cohort includes adults with ADHD exhibiting a high level of psychiatric comorbidity and psychopharmacological treatment, as well as healthy individuals. For detailed information about individual studies, recruitment and inclusion/exclusion criteria, see Supplementary Chapter 1.1. Information about fecal sample collection, storing, and sequencing are provided in Supplementary Chapter 2. Our report follows STORMS guidelines for human microbiome research, whenever possible [30], see the Supplementary Table 14.

Participants with gut-related diseases (irritable bowel disease) and those with an unclear ADHD diagnosis were excluded, and overlapping samples between MIND-

Set and IMpACT2-NL were removed from the MIND-Set cohort, resulting in a sample of 312 adults with and 305 adults without ADHD (56 exclusions, see Supplementary Figure 1). Table 1 provides a demographic overview of the included sample.

Table 1. Demographic description and characteristics of the included studies

Cohort Recruitment period Recruitment Country	**IMpACT2-NL** 2017-2020 The Netherlands		**NeuroIMAGE** 2009- 2012 The Netherlands		**MIND-Set** 2016-2021 The Netherlands		**Mental-Cat** 2016-2018 Spain	
Diagnostic group	Control	ADHD	Control	ADHD	Control	ADHD	Control	ADHD
Number of subjects	78	78	38	29	90	104	100	100
Sex, proportion female participants	0.51	0.57	0.47	0.31	0.54	0.42	0.53	0.49
Age mean years (SD)	34.49 (13.08)	33.86 (10.31)	21.84 (2.54)	22.21 (3.05)	38.55 (16.87)	37.38 (12.55)	30.3 (8.47)	33.17 (11.41)
BMI mean (SD)	24.85 (4.25)	24.86 (4.49)	22.84* (3.05)	24.39 (3.79)	23.85** (4.46)	28.01*** (15.19)	22.13 (2.91)	24.67 (4.25)
Smoking, proportion non-smokers	0.90	0.65	NA	NA	0.93	0.76	NA	NA
ADHD medication proportion currently using ADHD medication	0	0.51	0	0.42	0	0.84 [1]	0	Exclusion criterium
Inattentive symptoms mean(SD)	0.87 (1.26)	5.65 (2.17)	41.74 (10.67)	58.45 (12.12)	2.31* (1.98)	8.86**** (2.81)	NA	17.86 (4.33)
Hyperactive/ impulsive symptoms mean(SD)	0.79 (1.10)	7.38 (1.83)	45.42 (9.29)	66.86 (11.32)	2.51 (1.66)	8.09 (2.27)	NA	13.26 (6.08)
Instrument symptom assessment [2]	DIVA adult self-report		CAARS-DSM-IV adult self-report		CAARS-S.S short adult self-report		ADHD-RS adult self-report	
Sequencing Region	V4		V1/V2		V4		V3/V4	
Sequencing platform	Illumina NovaSeq 6000		Illumina HiSeq PE300		Illumina NovaSeq 6000		Illumina MiSeq 300-nt	
Antibiotic treatment, proportion using antibiotics	0.01 [3]		No information		0		Exclusion criterium	
Psychiatric comorbidity, proportion of ADHD + additional current psychiatric diagnoses	Exclusion criterium [4]		Exclusion criterium [5]		0.83		Exclusion criterium	

Footnote: * 1 missing value,** 27 missing values, ***15 missing values, ****11 missing values.

[1] 65.5 % of MIND-Set participants with an ADHD diagnosis received additional pharmacological treatment targeting the central nervous system (mostly antidepressants), with a maximum of 6 different drugs prescribed to an individual.

[2] Symptom assessment tools are described in more detail in Supplementary Chapter 1.1.

[3] IMpACT2-NL assessed frequency of antibiotics use (frequent, sometimes, rarely or never; 96.4% of participants answered never or rarely, one control participant reported frequent antibiotics usage.

[4] Diagnoses of neurodevelopmental and current psychiatric disorders were excluded.

[5] Diagnoses of autism were excluded.

Microbiome preprocessing

Preprocessing was harmonized and performed per study using QIIME2 pipeline defaults [31], Figure 1 summarizes the preprocessing steps. For all studies, the raw forward and reverse reads were demultiplexed and then denoised using DADA2 [32], where the primers were trimmed off, sequencing errors and erroneous read combinations were removed and clusters of representative sequences (amplicon sequence variants, ASVs) were identified. We assured high sequencing quality by truncating the reads displaying a signal drop below a median phred-score of 30 (marking 99.9% base-call accuracy [33]) towards the end of the reads resulting in a truncation from basepair 260 in NeuroIMAGE and VHIR. For IMpACT and MIND-Set no truncation was applied (no signal drop). The sequencing data for NeuroIMAGE was delivered in four batches and merged after denoising. The ASVs were aligned to their phylogenic tree (fasttree2 [34]). Taxonomy was assigned using a naive bayes classifier, pre-trained on a SILVA reference database of the full-length 16S gene (version 138, 99% OTUs full-length sequences, https://docs.qiime2.org/2022.2/data-resources/), to assure coherent classification irrespective of the sequencing region of the study. The resulting feature table with taxonomic assignments and the phylogenic tree were imported in R (version 4.2.1 [35]). We removed non-bacterial ASVs and screened for read depth (plateau in the rarefaction curve) and summarized sequencing data after each step, see Supplementary Chapter 2. We then investigated potential differences in alpha and beta diversity as well as composition per study, correcting for age and sex. Other common confounders were assessed post-hoc (ADHD medication, diet) or not included due to (excess) missing information (body-mass-index (BMI), smoking, general medication, anti-/probiotics) or high co-linearity with ADHD-control grouping (smoking, psychopharmacological treatment, comorbidities).

Alpha Diversity Analysis

We estimated three indices of alpha diversity, considering 1) number of observed ASVs, 2) abundance of ASVs (Shannon index), and 3) phylogenic relationships between ASVs (phylogenic distance) (*microbiome* package, [36]). We applied rank-based nonparametric regression analysis (*Rfit,* [37]) for each study (alpha diversity ~ ADHD diagnosis). We further extracted the standardized correlation coefficient R as effect size measure per study and meta analyzed over the four cohorts with a random-effects models, which estimates study heterogeneity (*metafor* package [38]).

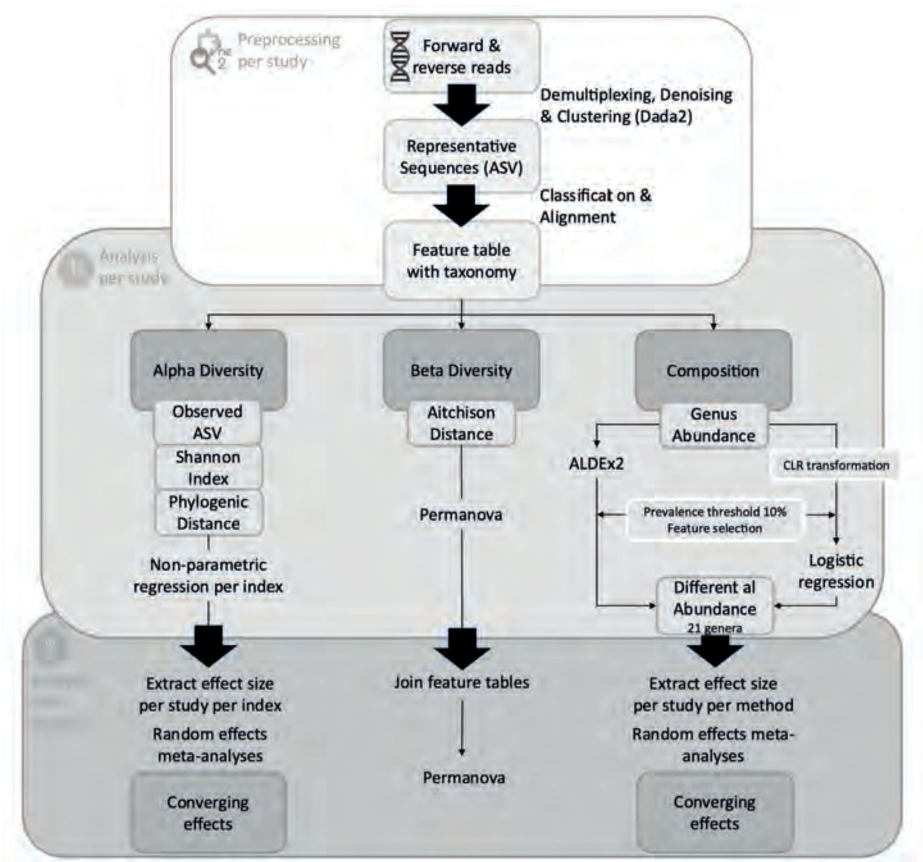


Figure 1. Workflow diagram of bioinformatic pipelines for preprocessing and statistical analyses.
Section 1 (top) describes the preprocessing per study, section 2 (middle) describes the statistical analysis of diversity and composition per study and section 3 (bottom) describes the statistical analysis over all studies. Abbreviations: ASV: amplicon sequence variants, ALDEx2: ANOVA-like differential expression, CLR: center log ratio.

Beta Diversity Analysis

Beta diversity (the similarity of the microbiome between samples) was investigated per study, applying the Permanova (*adonis2)* with 999 permutations in order to estimate Aitchison distance by ADHD diagnosis (*vegan* package [39]). We additionally investigated beta diversity differences over all studies in a mega-analytic fashion by combining all feature tables and including the cohort as a nominal dummy variable. We visualized the effect of ADHD diagnosis on beta diversity using Canonical Analysis of Principal coordinates (CAP) plots, supervised for group effects (*phyloseq* package [40]).

Composition

To investigate microbial composition, we applied a prevalence threshold of 10% (improves comparability over statistical tools, reduces the number of statistical tests and zero-biases [25]) and aggregated the data to the genus level, see Supplementary Table 2 for counts per study. We applied CLR transformations to the count data to account for zero-inflation and compositionality biases [24].

Feature selection

To test only genera with potential informative value for the outcome of interest (ADHD diagnosis), we performed feature selection using randomized lasso stability selection in each cohort (*monaLisa* package, [41]). In a random subsample of n/2, ADHD diagnosis was regressed against all genera in a lasso-penalized regression (repeated for 999 subsamples). The selection probability was calculated as the number of permutations in which a genus was selected (i.e., $\beta \neq 0$) divided by the total number of permutations. Due to small individual sample sizes and high interindividual variability in the gut-microbiome, the selection probabilities per genus are expected to be small. We applied a lenient threshold of 10% stability selection probability within each study, to assure that genera with potentially small within-study relevance will be picked up, as they might accumulate across studies. We disregarded all genera from further analysis whose selection probability stability paths showed low to no informative value, see Supplementary Chapter 5.1. Selected genera, prevalent across cohorts were introduced to differential abundance analysis.

Differential abundance analysis

We applied differential abundance analysis per study, using logistic regressions associating ADHD diagnosis with the CLR-transformed abundance. We meta-analyzed over the standardized effect size (log odds ratio) of all four studies for each genus. To account for potential inconsistencies across statistical tools, we additionally performed differential abundance analysis with ALDEx2. We applied ALDEx2 per study before prevalence thresholding and feature selection, as the correction for feature variation is estimated most accurately taking all features into account. We subsequently estimated standardized effect sizes (standardized correlation coefficient R and variance) and performed meta-analysis on only the prevalent, feature selected genera. We applied a significance threshold of $p < 0.05$, where *p-values* were false discovery rate (fdr) corrected.

Associations with symptoms

To investigate if compositional differences in the gut-microbiome of adults with ADHD were associated with the symptoms of hyperactivity/impulsivity or inattention, we employed rank-based regression (*Rfit*) on the mean centered number of symptoms (see supplementary chapter 1.1) and the (CLR-transformed) abundance of those genera, that were robustly associated with ADHD diagnosis abundance, corrected for age and sex. We extracted the standardized effect size measure R (*metafor*) for each association per study and meta analyzed (N = 505). We applied a significance threshold of $p < 0.05$, *p-values* were false discovery rate (fdr) corrected.

RESULTS

Alpha Diversity

We found no significant differences of alpha diversity between adults with and without ADHD, neither on the individual study level nor in the meta-analysis, in terms of observed ASV, Shannon index, or Faith's phylogenic diversity, see Supplementary Chapter 3.

Beta Diversity

At the individual study level, three out of four studies showed differences in beta diversity between people with and without ADHD (Supplementary Chapter 4). The Permanova over all studies showed a significant association of ADHD diagnosis with beta diversity ($p = 4.7E^{-2}$, F=1.77) explaining 0.2% of variance, despite pronounced differences between the cohorts (explaining ca. 25%, $p = 1.0E^{-2}$, F=67.94, Table 2). Figure 2 shows a separation of individuals with (blue) and without ADHD (red) as well as separation of MIND-Set and IMpACT2-NL, using the same sequencing technique and region, from the other two cohorts, which applied different wet-lab techniques.

Table 2. Beta Diversity across the samples of all four studies combined

	Sum of Squares	R^2	F	*p*-value
ADHD diagnosis	12890	0.002	1.77	4.7E-02
Age	23665	0.003	3.24	1.0E-03
Sex	11763	0.002	1.61	6.4E-02
Cohort	1486900	0.244	67.94	1.0E-03
Residual	4450154	0.731		
Total	6087500	1		

Table contains the sum of squares, the proportion of variance in beta diversity explained by the regressors diagnosis, age, sex, and effect of the study (R^2), as well as test statistics F and permutation p-value.

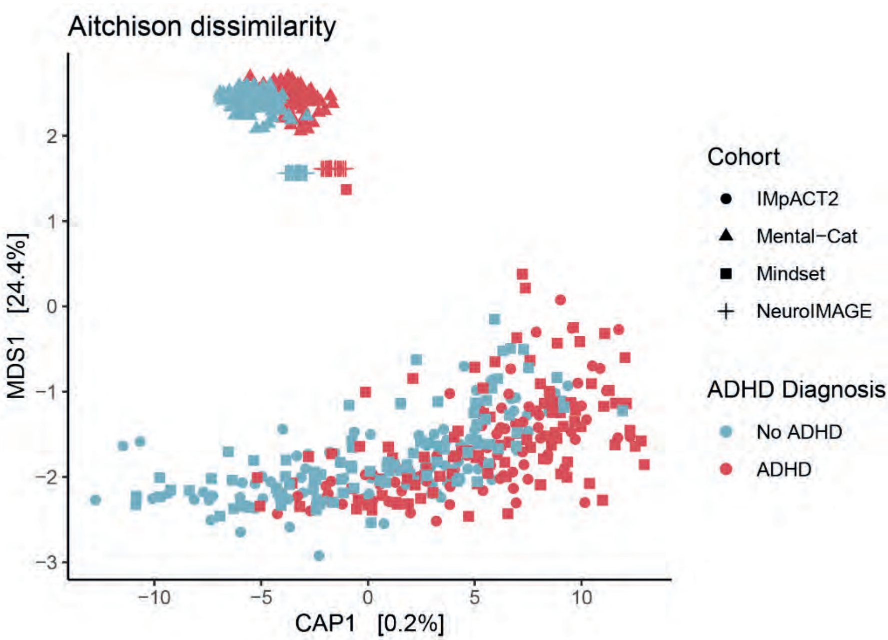


Figure 2. Beta-diversity of gut microbial communities in 312 people with ADHD (marked in blue) and 305 without ADHD (marked in red) over four cohorts.

The CAP plot is supervised for differences in Aitchison dissimilarity between diagnostic groups (ADHD diagnosis), the cohort is marked by shapes (dot for IMpACT2-NL, triangle for MIND-Set, square for NeuroIMAGE and + for Mental-Cat).

Composition

Feature selection

A total of 27 genera were selected over all four studies in the randomized Lasso stability selection, 20 of which exceeded the prevalence threshold of 10% in all four cohorts; see Supplementary Chapter 5 for the selected features, selection probability, and the stability paths per study.

Differential abundance analysis

Logistic regression-based meta-analyses of the 20 selected genera identified 5 significantly different genera between adults with and without ADHD (fdr-corrected): *Ruminococcus_torques_group* (p_{fdr}=4.4E^{-2}, Log odds ratio (LOR) = 0.17), *Eubacterium_xylanophilum_group* (p_{fdr}=6.9E^{-3}, LOR = -0.12), *Eubacterium_ruminantium_group* (p_{fdr}=4.4E^{-2}, LOR = -0.06), *Eisenbergiella* (p_{fdr}=2.0E^{-2}, LOR = 0.14), and *Clostridia_UCG_014* (p_{fdr}=4.4E^{-2}, LOR = -0.07). Figure 3 displays the effect size, direction of effects, and significance level for each genus.

By repeating the meta-analyses on the ALDEx2-based effects of the selected genera, we identified two fdr-corrected significantly different genera, both overlapping with the results from logistic regression; *Ruminococcus_torques_group* (p_{fdr}=1.9E^{-2}, r=0.13) was more abundant, while *Eubacterium_xylanophilum_group* (p_{fdr}=1.9E^{-2}, r=-0.12) was less abundant in people with ADHD compared to those without ADHD. At an uncorrected significance level, ALDEx2 identified four out of the five genera identified in logistic regression (along with *Eisenbergiella* and *Clostridia_UCG_014*), which were subsequently introduced to post-hoc symptom associations (see below). The significant results showed small effect sizes, with no significant influence of study heterogeneity. Forest plots of significant results, and results tables of the meta-analyses are described in Supplementary Chapter 6.1 and 6.2. We further visualized relative abundance and the number of non-zero samples for the 20 selected genera, see Supplementary Chapter 6.3.

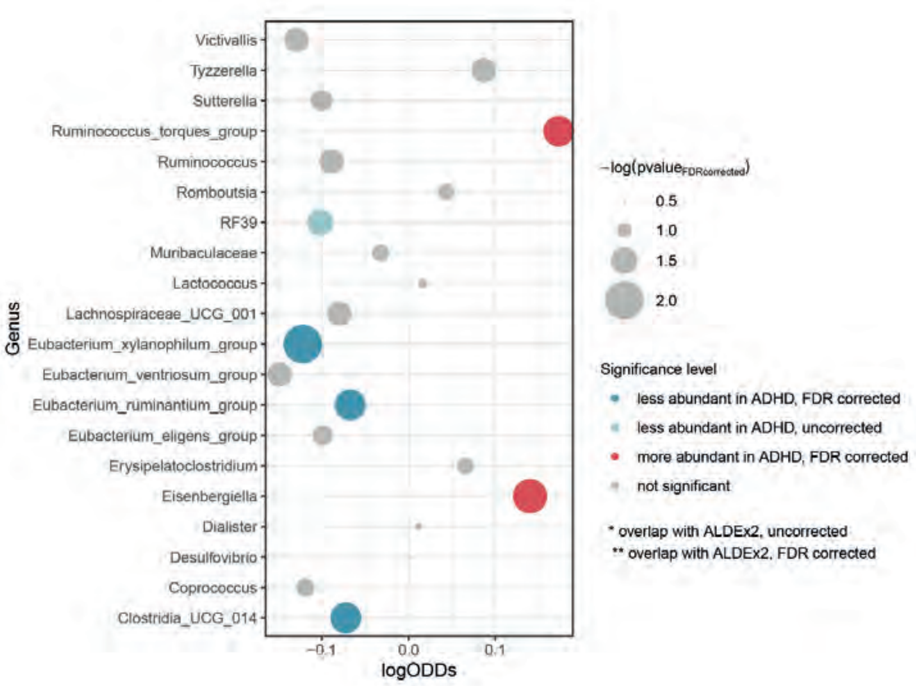


Figure 3. Results from the meta-analyses on all 21 genera (y-axis) from logistic regression, displaying significance level (bubble size), effect size (x-axis) and direction of effects (red for higher, blue for lower abundance in ADHD). Converging results with ALDEx2 are marked with *(uncorrected), **(fdr corrected).

Post-hoc associations with ADHD symptoms

More inattention symptoms were significantly associated with higher abundance of *Eisenbergiella* (p_{fdr}=1.1E^{-3}, r=0.16), and more hyperactivity/impulsivity symptoms were significantly associated with higher abundance of *Eisenbergiella* (p_{fdr}=4.2E^{-3}, r=0.14) and *Ruminococcus_torques_group* (p_{fdr}=1.2E^{-2}, r=0.13). The results of the meta-analyses are available in the Supplementary Chapter 7.1.

DISCUSSION

Study summary and results

In study, we aimed to identify robust gut-microbiome alterations in adult ADHD by harmonizing the bioinformatic pipelines of four case-control cohorts (N=617), investigating diversity and differential abundance across tools and indices, correcting for common confounders per study, meta-analyzing and interpreting converging results across tools and studies. While alpha diversity was not significantly different between adults with and without ADHD, we found differences in beta diversity and identified robust microbiome-compositional alterations in adult ADHD and associations with the severity of inattention and hyperactivity/impulsivity symptoms.

Interestingly, beta diversity was additionally influenced by the individual cohort setup, despite harmonized preprocessing. The clustering of two studies with overlapping wet-lab procedures and separation of two studies who applied different wet-lab strategies underlines the impact on detected composition and supports the need for meta-analytic approaches and harmonized wet-lab protocols to account for influences of collection, storing and sequencing. While diversity is an unspecific measure for disease-related processes, indicating only global tendencies of potentially disrupted ecosystems, compositional differences can add information on individual features involved and help identifying relevant functional pathways.

On a compositional level, *Eubacterium_xylanophilum_group* was significantly less abundant in adults with ADHD, while *Ruminococcus_torques_group* was more abundant and associated with more hyperactivity/impulsivity symptoms, converging over meta-analyses. Additionally, both methods identified higher abundance of *Eisenbergiella*, associated with hyperactivity/impulsivity and inattention symptoms, and lower abundance of *Clostridia_UCG_014* in adults with ADHD.

Functional properties

The Ruminococcus_torques_group has earlier been found enriched in children with ASD [42], Irritable Bowel Syndrome [43], Crohn's disease [44], and influenza-like illness [45]; it was also found associated with the degradation of the intestinal mucosal layer [46]. *Ruminococcus_torques* species were further associated with a western diet (high energy, low nutrition) and neuroinflammation; it might also be related to reduction in striatal dopamine in Parkinson's Disease (for a review, see [47]). These studies suggest a role of *Ruminococcus_torques_group* in gut-barrier functioning and pro-inflammatory processes, which have previously been implicated in the etiology of psychiatric, neurodevelopmental, and neurological disorders [48, 49].

Eisenbergiella has also been found enriched in individuals with neuropsychiatric disorders, such as ASD, depression, and Parkinson's disease [50-52], in chickens and mice infected with pathogens [53, 54], and in children with an allergy to cow's milk [55]. It was found reduced in rats after immunosuppressive/anti-inflammatory treatment [56], suggesting general associations with pro-inflammatory processes and immune activation. Higher abundance of *Eisenbergiella* has also been linked to a high energy diet (rich in carbohydrates, fat, and protein) [57] and to metabolic disorders, such as gestational diabetes mellitus [58].

Eubacterium_xylanophilum_group seems to play an anti-inflammatory role. It is considered a producer of SCFAs and was found enriched after intervention with polyphenols in piglets [59]. Metabolism of polyphenols into SCFAs by the gut-microbiome is discussed as a potential mechanism through which polyphenols might unfold their anti-inflammatory properties [60]. *Eubacterium_xylanophilum_group* was also found enriched in mice with colorectal cancer after supplementation with sodium butyrate, associated with SCFA production and an improved immune response [61]. Associations of *Eubacterium_xylanophilum_group* with beneficial effects on immune functioning might be relevant for ADHD, potentially supporting favorable health outcomes, but these interpretations are highly speculative, as human and functional studies are lacking.

In summary, the idea that *Ruminococcus_torques_group* and *Eisenbergiella* could play a role in the pathophysiology of adult ADHD is supported by the described associations with other brain disorders characterized by altered monoamine neurotransmission (e.g. ASD, depression, Parkinson's disease) and by the observed influences on inflammation and immune functioning. Immune activation, for example caused by increased intestinal permeability, is considered as a potential mechanism causing and maintaining psychiatric and somatic symptoms, reflecting in shared genetic risk of immune and psychiatric disorders [62]

Notably, none of these genera had been reported in any previous studies of gut microbiome composition in ADHD (see table ST1), potentially due to the differences in origin, developmental stage and power issues of the published studies.

Strengths and Limitations

The results of this study have to be viewed in the light of its strengths and limitations. Even though we provide a comparably big sample size, the expected small effect sizes and high inter- and intra-individual variability in gut-microbial signatures require replication of these findings in even larger samples. Collecting data from different studies comes with limitations of the included individual studies (quality, technical variation). We reduced and accounted for effects of individual study differences by harmonizing pipelines and using random effects meta-analyses. However, different sequencing region, for example, might still limit the detection of the same genera across studies. Inconsistently detected genera would not be considered or introduced to meta-analysis.

All included datasets were based on 16S sequencing, which picks up on low abundant features, but provides comparably low resolution. The taxonomic identification at this resolution does not provide information about functional properties of the identified taxa. Discussions of potential disorder-related mechanistic pathways (e.g. a potential role of inflammatory processes in ADHD) or functional properties of genus-level data are based on a narrative summary of associations with other phenotypes in the literature, and are therefore necessarily speculative and unspecific to ADHD. The gut-microbiome is sensitive to environmental, behavioral, and dietary changes. For people with psychiatric disorders, disorganized thoughts and behaviors or impulsive food choices, for example, might be responsible for the observed difference in abundance of particular genera. Similarly, the decrease of *Eisenbergiella* after immune-suppressive treatment supports the idea that - rather than a cause - increases in abundance could be seen as an epiphenomenon or consequence of inflammation. Through the association studies performed here, we therefore cannot give insights on causality or consequences of the differential microbial abundance in ADHD.

While we corrected for age and sex effects on the gut-microbiome, diet information was only provided for IMpACT2-NL and MIND-Set. After post-hoc analyses, correcting for diet on an individual study level in MIND-Set and IMpACT2-NL, all significant associations remained significant, see Supplementary Chapter 7.2. However, diet was significantly associated with ADHD diagnosis, confirming the relevance of diet as either behavioral epiphenomenon of or influence on ADHD. Dietary habits should be assessed thoroughly using standardized tools and accounted for in each study. Another important confounder is medication. Drugs can impact the gut-microbiome; in turn, the gut-microbiome might also impact the metabolism of medications and modulate the treatment response (for an overview, see [63]). However, in psychiatric case-control cohorts, pharmacological treatment is highly colinear with the diagnostic group, and issues like multi-medication resulting from high comorbidity and treatment adherence further complicate this picture. Significant contributions of the medication naïve sample (Mental-Cat) as well as the highly medicated sample (MIND-Set), point towards low sensitivity of these results for ADHD medication. Post-hoc analyses of medication effects was performed on cases only in IMpACT2-NL and NeuroIMAGE. Eisenbergiella was more abundant in participants with ADHD in the NeuroIMAGE sample, but not on IMpACT and no other association of genus abundance with current use of ADHD medication was found, see Supplementary Chapter 7.3. The interplay of gut-microbiota with psychopharmacological treatment should be investigated in future studies.

Conclusion and future directions

In summary, we identified alterations of the gut-microbiome in adult ADHD that were robust to statistical approach and study heterogeneity. The ADHD-associated genera suggested potential relevance of inflammatory and immune processes for ADHD symptoms. However, more human and functional studies are needed to support potential interpretations.

To exhaust the potential of 16S sequencing studies and increase comparability we emphasize the need for standardized pre-processing and statistical pipelines, large samples, and deep phenotyping. To extend this work to the lifespan of ADHD, future studies should integrate studies in children, adolescents, and adults in meta-regression or in longitudinal designs. These approaches could account for natural changes in the gut-microbiome in the different developmental stages and help to provide insight in potential influences of the gut-microbiome in early life on later neurodevelopment within ADHD (e.g. to identify potential remission profiles). To distinguish disorder-specific, symptom-/trait-specific, and transdiagnostic effects, studies across psychiatric categories and in population-based cohorts are needed. Additionally, the combination of 16S studies with metagenomic sequencing, clustering of sequences based on their involvement in functional mechanistic pathways as well as wet-lab culturing studies investigating functions of the bacteria are needed to gain mechanistic insights. Intervention studies, targeting the reduction of genera enriched in ADHD (and/or the enrichment of reduced genera), could be implemented to infer causal relationships between ADHD symptoms and gut-microbial alterations; such studies could evaluate the potential of the gut-microbiome as a biomarker as well as for treatment support. Investigating the gut-microbial changes in pharmacological randomized control trials could help disentangle disorder-related effects from medication-related effects, additionally providing a perspective to improve treatment response.

Acknowledgements

NeuroIMAGE: This project was supported by grants from National Institutes of Health (grant R01MH62873 to SV Faraone) for initial sample recruitment, and from NWO Large Investment (grant 1750102007010 to JK Buitelaar), NWO Brain & Cognition (grant 433-09-242 to JK Buitelaar), and grants from Radboud University Medical Center, University Medical Center Groningen, Accare, and VU University

Amsterdam for subsequent assessment waves. NeuroIMAGE also received funding from the European Community's Seventh Framework Programme (FP7/2007 – 2013) under grant agreements n° 602805 (Aggressotype), n° 278948 (TACTICS), and n° 602450 (IMAGEMEND), and from the European Community's Horizon 2020 Programme (H2020/2014 – 2020) under grant agreements n° 643051 (MiND), n° 667302 (CoCA), and n° 728018 (Eat2beNICE).

IMpACT: We acknowledge funding from the Netherlands Organization for Scientific Research (NWO), i.e. the Veni Innovation Program (grant 016-196-115 to MH) and the Vici Innovation Program (grant 016–130-669 to BF). The work was also supported by the European College of Neuropsychopharmacology (ECNP) Network "ADHD Across the Lifespan".

BJ and BF were also supported by funding from the European Community's Horizon 2020 Programme (H2020/2014 – 2020) under grant agreement n° 847879 (PRIME).

Conflict of Interests

J.A.R.Q was on the speakers' bureau and/or acted as consultant for Biogen, Janssen-Cilag, Novartis, Shire, Takeda, Bial, Shionogi, Sincrolab, Novartis, BMS, Medice, Rubió, Uriach, Technofarma and Raffo in the last 3 years. He also received travel awards (air tickets + hotel) for taking part in psychiatric meetings from Janssen-Cilag, Rubió, Shire, Takeda, Shionogi, Bial and Medice. The Department of Psychiatry chaired by him received unrestricted educational and research support from the following companies in the last 3 years: Janssen- Cilag, Shire, Oryzon, Roche, Psious, and Rubió.

BF has received educational speaking fees from Medice.
IT receives funding from the Dutch Organisation of research (ZONMW project numbers 80-86200-98-20006, 80-85200-98-20006, 60-63600-98-903)
VR has served as a speaker for Rubió and Shire/Takeda in the last 5 years. She has received travel awards from Shire/Takeda for participating in psychiatric meetings.
JKB has been in the past 3 years a consultant to / member of advisory board of / and/or speaker for Takeda, Roche, Medice, Angelini, Janssen, and Servier. He is not an employee of any of these companies, and not a stock shareholder of any of these companies. He has no other financial or material support, including expert testimony, patents, royalties.
BJ, PV, DM, PvE, MR, JV, MB, AAV and MH have nothing further to disclose.

REFERENCES

1. Faraone, S.V., *ADHD*. Nature Reviews Disease Primers, 2015. **15027**.
2. Kittel-Schneider, S., et al., *Non-mental diseases associated with ADHD across the lifespan: Fidgety Philipp and Pippi Longstocking at risk of multimorbidity?* Neuroscience & Biobehavioral Reviews, 2021.
3. Franke, B., et al., *Live fast, die young? A review on the developmental trajectories of ADHD across the lifespan*. European Neuropsychopharmacology, 2018. **28**(10): p. 1059-1088.
4. Núñez-Jaramillo, L., A. Herrera-Solís, and W.V. Herrera-Morales, *ADHD: reviewing the causes and evaluating solutions*. Journal of personalized medicine, 2021. **11**(3): p. 166.
5. Warner, B.B., *The contribution of the gut microbiome to neurodevelopment and neuropsychiatric disorders*. Pediatric Research, 2019. **85**(2): p. 216-224.
6. Bull-Larsen, S. and M.H. Mohajeri, *The potential influence of the bacterial microbiome on the development and progression of ADHD*. Nutrients, 2019. **11**(11): p. 2805.
7. Dam, S.A., et al., *The role of the gut-brain axis in attention-deficit/hyperactivity disorder*. Gastroenterology Clinics, 2019. **48**(3): p. 407-431.
8. Liu, F., et al., *Altered composition and function of intestinal microbiota in autism spectrum disorders: a systematic review*. Translational psychiatry, 2019. **9**(1): p. 1-13.
9. Nikolova, V.L., et al., *Perturbations in gut microbiota composition in psychiatric disorders: a review and meta-analysis*. JAMA psychiatry, 2021. **78**(12): p. 1343-1354.
10. Karlsson, F., et al., *Assessing the human gut microbiota in metabolic diseases*. Diabetes, 2013. **62**(10): p. 3341-3349.
11. Ding, J.-H., et al., *Role of gut microbiota via the gut-liver-brain axis in digestive diseases*. World Journal of Gastroenterology, 2020. **26**(40): p. 6141.
12. Checa-Ros, A., et al., *Current evidence on the role of the gut microbiome in ADHD pathophysiology and therapeutic implications*. Nutrients, 2021. **13**(1): p. 249.
13. Shirvani-Rad, S., et al., *The Role of Gut Microbiota-Brain Axis in Pathophysiology of ADHD: A Systematic Review*. Journal of Attention Disorders, 2022: p. 10870547211073474.
14. Sukmajaya, A.C., M.I. Lusida, and Y. Setiawati, *Systematic review of gut microbiota and attention-deficit hyperactivity disorder (ADHD)*. Annals of general psychiatry, 2021. **20**(1): p. 1-12.
15. Bundgaard-Nielsen, C., et al., *Gut microbiota profiles of autism spectrum disorder and attention deficit/hyperactivity disorder: A systematic literature review*. Gut Microbes, 2020. **11**(5): p. 1172-1187.
16. Gkougka, D., et al., *Gut microbiome and attention deficit/hyperactivity disorder: a systematic review*. Pediatric Research, 2022: p. 1-13.
17. Wan, L., et al., *Case-control study of the effects of gut microbiota composition on neurotransmitter metabolic pathways in children with attention deficit hyperactivity disorder*. Frontiers in Neuroscience, 2020. **14**: p. 127.
18. Jiang, H.-y., et al., *Gut microbiota profiles in treatment-naïve children with attention deficit hyperactivity disorder*. Behavioural brain research, 2018. **347**: p. 408-413.
19. Richarte, V., et al., *Gut microbiota signature in treatment-naive attention-deficit/hyperactivity disorder*. Translational psychiatry, 2021. **11**(1): p. 1-7.
20. Aarts, E., et al., *Gut microbiome in ADHD and its relation to neural reward anticipation*. PloS one, 2017. **12**(9): p. e0183509.

21. Szopinska-Tokov, J., et al., *Correction: Szopinska-Tokov et al. Investigating the Gut Microbiota Composition of Individuals with Attention-Deficit/Hyperactivity Disorder and Association with Symptoms. Microorganisms 2020, 8, 406.* Microorganisms, 2021. **9**(7): p. 1358.
22. Hiergeist, A., J. Gessner, and A. Gessner, *Current limitations for the assessment of the role of the gut microbiome for attention deficit hyperactivity disorder (ADHD).* Frontiers in Psychiatry, 2020. **11**: p. 623.
23. Abellan-Schneyder, I., et al., *Primer, pipelines, parameters: issues in 16S rRNA gene sequencing.* Msphere, 2021. **6**(1): p. e01202-20.
24. Gloor, G.B., et al., *Microbiome datasets are compositional: and this is not optional.* Frontiers in microbiology, 2017. **8**: p. 2224.
25. Nearing, J.T., et al., *Microbiome differential abundance methods produce different results across 38 datasets.* Nature communications, 2022. **13**(1): p. 1-16.
26. Gloor, G.B. and G. Reid, *Compositional analysis: a valid approach to analyze microbiome high-throughput sequencing data.* Canadian journal of microbiology, 2016. **62**(8): p. 692-703.
27. van Eijndhoven, P., et al., *Measuring Integrated Novel Dimensions in Neurodevelopmental and Stress-Related Mental Disorders (MIND-SET): protocol for a cross-sectional comorbidity study from a Research Domain Criteria perspective.* JMIRx Med, 2022. **3**(1): p. e31269.
28. Jakobi, B., et al., *Neural Correlates of Reactive Aggression in Adult ADHD.* Frontiers in psychiatry, 2022: p. 1019.
29. von Rhein, D., et al., *The NeuroIMAGE study: a prospective phenotypic, cognitive, genetic and MRI study in children with attention-deficit/hyperactivity disorder. Design and descriptives.* European child & adolescent psychiatry, 2015. **24**(3): p. 265-281.
30. Mirzayi, C., et al., *Reporting guidelines for human microbiome research: the STORMS checklist.* Nature medicine, 2021. **27**(11): p. 1885-1892.
31. Bolyen, E., et al., *Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2.* Nature biotechnology, 2019. **37**(8): p. 852-857.
32. Callahan, B.J., et al., *DADA2: High-resolution sample inference from Illumina amplicon data.* Nature methods, 2016. **13**(7): p. 581-583.
33. Ewing, B. and P. Green, *Base-calling of automated sequencer traces using phred. II. Error probabilities.* Genome research, 1998. **8**(3): p. 186-194.
34. Price, M.N., P.S. Dehal, and A.P. Arkin, *FastTree 2–approximately maximum-likelihood trees for large alignments.* PloS one, 2010. **5**(3): p. e9490.
35. R Core Team, R. and R.C. Team, *R: a language and environment for statistical computing. R Foundation for Statistical Computing; 2020*. 2021.
36. Lahti, L. and S. Shetty, *Tools for microbiome analysis in R. Microbiome package version 1.7. 21.* Bioconductor. Available at: http://microbiome. github. com/microbiome.[Google Scholar], 2017.
37. Kloke, J.D. and J.W. McKean, *Rfit: rank-based estimation for linear models.* R J., 2012. **4**(2): p. 57.
38. Balduzzi, S., G. Rücker, and G. Schwarzer, *How to perform a meta-analysis with R: a practical tutorial.* BMJ Ment Health, 2019. **22**(4): p. 153-160.
39. Oksanen, J., F. Blanchet, and M. Friendly, *vegan: community ecology package. R package version 2.4–4.2*. 2017.
40. McMurdie, P.J. and S. Holmes, *phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data.* PloS one, 2013. **8**(4): p. e61217.
41. Meinshausen, N. and P. Bühlmann, *Stability selection.* Journal of the Royal Statistical Society: Series B (Statistical Methodology), 2010. **72**(4): p. 417-473.

42. Wang, L., et al., *Increased abundance of Sutterella spp. and Ruminococcus torques in feces of children with autism spectrum disorder.* Molecular autism, 2013. **4**(1): p. 1-4.

43. Malinen, E., et al., *Association of symptoms with gastrointestinal microbiota in irritable bowel syndrome*. World journal of gastroenterology: WJG, 2010. **16**(36): p. 4532.

44. Joossens, M., et al., *Dysbiosis of the faecal microbiota in patients with Crohn's disease and their unaffected relatives.* Gut, 2011. **60**(5): p. 631-637.

45. Fuentes, S., et al., *Associations of faecal microbiota with influenza-like illness in participants aged 60 years or older: an observational study.* The Lancet Healthy Longevity, 2021. **2**(1): p. e13-e23.

46. Hoskins, L.C., et al., *Mucin degradation in human colon ecosystems. Isolation and properties of fecal strains that degrade ABH blood group antigens and oligosaccharides from mucin glycoproteins.* The Journal of clinical investigation, 1985. **75**(3): p. 944-953.

47. Hamamah, S., A. Hajnal, and M. Covasa, *Impact of Nutrition, Microbiota Transplant and Weight Loss Surgery on Dopaminergic Alterations in Parkinson's Disease and Obesity.* International journal of molecular sciences, 2022. **23**(14): p. 7503.

48. Pellegrini, C., et al., *The intestinal barrier in disorders of the central nervous system*. The Lancet Gastroenterology & Hepatology, 2022.

49. Bauer, M.E. and A.L. Teixeira, *Inflammation in psychiatric disorders: what comes first?* Annals of the New York Academy of Sciences, 2019. **1437**(1): p. 57-67.

50. Ye, F., et al., *Comparison of gut microbiota in autism spectrum disorders and neurotypical boys in China: a case-control study.* Synthetic and Systems Biotechnology, 2021. **6**(2): p. 120-126.

51. Kim, C.-H., et al. *Comparison of Metabolites and Gut Microbes between Patients with Parkinson's Disease and Healthy Individuals—A Pilot Clinical Observational Study (STROBE Compliant)*. in *Healthcare*. 2022. MDPI.

52. Fontana, A., et al., *Exploring the role of gut microbiota in major depressive disorder and in treatment resistance to antidepressants.* Biomedicines, 2020. **8**(9): p. 311.

53. Bao, J., et al., *Echinococcus granulosus infection results in an increase in Eisenbergiella and Parabacteroides genera in the gut of mice.* Frontiers in Microbiology, 2018. **9**: p. 2890.

54. Li, H.-Y., et al., *Modulation of gut microbiota, short-chain fatty acid production, and inflammatory cytokine expression in the cecum of porcine Deltacoronavirus-infected chicks.* Frontiers in microbiology, 2020. **11**: p. 897.

55. Mauras, A., et al., *Gut microbiota from infant with cow's milk allergy promotes clinical and immune features of atopy in a murine model.* Allergy, 2019. **74**(9): p. 1790.

56. Zhang, J., et al., *Integrative Analysis of Gut Microbiota and Fecal Metabolites in Rats after Prednisone Treatment.* Microbiology spectrum, 2021. **9**(3): p. e00650-21.

57. Surono, I.S., et al., *Differences in immune status and fecal SCFA between Indonesian stunted children and children with normal nutritional status.* PloS one, 2021. **16**(7): p. e0254300.

58. Ma, S., et al., *Alterations in gut microbiota of gestational diabetes patients during the first trimester of pregnancy.* Frontiers in cellular and infection microbiology, 2020. **10**: p. 58.

59. Hu, R., et al., *Dietary ferulic acid and vanillic acid on inflammation, gut barrier function and growth performance in lipopolysaccharide-challenged piglets.* Animal Nutrition, 2022. **8**(1): p. 144-152.

60. Diotallevi, C., et al., *Healthy dietary patterns to reduce obesity-related metabolic disease: polyphenol-microbiome interactions unifying health effects across geography.* Current Opinion in Clinical Nutrition & Metabolic Care, 2020. **23**(6): p. 437-444.

61. Ma, X., et al., *Sodium butyrate modulates gut microbiota and immune response in colorectal cancer liver metastatic mice.* Cell Biology and Toxicology, 2020. **36**(5): p. 509-515.

62. Rudzki, L. and A. Szulc, *"Immune gate" of psychopathology—The role of gut derived immune activation in major psychiatric disorders.* Frontiers in psychiatry, 2018. **9**: p. 205.

63. Maier, L. and A. Typas, *Systematically investigating the impact of medication on the gut microbiome.* Current opinion in microbiology, 2017. **39**: p. 128-135.

SUPPLEMENTARY MATERIAL

1. Study identification and description

We identified potentially relevant studies by screening recent systematic reviews [13, 14] and through literature search for original case-control studies including fecal microbiome samples from participants with and without ADHD diagnoses.

1.1 Recruitment and inclusion criteria per study

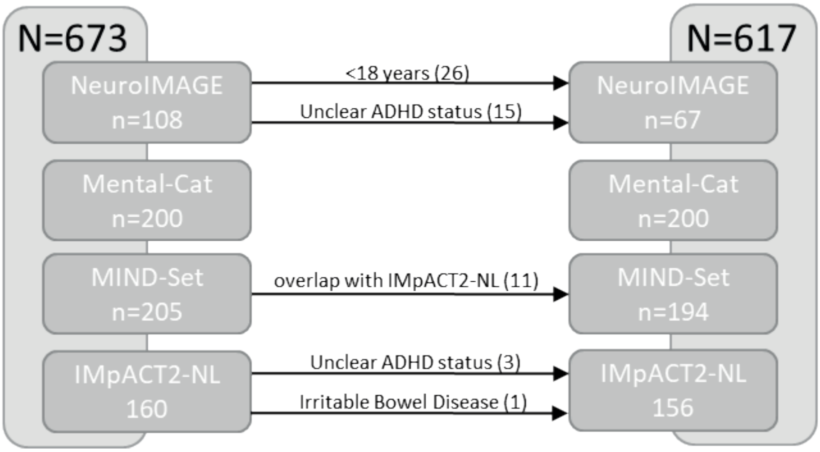
The Mental-Cat cohort consists of 100 treatment naïve adults with and 100 adults without ADHD. Participants with ADHD were recruited from an outpatient program in Catalonia, Spain, by a clinical group from the Hospital Universitari Vall d'Hebron of Barcelona, Spain. The study was approved by the Clinical Research Ethics Committee (CREC) of Hospital Universitari Vall d'Hebron. Exclusion criteria: intelligence quotient < 70, current or past psychiatric, neurologic or systemic disorders, other developmental disorders, current or past neurologic, metabolic, cardiac, liver, kidney, or respiratory conditions, chronic pharmacological treatment of any kind, birth weight ≤ 1.5 kg, treatment with probiotics or antibiotics prior to fecal sample collection. For the control group, participants with ADHD or other psychiatric disorders (current or past) were excluded. Assessment of **ADHD symptoms** was conducted with the Adult Self-Report Scale A.S.R. S v1.1 in the participants with ADHD only [64]. This scale consists of 18 questions about frequency of symptoms of adult ADHD in the scale of never, rarely, sometimes, often and very often. 7 of these questions are scored with 1 when answered with often or very often, the remaining 11 questions also if the answer is sometimes. See [65] for further information.

The IMpACT2-NL cohort consists of 83 adults with and 79 adults without ADHD, of which 77 with and 79 without ADHD provided fecal samples. Participants were recruited via local newspapers, advertisements in sports clubs in and around Nijmegen, The Netherlands, and via patient organizations. Participants received monetary compensation and gave written informed consent. The study was approved by the local medical ethical committee (Central Commission for Human Rights Research (CCMO)). Exclusion criteria for all participants comprised: younger than 18 or older than 60 years, neurological disorders, psychosis or substance abuse in the last 6 months, current major depression, psycho-pharmaceutical therapy (other than stimulants for participants with ADHD), impairments of hearing, seeing and sensorimotor abilities and no knowledge of the Dutch language. Exclusion criteria in the control group comprised no current or previous diagnoses of ADHD, and no first-degree family members with ADHD. Assessment of **ADHD symptoms** was conducted with the Diagnostic Interview for Adult ADHD (DIVA 2.0 [66]], based on the Diagnostic and Statistical Manual of Mental Disorders (DSM), assessing the presence or absence of 9 symptoms of inattention and 9 symptoms of hyperactivity/impulsivity providing every-day examples for each symptom. See [28] for further information.

The **MIND-Set** cohort comprises ca 650 individuals with a psychiatric disorder and 150 neurotypical controls (by 2021). Oh those, 104 individuals with a diagnosis of ADHD and 90 neurotypical individuals provided fecal samples. Participants were recruited at the inpatient and outpatient unit of the psychiatric department of the Radboud University Medical Center (Radboudumc), Nijmegen, the Netherlands, provided written informed consent and received monetary compensation. The study was approved by the local medical ethical committee (Commissie Mensgebonden Onderzoek Arnhem-Nijmegen). Individuals of at least 18 years were included in the neurotypical control group if they had no psychiatric diagnoses, or in the psychiatric patient group if they had a diagnosis of a stress-related (mood disorder, anxiety disorder, or substance use disorder) or neurodevelopmental disorder (ASD or ADHD). In this analyses, only participants with ADHD were included, presenting with a high level of comorbidity (84% had another psychiatric diagnosis), and often pharmacological treatment with several medications (most frequently ADHD medication and antidepressants). Exclusion criteria for the psychiatric patient group were diseases of the central nervous system, (permanent) sensorimotor or (neuro)cognitive impairments, current psychosis, IQ <70, no knowledge of the Dutch language. **ADHD symptoms** were assessed with the CAARS-S:S, a short self-report rating scale covering the frequency of symptoms of Inattention/ Memory Problems (five items), Hyperactivity/Restlessness (five items) and Impulsivity/ Emotional Lability (five items), scored on a 4-point scale (0 = never, 1 = sometimes, 2 = often, and 3 = very often). We used the mean of Impulsivity/Emotional Lability and Hyperactivity/Restlessness symptoms as a measure of Hyperactivity/ Impulsivity symptoms.

The **NeuroIMAGE** cohort is a Dutch multicenter study that recruited families with members with ADHD and families without members with ADHD in Nijmegen and Amsterdam, The Netherlands. The study was approved by the regional ethics committee (Centrale Commissie Mensgebonden Onderzoek: CMO Regio Arnhem Nijmegen; 2008/163; ABR: NL23894.091.08). A small subset of the study-arm in Nijmegen provided fecal samples: 41 participants with ADHD, 48 healthy controls as well as 15 participants with so-called subthreshold ADHD (e.g. control participants with high symptom scores or individuals with an ADHD diagnosis who scored low on symptom scales). For more information on the definition of ADHD in NeuroIMAGE, see [67]. Inclusion criteria were: An age of 5 to 30 years, European

Caucasian descent, IQ ≥70, no diagnosis of ASD, epilepsy, neurological disorders and learning disabilities. Only adult participants with unquestionable ADHD status were included in this study. **ADHD symptom** scores were derived from the K-SADS and the different versions of Conners' rating scale (in adults the CAARS:L, the long version of the self-report), including the DSM-5 subscales for Inattentive and Hyperactive/Impulsive symptoms, for a detailed description of the calculation of symptom scores, see [67].



Supplementary Figure 1. Flowchart of reasons for exclusions of participants from this study.

Supplementary Table 1. Summary of gut-microbiome studies in ADHD

Authors	Year	Country	Sequencing	Sample size ADHD: Control
Published case-control comparison studies				
Richarte et al.	2021	Spain	16S V3/V4	100:100
Szopinska-Tokov et al.	2020	Netherlands	16S V1/V2	41:48
Aarts et al.	2017	Netherlands	16S V1/V2	19:77
Wan et al.	2020	China	Shotgun metagenomics	17:17
Wang et al.	2020	Taiwan	16S V3/V4	30:30
Prehn-Kristensen et al.	2018	Germany	16S V1/V2	14:17
Jiang et al.	2018	China	16S V3/V4	51:32
Non-peer reviewed reports/preprints				
Li et al.	2020	China	metagenomics	98:109
Fan et al.	2019	China	16S V3/V4	49:24
Akram et al.	2017	NA	16S V3/V4	14:20

Summary of results of previous studies on the fecal gut-microbiome samples in people with and without ADHD, including description of the study population and summary of the main findings, synthesized from recent systematic reviews on the gut-microbiome in ADHD [13, 14]. Data of the grey-shaded studies was included in this meta-analysis, data of all studies was requested. Results of the non-peer-reviewed studies were not interpreted in the manuscript.

Age	Alpha diversity	Beta diversity	Genus level differential abundance
Adult	No difference	No difference	***Dialister***, *Megamonas* **up** *Gracilibacter*, *Anaerotaenia* down
Adolescent - adult	No difference	No difference	*Intestinibacter* up, *Coprococcus_2*, *Prevotella_9* down
Adolescent - adult	No difference	NA	*Bifidobacterium* up
Children	No difference	NA	***Faecalibacterium* down**, *Veillonellaceae* down, ***Odoribacter* up**, *Enterococcus* up
Children	Simpson down, Shannon up, Chao2 up	NA	*Lactobacillus* up, *Fusobacterium* up
Children	Shannon down	Difference	***Prevotella***, *Parabacteroides* down, *Neisseria* up
Children	No difference	No difference	***Faecalibacterium***, *Lachnoclostridium*, and ***Dialister* down**
Children	Lower observed features, Shannon no difference	No difference	No genus level results but species belonging to *Bacteroides*, *Prevotella* and *Bifidobacterium* genus
Children	lower across indices in inattention		***Prevotella***, *Intestinimonas*, *Marvinbryantia*, *Eggerthella* (hyperactivity), 14 genera (inattention), *Megamonas*, *Corprococcus_2*, *Paraprevotella* (combined)
Young adults	NA	NA	*Phascolarctobacterium*, *Paraprevotella*, *Veillonella*, ***Odoribacter***

2. Sample collection, storing and sequencing

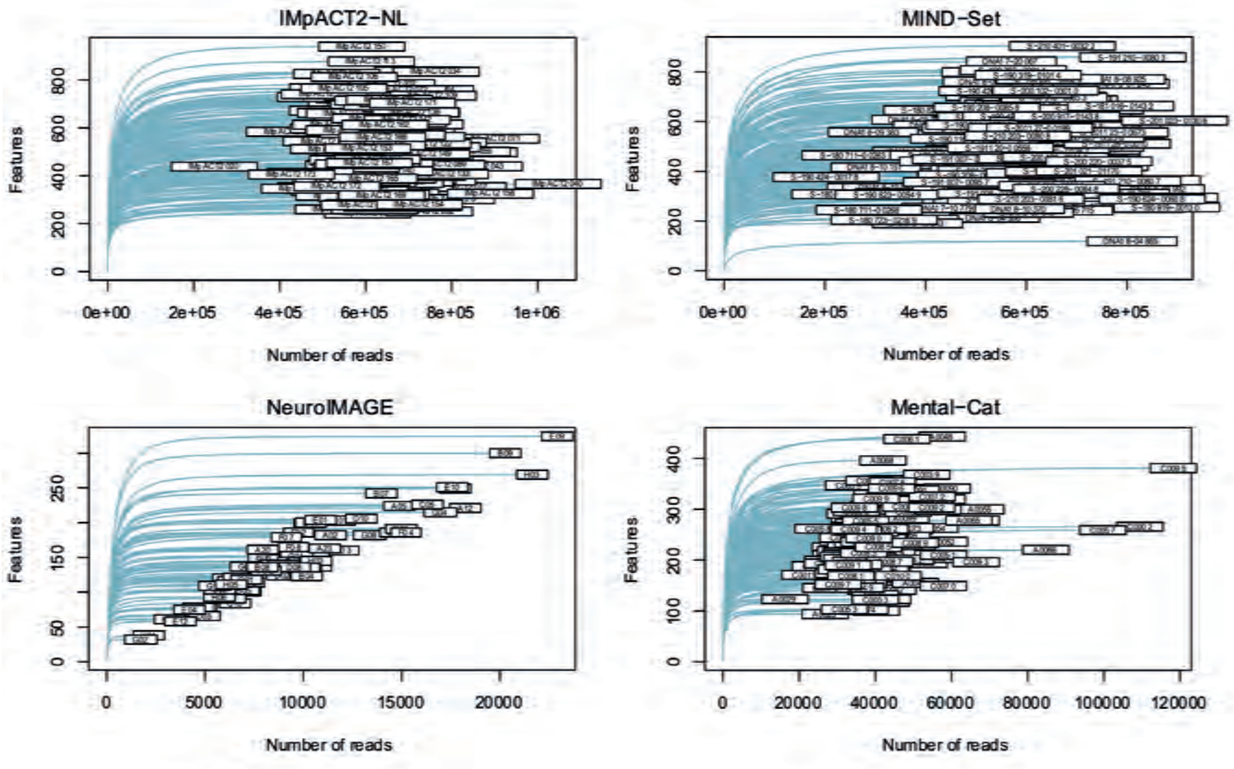
For detailed information on the sample collection, storing, and sequencing of the NeuroIMAGE and VHIR cohorts please refer to the original papers [19, 20]. The fecal samples of the MIND-Set and IMpACT study were collected, stored, and processed according to our lab-routine (as described in Bloemendaal et al, 2022). Participants were instructed to collect their fecal samples at home using a validated protocol by OMNIgene•GUT kit (DNAGenotek, Ottawa, CA) and send them back to our laboratory. Samples received in good order were aliquoted into 1.5 ml Eppendorf tubes and stored in -80˚C. Further processing was done by Baseclear B.V by aliquoting 150 mg feces, isolating and purifying DNA using a bead-beating procedure, using ZymoBIOMICS DNA 96 MagBead kit in conjunction with Kingfisher. To identify bacterial DNA, the V4 region of 16S ribosomal RNA (rRNA) gene was targeted. Amplicons were built using the primers 515-F: TCGTCGGCAGCGTCAGATGT-GTATAAGACAGGTGYCAGCMGCCGCGGTAA, 806Rb: GT CTCGTGGGCTCGGAGATGTGTATAAGACAGAGG-GACTACNVGGGTWTCTAAT) using the Phusion High-Fidelity PCR Master Mix with an HF Buffer (Thermo Scientific). Illumina indexes were then ligated to the amplicons. The libraries were sequenced on the Illumina Novaseq 6000 platform with the Novaseq 6000 SP reagent kit v1 with 500 cycles (paired-end, 250 bp)(Baseclear B.V., Leiden, the Netherlands). Both a PCR negative control (water control for PCR and library preparation) was included sample to assess contamination introduced during the PCR step as well as a positive control (ZymoBIOMICS Gut Microbiome Stdrd (ZY-D6331)) to assess correct detection of a sample with a known microbial distribution. Finally, reads were demultiplexed, filtered, and adapter sequences and control signals removed.

2.1 Supplementary Table 2. Sequencing data summary

	IMPACT2-NL	MIND-Set	VHIR	NeuroIMAGE
Quality				
Average Phred score across all basepairs (forward and reverse) in Median percentile	37	37	35.62	33.69
Denoising stats				
Input sequences	776796.6	762115.9	75640.5	91257.3
Filtered sequences	693940.1	672774.9	48995	61604.4
Percentage of input passed filter	89.33 %	88.28 %	64.77 %	67.50 %
Denoised sequences	690254	667342.3	47970.7	56613.5
Merged sequences	678190	652028.4	43971.0	38715.7
Percentage of input merged	87.30 %	85.55 %	58.13 %	42.42 %
Non-chimeric sequences	633693.7	585221.7	39889.2	16667.1
Percentage of input non-chimeric	81.58 %	76.78 %	52.74 %	18.26 %
ASV level				
ASV count	14408	21699	6535	20459
Mean sequence length	238.72	231.31	506.38	362.7
Mean frequency per sample	641512.5	601319.9	41252.5	19016
Median frequency per feature	23	12	58	50
Resulting datasets				
Bacteria count	10422	14000	6466	11406
Genera count	613	564	334	234
Prevalent genera count (10%)	234	226	159	116

Summary of sequencing data before and after each preprocessing step: denoising, clustering, genus aggregation and prevalence filtering.

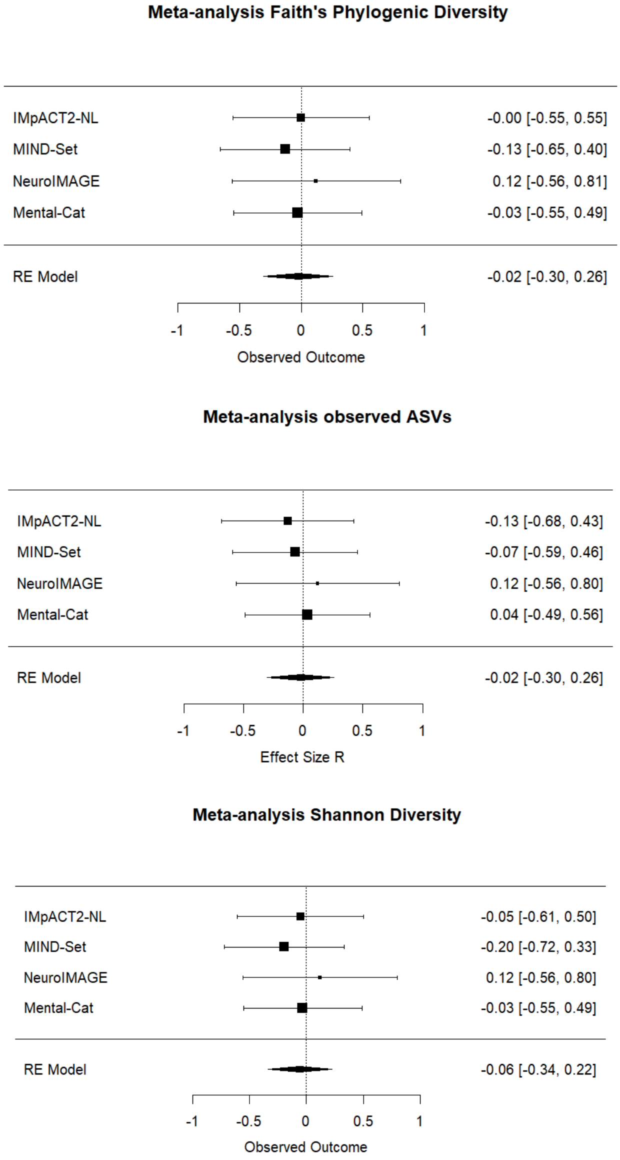
2.2 Rarefaction curves



Supplementary Figure 2. Rarefaction curve of each study (IMpACT2-NL top left, Mental-Cat top right, NeuroIMAGE bottom left, MIND-Set bottom right).

3. Alpha diversity

3.1 Meta-analyses results of alpha diversity

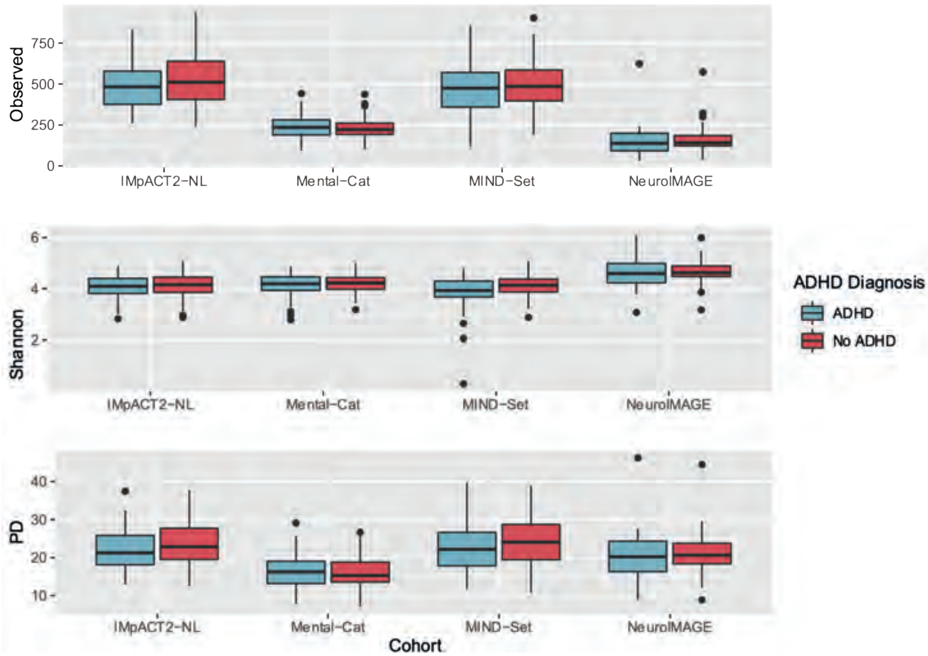


Supplementary Figure 3. Forest plots of the alpha diversity meta-analyses of observed ASVs (top left), Shannon index (top right), and phylogenic distance (bottom).

Supplementary Table 3. Results of the alpha diversity meta-analyses

	Observed ASVs	Shannon index	Phylogenic distance
Estimate	-0.02	-0.06	-0.02
Standard Error	0.14	0.14	0.14
z-value	-0.15	-0.41	-0.17
p-value	0.87	0.68	0.86
CI lower	-0.30	-0.33	-0.30
CI upper	0.21	0.22	0.25
p-value heterogeneity	0.94	0.91	0.95
Q-value heterogeneity	0.39	0.54	0.33

3.2 Individual study results of alpha diversity



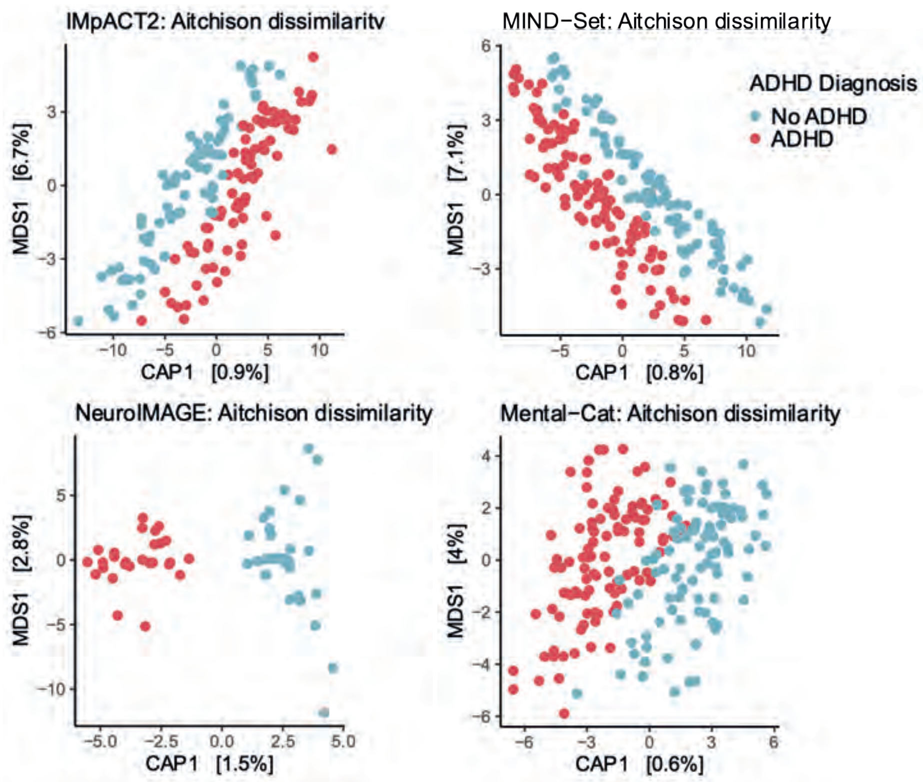
Supplementary Figure 4. Alpha Diversity per study of observed ASVs (top), Shannon index (middle) and phylogenic diversity (bottom). Participants with ADHD are marked in red, without ADHD are marked in blue.

Supplementary Table 4. Individual study results of alpha diversity

		Observed ASV			Shannon index			Phylogenic Diversity		
Cohort		**ADHD**	**age**	**gender**	**ADHD**	**age**	**gender**	**ADHD**	**age**	**gender**
IMpACT2	Estimate	-37.92	6.26	-50.86	-0.05	0.01	-0.22	-1.42	0.18	-1.72
	Std.Error	23.36	1.00	23.44	0.07	0.00	0.07	0.79	0.03	0.79
	T value	-1.62	6.27	-2.17	-0.66	4.47	-3.02	-1.80	5.45	-2.17
	P value	$1.07E^{-01}$	$3.51E^{-09}$	$3.16E^{-02}$	$5.12E^{-01}$	$1.50E^{-05}$	$2.99E^{-03}$	$7.33E^{-02}$	$2.01E^{-07}$	$3.13E^{-02}$
MIND-Set	Estimate	-21.08	1.93	-9.50	-0.19	0.00	-0.03	-1.57	0.04	-0.40
	Std.Error	22.16	0.76	22.42	0.07	0.00	0.07	0.95	0.03	0.96
	T value	-0.95	2.54	-0.42	-2.76	0.35	-0.49	-1.65	1.32	-0.42
	P value	$3.43E^{-01}$	$1.20E^{-02}$	$6.72E^{-01}$	$6.38E^{-03}$	$7.26E^{-01}$	$6.24E^{-01}$	$1.01E^{-01}$	$1.88E^{-01}$	$6.78E^{-01}$
NeuroIMAGE	Estimate	-13.71	-4.35	2.59	-0.11	-0.02	0.02	-0.55	-0.83	0.20
	Std.Error	15.71	15.85	2.81	0.12	0.12	0.02	1.44	1.45	0.26
	T value	-0.87	-0.27	0.92	-0.90	-0.18	0.84	-0.38	-0.57	0.79
	P value	$3.86E^{-01}$	$7.84E^{-01}$	$3.60E^{-01}$	$3.73E^{-01}$	$8.55E^{-01}$	$4.02E^{-01}$	$7.03E^{-01}$	$5.71E^{-01}$	$4.34E^{-01}$
Mental-Cat	Estimate	4.37	-0.95	1.79	-0.02	-0.01	0.01	0.00	-0.70	0.10
	Std.Error	8.65	8.57	0.43	0.05	0.05	0.00	0.59	0.58	0.03
	T value	0.50	-0.11	4.18	-0.47	-0.27	3.13	-0.01	-1.21	3.47
	P value	$6.14E^{-01}$	$9.12E^{-01}$	$4.37E^{-05}$	$6.41E^{-01}$	$7.91E^{-01}$	$1.99E^{-03}$	$9.95E^{-01}$	$2.29E^{-01}$	$6.30E^{-04}$

4. Beta diversity

4.1 Individual study results of beta diversity: CAP Plots



Supplementary Figure 5. CAP plots supervised for effects of ADHD diagnosis per study.

Supplementary Table 5. Individual study results of beta diversity permanova

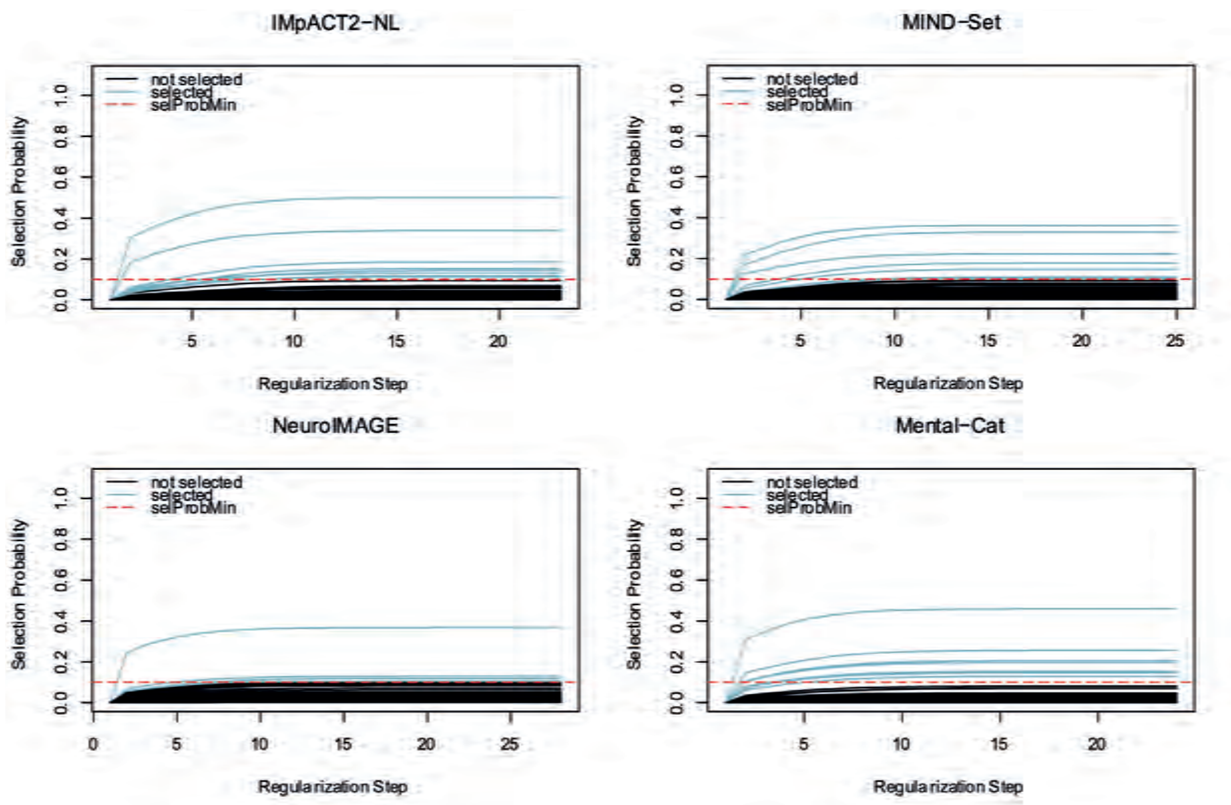
Cohort	Regressors	Sum Of Squares	R^2	F	*P* value
IMpACT2	ADHD diagnosis	12637.89	0.01	1.41	$8.00E^{-03}$
	Age	22960.25	0.02	2.56	$1.00E^{-03}$
	Sex	13255.37	0.01	1.48	$3.00E^{-03}$
MIND-Set	ADHD diagnosis	13463.10	0.01	1.50	$5.00E^{-03}$
	Age	17776.96	0.01	1.99	$1.00E^{-03}$
	Sex	10074.13	0.01	1.13	$1.19E^{-01}$
NeuroIMAGE	ADHD diagnosis	5176.29	0.01	0.95	$9.61E^{-01}$
	Age	5612.08	0.02	1.03	$2.80E^{-01}$
	Sex	5456.44	0.02	1.00	$4.61E^{-01}$
Mental-Cat	ADHD diagnosis	5282.24	0.01	1.19	$1.10E^{-02}$
	Age	7021.62	0.01	1.45	$2.00E^{-03}$
	Sex	6565.05	0.01	1.36	$4.00E^{-03}$

5 Feature selection

Supplementary Table 6. Summary of the selected genera across studies

Genus	Selection probability
Tyzzerella	0.4992
Eubacterium_eligens_group	0.4581
Romboutsia	0.3682
RF39	0.3384
Victivallis	0.3398
Lachnospiraceae UCG 001	0.2562
Ruminococcus torques group	0.2222
Eubacterium ventriosum group	0.2059
Ruminococcus	0.1962
Sutterella	0.1849
Eubacterium xylanophilum group	0.1523
Eisenbergiella	0.1303
Erysipelatoclostridium	0.1294
Dialister	0.1289
Clostridia UCG 014	0.1186
Eubacterium ruminantium group	0.1123
Coprococcus	0.1098
Desulfovibrio	0.1059
Lactococcus	0.1052
Muribaculaceae	0.1041

Feature selection results over all studies. Selection probability of all selected genera are shown across studies.



Supplementary Figure 6. Stability path plots of the feature selection for Mental-Cat (top-left),IMpACT2-NL (top-right), MIND-Set (bottom-left) and NeuroIMAGE (bottom-right). Randomized Lasso stability selection starts with a probability of 0 for all genera. With each regularization step (penalized regression on a different subsample), the selection probability is updated. The selection probability threshold of 10% (marked in red) was chosen in order to pick up signals deviating visibly from the center of mass around 0 across all four studies. Stability paths of genera with selection probability of 10% or higher are marked in blue. Unselected genera are marked in black.

6 Differential abundance analysis

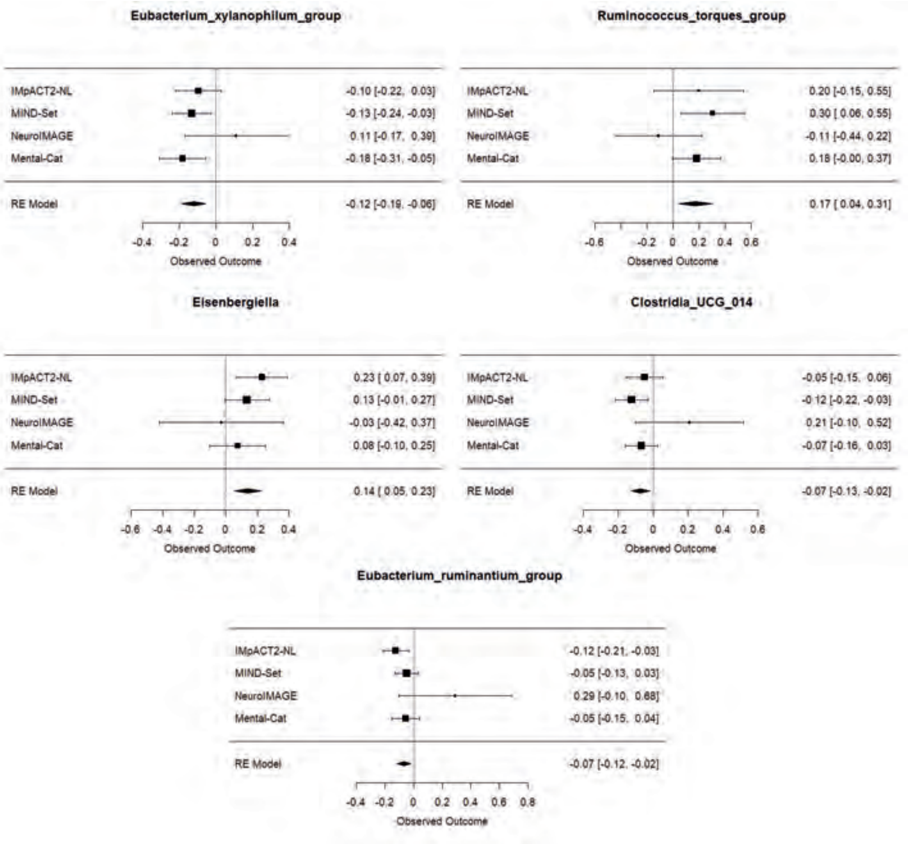
6.1 Logistic regression Meta-analyses

Supplementary Table 7. Results of the logistic regression meta-analyses

Genus	uncorrected p-value Logistic Regression	fdr corrected p-value Logistic Regression	Effect Size R Logistic Regression	Standard Error of R Logistic Regression	I2 Logistic Regression	pvalue test for heterogeneity Logistic Regression	Q value test for heterogeneity Logistic Regression
Eubacterium xylanophilum group	3.31E-04	6.95E-03	-0.12	0.03	0.04	3.04E-01	3.63
Eisenbergiella	1.91E-03	2.00E-02	0.14	0.05	0.00	4.91E-01	2.41
Clostridia UCG 014	1.05E-02	4.42E-02	-0.07	0.03	0.23	2.15E-01	4.47
Eubacterium ruminantium group	9.68E-03	4.42E-02	-0.07	0.03	0.01	1.77E-01	4.93
Ruminococcus torques group	1.05E-02	4.42E-02	0.17	0.07	7.54	2.64E-01	3.98
Eubacterium brachy group	3.22E-02	1.03E-01	0.11	0.05	0.00	6.83E-01	1.50
RF39	3.44E-02	1.03E-01	-0.10	0.05	51.08	1.45E-01	5.40
Eubacterium ventriosum group	5.69E-02	1.39E-01	-0.15	0.08	56.72	7.66E-02	6.86
Lachnospiraceae UCG 001	5.95E-02	1.39E-01	-0.08	0.04	33.50	2.38E-01	4.23
Ruminococcus	7.41E-02	1.41E-01	-0.09	0.05	27.30	3.03E-01	3.64
Victivallis	6.87E-02	1.41E-01	-0.13	0.07	66.43	3.14E-02	8.85
Tyzzerella	8.49E-02	1.49E-01	0.09	0.05	59.95	6.10E-02	7.37
Sutterella	1.51E-01	2.44E-01	-0.10	0.07	73.79	1.32E-02	10.74
Eubacterium eligens group	1.84E-01	2.76E-01	-0.10	0.07	70.75	2.08E-02	9.76
Coprococcus	2.55E-01	3.58E-01	-0.12	0.10	59.90	6.42E-02	7.26
Muribaculaceae	3.19E-01	4.19E-01	-0.03	0.03	41.84	2.12E-01	4.51

Supplementary Table 7. Continued

Genus	uncorrected p-value Logistic Regression	fdr corrected p-value Logistic Regression	Effect Size R Logistic Regression	Standard Error of R Logistic Regression	I2 Logistic Regression	pvalue test for heterogeneity Logistic Regression	Q value test for heterogeneity Logistic Regression
Erysipelatoclostridium	3.67E-01	4.31E-01	0.07	0.07	65.97	4.67E-02	7.97
Romboutsia	3.69E-01	4.31E-01	0.04	0.05	30.29	2.31E-01	4.30
Lactococcus	7.06E-01	7.80E-01	0.02	0.04	0.07	2.38E-01	4.23
Dialister	8.18E-01	8.59E-01	0.01	0.05	73.97	1.49E-02	10.48
Desulfovibrio	9.31E-01	9.31E-01	0.00	0.03	0.01	6.02E-01	1.86



Supplementary Figure 7. Forest plots of the genera showing significant associations with ADHD diagnosis in the meta-analyses based on logistic regression.

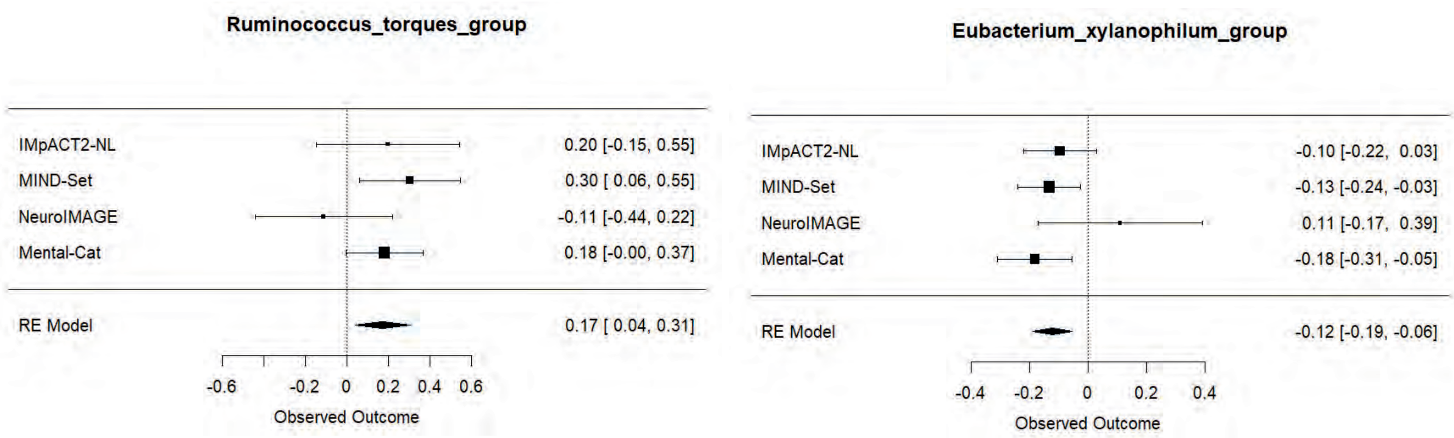
6.2 ALDEx2 Meta-analyses

Supplementary Table 8. Results of the ALDEx2 meta-analyses

Genus	Uncorrected p-value ALDEx2	Effect Size R ALDEx2	Standard Error of R ALDEx2
Eubacterium xylanophilum group	1.87E-03	-0.13	0.04
Ruminococcus torques group	1.46E-03	0.13	0.04
Eisenbergiella	7.86E-03	0.11	0.04
Clostridia UCG 014	1.23E-02	-0.11	0.04
Erysipelatoclostridium	3.22E-02	-0.09	0.04
Eubacterium brachy group	1.07E-01	0.06	0.04
Tyzzerella	1.16E-01	0.11	0.07
Eubacterium ruminantium group	1.50E-01	-0.08	0.06
Romboutsia	1.41E-01	0.07	0.04
Ruminococcus	1.76E-01	-0.07	0.05
Lachnospiraceae UCG 001	2.11E-01	-0.07	0.06
RF39	2.95E-01	-0.09	0.08
Victivallis	3.73E-01	-0.07	0.08
Coprococcus	4.97E-01	-0.04	0.06
Eubacterium eligens group	5.46E-01	-0.04	0.07
Eubacterium ventriosum group	4.34E-01	-0.05	0.06
Lactococcus	4.67E-01	0.03	0.04
Muribaculaceae	5.23E-01	-0.04	0.06
Sutterella	4.98E-01	-0.06	0.08
Dialister	7.38E-01	0.02	0.07
Desulfovibrio	9.17E-01	0.00	0.04

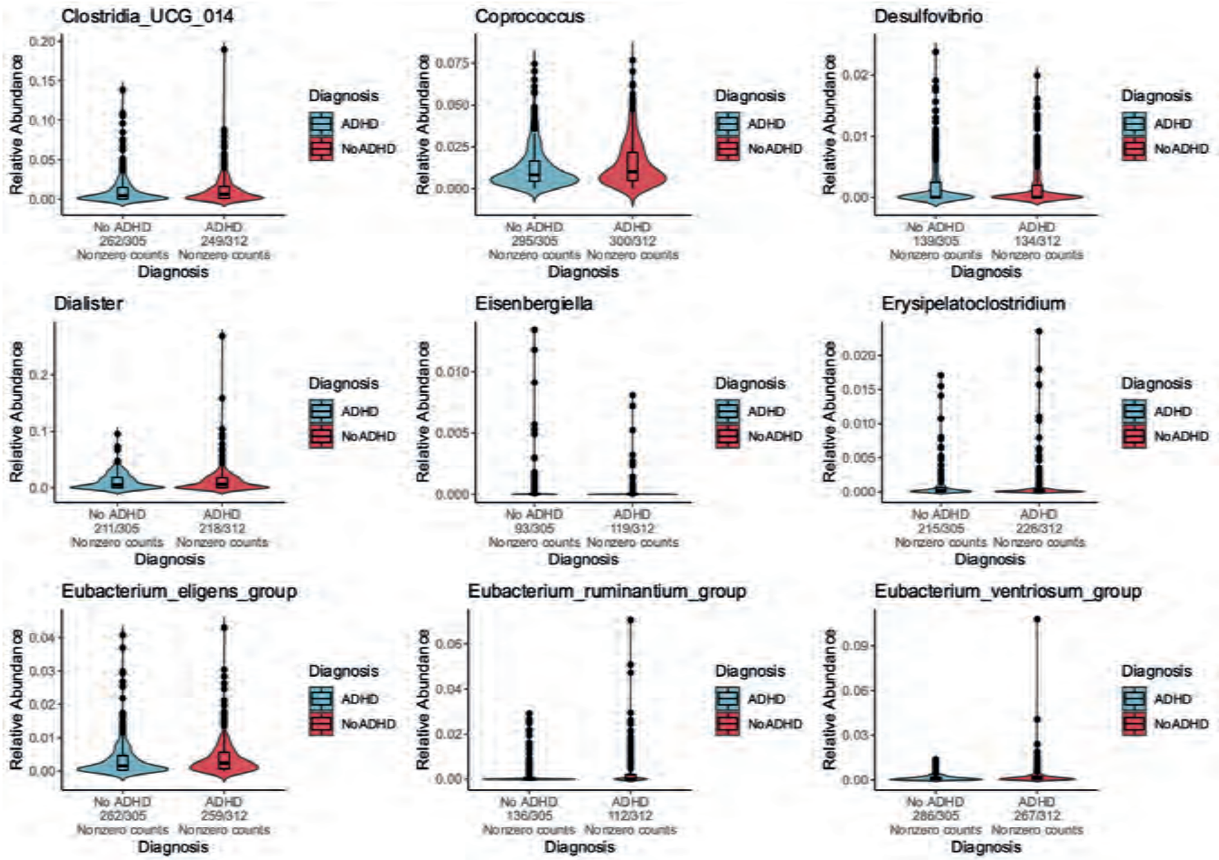
I^2 ALDEx2	p-value test for heterogeneity ALDEx2	Q value test for heterogeneity ALDEx2	fdr corrected p-value ALDEx2
0.03	1.77E-01	4.93	1.96E-02
0.00	8.81E-01	0.67	1.96E-02
0.00	5.13E-01	2.30	5.50E-02
15.27	4.05E-01	2.91	6.48E-02
4.15	2.29E-01	4.32	1.35E-01
0.00	4.96E-01	2.39	3.48E-01
63.06	4.27E-02	8.17	3.48E-01
45.24	1.22E-01	5.80	3.51E-01
15.71	3.74E-01	3.12	3.51E-01
33.39	1.78E-01	4.92	3.69E-01
50.65	1.02E-01	6.21	4.03E-01
75.75	9.02E-03	11.57	5.16E-01
69.98	1.94E-02	9.90	6.03E-01
54.19	8.54E-02	6.61	6.03E-01
68.09	2.39E-02	9.45	6.03E-01
53.27	9.27E-02	6.42	6.03E-01
0.00	6.72E-01	1.55	6.03E-01
46.10	1.29E-01	5.66	6.03E-01
75.62	7.78E-03	11.89	6.03E-01
65.95	2.92E-02	9.01	7.75E-01
0.00	7.41E-01	1.25	9.17E-01

3

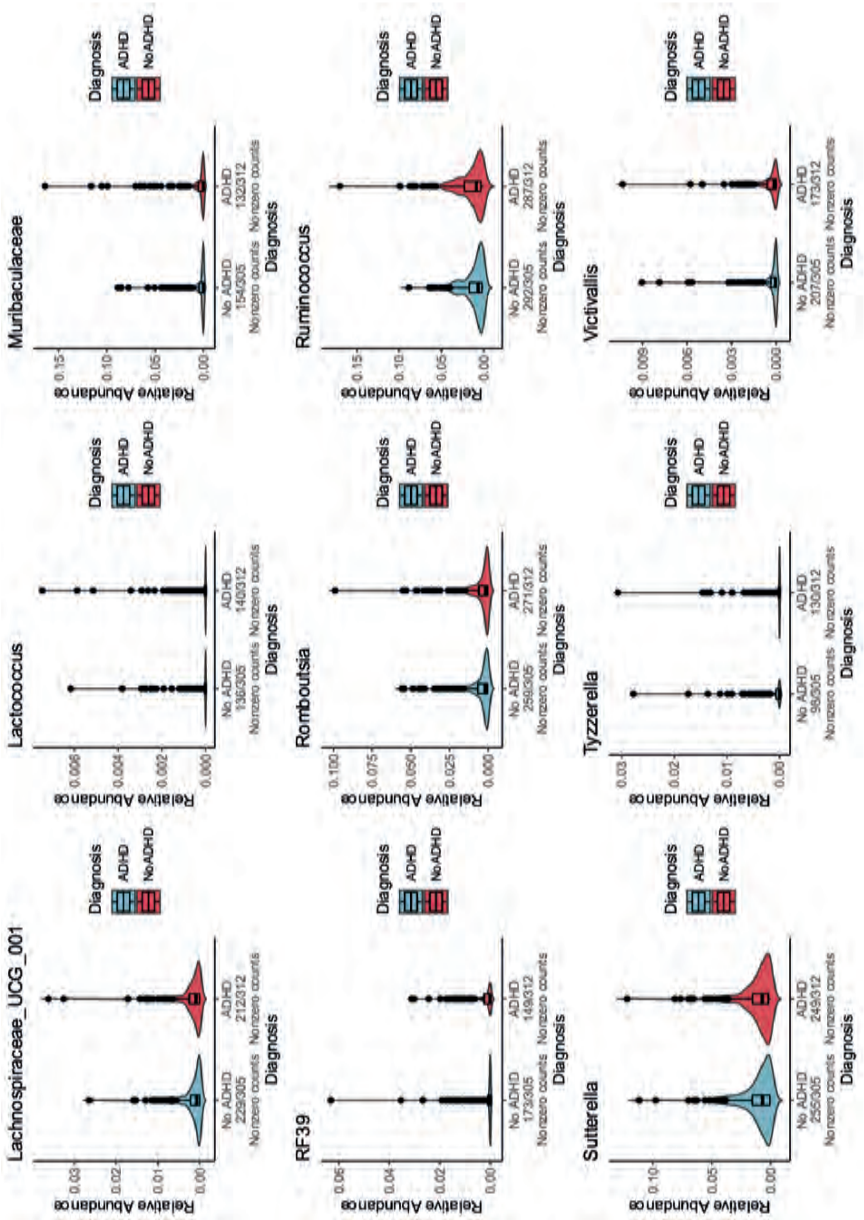


Supplementary Figure 8. Forest plots of the genera showing significant associations with ADHD diagnosis in the meta-analyses based on ALDEx2.

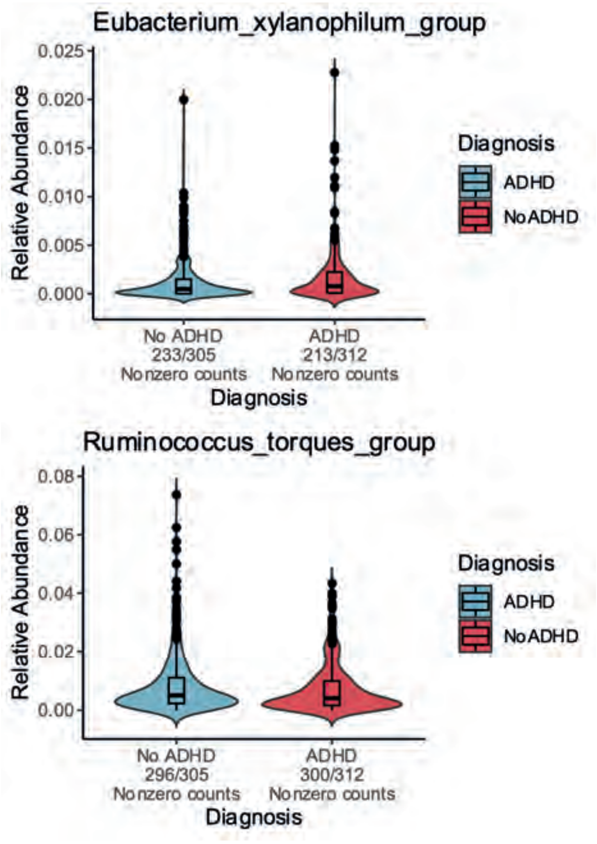
6.3 Relative Abundance Plots



Supplementary Figure 9. Relative Abundance aggregated over all studies of the 21 prevalent selected genera..



Supplementary Figure 9. Continued



Supplementary Figure 9. Continued

7. Post-Hoc analyses

7.1 Meta-analyses of symptom associations

Supplementary Table 9. Meta-analyses results on inattention symptoms

Genus	p-value	Std R	SE R	I^2	Het. Pvalue	Het. Q	*p*-value fdr
Eisenbergiella	2.90^{E}-04	0.16	0.04	0.00	6.69^{E}-01	1.56	1.16^{E}-03
Ruminococcus torques group	3.30^{E}-01	0.09	0.04	0.00	7.99^{E}-01	1.01	6.61^{E}-02
Clostridia UCG 014	5.96^{E}-01	-0.04	0.07	53.45	9.01^{E}-02	6.49	5.97^{E}-01
Eubacterium xylanophilum group	5.96^{E}-01	-0.02	0.04	0.00	4.21^{E}-01	2.82	5.97^{E}-01

Supplementary Table 10. Meta-analyses results on hyperactivity/impulsivity symptoms

Genus	p-value	Std R	SE R	I^2	Het. pvalue	Het. Q	*p*-value fdr
Eisenbergiella	1.07E-04	0.14	0.04	0.00	4.37E-01	2.72	4.27E-03
Ruminococcus torques group	6.18E-03	0.13	0.05	8.00	3.96E-01	2.97	1.24E-02
Clostridia UCG 014	3.78E-01	-0.05	0.04	44.89	1.41E-01	5.46	3.78E-01
Eubacterium xylanophilum group	2.09E-01	-0.05	0.06	0.06	3.78E-01	3.08	2.79E-01

7.2 Post-hoc correction for diet

194 Participants of the MIND-Set and 157 participants of the IMpACT2-NL study additionally filled in a semiquantitative food questionnaire, asking about the frequency of meat, fruit, vegetables, legumes and sweetened beverage consumption (in the scale of never, monthly, weekly or daily). No dietary information was provided for the NeuroIMAGE and Mental-Cat sample. Polychoric correlations between variables were computed, on which exploratory factor analysis (maximum likelihood estimation and varimax rotation) was performed. Parallel analysis suggested two factors in each cohort. The resulting factors were similar in both cohorts. One factor represented the consumption of 'healthier' foods (vegetables, legumes and fruit), while the other factor reflected the consumption of less healthy foods (meat and sweetened beverages) (see table ST11 for factor loadings of the individual food items). We computed a diet index by taking the absolute difference in scores of the first and the second factor. This index was intended to reflect the relative intake of healthier foods compared to less healthy foods. We introduced this diet index as a covariate into the logistic regressions at the individual study level in IMpACT2-NL and MIND-Set for the four significantly associated genera from the meta-analyses (Ruminococcus torques group, Eubacterium xylanophilum group, Eisenbergiella and Clostridia_UCG_014).

Supplementary Table 11. Factor loadings for the diet analyses

Food Items	MIND-Set		IMpACT2-NL	
	Factor 1	Factor 2	Factor 1	Factor 2
Meat	0.02	0.38	-0.22	0.47
Fruit	0.47	-0.38	0.64	-0.37
Vegetables	0.99	-0.09	0.71	-0.22
Legumes	0.43	-0.04	0.55	0.07
Sweet beverages	-0.13	0.35	0.03	0.65

Notably, on the individual study level, only Eisenbergiella abundance was significantly associated with ADHD diagnosis in IMpACT, the effect remained after correction for diet. All significant associations at the individual study level in MIND-Set remained significant after correction for the diet index. However, diet was significantly associated with ADHD diagnosis across all four associations in MIND-Set and subthreshold ($p<.1$) associated with ADHD diagnosis in IMpACT2-NL. Supplementary table 12 summarizes the results after correction for the diet index.

7.3 Post-hoc analyses of medication effects

We further investigated the potential effects of medication on the gut-microbiome. Significant associations of *Ruminococcus torques group, Eubacterium xylanophilum group* and *Eisenbergiella* with ADHD diagnosis in the medication naïve individuals of the Mental-Cat as well as the highly medicated sample of the MIND-Set cohort, point towards low sensitivity of these results for ADHD medication. We additionally performed a cases-only analysis of the potential effect of medication on genus abundance in IMpACT2-NL (38 of 78 participants indicated current simulant use) and NeuroIMAGE (12 of 29 participants indicated simulant use). Due to the extreme medication profiles (medication naïve participants in Mental-Cat and highly (and partly multi-)medicated individuals in MIND-Set) we could not perform this analysis in these cohorts. Supplementary Table 13 shows the results of the per study associations of genus-abundance with current use of ADHD medication in participants with ADHD. *Eisenbergiella* abundance was significantly higher in adults with ADHD medication in the NeuroIMAGE, but not the IMpACT2-NL study, while the other results showed no effect of medication. Supplementary Table 14 shows the results of the per study evaluation of associations of genus-abundance with current use of ADHD medication. Eisenbergiella abundance was significantly higher in adults with ADHD medication in the NeuroIMAGE, but not the IMpACT2-NL study.

Supplementary Table 12. Associations of genus abundance with ADHD diagnosis corrected for diet

Model **ADHD Diagnosis ~ Genus**		IMpACT2-NL				MIND-Set			
		Estimate	Std Error	t value	*p* value	Estimate	Std Error	t value	*p* value
Ruminococcus torques group	Diet	0.33	0.17	1.93	5.34 E^{-2}	-0.21	0.09	-2.19	2.81 E^{-2}
	Genus	-0.05	0.17	-0.28	7.77 E^{-1}	0.32	0.13	2.55	1.09 E^{-2}
Eubacterium xylanophilum group	Diet	0.34	0.17	1.99	4.62 E^{-2}	-0.21	0.09	-2.29	2.20 E^{-2}
	Genus	0.06	0.06	0.84	3.98 E^{-1}	-0.13	0.05	-2.21	2.70 E^{-2}
Eisenbergiella	Diet	0.30	0.17	1.71	8.7^{1} E^{-2}	-0.22	0.09	-2.42	1.57 E^{-2}
	Genus	0.17	0.08	2.07	3.89 E^{-2}	0.16	0.08	2.02	4.36 E^{-2}
Clostridia UCG 014	Diet	0.34	0.17	1.95	5.^{11} E^{-2}	-0.21	0.09	-2.25	2.47 E^{-2}
	Genus	0.07	0.05	1.33	^{1}.82 E^{-1}	-0.12	0.05	-2.38	1.70 E^{-2}

Supplementary Table 13. Associations of medication with genus abundance

Genus	**IMpACT2-NL**				**NeuroIMAGE**			
	Estimate	Std Error	t value	*p* value	Estimate	Std Error	t value	*p* value
Ruminococcus torques group	-0.32	0.19	-1.71	9.1 E^{-1}	0.17	0.39	0.42	6.7 E^{-1}
Eubacterium xylanophilum group	0.42	0.48	0.88	3.8 E^{-1}	-0.06	0.68	0.08	9.3 E^{-1}
Eisenbergiella	0.25	0.36	0.70	4.8 E^{-1}	0.56	0.25	2.23	3.5 E^{-2}
Clostridia UCG 014	0.01	0.52	0.01	9.9 E^{-1}	-0.02	0.53	-0.05	9.5 E^{-1}

8 STORMS guidelines

Supplementary Table 14. Storms Checklist

Number	Item	Recommendation	Item Source
Abstract			
1.0	Structured or Unstructured Abstract	Abstract should include information on background, methods, results, and conclusions in structured or unstructured format.	STORMS
1.1	Study Design	State study design in abstract.	STORMS
1.2	Sequencing methods	State the strategy used for metagenomic classification.	STORMS
1.3	Specimens	Describe body site(s) studied.	STORMS
Introduction			
2.0	Background and Rationale	Summarize the underlying background, scientific evidence, or theory driving the current hypothesis as well as the study objectives.	STORMS
2.1	Hypotheses	State the pre-specified hypothesis. If the study is exploratory, state any pre-specified study objectives.	STORMS
Methods			
3.0	Study Design	Describe the study design.	STORMS
3.1	Participants	State what the population of interest is, and the method by which participants are sampled from that population. Include relevant information on physiological state of the subjects or stage in the life history of disease under study when participants were sampled.	STORMS

Additional Guidance	Yes/No/NA	Comments or location in manuscript
	Yes	
See 3.0 for additional information on study design.	Yes	
For example, targeted 16S by qPCR or sequencing, shotgun metagenomics, metatranscriptomics, etc.	Yes	
	Yes	
	Yes	
	Yes	Exploratory
Observational (Case-Control, Cohort, Cross-sectional survey, etc.) or Experimental (Randomized controlled trial, Non-randomized controlled trial, etc.). For a brief description of common study designs see: DOI: 10.11613/BM.2014.022 If applicable, describe any blinding (e.g. single or double-blinding) used in the course of the study.	Yes	
Examples of the population of interest could be: adults with no chronic health conditions, adults with type II diabetes, newborns, etc. This is the total population to whom the study is hoped to be generalizable to. The sampling method describes how potential participants were selected from that population. If the participants are from a substudy of a larger study, provide a brief description of that study and cite that study. Clearly state how cases and controls are defined. An example of relevant physiological state might be pre/post menopausal for a vaginal microbiome study; examples of stage in the life history of disease could be whether specimens were collected during active or dormant disease, or before or after treatment.	Yes	Methods and Materials + Supplementary Chapter 1.1

Supplementary Table 14. Continued

Number	Item	Recommendation	Item Source
3.2	Geographic location	State the geographic region(s) where participants were sampled from.	MIxS: geographic location (country and/or sea,region)
3.3	Relevant Dates	State the start and end dates for recruitment, follow-up, and data collection.	STORMS
3.4	Eligibility criteria	List any criteria for inclusion and exclusion of recruited participants.	Modified STROBE
3.5	Antibiotics Usage	List what is known about antibiotics usage before or during sample collection.	STORMS
3.6	Analytic sample size	Explain how the final analytic sample size was calculated, including the number of cases and controls if relevant, and reasons for dropout at each stage of the study. This should include the number of individuals in whom microbiome sequencing was attempted and the number in whom microbiome sequencing was successful.	STORMS
3.7	Longitudinal Studies	For longitudinal studies, state how many follow-ups were conducted, describe sample size at follow-up by group or condition, and discuss any loss to follow-up.	STORMS
3.8	Matching	For matched studies, give matching criteria.	Modified STROBE
3.9	Ethics	State the name of the institutional review board that approved the study and protocols, protocol number and date of approval, and procedures for obtaining informed consent from participants.	STORMS

Additional Guidance	Yes/No/NA	Comments or location in manuscript
Geographic coordinates can be reported to prevent potential ambiguities if necessary.	Yes	Table 1
Recruitment is the period in which participants are recruited for the study. In longitudinal studies, follow-up is the date range in which participants are asked to complete a specific assessment. Finally, data collection is the total period in which data is being collected from participants including during initial recruitment through all follow-ups.	Yes	Table 1
Among potential recruited participants, how were some chosen and others not? This could include criteria such as sex, diet, age, health status, or BMI. If there is a primary and validation sample, describe inclusion/exclusion criteria for each.	Yes	Supplementary Chapter 1.1
If participants were excluded due to current or recent antibiotics usage, state this here. Other factors (e.g. proton pump inhibitors, probiotics, etc.) that may influence the microbiome should also be described as well.	Partly	Information with sufficient detail was only acquired in MIND-SET, exclusion criterium in Mental-Cat
Consider use of a flow diagram (see template at https://stormsmicrobiome.org/figures). Also state sample size in abstract. If power analysis was used to calculate sample size, describe those calculations.	Partly	See Methods section and supplementary chapter 1.1. Note, information of attempted sequencing was not available for Mental-Cat and NeuroIMAGE
If there is loss to follow-up, discuss the likelihood that drop-out is associated with exposures, treatments, or outcomes of interest.	NA	
"Matched" refers to matching between comparable study participants as cases and controls or exposed / unexposed. Indicate whether participants were individual or frequency matched and in what ratio were they matched (e.g. 1 case to 1 control).	NA	
	Partly	Note, protocol number and date of approval are not provided

3

Supplementary Table 14. Continued

Number	Item	Recommendation	Item Source
4.0	Laboratory methods	State the laboratory/center where laboratory work was done.	STORMS
4.1	Specimen collection	State the body site(s) sampled from and how specimens were collected.	MIxS: sample collection device or method; host body site
4.2	Shipping	Describe how samples were stored and shipped to the laboratory.	STORMS
4.3	Storage	Describe how the laboratory stored samples, including time between collection and storage and any preservation buffers or refrigeration used.	STORMS
4.4	DNA extraction	Provide DNA extraction method, including kit and version if relevant.	MIxS: nucleic acid extraction
4.5	Human DNA sequence depletion or microbial DNA enrichment	Describe whether human DNA sequence depletion or enrichment of microbial or viral DNA was performed.	STORMS
4.6	Primer selection	Provide primer selection and DNA amplification methods as well as variable region sequenced (if applicable).	MIxS: pcr primers
4.7	Positive Controls	Describe any positive controls (mock communities) if used.	STORMS
4.8	Negative Controls	Describe any negative controls if used.	STORMS
4.9	Contaminant mitigation and identification	Provide any laboratory or computational methods used to control for or identify microbiome contamination from the environment, reagents, or laboratory.	STORMS
4.10	Replication	Describe any biological or technical replicates included in the sequencing, including which steps were replicated between them.	STORMS
4.11	Sequencing strategy	Major divisions of strategy, such as shotgun or amplicon sequencing.	MIxS: sequencing method

Additional Guidance	Yes/No/NA	Comments or location in manuscript
Provide a reference to complete lab protocols if previously published elsewhere such as on protocols.io. Note any modifications of lab protocols and the reason for protocol modifications.	Partly	Information only available for MIND-Set and IMpACT2-NL, Supplementary Chapter 2
Use terms from the Uber-anatomy Ontology (https://www.ebi.ac.uk/ols/ontologies/uberon) to describe body sites in a standardized format.	Yes	Supplementary Chapter 2
Include length of time from collection to receipt by the lab and if temperature control was used during shipping.	Partly	Supplementary Chapter 2, information only available for IMpACT2-NL and MIND-Set
State where each procedure or lot of samples was done if not all in the same place. Include reagent/lot/catalogue #s for storage buffers.	Partly	Supplementary Chapter 2, information only available for IMpACT2-NL and MIND-Set
If any DNA quantification methods were used prior to DNA amplification or at the pooling step of library preparation, state so here.	Partly	Supplementary Chapter 2, information only available for IMpACT2-NL and MIND-Set
	NA	
	Yes	Methods section
If used, should be deposited under guidance provided in the 8.X items.	Yes	Supplementary Section 1.1
If used, should be deposited under guidance provided in the 8.X items.	Yes	Supplementary Section 1.1
Includes filtering of reagents and other steps to minimize contamination. It is relevant to state whether the specimens of interest have low microbial load, which makes contamination especially relevant.	NA	Baseclear inclusion of negative water control and sterile lab environment
Replication may be biological (redundant biological specimens) or technical (aliquots taken at different stages of analysis) and used in extraction, sequencing, preprocessing, and/or data analysis.	NA	
For amplicon sequencing (for example, 16S variable region), state the region selected. State the model of sequencer used.	Yes	Methods section

3

Supplementary Table 14. Continued

Number	Item	Recommendation	Item Source
4.12	Sequencing methods	State whether experimental quantification was used (QMP/cell count based, spike-in based) or whether relative abundance methods were applied.	STORMS
4.13	Batch effects	Detail any blocking or randomization used in study design to avoid confounding of batches with exposures or outcomes. Discuss any likely sources of batch effects, if known.	STORMS
4.14	Metatranscriptomics	Detail whether any mRNA enrichment was performed and whether/how retrotranscription was performed prior to sequencing. Provide size range of isolated transcripts. Describe whether the sequencing library was stranded or not. Provide details on sequencing methods and platforms.	STORMS
4.15	Metaproteomics	Detail which protease was used for digestion. Provide details on proteomic methods and platforms (e.g. LC-MS/MS, instrument type, column type, mass range, resolution, scan speed, maximum injection time, isolation window, normalised collision energy, and resolution).	STORMS
4.16	Metabolomics	Specify the analytic method used (such as nuclear magnetic resonance spectroscopy or mass spectrometry). For mass spectrometry, detail which fractions were obtained (polar and/or non-polar) and how these were analyzed. Provide details on metabolomics methods and platforms (e.g. derivatization, instrument type, injection type, column type and instrument settings).	STORMS
5.0	Data sources/ measurement	For each non-microbiome variable, including the health condition, intervention, or other variable of interest, state how it was defined, how it was measured or collected, and any transformations applied to the variable prior to analysis.	MIxS: host disease status

Additional Guidance	Yes/No/NA	Comments or location in manuscript
These include read length, sequencing depth per sample (average and minimum), whether reads are paired, and other parameters.	NA	
Sources of batch effects include sample collection, storage, library preparation, and sequencing and are commonly unavoidable in all but the smallest of studies.	NA	No randomization, batches are filled with samples according to the date they were received across MIND-Set and IMpACT, mixing control subjects and participants with ADHD
Provide details on any internal standards which may have been used as well as parameters and versions of any software or databases used.	NA	
Provide details on any internal standards which may have been used as well as parameters and versions of any software or databases used.	NA	
Provide details on any internal standards which may have been used as well as parameters and versions of any software or databases used.	NA	
State any sources of potential bias in measurements, for example multiple interviewers or measurement instruments, and whether these potential biases were assessed or accounted for in study design. Use terms from a standardized ontology such as the Experimental Factor Ontology (https://www.ebi.ac.uk/efo/) to describe variables of interest in a standardized format.	Yes	Methods section, Discussion, Supplementary Chapter 1.1

Supplementary Table 14. Continued

Number	Item	Recommendation	Item Source
6.0	Research design for causal inference	Discuss any potential for confounding by variables that may influence both the outcome and exposure of interest. State any variables controlled for and the rationale for controlling for them.	STORMS
6.1	Selection bias	Discuss potential for selection or survival bias.	STORMS
7.0	Bioinformatic and Statistical Methods	Describe any transformations to quantitative variables used in analyses (e.g. use of percentages instead of counts, normalization, rarefaction, categorization).	STORMS
7.1	Quality Control	Describe any methods to identify or filter low quality reads or samples.	MIxS: sequence quality check
7.2	Sequence analysis	Describe any taxonomic, functional profiling, or other sequence analysis performed.	MIxS: feature prediction; similarity search method

Additional Guidance	Yes/No/NA	Comments or location in manuscript
For causal inference, this item refers to describing the assumptions that would be required to draw causal inferences from observational data. See Vujkovic-Cvijin, I., Sklar, J., Jiang, L. et al. Host variables confound gut microbiota studies of human disease. Nature 587, 448–454 (2020). https://doi.org/10.1038/s41586-020-2881-9 for more details on confounding in observational microbiome studies. For example, hypothesized confounders may be controlled for by multivariable adjustment. Consider using a directed acyclic graph (DAG) to describe your causal model and justify any variables controlled for. DAGs can be made using www.dagitty.net.	NA	
Selection bias can occur when some members of the target study population are more likely to be included in the study/final analytic sample than others. Some examples include survival bias (where part of the target study population is more likely to die before they can be studied), convenience sampling (where members of the target study population are not selected at random), and loss to follow-up (when probability of dropping out is related to one of the things being studied).	NA	Feature selection was applied, while bias is introduced underestimating low prevalent features, the data-driven selection instead of hypothesis driven might on the other hand reduce bias
If a variable is analyzed using different transformations, state rationale for the transformation and for each analyses which version of the variable is used. In case of any complex or multistep transformations, give enumerated instructions for reproducing those transformations.	Yes	Methods section, Supplementary Chapter 2
If samples were excluded based on quality or read depth, list the criteria used, the number of samples excluded, and the final sample size after quality control.	Yes	Methods section
	Yes	Methods section

Supplementary Table 14. Continued

Number	Item	Recommendation	Item Source
7.3	Statistical methods	Describe all statistical methods.	Modified STROBE
7.4	Longitudinal analysis	If the study is longitudinal, include a section that explicitly states what analysis methods were used (if any) to account for grouping of measurements by individual or patterns over time.	STORMS
7.5	Subgroup analysis	Describe any methods used to examine subgroups and interactions.	STROBE
7.6	Missing data	Explain how missing data were addressed.	STROBE
7.7	Sensitivity analyses	Describe any sensitivity analyses.	STROBE
7.8	Findings	State criteria used to select findings for reporting.	STORMS
7.9	Software	Cite all software (including read mapping software) and databases (including any used for taxonomic reference or annotating amplicons, if applicable) used. Include version numbers.	Modified STREGA

Additional Guidance	Yes/No/NA	Comments or location in manuscript
Describe any statistical tests used, exploratory data analysis performed, dimension reduction methods/unsupervised analysis, alpha/beta metrics, and/or methods for adjusting for measurement bias. If multiple statistical methods are possible, discuss why the methods used were selected. If a multiple hypothesis testing correction method was used, describe the type of correction used. State which taxonomic levels are analyzed.	Yes	Methods section
	NA	
	NA	
"Missing data" refers to participant measurements such as covariates, exposures, outcomes, or time points that should have been collected but were not, not to zeros in taxonomic abundance tables or data points not applicable to that observation.	Yes	missing data was strictly excluded
	Yes	Supplementary Chapter 7
For example, false discovery rate with total number of tests, effect size threshold, significance threshold, microbes of interest.	Yes	Convergence across tools, meta-analysis, fdr corrected
Installed packages, add-ons or libraries should be stated and cited in addition to the software used. All parameters employed that differ from the default of that software/version should be provided. This is in addition to, not a replacement for, publishing of code as outlined in the section Reproducible Research.	Yes	Methods section

3

Supplementary Table 14. Continued

Number	Item	Recommendation	Item Source
8.0	Reproducible research	Make a statement about whether and how others can reproduce the reported analysis.	STORMS
8.1	Raw data access	State where raw data may be accessed including demultiplexing information.	STORMS
8.2	Processed data access	State where processed data may be accessed.	STORMS
8.3	Participant data access	State where individual participant data such as demographics and other covariates may be accessed, and how they can be matched to the microbiome data.	STORMS
8.4	Source code access	State where code may be accessed.	STORMS
8.5	Full results	Provide full results of all analyses, in computer-readable format, in supplementary materials.	STORMS
Results			

	Additional Guidance	Yes/No/NA	Comments or location in manuscript
	Any protected information that has been excluded or provided under controlled access should be listed along with any relevant data access procedures. "On request from authors" is not sufficiently detailed; formal data access procedures and conditions should be defined. If data are unavailable, state so clearly. Consider using a specialized rubric for reproducible research (such as:https://mbio.asm.org/content/9/3/e00525-18.short). Consider preregistering the study protocol (such as on osf.io orhttps://plos.org/open-science/preregistration/).	NA	We are not owners of the individual study data, raw sequences are available upon request, summary statistics are attached to the supplement
	Robust, long-term databases such as those hosted by NCBI and EBI are preferred. If using a private repository, provide rationale.	NA	
	Unfiltered data should be provided. Robust, long-term databases such as those hosted by NCBI and EBI-EMBL are preferred. Repositories like zenodo (https://zenodo.org/) or publisso (https://www.publisso.de/en/working-for-you/doi-service/) can be used to provide a DOI and long-term storage for processed datasets, even those which cannot be published openly.	NA	
	If re-categorized, transformed, or otherwise derived variables were used in the analysis, these variables or code for deriving them should be provided. Examples of how participant data can be matched to microbiome data are: using the same set of anonymized identifiers, or using different anonymized identifiers but providing a map. Provided data should be sufficient to independently replicate the current analysis.	NA	
	If a standard or formalized workflow was employed, reference it here.	Yes	Methods section
	For example, any fold-changes, p-values, or FDR values calculated, provided as a spreadsheet. Use a machine-readable, plain-text format such as csv or tsv.	Yes	Provided in supplementary materials (word format)

Supplementary Table 14. Continued

Number	Item	Recommendation	Item Source
9.0	Descriptive data	Give characteristics of study participants (e.g. dietary, demographic, clinical, social) and information on exposures and potential confounders.	STROBE
10.0	Microbiome data	Report descriptive findings for microbiome analyses with all applicable outcomes and covariates.	STORMS
10.1	Taxonomy	Identify taxonomy using standardized taxon classifications that are sufficient to uniquely identify taxa.	STORMS
10.2	Differential abundance	Report results of differential abundance analysis by the variable of interest and (if applicable) by time, clearly indicating the direction of change and total number of taxa tested.	STORMS
10.3	Other data types	Report other data analyzed--e.g. metabolic function, functional potential, MAG assembly, and RNAseq.	STORMS
10.4	Other statistical analysis	Report any statistical data analysis not covered above.	STORMS
Discussion			
11.0	Key results	Summarise key results with reference to study objectives	STROBE

Additional Guidance	Yes/No/NA	Comments or location in manuscript
Typically reported in a table included in the paper or as a supplementary table. Indicate number of participants with missing data for each variable of interest. This includes environmental and lifestyle factors that may affect the relationship between the microbiome and the condition of interest. Participant diet and medication use should be summarized, if known. At minimum, age and sex of all participants should be summarized.	Yes	Methods section
This includes measures of diversity as well as relative abundances. These descriptive findings should be reported both for the sample overall and for individual groups.	Yes	Results section, Supplementary Materials
If not using full taxonomic hierarchy, make sure it is clear whether names stated are species, genera, family, etc. Italicize genus/species pairs. Consult journal guidelines or standardized references on taxonomic nomenclature. For instance,https://wwwnc.cdc.gov/eid/page/scientific-nomenclature	Yes	Genus only
If there are more than two groups, include omnibus (multigroup) test results if applicable to the research question. If applicable, reported effect sizes should include a measure of uncertainty such as the confidence interval.	Yes	Results, Supplementary chapter 6
	NA	
This could include subgroup analysis, sensitivity analyses, and cluster analysis. Visualizations should be easily interpretable and colorblind-friendly. The caption and/or main text should provide a detailed description of visualizations for visually-impaired readers.	Yes	
	Yes	

Supplementary Table 14. Continued

Number	Item	Recommendation	Item Source
12.0	Interpretation	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence.	STROBE
13.0	Limitations	Discuss limitations of the study, taking into account sources of potential bias or imprecision.	STROBE
13.1	Bias	Discuss any potential for bias to influence study findings.	STORMS
13.2	Generalizability	Discuss the generalisability (external validity) of the study results	STROBE
14.0	Ongoing/future work	Describe potential future research or ongoing research based on the study's findings.	STORMS
Other information			
15.0	Funding	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	STROBE
15.1	Acknowledgements	Include acknowledgements of those who contributed to the research but did not meet critera for authorship.	STORMS
15.2	Conflicts of Interest	Include a conflicts of interest statement.	STORMS
16.0	Supplements	Indicate where supplements may be accessed and what materials they contain.	STORMS
17.0	Supplementary data	Provide supplementary data files of results with for all taxa and all outcome variables analyzed. Indicate the taxonomic level of all taxa.	STORMS

Additional Guidance	Yes/No/NA	Comments or location in manuscript
Define or clarify any subjective terms such as "dominant," "dysbiosis," and similar words used in interpretation of results. When interpreting the findings, consider how the interpretation of the findings may be summarized or quoted for the general public such as in press releases or news articles. If causal language is used in the interpretation (such as "alters," "affects," "results in," "causes," or "impacts"), assumptions made for causal inference should be explicitly stated as part of 6.0 and 13.0. Distinguish between function potential (ie inferred from metagenomics) and observed activity (ie metatranscriptomic, metabolomic, proteomic) if discussing microbial function.	Yes	
Also consider limitations resulting from the methods (especially novel methods), the study design, and the sample size.	Yes	
May include sampling method, representativeness of study participants, or potential confounding.	Yes	
To what populations or other settings do you expect the conclusions to generalize?	NA	
	Yes	
	Partly	Acknowledgements, Where information was provided
For general guidelines on authorship, seehttp://www.icmje.org andhttps://www.elsevier.com/authors/journal-authors/policies-and-ethics/credit-author-statement	NA	
	Yes	Disclosure
	Yes	In text
Depending on the analysis performed, examples of the supplemental results included could be mean relative abundance, differential abundance, raw p-value, multiple hypothesis testing-adjusted p-values, and standard error. All discussed taxa should include the taxonomic level (e.g. class, order, genus).	Yes	

3

Supplementary Table 15. PRISMA Checklist

Section and Topic	Item	Checklist item	Location where item is reported
TITLE			
Title	1	Identify the report as a systematic review.	NA, report is a meta-analysis
ABSTRACT			
Abstract	2	See the PRISMA 2020 for Abstracts checklist.	Abstract
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of existing knowledge.	NA
Objectives	4	Provide an explicit statement of the objective(s) or question(s) the review addresses.	NA
METHODS			
Eligibility criteria	5	Specify the inclusion and exclusion criteria for the review and how studies were grouped for the syntheses.	Supplementary section 1
Information sources	6	Specify all databases, registers, websites, organisations, reference lists and other sources searched or consulted to identify studies. Specify the date when each source was last searched or consulted.	Supplementary figure 15
Search strategy	7	Present the full search strategies for all databases, registers and websites, including any filters and limits used.	Supplementary figure 15
Selection process	8	Specify the methods used to decide whether a study met the inclusion criteria of the review, including how many reviewers screened each record and each report retrieved, whether they worked independently, and if applicable, details of automation tools used in the process.	Supplementary figure 15
Data collection process	9	Specify the methods used to collect data from reports, including how many reviewers collected data from each report, whether they worked independently, any processes for obtaining or confirming data from study investigators, and if applicable, details of automation tools used in the process.	Supplementary figure 15

Supplementary Table 15. Continued

Section and Topic	Item	Checklist item	Location where item is reported
Data items	10a	List and define all outcomes for which data were sought. Specify whether all results that were compatible with each outcome domain in each study were sought (e.g. for all measures, time points, analyses), and if not, the methods used to decide which results to collect.	Supplementary figure 15, supplementary section 1
	10b	List and define all other variables for which data were sought (e.g. participant and intervention characteristics, funding sources). Describe any assumptions made about any missing or unclear information.	NA
Study risk of bias assessment	11	Specify the methods used to assess risk of bias in the included studies, including details of the tool(s) used, how many reviewers assessed each study and whether they worked independently, and if applicable, details of automation tools used in the process.	NA (not a review, only 2 studies received)
Effect measures	12	Specify for each outcome the effect measure(s) (e.g. risk ratio, mean difference) used in the synthesis or presentation of results.	Re-analyzed, see Methods section Manuscript
Synthesis methods	13a	Describe the processes used to decide which studies were eligible for each synthesis (e.g. tabulating the study intervention characteristics and comparing against the planned groups for each synthesis (item #5)).	Supplementary figure 15
	13b	Describe any methods required to prepare the data for presentation or synthesis, such as handling of missing summary statistics, or data conversions.	Data request, see supplementary section 1
	13c	Describe any methods used to tabulate or visually display results of individual studies and syntheses.	NA
	13d	Describe any methods used to synthesize results and provide a rationale for the choice(s). If meta-analysis was performed, describe the model(s), method(s) to identify the presence and extent of statistical heterogeneity, and software package(s) used.	See Methods section Manuscript
	13e	Describe any methods used to explore possible causes of heterogeneity among study results (e.g. subgroup analysis, meta-regression).	See Methods section Manuscript
	13f	Describe any sensitivity analyses conducted to assess robustness of the synthesized results.	See supplementary section 7

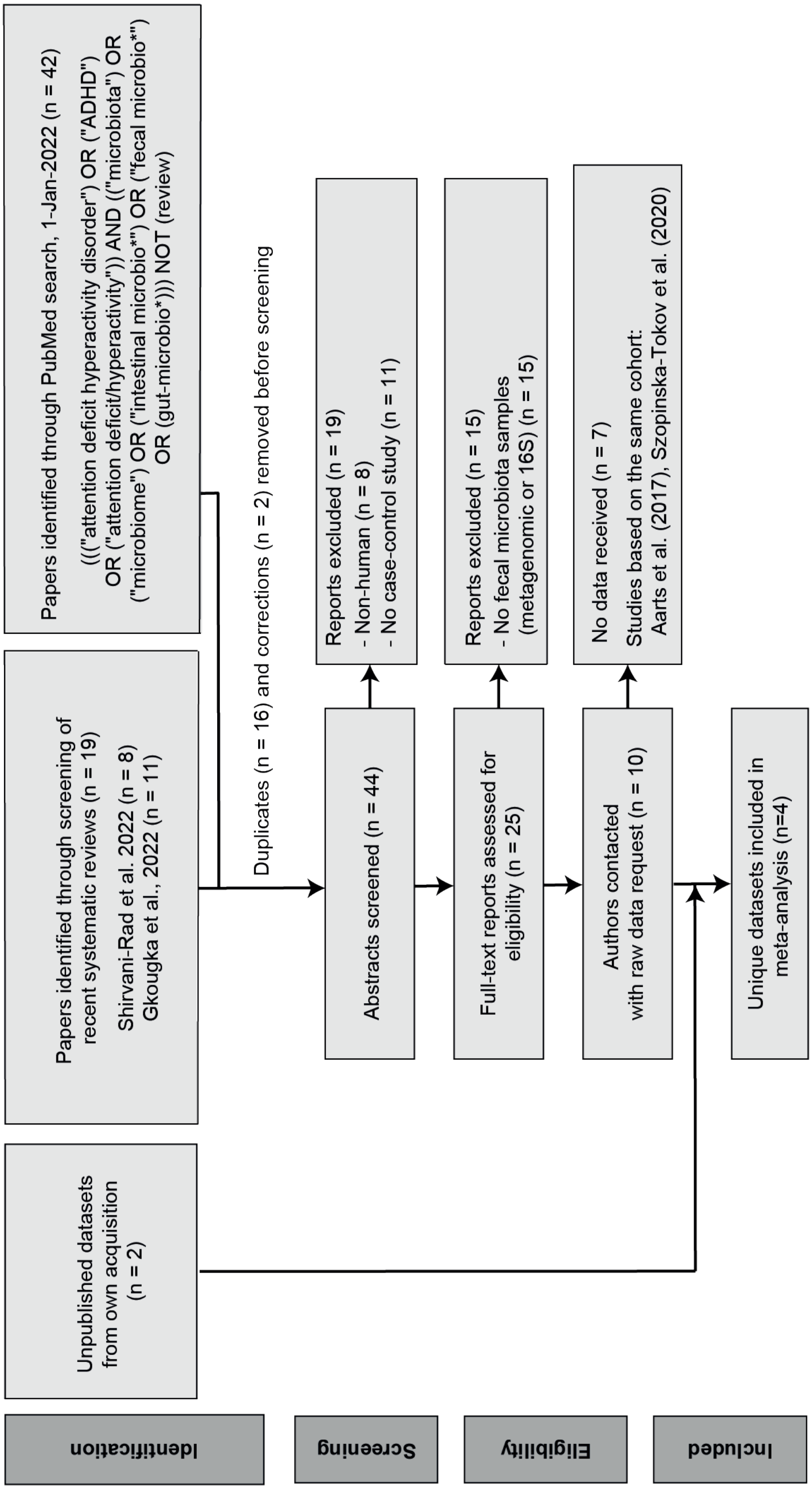
Supplementary Table 15. Continued

Section and Topic	Item	Checklist item	Location where item is reported
Reporting bias assessment	14	Describe any methods used to assess risk of bias due to missing results in a synthesis (arising from reporting biases).	Only 2 included studies,assessment skew
Certainty assessment	15	Describe any methods used to assess certainty (or confidence) in the body of evidence for an outcome.	Convergence, see Methods and Discussion section
RESULTS			
Study selection	16a	Describe the results of the search and selection process, from the number of records identified in the search to the number of studies included in the review, ideally using a flow diagram.	See Methods section Manuscript
	16b	Cite studies that might appear to meet the inclusion criteria, but which were excluded, and explain why they were excluded.	See Methods section Manuscript
Study characteristics	17	Cite each included study and present its characteristics.	See supplementary section 1
Risk of bias in studies	18	Present assessments of risk of bias for each included study.	NA
Results of individual studies	19	For all outcomes, present, for each study: (a) summary statistics for each group (where appropriate) and (b) an effect estimate and its precision (e.g. confidence/credible interval), ideally using structured tables or plots.	See supplementary section 3, 4 and 5
Results of syntheses	20a	For each synthesis, briefly summarise the characteristics and risk of bias among contributing studies.	NA
	20b	Present results of all statistical syntheses conducted. If meta-analysis was done, present for each the summary estimate and its precision (e.g. confidence/credible interval) and measures of statistical heterogeneity. If comparing groups, describe the direction of the effect.	See Supplementary results and results section
	20c	Present results of all investigations of possible causes of heterogeneity among study results.	See Supplementary results and results section
	20d	Present results of all sensitivity analyses conducted to assess the robustness of the synthesized results.	See Supplementary section 7
Reporting biases	21	Present assessments of risk of bias due to missing results (arising from reporting biases) for each synthesis assessed.	NA
Certainty of evidence	22	Present assessments of certainty (or confidence) in the body of evidence for each outcome assessed.	See supplementary section 6 and Results section in Manuscript

Supplementary Table 15. Continued

Section and Topic	Item	Checklist item	Location where item is reported
DISCUSSION			
Discussion	23a	Provide a general interpretation of the results in the context of other evidence.	See Discussion in Manuscript
	23b	Discuss any limitations of the evidence included in the review.	See Discussion in Manuscript
	23c	Discuss any limitations of the review processes used.	See Discussion in Manuscript
	23d	Discuss implications of the results for practice, policy, and future research.	See Discussion in Manuscript
OTHER INFORMATION			
Registration and protocol	24a	Provide registration information for the review, including register name and registration number, or state that the review was not registered.	NA
	24b	Indicate where the review protocol can be accessed, or state that a protocol was not prepared.	NA
	24c	Describe and explain any amendments to information provided at registration or in the protocol.	NA
Support	25	Describe sources of financial or non-financial support for the review, and the role of the funders or sponsors in the review.	See Funding in Manuscript
Competing interests	26	Declare any competing interests of review authors.	See Financial Disclosures in Manuscript
Availability of data, code and other materials	27	Report which of the following are publicly available and where they can be found: template data collection forms; data extracted from included studies; data used for all analyses; analytic code; any other materials used in the review.	NA, all data was accessed after motivated request to the data owners

From: Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ 2021;372:n71. doi: 10.1136/bmj.n71



Supplementary Figure 10. Flow diagram of study selection for the meta-analysis.

Chapter 4

The role of diet and the gut-microbiota in reactive aggression and adult ADHD – An exploratory analysis

Jakobi, B., Cimetti, C., Mulder, D., Vlaming, P., Franke, B., Hoogman, M., Arias-Vasquez, A.

The role of diet and the gut-microbiota in reactive aggression and adult ADHD –
An exploratory analysis.

published as: Jakobi, B., Cimetti, C., Mulder, D., Vlaming, P., Franke, B., Hoogman, M., & Arias-Vasquez, A. (2024). The Role of Diet and the Gut Microbiota in Reactive Aggression and Adult ADHD— An Exploratory Analysis. Nutrients, 16(14), 2174.

ABSTRACT

Attention-deficit/hyperactivity disorder (ADHD) is a common neurodevelopmental condition. Symptoms persist into adulthood in more than half of the affected individuals, often accompanied by emotion dysregulation such as reactive aggression. Recent research has suggested that diet and gut-microbial changes might modulate ADHD symptoms as well as emotional behaviors; however, existing research results are inconclusive, and an integration of research on dietary and microbial patterns with ADHD is missing.

In this study, we investigated the role of diet and gut-microbiota in adult ADHD and reactive aggression in 77 adults with ADHD and 76 neurotypical individuals. We applied exploratory factor analysis on a dietary questionnaire and studied the relationships between ADHD and reactive aggression with diet. We furthermore estimated bacterial community and taxonomic differences of 16S-sequenced fecal microbiome samples, and potential mediating effects of bacterial genus abundance on significant diet-behavior associations.

The exploratory factor analysis yielded three dietary patterns: an *high-alcohol* factor, a *high-energy* factor, and a *high-fiber* factor. While neither factor was associated with ADHD diagnosis, the *high-energy* factor was associated with reactive aggression scores ($t = 2.75$, $p_{FDR} = 4.01E-2$). We additionally identified several genera associated with either reactive aggression (e.g. *Eubacterium xylanophilum*, *Lactobacillus*, and *Slackia*) or ADHD diagnosis (e.g. *Eisenbergiella*); neither overlap nor significant mediation effects of the selected genera on the association of reactive aggression with the *high-energy* diet were observed. Our results suggest that diet and the microbiome are linked to reactive aggression and/or ADHD, and highlight the need to further study the way diet and the gut-microbiome interact.

INTRODUCTION

Attention-deficit/hyperactivity disorder (ADHD) is a common neurodevelopmental condition [1]. Symptoms of hyperactivity/impulsivity and inattention persist into adulthood in more than half of the affected individuals, and at least 15% still meet full diagnostic criteria [2]. Up to 70% of adults with persistent ADHD symptoms are affected by emotion regulation problems, such as reactive aggression [2]. Aggressive behavior is a frequent catalyst for diagnostic consultation [3] and has a large impact on social and functional impairment, like dysfunctional relationships, peer rejection, impairments in school/occupation, and higher risk of engaging in criminal behavior or suicidal attempts [4-7]. Little is known about potential mechanisms underlying the co-occurrence of ADHD with reactive aggression. Alterations of immune response, inflammatory processes affecting brain function and development [8, 9], and altered neurotransmission and brain functioning in ADHD might play a role in the development of aggressive behavior. People with ADHD were shown to exhibit altered brain development in regions of emotion regulation [10, 11] and altered brain functioning during emotion processing in relation to elevated reactive aggression [12]. Next to genetic predisposition, these processes are likely influenced by environmental factors [13-15]. Recently, diet and the gut-microbiome have received attention in the research of ADHD and emotional behavior, representing potential targets for prevention and treatment support [16-19].

Multiple studies have reported altered eating behavior in children and adolescents with ADHD compared to neurotypical peers (for a review, see [20]). While symptoms of ADHD, inattention and impulsivity, as well as poor planning skills, may influence food choices and cause difficulties in adhering to a healthy eating pattern [17], diet might influence ADHD symptoms as well. For example, a Western diet, high in energy sources (fats, proteins and sugars), as well as low consumption of nutritious foods (fruits, vegetables, and foods that are rich in fiber, polyunsaturated fatty acids (PUFAs), and minerals), were associated with an increased risk for ADHD symptoms (for a review, see [17]). Furthermore, some dietary interventions could partially ameliorate the symptoms of ADHD by either restricting sugar consumption, imposing additive- and preservative-free, or hypoallergenic diets (for a review see [21]) or adding supplements (e.g. omega-3 PUFAs, minerals like zinc and iron, and multivitamins [20]).

Similar dietary patterns - increased consumption of sweet drinks and foods, and lower consumption of fruits and vegetables - have been associated with (emotional) self-regulation difficulties and negative emotions [22-25]. Studies on the supplementation of vitamins, minerals, and in particular omega-3 PUFAs have shown a reduction in reported incidents and aggressive behavior in imprisoned adults and in children displaying behavioral problems (for a review see [16]). Low omega-3 PUFA plasma-levels in adolescents with ADHD have also been associated with atypical brain functioning during emotion processing, proposing a mechanism in which diet influences the emergence of emotion dysregulation leading to reactive aggression in ADHD [26]. However, research on the role of diet in (reactive) aggression is still underrepresented and fails to integrate the potential role of key mechanisms such as the gut-microbiota [16].

Diet might affect reactive aggression and ADHD either via the enteric nervous system or indirectly by mediating changes in the gut-microbiota [27]. The gut-microbiota can influence brain functioning [28], development, and behavior relevant for ADHD and reactive aggression, by e.g. modulating the synthesis and bioavailability of key neurotransmitters such as dopamine and serotonin [29]), or neuroinflammatory processes. These pathways have been associated with ADHD as well as aggressive behaviors [30]. Studies in children and adolescents with ADHD have reported differences in the gut-microbiota composition and diversity compared to neurotypical individuals and associations of the abundance of specific bacterial genera with symptom severity (for a review, see [18]). Alterations of gut-microbiota in ADHD and their potential effects on biological pathways relevant for reactive aggression suggest shared gut-microbial alterations with reactive aggression.

Despite implications of the gut-microbiota as risk factor for the development of emotion dysregulation in infants [31], associations with affective disorders [32], and aggression in other species (e.g. dogs, rodents, and Drosophila [33-35]), there are no empirical studies in humans investigating this topic (for a review see [15]). However, Carbia and colleagues (2021) proposed a microbiome-neuro-immuno-affective framework, linking the effects of microbial alterations, inflammation, and alcohol consumption to emotional dysregulation through fronto-limbic circuits and induction of addiction [36]. This framework supports the role of alterations of gut-microbiota, inflammation, and dietary effects in emotion dysregulation.

Despite the potential relevance of diet for reactive aggression in ADHD and the narrative overlap between dietary patterns relevant for both behaviors, to our knowledge, no study has investigated the role of diet in reactive aggression and ADHD together, or potential effects of the gut-microbiota on their relationship [16, 19]. In this study, we therefore investigated the *direct* associations between diet and behavior (reactive aggression and ADHD) and the potential *mediator* role of the gut-microbiota in diet-behavior relationships. We aimed to answer the following research questions: 1) Are there unique or shared dietary patterns that are associated with ADHD and reactive aggression? 2) Are there unique or shared patterns of gut-microbiome diversity and composition related to ADHD and to reactive aggression? 3) Do gut-microbiota mediate potential diet-ADHD and/or diet-reactive aggression associations?

METHODS AND MATERIALS

Participants and Experimental Procedure

A total of 83 adults with and 79 without ADHD participated in the IMpACT2-NL study, for a description of the study, see [12]. Participants were recruited from the area of Nijmegen, The Netherlands (2017 – 2020). Exclusion criteria were neurological disorders, psychosis, and/or substance abuse in the last 6 months, current major depression, and psycho-pharmaceutical therapy other than ADHD medication. Participants were recruited for the ADHD group if they had been diagnosed with ADHD by a clinician. To confirm diagnoses and assess the number of previous and current symptoms, we conducted the Diagnostic Interview for Adult ADHD (DIVA 2.0 [37]) in all participants. This questionnaire consists of 2 subscales of 8 inattention symptoms and 8 hyperactivity/impulsivity symptoms. Participants were included in the ADHD group if they scored ≥ 5 symptoms in one subscale and in the control group if they had no previous diagnoses of ADHD, no first-degree family members with ADHD, and < 5 symptoms over both DIVA subscales [38]. Detailed recruitment information can be found in Jakobi et al. (2022) [12]. All participants provided written informed consent before participating in the study. The study was approved by the local medical ethical committee (Central Commission for Human Rights Research (CCMO), NL47721.091.14, protocol 2014-290). Among a battery of neuropsychological tests and questionnaires, all participants completed a short semiquantitative food questionnaire and the Reactive-Proactive Aggression Questionnaire (RPQ) [39]. For a description of these questionnaires and how they were coded, see Supplementary Methods 1. Participants were instructed to collect their fecal samples at home using a validated kit and protocol (OMNIgene•GUT, DNAGenotek, Ottawa, CA) and send them back to our laboratory for gut-microbiota analyses [40]. We excluded participants with missing fecal samples, irritable bowel syndrome (IBS), > 30% missing answers in the relevant online questionnaires, frequent antibiotics usage (frequent, sometimes, rarely or never; 96.4% of participants answered never or rarely, one control participant reported frequent antibiotics usage) resulting in 77 participants with and 76 without ADHD. All analyses were performed in R (version 4.2.1 (R Core Team & Team, 2021)).

This report follows the STORMS guidelines for reporting microbiome research where possible, see Supplementary Table 8 [41].

Statistical Analysis

Dietary patterns

To identify patterns of dietary habits, we applied Exploratory Factor Analysis (EFA) (*psych* package [42]) on the weekly quantities of the food items from the semiquantitative food questionnaire, see Supplementary Methods 1. We used the heterogeneous correlation matrix of questionnaire items to assess the factor loadings, accounting for mixed ordinal categorical and continuous input [39, 40]. Parallel analysis determined the number of factors. Bartlett scores were extracted and mean-centered for each diet factor.

Diet-Behavior associations

To investigate associations of the resulting diet factors with ADHD diagnosis we applied logistic regression due to the bivariate distribution. For associations of diet with reactive aggression, we chose nonparametric rank-based regression (*Rfit* package [43]) across all participants, and corrected for ADHD diagnosis.

Microbiota-Behavior associations

The fecal sample wet-lab procedures, 16S rRNA sequencing of the V4 region, and data preprocessing are described in Supplementary Methods 2. We investigated associations of alpha diversity (e.g. observed number of amplicon-sequence-variants (ASVs), Shannon diversity, and Faith's phylogenic diversity) with reactive aggression using rank-based regression, and with ADHD diagnosis using logistic regression. We applied permanova to test the associations of beta diversity (Aitchison distance on ASV level) with both behaviors (adonis2, *vegan* package [44]). For compositional analyses, the ASV table was aggregated to the genus-level, and counts were center-log-ratio (CLR) transformed. To reduce the number of tests, we further applied randomized Lasso feature selection (*monaLisa* package, 10% selection probability [45]). This method selected genera for subsequent association tests with reactive aggression and ADHD. We investigated associations between CLR-transformed abundances and reactive regression and ADHD diagnosis, with rank-based and logistic regression, respectively. We additionally analyzed abundance-behavior associations with a commonly used differential abundance analysis tool (ANOVA-like Differential Expression (ALDEx2) [46], as converging results across statistical tools are more likely to reflect true effects. For detailed descriptions of the statistical analyses and feature selection, see Supplementary Methods 3.

Mediation analysis of the gut-microbiota on diet and behavior

To investigate mediating effects of the gut-microbiota on significant diet-behavior associations, we first identified genera that were potential mediators (using the *mma* package [47], default p < .1 for correlations with diet and behavior). Then, we applied nonparametric mediation analysis of the diet-behavior relationship (*mediation* package [48], see Figure 1). This function applies quantile regression to assess the direct effect behavior~diet (A) and indirect effects, accounted for by the mediator (diet~mediator (B) * mediator~behavior (C)) and estimates significance of this mediation effect with a bootstrapping procedure [48].

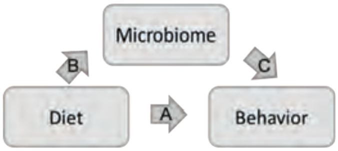


Figure 1. Diagram of potential mediating effects of the gut-microbiota on the relationship between the diet factors and behavioral outcome measures such as ADHD diagnosis and reactive aggression.

All analyses on diet and the gut-microbiota were corrected for age, sex, body mass index (BMI), and current smoking. The significance threshold was set at p<.05 and FDR-adjusted for relevant tests. We investigated associations with reactive aggression across all participants. Due to the case-control design of the study, significant associations with reactive aggression were additionally analyzed for associations with ADHD diagnosis.

RESULTS

Demographic description of the sample

Demographic descriptions of the sample are presented in Table 1. A total of 77 participants with and 76 without ADHD were included. Both groups had comparable distributions of age, sex, and body mass index (BMI), but participants with ADHD more often reported current smoking and showed higher reactive aggression scores compared to neurotypical individuals.

Diet

Dietary patterns

Parallel analysis in the EFA suggested a 3-factor solution (Supplementary Figure 2). *Factor1* was characterized by high consumption of alcohol and meat and low consumption of sweetened beverages and chocolate – we describe this factor as *high-alcohol*; *Factor2* was defined by a high consumption of sweetened beverages, milk, and meat and low consumption of vegetables – resembling a *high-energy*diet similar to a Western diet; *Factor3* showed high consumption of legumes, fruits, and vegetables and a low consumption of meat, milk, and chocolate – describing a plant-based *high-fiber*diet (RMSE = 0.06, corrected for degrees of freedom). The factors were not correlated, see Supplementary Table 1 for further information. Figure 2 shows the loadings of each food item on the three factors.

Table 1. Demographic description of the sample

Measure	ADHD	No ADHD	*p*-value
N	77	76	
Age (SD)	34.09 (10.37)	34.43 (12.9)	8.43 E-01
Sex, % male	42.86%	47.82%	6.27 E-01
BMI (SD)	24.90 (4.51)	24.82 (4.1)	9.71 E-01
Smoking,% current non-smokers	70.13%	90.91%	5.56 E-04
Stimulant medication, % current users	57.14%	0%	n.a.
Reactive aggression scores (SD)	8.23 (4.04)	5.64 (3.2)	1.73 E-05
Number of inattentive symptoms DIVA (SD)	7.34 (1.90)	0.83 (1.2)	2.20 E-16
Number of hyperactive/impulsive symptoms DIVA (SD)	5.62 (2.22)	0.81 (1.1)	2.20 E-16

Demographic description of the sample including mean and standard deviation of age, BMI, mean centered diet scores, and reactive aggression scores as well as percentage of current stimulant users, current non-smokers and male participants. Antibiotic and probiotic usage was assessed in the scale of often, sometimes, rarely, or never: no participant used antibiotics frequently, 7 participants used probiotics frequently. Statistical testing was performed using the Mann Whitney test as well as the Chi-squared test for distribution free comparisons of independent samples. SD=standard deviation, n.a.=not applicable.

Diet-Behavior associations

Diet and reactive aggression

We found reactive aggression scores to be associated with the ***high-energy*** diet Factor2; ADHD diagnosis and male sex were of relevance for this relationship, see Table 2 (top).

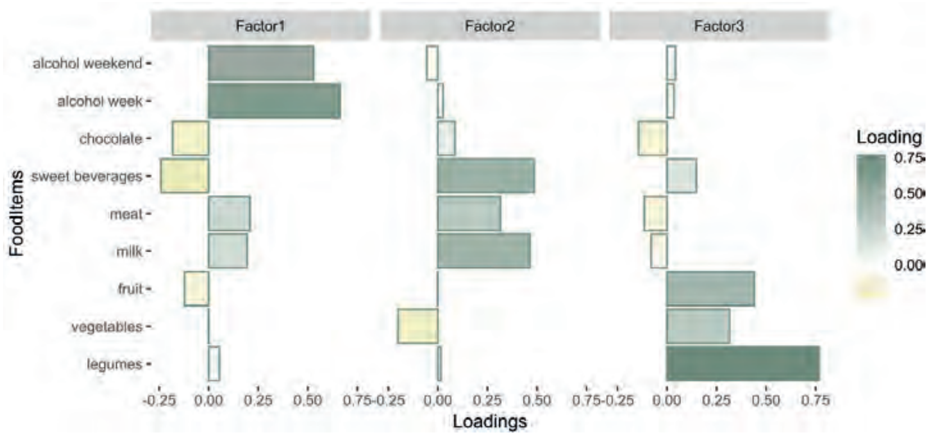


Figure 2. Factor loadings of the food items for the three diet factors suggested by EFA. Factor1, high-alcohol (left); Factor2, high-energy (middle); Factor3, high-fiber (right).

Diet and ADHD diagnosis

None of the dietary factors were associated with ADHD diagnosis. Confounders age, sex, and BMI did not affect the outcome, while current smoking showed a positive association with ADHD diagnosis, see Table 2 (bottom).

Microbiota

Microbiota-Behavior associations

Alpha diversity was not significantly associated with reactive aggression or ADHD diagnosis. Current smoking was not a relevant predictor for reactive aggression, but age, sex, and BMI did show effects in both models, see Supplementary Results 2.1. Beta diversity was associated with ADHD diagnosis ($F = 1.24$, $R^2 = 0.008$, $p = 2.9E-2$) but not with reactive aggression, see Supplementary Results 2.2.

Table 2. Diet-behavior associations

	Standard Estimate	Estimate	Std. Error	z-value/ t-value	*p*-value	*p*-value FDR
Reactive Aggression ~ Factor1 + Factor2 + Factor3 + age + sex + BMI + smoking + ADHD diagnosis						
Factor1	0.37	0.16	0.22	0.74	4.6E-01	1
Factor2	0.82	0.51	0.19	2.73	7.0E-03	4.22E-02
Factor3	0.30	0.12	0.21	0.60	5.5E-01	1
Age	0.06	0.00	0.02	0.11	9.1E-01	NA
Sex	-0.76	-2.03	0.55	-3.72	2.8E-04	NA
BMI	0.33	0.04	0.06	0.67	5.0E-01	NA
Smoke	0.07	0.05	0.40	0.13	8.9E-01	NA
ADHD diagnosis	0.75	2.22	0.55	4.04	8.4E-05	5.85E-04
ADHD Diagnosis ~ Factor1 + Factor2 + Factor3 + age + sex + BMI + smoking						
Factor1	-0.26	-0.11	0.15	-0.73	4.7E-01	1
Factor2	-0.01	0.00	0.12	-0.03	9.7E-01	1
Factor3	0.02	0.01	0.13	0.06	9.5E-01	1
Age	0.02	0.00	0.01	0.04	9.7E-01	NA
Sex	0.36	0.36	0.36	1.01	3.2E-01	NA
BMI	0.02	0.00	0.04	0.05	9.6E-01	NA
Smoke	1.13	0.87	0.27	3.21	1.6E-03	NA

Results from the logistic regression analysis of dietary factors with ADHD diagnosis (bottom), and the rank-based regression with reactive aggression (top), corrected for age, sex, BMI, current smoking, and ADHD diagnosis, showing standardized regression estimates, estimates, standard errors, z-statistic (for the nonparametric regression t-statistic), and p-values. FDR correction was applied to p-values of associations of the dietary factors with ADHD diagnosis or reactive aggression. Significant results of interest at an FDR-corrected threshold of $p< .05$ are highlighted in grey shading.

Our feature selection step selected nine genera for reactive aggression and nine genera for ADHD diagnosis, see Supplementary Results 3. The genera selected for reactive aggression scores and ADHD diagnosis did not overlap. Eight out of nine selected genera were significantly associated with reactive aggression in regression analysis, and additional ALDEx2 identified three converging results: ***Eubacterium xylanophilum group, Lactobacillus,*** and ***Slackia,*** see Supplementary Figure 6. *Lactobacillus* (p_{FDR} = 3.9E-02) and *Slackia* (p_{FDR} = 9.0E-03) remained significant after correction for ADHD diagnosis, while *Eubacterium xylanophilum group* did not (p_{FDR} = 1.3E-01), see Supplementary Figure 7 and Supplementary Table 4. All selected genera for ADHD remained significantly associated after FDR correction using logistic regression. Six of these genera (*Tyzzerella, RF39, Sutterella, uncultured 6, Eisenbergiella,* and *Eubacterium fissicatena group)* were additionally identified using ALDEx2, see Supplementary Figure 8 for relative abundance plots. Table 3 summarizes the results of the feature selection and differential abundance analysis.

The gut-microbiota as a mediator of diet and behavior

Two genera, *Eubacterium nodatum group* and *Lachnospiraceae UCG 010,* were identified as potential mediators, based on suggested correlations (p <.1) with both, reactive aggression and the high-energy diet factor (see Supplementary Table 5) [47]. These two genera were not previously tested for association with reactive aggression, as they were not selected in the feature selection step. The genera associated with reactive aggression did not show a correlation with the high-energy diet Factor2. Mediation analyses of the two selected genera on the association of the reactive aggression with the high-energy diet showed no significant mediation effect, see Supplementary Table 6 for a summary of the mediation analysis.

Table 3. Microbiota-behavior associations

Feature selection		**Logistic regression / rank-based regression**					**ALDEx2**				
Genus	Sel. Prob.	Std. Error	Estimate	z	*p*-value	*p*-value FDR	Estimate	Std. Error	t	*p*-value	*p*-value FDR
Reactive Aggression											
Lactobacillus	0.27	0.11	0.28	2.54	1.2E-02	1.7E-02	-0.23	0.1	-2.42	1.9E-02	5.2E-02
Slackia	0.35	0.08	0.24	2.88	4.5E-03	1.4E-02	-0.32	0.13	-2.52	1.7E-02	5.2E-02
Eubacterium xylanophilum group *	0.11	0.12	-0.26	-2.23	2.7E-02	2.9E-02	0.21	0.1	2.23	3.3E-02	5.3E-02
Dialister	0.11	0.07	-0.16	-2.28	2.4E-02	2.9E-02	0.28	0.15	1.95	5.8E-02	9.1E-02
Succiniclasticum	0.19	0.15	0.33	2.25	2.6E-02	2.9E-02	-0.24	0.1	-2.46	4.4E-02	9.9E-02
Allhorhizobium Neorhizobium Pararhizobium Rhizobium	0.26	0.38	1.14	3.03	2.9E-03	1.4E-02	-0.12	0.08	-1.53	2.3E-01	2.5E-01
Murdochiella	0.21	0.35	0.91	2.6	1.0E-02	1.6E-02	-0.11	0.08	-1.51	2.3E-01	2.5E-01
Lachnospiraceae	0.13	0.22	0.51	2.31	2.2E-02	2.9E-02	-0.11	0.09	-1.29	2.7E-01	2.8E-01
Atopobium	0.11	0.26	-0.18	-0.69	4.9E-01	4.9E-01	0.09	0.08	1.16	3.3E-01	3.4E-01
ADHD Diagnosis											
Tyzzerella	0.59	0.06	0.22	3.82	1.3E-04	2.4E-03	-3.6	0.99	-3.65	9.7E-04	9.4E-03
RF39	0.25	0.06	-0.19	-3.39	7.1E-04	6.4E-03	3.13	0.9	3.49	1.2E-03	9.4E-03
Eubacterium fissicatena group	0.11	0.11	0.28	2.55	1.1E-02	1.6E-02	-1.48	0.59	-2.53	2.4E-02	5.0E-02
Sutterella	0.19	0.08	-0.24	-2.85	4.4E-03	1.4E-02	1.75	0.66	2.66	9.3E-03	5.2E-02
uncultured.6	0.15	0.06	-0.18	-2.78	5.4E-03	1.4E-02	2.3	0.9	2.57	2.1E-02	5.2E-02
Eisenbergiella	0.12	0.09	0.25	2.77	5.6E-03	1.4E-02	-1.79	0.67	-2.67	1.6E-02	5.2E-02
Ruminiclostridium	0.15	0.11	0.3	2.63	8.7E-03	1.6E-02	-1.47	0.71	-2.07	9.3E-02	1.1E-01
Caulobacter	0.11	0.16	0.43	2.64	8.3E-03	1.6E-02	-0.97	0.56	-1.74	1.4E-01	1.5E-01
Sanguibacteroides	0.16	0.15	-0.4	-2.58	9.8E-03	1.6E-02	1.25	0.65	1.93	1.3E-01	2.1E-01

Table 3 summarizes the results of the feature selection, namely selected genera and selection probability, and the subsequent differential abundance analyses of ADHD Diagnosis (top) and reactive aggression (bottom) with two statistical approaches, logistic regression (left) and ALDEx2(right), Estimate, standard error, z/t value, raw and FDR adjusted p value corrected for all tests (18 for logistic regression, 36 when repeating the analyses in ALDEx2). Convergent results, significant in both and FDR-corrected in at least one method are highlighted. * Eubacterium xylanophilum group did not remain significant after correction for ADHD diagnosis (p_{FDR} = 1.3E-01).

DISCUSSION

To our knowledge, this is the first study investigating the role of diet and the gut-microbiome in reactive aggression, together with potential mediating effects of the gut-microbiota on the relationships between diet, ADHD and reactive aggression. We found a positive association of a high-energy dietary pattern with reactive aggression, and we observed gut-microbial alterations in reactive aggression and ADHD. No mediation effects were seen.

Diet

We identified three dietary factors in our study population, resembling an high-alcohol, a high-energy, and a high-fiber dietary pattern. Our results resemble the dietary patterns from Shi and colleagues (2022) [49], who used a similar questionnaire in a healthy population sample. ADHD diagnosis was not associated with any of these dietary patterns, suggesting a generally similar diet in adults with and without ADHD. Other factors, for example the gut-microbiota that could result in different bioavailability of nutrients between individuals with and without ADHD, have to be investigated. While recent meta analyses on studies in children had reported an unhealthier "Western diet" with high caloric and low nutritional food intake [50], the few published studies on adults had inconsistent results [51-53].

Reactive aggression was positively associated with the *"high-energy"* diet factor. Despite the lack of research on the relationship of reactive aggression with diet, this finding matches the literature on (emotion) regulation and negative affect [22, 23]. Western diet, and sweetened foods and beverages in particular, have been associated with (chronic) pro-inflammatory processes (for a review see [54]), which might be relevant for altered brain functioning [55]and reactive aggression [16]. Interventions reducing sweetened foods/drinks and improving the nutritional value by introducing more vegetables could be particularly beneficial for reactive aggression.

Gut-microbiota

The associations of gut-microbiota with ADHD diagnosis are largely similar to our recent meta-analysis on this topic (N_{total}= 617, Jakobi et al. (2023) [56]), in which the IMpACT2-NL cohort was included: While alpha diversity was not associated with reactive aggression, beta diversity showed significant differences between adults with and without ADHD. Higher abundance of *Eisenbergiella*, observed in adults with ADHD compared to neurotypical peers, was also seen in our recent meta-analysis. *Eisenbergiella* has been previously associated with pro-inflammatory processes and immune activation [57-59] and was shown to be enriched in psychiatric [60, 61] and metabolic disorders such as gestational diabetes mellitus [62]. This associations might suggest a pathogenic role. Alternatively, the higher abundance could be a consequence/epiphenomenon of disadvantageous health outcomes.

Reactive aggression was neither associated with alpha nor beta diversity in this study. However, we identified several genera associated with reactive aggression. *Slackia* and *Lactobacillus* were associated with reactive aggression scores across participants and *Eubacterium xylanophilum group* with lower scores. Studies reporting, e.g. effects of probiotic intervention including Lactobacillus on cognition and brain functioning during emotion processing [63, 64], and enriched Slackia abundance in association with low female sex-hormone levels [65] support the potential involvement of these genera in biological pathways relevant for reactive aggression, but these results have to be replicated.

In this study we found no overlap in selected genera for ADHD diagnosis and reactive aggression. However, lower abundance of *Eubacterium xylanophilum group* - associated with high reactive aggression scores - was also associated with ADHD diagnosis in our meta-analysis [56]; a small effect size may have led to the negative result in the current study. *Eubacterium xylanophilum group* is a producer of short-chain-fatty acids (SCFAs) [66] from polyphenols (nutritional compounds of plant-based food items such as nuts, legumes, vegetables, and fruits) [67, 68] and shows reduced abundance in women with gestational diabetes [62]. This bacterial genus may therefore have beneficial effects on immune functioning and inflammation [69].

Mediation

We identified two genera as potential mediators of the association between the "high-energy" diet factor and reactive aggression, *Lachnospiraceae UCG 010* and *Eubacterium nodatum group*. Both genera tended to be less abundant in individuals with higher reactive aggression scores and less abundant in relation to higher scores on the **high-energy** diet (low in vegetables). Indeed, these taxa have been shown to be enriched in plant-based diets [66, 70, 71]. *Eubacterium nodatum group* is involved in inflammatory processes in different ways (SCFA producer [66], pathogen in oral infections [72], and increased *Lachnospiraceae UCG 010* abundance was associated with lower cholesterol and alterations in tryptophane metabolism after cholesterol intervention using grape powder in healthy individuals [73]. While general health benefits suggest that these genera might mediate positive effects of diet on behavior, causal mediation analysis showed no significant mediation effects. This could result from taxonomic clustering; at the genus-level, several species and strains with various functional properties can be clustered together. Higher functional resolution (e.g. achieved by metagenomic sequencing to the species level, or clustering of sequences for functional pathways) could provide more meaningful associations and a more powerful way to investigate this question. Other than influencing the microbiome, diet might also affect behavior by directly acting on the enteric nervous system, which we did not investigate in the current study.

Strengths and Limitations

The current study shows strengths and limitations that should be considered when interpreting the results. This is the first study to investigate diet and the gut-microbiome in reactive aggression and the first one to investigate potential mediations of gut-microbiota on diet - reactive aggression and diet – ADHD associations. While we have highlighted the importance of including diet information in the study of mental health, detailed and reliable nutritional data can be difficult to obtain.

As in most nutritional studies, the dietary questionnaire implemented in this study was a self-report measure in which we traded acceptability by the participants of the study with reduced reliability compared to more laborious measures [69]. Our questionnaire had low resolution answer options ("portions", "pieces", "glasses") and concentrated on a limited number of major food groups. Using a selection of food items that could be categorized as generally beneficial/healthy or disadvantageous/unhealthy, we described our dietary factors in terms of nutritional value. The limitations of the self-reports of diet in adults in this and other studies might hamper the detection of consistent effects of diet in adult ADHD (in contrast to studies in children with ADHD [REFs]) and related behaviors. Despite the low resolution of our diet measure, we identified meaningful dietary patterns, replicating the results of other studies with similar questionnaires, and found a significant association of a 'high-energy' diet factor with reactive aggression scores, potentially highlighting the relevance of nutrition for healthy, situation-appropriate behavior.

On the side of strengths, the identification of food items and dietary patterns that ameliorate or aggravate reactive aggression, as provided in this study, and/or symptoms of ADHD are prerequisites for the development of targeted dietary interventions – an affordable treatment support with little side effects, benefitting individuals beyond the behavioral symptoms (e.g., overall health and metabolic comorbidities). While some nutritional intervention studies in ADHD have shown promising results in ADHD treatment support [21, 74], more evidence for treatment success of targeted nutritional interventions is needed [75]. Higher resolution diet data, stemming from standardized food frequency questionnaires (FFQ, e.g. [76]), combined with randomized controlled trials for nutritional intervention studies should be applied to investigate the potential disadvantageous effects of high-energy diets and potential protective roles of food groups, e.g. PUFAs, for the emergence of reactive aggression and adult ADHD. Other confounders of diet and overall health should be considered when studying diet and the gut-microbiome. Socio-economic status, education level, physical exercise and sleep, for example, have been reported to influence dietary choices and overall health [71, 72].

A final set of both strengths and limitations is related to microbiota research. This study is the second-largest currently available study of gut-microbiota - ADHD associations and the only one of gut-microbiota - reactive aggression associations to date. Effect sizes in the zero-inflated data-masses of microbial sequencing data are expectedly small. For robust results, bigger study populations would be beneficial. We applied a threefold strategy to mitigate these limitations: Firstly, we reduced the statistical testing burden of uninformative genera by applying a feature selection step prior to differential abundance analysis; Secondly we corrected all analyses for common confounders; thirdly, we performed analyses using several statistical tools (logistic regression, ALDEx2) and indices (observed ASVs, Shannon index, Faith's phylogenic diversity) to identify converging results.

Conclusion

In the current study, we showed that diet and the gut-microbiota play a role in reactive aggression and adult ADHD. If replicated, these results could help identify targets for nutritional interventions or microbiota targeted pre-/probiotics as treatment support for reactive aggression, especially in the context of adult ADHD. While inflammatory processes might play a role in both reactive aggression and ADHD, the mechanisms at play in the interaction of diet, the gut-microbiota, and these behaviors deserve more investigation. To do so, large studies with detailed dietary phenotyping are needed to robustly identify dietary and microbial signatures of ADHD or ADHD-related behaviors. In addition, the use of metagenomic sequencing, pathway analysis and clustering based on functional capacities instead of phylogenetic relationship, as well as microbial culturing and basic research into gastrointestinal environments are needed to characterize the mechanisms involved.

Author Contributions

B.J. first author: conceptualization, methodology, analysis, data curation, visualization, writing—original draft. C.C.: formal analysis, writing—review and editing. D.M.: methodology, writing—review and editing. P.V.: project administration, methodology, writing—review and editing. B.F.: supervision, funding acquisition, writing—review and editing. M.H.: supervision, funding acquisition, writing—review and editing. A.A.-V.: conceptualization, methodology, writing—review and editing, supervision, funding acquisition. All authors have read and agreed to the published version of the manuscript.

Funding

We acknowledge funding from the Netherlands Organization for Scientific Research (NWO), i.e., the Veni Innovation Program (grant 016-196-115 to M.H.) and the Vici Innovation Program (grant 016–130-669 to B.F.). The work was also supported by the European College of Neuropsychopharmacology (ECNP) Network "ADHD Across the Lifespan". B.J. and B.F. were also supported by funding from the European Community's Horizon 2020 Programme (H2020/2014–2020) under grant agreement n° 847879 (PRIME). Concurrently, the research contributing to these results was also supported by funding from the European Community's Horizon 2020 research and innovation program, specifically through the Eat2beNICE project (grant agreement no. 728018), the CANDY project (grant agreement no. 847818) and the DISCOvERIE project (grant agreement no. 848228).

REFERENCES

1. Faraone, S., et al., *482 ADHD*. Nature Reviews Disease Primers, 2015. **1**: p. 15027.
2. Shaw, P., et al., *Emotion dysregulation in attention deficit hyperactivity disorder*. Focus, 2016. **14**(1): p. 127-144.
3. King, S. and D.A. Waschbusch, *Aggression in children with attention-deficit/hyperactivity disorder*. Expert Review of Neurotherapeutics, 2010. **10**(10): p. 1581-1594.
4. Abel, M.R., et al., *Reactive aggression and suicidal behaviors in children receiving outpatient psychological services: the moderating role of hyperactivity and inattention*. Child Psychiatry & Human Development, 2020. **51**: p. 2-12.
5. Slaughter, K.E., et al., *Reactive and proactive aggression in children with and without ADHD and negative emotional lability*. Social Development, 2020. **29**(1): p. 320-338.
6. Evans, S.C., et al., *The role of reactive aggression in the link between hyperactive–impulsive behaviors and peer rejection in adolescents*. Child Psychiatry & Human Development, 2015. **46**: p. 903-912.
7. Martel, M.M., *Research review: A new perspective on attention-deficit/hyperactivity disorder: Emotion dysregulation and trait models*. Journal of Child Psychology and Psychiatry, 2009. **50**(9): p. 1042-1051.
8. Lenz, K.M. and L.H. Nelson, *Microglia and beyond: innate immune cells as regulators of brain development and behavioral function*. Frontiers in immunology, 2018. **9**: p. 698.
9. Dantzer, R., et al., *From inflammation to sickness and depression: when the immune system subjugates the brain*. Nature reviews neuroscience, 2008. **9**(1): p. 46-56.
10. Hoogman, M., et al., *Subcortical brain volume differences in participants with attention deficit hyperactivity disorder in children and adults: a cross-sectional mega-analysis*. The Lancet Psychiatry, 2017. **4**(4): p. 310-319.
11. Hoogman, M., et al., *Brain imaging of the cortex in ADHD: a coordinated analysis of large-scale clinical and population-based samples*. American Journal of Psychiatry, 2019. **176**(7): p. 531-542.
12. Jakobi, B., et al., *Neural Correlates of Reactive Aggression in Adult ADHD*. Frontiers in psychiatry, 2022: p. 1019.
13. Tylee, D.S., et al., *Genetic correlations among psychiatric and immune-related phenotypes based on genome-wide association data*. American Journal of Medical Genetics Part B: Neuropsychiatric Genetics, 2018. **177**(7): p. 641-657.
14. Seroczynski, A.D., C. Bergeman, and E.F. Coccaro, *Etiology of the impulsivity/aggression relationship: genes or environment?* Psychiatry research, 1999. **86**(1): p. 41-57.
15. Kim, J.H., et al., *Environmental risk factors, protective factors, and peripheral biomarkers for ADHD: an umbrella review*. The Lancet Psychiatry, 2020. **7**(11): p. 955-970.
16. Tcherni-Buzzeo, M., *Dietary interventions, the gut microbiome, and aggressive behavior: Review of research evidence and potential next steps*. Aggressive behavior, 2023. **49**(1): p. 15-32.
17. Pinto, S., et al., *Eating Patterns and Dietary Interventions in ADHD: A Narrative Review*. Nutrients, 2022. **14**(20): p. 4332.
18. Gkougka, D., et al., *Gut microbiome and attention deficit/hyperactivity disorder: a systematic review*. Pediatric Research, 2022: p. 1-13.
19. Langmajerová, M., et al., *The effect of microbiome composition on impulsive and violent behavior: a systematic review*. Behavioural brain research, 2022: p. 114266.

20. Del-Ponte, B., et al., *Dietary patterns and attention deficit/hyperactivity disorder (ADHD): a systematic review and meta-analysis*. Journal of affective disorders, 2019. **252**: p. 160-173.
21. Uldall Torp, N.M. and P.H. Thomsen, *The use of diet interventions to treat symptoms of ADHD in children and adolescents–a systematic review of randomized controlled trials*. Nordic Journal of Psychiatry, 2020. **74**(8): p. 558-568.
22. Lipsanen, J., et al., *Temperament profiles are associated with dietary behavior from childhood to adulthood*. Appetite, 2020. **151**: p. 104681.
23. Riggs, N.R., et al., *Executive cognitive function and food intake in children*. Journal of nutrition education and behavior, 2010. **42**(6): p. 398-403.
24. Vollrath, M.E., et al., *Associations between temperament at age 1.5 years and obesogenic diet at ages 3 and 7 years*. Journal of developmental and behavioral pediatrics: JDBP, 2012. **33**(9): p. 721.
25. Holt, M., *Association of Dietary Intake Patterns with Emotion Regulation*. 2013.
26. Gow, R.V., et al., *Omega-3 fatty acids are related to abnormal emotion processing in adolescent boys with attention deficit hyperactivity disorder*. Prostaglandins, Leukotrienes and Essential Fatty Acids, 2013. **88**(6): p. 419-429.
27. Sarkar, A., et al., *The role of the microbiome in the neurobiology of social behaviour*. Biological Reviews, 2020. **95**(5): p. 1131-1166.
28. Mulder, D., et al., *A systematic review exploring the association between the human gut microbiota and brain connectivity in health and disease*. Molecular Psychiatry, 2023: p. 1-25.
29. Strandwitz, P., *Neurotransmitter modulation by the gut microbiota*. Brain research, 2018. **1693**: p. 128-133.
30. Dam, S.A., et al., *The role of the gut-brain axis in attention-deficit/hyperactivity disorder*. Gastroenterology Clinics, 2019. **48**(3): p. 407-431.
31. Fox, M., et al., *Development of the infant gut microbiome predicts temperament across the first year of life*. Development and psychopathology, 2022. **34**(5): p. 1914-1925.
32. Capuco, A., et al., *Gut microbiome dysbiosis and depression: A comprehensive review*. Current pain and headache reports, 2020. **24**: p. 1-14.
33. Kirchoff, N.S., M.A. Udell, and T.J. Sharpton, *The gut microbiome correlates with conspecific aggression in a small population of rescued dogs (Canis familiaris)*. PeerJ, 2019. **7**: p. e6103.
34. Jia, Y., et al., *Gut microbiome modulates Drosophila aggression through octopamine signaling*. Nature communications, 2021. **12**(1): p. 2698.
35. Ren, C.C., et al., *Photoperiod modulates the gut microbiome and aggressive behavior in Siberian hamsters*. Journal of Experimental Biology, 2020. **223**(3): p. jeb212548.
36. Carbia, C., et al., *A biological framework for emotional dysregulation in alcohol misuse: from gut to brain*. Molecular psychiatry, 2021. **26**(4): p. 1098-1118.
37. Kooij, J. and M. Francken, *DIVA 2.0.* Diagnostic Interview Voor ADHD in Adults bij volwassenen [DIVA 2 0 Diagnostic Interview ADHD in Adults]. DIVA Foundation (http://www. divacenter. eu/ DIVA. aspx), 2010.
38. Kooij, J., et al., *Updated European Consensus Statement on diagnosis and treatment of adult ADHD.* European psychiatry, 2019. **56**(1): p. 14-34.

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39. Raine, A., et al., *The reactive–proactive aggression questionnaire: Differential correlates of reactive and proactive aggression in adolescent boys.* Aggressive Behavior: Official Journal of the International Society for Research on Aggression, 2006. **32**(2): p. 159-171.
40. Bloemendaal, M., et al., *The role of the gut microbiota in patients with Kleefstra syndrome.* American Journal of Medical Genetics Part B: Neuropsychiatric Genetics, 2023.
41. Mirzayi, C., et al., *Strengthening the Organization and Reporting of Microbiome Studies (STORMS): a reporting checklist for human microbiome research.* BioRxiv, 2020: p. 2020.06. 24.167353.
42. Revelle, W.R., *psych: Procedures for personality and psychological research.* 2017.
43. Kloke, J.D. and J.W. McKean, *Rfit: rank-based estimation for linear models.* R J., 2012. **4**(2): p. 57.
44. Oksanen, J., et al., *The vegan package.* Community ecology package, 2007. **10**(631-637): p. 719.
45. Meinshausen, N. and P. Bühlmann, *Stability selection.* Journal of the Royal Statistical Society: Series B (Statistical Methodology), 2010. **72**(4): p. 417-473.
46. Gloor, G., *ALDEx2: ANOVA-Like Differential Expression tool for compositional data.* ALDEX manual modular, 2015. **20**: p. 1-11.
47. Yu, Q. and B. Li, *mma: an R package for mediation analysis with multiple mediators.* Journal of Open Research Software, 2017. **5**(1).
48. Tingley, D., et al., *Mediation: R package for causal mediation analysis.* 2014.
49. Shi, H., et al., *The gut microbiome as mediator between diet and its impact on immune function.* Scientific Reports, 2022. **12**(1): p. 5149.
50. Shareghfarid, E., et al., *Empirically derived dietary patterns and food groups intake in relation with Attention Deficit/Hyperactivity Disorder (ADHD): A systematic review and meta-analysis.* Clinical nutrition ESPEN, 2020. **36**: p. 28-35.
51. Holton, K.F., et al., *Evaluation of dietary intake in children and college students with and without attention-deficit/hyperactivity disorder.* Nutritional neuroscience, 2019. **22**(9): p. 664-677.
52. Li, L., et al., *Attention-deficit/hyperactivity disorder symptoms and dietary habits in adulthood: A large population-based twin study in Sweden.* American Journal of Medical Genetics Part B: Neuropsychiatric Genetics, 2020. **183**(8): p. 475-485.
53. Weissenberger, S., et al., *ADHD and lifestyle habits in Czech adults, a national sample.* Neuropsychiatric disease and treatment, 2018: p. 293-299.
54. Della Corte, K.W., et al., *Effect of dietary sugar intake on biomarkers of subclinical inflammation: a systematic review and meta-analysis of intervention studies.* Nutrients, 2018. **10**(5): p. 606.
55. Wärnberg, J., et al., *Nutrition, inflammation, and cognitive function.* Annals of the New York Academy of Sciences, 2009. **1153**(1): p. 164-175.
56. Jakobi, B., et al., *The gut-microbiome in adult Attention-deficit/hyperactivity disorder-A Meta-analysis.* medRxiv, 2023: p. 2023.12. 18.23300126.
57. Bao, J., et al., *Echinococcus granulosus infection results in an increase in Eisenbergiella and Parabacteroides genera in the gut of mice.* Frontiers in microbiology, 2018. **9**: p. 2890.
58. Zhang, Y., et al., *Dietary resistant starch modifies the composition and function of caecal microbiota of broilers.* Journal of the Science of Food and Agriculture, 2020. **100**(3): p. 1274-1284.
59. Zhang, J., et al., *Integrative Analysis of Gut Microbiota and Fecal Metabolites in Rats after Prednisone Treatment.* Microbiology Spectrum, 2021. **9**(3): p. e00650-21.
60. Ye, F., et al., *Comparison of gut microbiota in autism spectrum disorders and neurotypical boys in China: A case-control study.* Synthetic and Systems Biotechnology, 2021. **6**(2): p. 120-126.

61. Wan, X., et al., *Gut–microbiota–brain axis in the vulnerability to psychosis in adulthood after repeated cannabis exposure during adolescence.* European Archives of Psychiatry and Clinical Neuroscience, 2022. **272**(7): p. 1297-1309.

62. Ma, S., et al., *Alterations in gut microbiota of gestational diabetes patients during the first trimester of pregnancy.* Frontiers in cellular and infection microbiology, 2020. **10**: p. 58.

63. Turroni, F., et al., *Molecular dialogue between the human gut microbiota and the host: a Lactobacillus and Bifidobacterium perspective.* Cellular and Molecular Life Sciences, 2014. **71**: p. 183-203.

64. Rode, J., et al., *Probiotic mixture containing lactobacillus helveticus, Bifidobacterium longum and Lactiplantibacillus plantarum affects brain responses toward an emotional task in healthy subjects: a randomized clinical trial.* Frontiers in nutrition, 2022. **9**: p. 865.

65. Shin, J.-H., et al., *Serum level of sex steroid hormone is associated with diversity and profiles of human gut microbiome.* Research in microbiology, 2019. **170**(4-5): p. 192-201.

66. Wade, W.G., *The genus Eubacterium and related genera.* Prokaryotes, 2006. **4**: p. 823-835.

67. Hu, R., et al., *Dietary ferulic acid and vanillic acid on inflammation, gut barrier function and growth performance in lipopolysaccharide-challenged piglets.* Animal Nutrition, 2022. **8**: p. 144-152.

68. Diotallevi, C., et al., *Healthy dietary patterns to reduce obesity-related metabolic disease: polyphenol-microbiome interactions unifying health effects across geography.* Current Opinion in Clinical Nutrition & Metabolic Care, 2020. **23**(6): p. 437-444.

69. Ma, X., et al., *Sodium butyrate modulates gut microbiota and immune response in colorectal cancer liver metastatic mice.* Cell Biology and Toxicology, 2020. **36**: p. 509-515.

70. Calderón-Pérez, L., et al., *Interplay between dietary phenolic compound intake and the human gut microbiome in hypertension: A cross-sectional study.* Food chemistry, 2021. **344**: p. 128567.

71. Calvete-Torre, I., et al., *Prebiotic potential of apple pomace and pectins from different apple varieties: Modulatory effects on key target commensal microbial populations.* Food Hydrocolloids, 2022. **133**: p. 107958.

72. Holdeman, L.V., et al., *Descriptions of Eubacterium timidum sp. nov., Eubacterium brachy sp. nov., and Eubacterium nodatum sp. nov. isolated from human periodontitis.* International Journal of Systematic and Evolutionary Microbiology, 1980. **30**(1): p. 163-169.

73. Yang, J., et al., *Effect of standardized grape powder consumption on the gut microbiome of healthy subjects: a pilot study.* Nutrients, 2021. **13**(11): p. 3965.

74. Ly, V., et al., *Elimination diets' efficacy and mechanisms in attention deficit hyperactivity disorder and autism spectrum disorder.* European child & adolescent psychiatry, 2017. **26**: p. 1067-1079.

75. Breda, V., et al., *Is there a place for dietetic interventions in adult ADHD?* Progress in Neuro-Psychopharmacology and Biological Psychiatry, 2022: p. 110613.

76. Molag, M., *Towards transparent development of food frequency questionnaires: scientific basis of the Dutch FFQ-TOOL tm: a computer system to generate, apply and process FFQs*. 2010: Wageningen University and Research.

77. Callahan, B.J., et al., *DADA2: High-resolution sample inference from Illumina amplicon data.* Nature methods, 2016. **13**(7): p. 581-583.

78. Gloor, G.B., et al., *Microbiome datasets are compositional: and this is not optional.* Frontiers in microbiology, 2017. **8**: p. 2224.

79. Nearing, J.T., et al., *Microbiome differential abundance methods produce different results across 38 datasets.* Nature Communications, 2022. **13**(1): p. 342.

80. Lahti, L. and S. Shetty, *microbiome R package.* 2017.

SUPPLEMENTARY MATERIAL

1 Supplementary Methods

1.1 Description of the questionnaires

The reactive-proactive questionnaire (RPQ) is a 23-item self-report questionnaire inquiring 11 example sentences of reactive and 12 of proactive aggression [39]. The questions are rated by the participants based on how often they have experienced the situation/feeling or acted in the way that the example sentence describes in a scale of 0 (never), 1 (sometimes), and 2 (often). For each subscale, the sum of the points over the subscale-relevant questions represent the subscale score. We focus on the reactive aggression questionnaire as this behavior is particularly associated with ADHD and represents a severe form or emotion dysregulation.

The semiquantitative food questionnaire assesses the frequency (never, monthly, weekly, daily) of eight food items and beverages (meat, fruit, vegetables, legumes, chocolate, milk, sugary drinks, alcohol), Supplementary Figure 1 is a copy of this questionnaire in Dutch. Except for meat, the amount per indicated frequency was assessed (in pieces, portions, glasses, or bars) and normalized to weekly quantities.

1.2 Microbiota preprocessing

1.2.1 Fecal sample processing

The fecal samples were received in our laboratory, aliquoted into 1.5 ml Eppendorf tubes, and stored in -80˚C. Further processing was done by Baseclear B.V by aliquoting 150 mg feces, isolating and purifying DNA using a bead-beating procedure with the ZymoBIOMICS DNA 96 MagBead kit in conjunction with Kingfisher. The V4 region of 16S ribosomal RNA (rRNA) gene was targeted for sequencing. Amplicons were built using the primers 515-F: TCGTCGGCAGCGTCAGATGTGTATAAGACAGGTGYCAGCMGCCGCGGTAA, 806Rb: GTC TCGTGGGCTCGGAGATGTGTATAAGACAGAGG-GACTACNVGGGTWTCTAAT using the Phusion High-Fidelity PCR Master Mix with an HF Buffer (Thermo Fisher Scientific). The Illumina NovaSeq 6000 platform with the Novaseq 6000 SP reagent kit v1 with 500 cycles (paired-end, 250 bp) was used. Reads were demultiplexed, filtered, and adapter sequences and control signals were removed [56]. The total number of reads was 126.015.919 across all samples (median per sample = 781. 345, range 288.695 – 1.204.337) with a median phred score of Q > 30 for all samples, indicating 99.9% sequencing accuracy.

SECTIE C : VOEDING

In deze sectie vragen wij u om vragen te beantwoorden over **uw eetgewoontes en voedingspatronen.** Al de informatie die wij van u ontvangen zal vertrouwelijk blijven en dusdanig behandeld worden.

* () Bent u vegetariër (geen vlees) of veganist (geen vlees en andere dierlijke producten, zoals kaas, melk, eieren, ...)?

Kies één van de volgende mogelijkheden:

- Nee
- Ja
- Weet ik niet

* () Hoe vaak eet u vlees? Dit mag alle soorten vlees zijn.

Beantwoord deze vraag alleen als aan de volgende voorwaarden is voldaan:
VOE1 != 'Y'

Kies één van de volgende mogelijkheden:

- Dagelijks
- Wekelijks
- Maandelijks
- Weet ik niet

* () Hoe vaak eet u fruit? Dit mag zowel vers fruit, als ingevroren, als in blik zijn.

Kies één van de volgende mogelijkheden:

- Dagelijks
- Wekelijks
- Maandelijks
- Nooit
- Weet ik niet

* () Hoe vaak eet u bonen, erwten, kolen (bloemkool, savooikool, witte kool, ...), broccoli en andere vezelbevattende groenten?

Kies één van de volgende mogelijkheden:

- Dagelijks
- Wekelijks
- Maandelijks
- Nooit
- Weet ik niet

* () Aantal porties [per aangegeven tijdseenheid]:

Beantwoord deze vraag alleen als aan de volgende voorwaarden is voldaan:
VOE5 == '0' || VOE5 == 1 || VOE5 == 2

In dit veld mogen alleen cijfers ingevoerd worden.

Vul uw antwoord hier in:

* () Hoe vaak gebruikt u suikerbevattende dranken? (koud: Coca Cola, Sprite, Fanta, Ice-tea, Nestea, energiedrankjes zoals Red Bull, ...) (warm: cappuccino, koffie met suiker, lattes, ...)

Kies één van de volgende mogelijkheden:

- Dagelijks
- Wekelijks
- Maandelijks
- Nooit
- Weet ik niet

* () Hoeveel chocolade eet u per maand?

Kies één van de volgende mogelijkheden:

- Ik eet geen chocolade
- 0 -1 reep (a 200 gram)
- 1 tot 5 repen (a 200 gram)
- 5 - 10 repen (a 200 gram)
- Meer dan 10 repen (a 200 gram)
- Weet ik niet

* () Is dit voornamelijk:

Beantwoord deze vraag alleen als aan de volgende voorwaarden is voldaan:
VOE9 > 0

Kies één van de volgende mogelijkheden:

- Puur
- Melk
- Wit

* () Aantal stuks:

Beantwoord deze vraag alleen als aan de volgende voorwaarden is voldaan:
VOE3 == '0' || VOE3 == 1 || VOE3 == 2

In dit veld mogen alleen cijfers ingevoerd worden.

Vul uw antwoord hier in:

* () Hoe vaak eet u groenten?

Kies één van de volgende mogelijkheden:

- Dagelijks
- Wekelijks
- Maandelijks
- Nooit
- Weet ik niet

* () Aantal porties [per aangegeven tijdseenheid]:

Beantwoord deze vraag alleen als aan de volgende voorwaarden is voldaan:
VOE4 == '0' || VOE4 == 1 || VOE4 == 2

In dit veld mogen alleen cijfers ingevoerd worden.

Vul uw antwoord hier in:

* () Aantal glazen/koppen:

Beantwoord deze vraag alleen als aan de volgende voorwaarden is voldaan:
VOE6 == '0' || VOE6 == 1 || VOE6 == 2

In dit veld mogen alleen cijfers ingevoerd worden.

Vul uw antwoord hier in:

* () Hoeveel alcohol gebruikt u doordeweeks per dag (wijn, bier, ... ; geen sterke dranken)?

Kies één van de volgende mogelijkheden:

- 0 glazen per dag
- 1 - 2 glazen per dag
- 3 - 4 glazen per dag
- 5 -6 glazen per dag
- > 6 glazen per dag
- Weet ik niet

* () Hoeveel alcohol gebruikt u in het weekend per dag (wijn, bier, ... ; geen sterke dranken)?

Kies één van de volgende mogelijkheden:

- 0 glazen per dag
- 1 - 2 glazen per dag
- 3 - 4 glazen per dag
- 5 -6 glazen per dag
- > 6 glazen per dag
- Weet ik niet

* () Hoeveel melk drinkt u per week?

Kies één van de volgende mogelijkheden:

- Geen
- 1 -2 bekers per dag (a 200 ml)
- 2 - 4 bekers per dag (a 200 ml)
- Meer

* () Is deze melk:

Beantwoord deze vraag alleen als aan de volgende voorwaarden is voldaan:
VOE10 > 0

Kies één van de volgende mogelijkheden:

- Gepasteuriseerd
- Lang houdbaar

Supplementary Figure 1. Semiquantitative questionnaire on eating habits, copy of the Dutch original *version.*

1.2.2 Preprocessing of sequencing data

Raw sequences were demultiplexed and denoised in DADA2 (QIIME2, [77]). The resulting 14.408 Amplicon Sequence Variants (ASVs) were classified (naive Bayes classifier pre-trained on the SILVA database 138 for the V3/V4 Region, https://docs.qiime2.org/2022.2/data-resources/). We filtered bacterial DNA (10.428 bacteria over 153 samples) and analyzed alpha and beta diversity on bacterial ASV level. For compositional analysis and the feature selection step in which we determined the taxa to investigate, we aggregated the sequences to the genus level (resulting in 624 genera) and applied a prevalence threshold of 10% - reducing the number of tests and increasing comparability between studies – (resulting in 240 genera) and a center-log-ratio (CLR) transformation to account for bias of compositionality and sequencing [78, 79].

1.3 Microbiota-behavior analyses

1.3.1 Alpha and beta diversity

We assessed three indices of alpha diversity: 1) the number of observed ASVs describes the richness; 2) the Shannon index combines information of the richness of identified taxa as well as the evenness of their distribution within a sample; 3) Faith's phylogenic diversity further integrates the phylogenic relationship between the taxa (*microbiome* package [80]). We applied rank-based regression models [43] to investigate associations of reactive aggression, and we applied logistic regression models to identify associations of ADHD diagnosis with alpha diversity indices. To investigate associations of both behaviors with beta diversity, we applied principal coordinate analysis on the CLR-transformed ASV counts (Aitchison distance) using two separate permanova models (*vegan* package [44]) for ADHD diagnosis and reactive aggression, respectively. All models were corrected for age, sex, BMI, and current smoking.

1.3.2 Feature selection and differential abundance analysis

To reduce the number of tests in compositional analyses, we first applied a feature selection step on the genus level data using random Lasso stability selection (*monaLisa* package, [45]). Randomized Lasso selection is based on stability rather than effect size. In a random subsample of n/2, the respective behavior was regressed against all genera in a lasso-penalized regression, and the procedure was repeated for 999 subsamples (the source code was adapted for logistic regression for the selection based on ADHD diagnosis). The selection probability was calculated as the number of permutations in which a genus was selected (i.e., $\beta \neq 0$) divided by the total number of permutations. Due to the high interindividual variability and a relatively small sample of 153 participants, this permutation on subsamples of zero-inflated data will lead to expectedly small stability and selection probabilities per genus. We plotted the stability paths over all permutations and genera, based on those plots we decided on the lenient selection probability threshold for both behaviors of ≥ 10%, to only test associations of those genera with a stability path deviating from the center of mass (see Supplementary Figure 4). On these genera we performed univariate association tests with the respective outcome measures (logistic regression for ADHD diagnosis and rank-based regression for reactive aggression scores). In addition, we performed differential abundance analysis with ALDEx2, a commonly applied tool that uses CLR transformation and dirichlet sampling prior to statistical testing. We corrected for age, sex, BMI, and current smoking in all models. P-values were FDR-corrected for all employed models over both behavioral outcomes. We further investigated effects of ADHD diagnosis on significant associations with reactive aggression, by adding ADHD diagnosis to the model across all participants and effects of current use of ADHD medication on significant associations with ADHD diagnosis in participants with ADHD only.

2 Supplementary Results

2.1 Diet

2.1.1 Diet Exploratory Factor Analysis

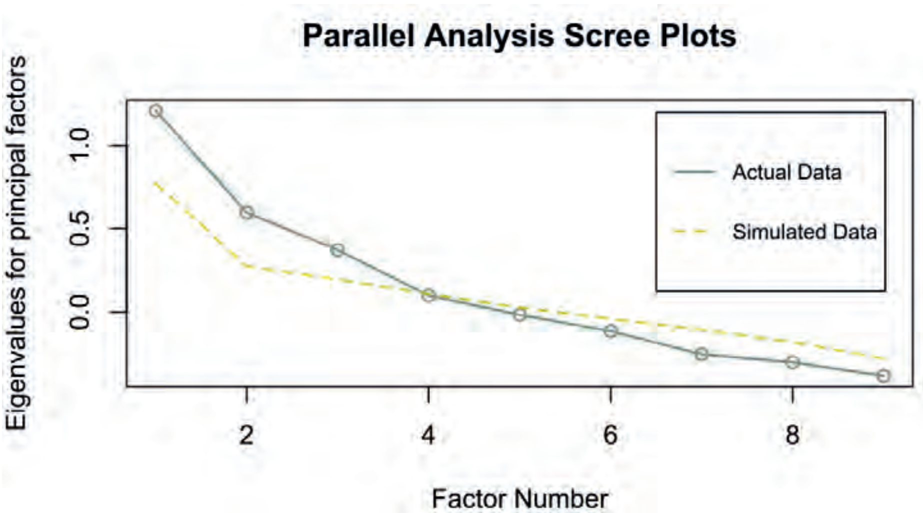

Figure 2. Scree plot, results of the parallel analysis. Actual data explains more variance compared to random simulated data between 1 and 3 factors, suggesting a 3-factor solution.

Table 1. Factor correlations

	Factor1	Factor2	Factor3
Factor1	1	-0.03	0.08
Factor2	-0.03	1	-0.07
Factor3	0.08	-0.07	1

Correlations between the factors resulting from the EFA are low, Factor1 describes a high-alcohol diet, Factor2 describes a high-energy diet, Factor3 describes a high-fiber diet.

2.1.2 Diet - behavior associations

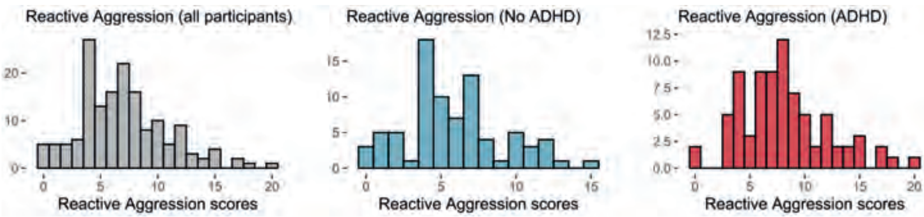

Figure 3. Distribution of reactive aggression scores across participants with and without ADHD.

2.2 Microbiome - behavior associations

2.2.1 Alpha Diversity

Alpha diversity (observed ASVs, Shannon index and Faith's Phylogenic Diversity) was neither associated with reactive aggression, see Supplementary Table 2 (top), nor with ADHD diagnosis, see Supplementary Table 2 (bottom). However, as previously shown, age, sex, and BMI in both models. Current smoking was not associated.

2.2 Beta Diversity

Beta diversity was associated with age and sex in both models, but not with reactive aggression scores. ADHD diagnosis was associated with beta diversity before FDR correction, but the effect did not survive the correction, see Supplementary Table 3 for all results and Supplementary Figure 4 for the supervised Canonical analysis of principal coordinates (CAP) plot of beta diversity for ADHD diagnosis.

Table 2. Associations of alpha diversity with ADHD and reactive aggression

	Observed ASVs				Shannon Diversity				Faiths Phylogenic Diversity			
	Estimate	Std. Error	t-value/ z-value	*p*-value	Estimate	Std. Error	t-value/ z-value	*p*-value	Estimate	Std. Error	t-value/ z-value	*p*-value
Reactive Aggression scores ~ alpha + age + sex + BMI + smoking + ADHD Diagnosis												
Alpha	0.00	0.00	-0.19	6.8E-01	0.02	0.60	0.04	9.7E-01	0.02	0.07	0.32	7.5E-01
Age	0.01	0.02	0.52	5.1E-01	0.01	0.02	0.45	6.5E-01	0.00	0.03	0.35	7.2E-01
Sex	-2.27	0.52	-4.37	5.5E-04	-2.30	0.55	-4.33	4.2E-05	-2.29	0.54	-4.28	3.3E-05
BMI	0.05	0.06	0.89	3.3E-01	0.05	0.06	0.89	3.7E-01	0.06	0.06	0.95	3.4E-01
Smoking	0.19	0.37	0.53	8.1E-02	0.19	0.38	0.52	6.0E-01	0.37	0.38	0.51	6.1E-02
ADHD Diagnosis	2.48	0.52	4.69	6.1E-06	0.53	0.54	4.61	8.5E-06	2.51	0.53	4.66	7.1E-06
ADHD Diagnosis ~ alpha + age + sex + BMI + smoking												
Alpha	0.00	0.00	-1.65	9.8E-02	-0.30	0.39	-0.78	4.4E-01	-0.08	0.04	-1.93	5.3E-02
Age	0.01	0.02	0.80	4.2E-01	0.00	0.02	0.29	7.7E-01	0.01	0.02	0.91	3.6E-01
Sex	0.46	0.35	1.32	1.9E-01	0.44	0.36	1.22	2.2E-01	0.51	0.36	1.44	1.5E-01
BMI	-0.01	0.04	-0.23	8.2E-01	-0.01	0.04	-0.13	9.0E-01	-0.01	0.04	-0.25	8.1E-01
Smoking	0.83	0.26	3.23	1.2E-03	0.82	0.26	3.19	1.4E-03	0.83	0.26	3.24	1.2E-03

Results of the regression analysis of alpha diversity indices with reactive aggression scores (top) and ADHD diagnosis (bottom), corrected for age, sex, BMI, smoking, and ADHD diagnosis, showing estimates, standard errors, t / z -statistic and p-values.

4

Table 3. Associations of beta diversity with ADHD and reactive aggression

	SumOfSqs	R^2	F	*p*-value
Beta ~ Reactive Aggression + age + sex + BMI + smoking + ADHD Diagnosis				
RPQ Reactive Aggression	6367	0.007	1.01	3.8E-01
Age	16853	0.017	2.69	1.0E-03
Sex	9080	0.009	1.45	6.0E-03
BMI	7423	0.008	1.18	7.9E-02
Smoking	7411	0.007	1.14	1.2E-01
ADHD diagnosis	8054	0.008	1.28	2.4E-02
Beta ~ ADHD Diagnosis + age + sex + BMI + smoking				
ADHD	7825472	0.008	1.25	2.9E-02
Age	16914176	0.017	2.69	1.0E-03
Sex	9053954	0.009	1.44	8.0E-03
BMI	7454836	0.008	1.19	6.9E-02
Smoking	7143587	0.007	1.14	1.1E-01

Results of the permanova with 999 permutations of Aitchison distance between samples with and without ADHD (top) and reactive aggression scores (bottom), corrected for age, sex, SMI, smoking, and ADHD medication, where appropriate. We report sums of squares (SumOfSqs), R-squared, F statistic, and p values. Relevant p-values for the effects of interest were FDR-corrected over both models. Significant results are highlighted.

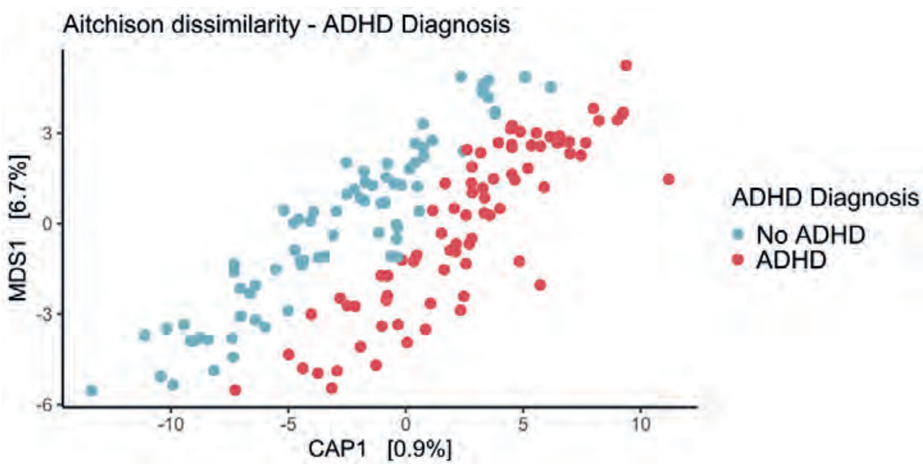

Figure 4. CAP plot supervised for ADHD diagnosis. Individuals without ADHD are marked red. Individuals with ADHD are marked in blue.

2.3 Feature selection and Differential abundance analysis

2.3.1 Feature selection stability path plots behavior

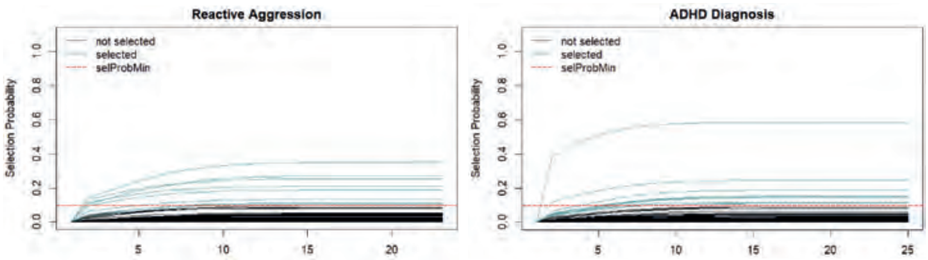

Figure 5. Stability path plots from random Lasso stability selection for ADHD diagnosis (left) and reactive aggression scores (right). The red line marks the chosen selection probability of 10% over both behaviors, selecting genera whose stability path deviated visually from the majority of results close to zero over all outcomes.

2.3.2 Reactive Aggression – Composition: relative abundance plots of significant genera

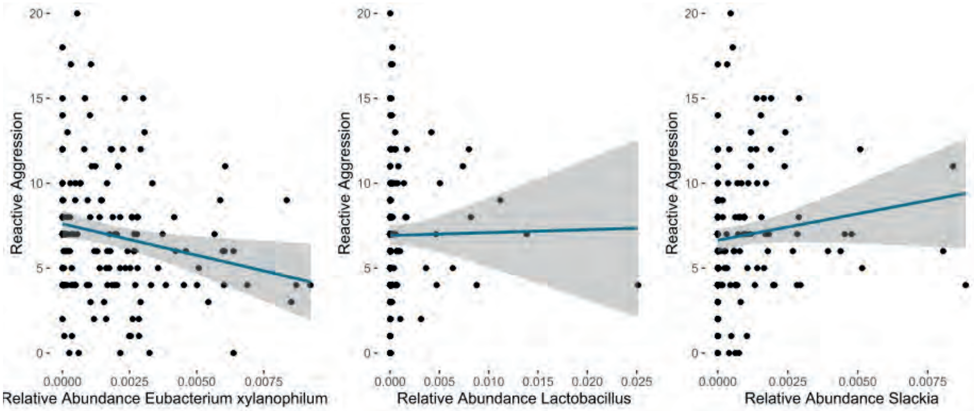

Figure 6. Relative abundance of the genera that were significantly associated with reactive aggression across all participants and Eubacterium xylanophilum group in participants with (red) and without ADHD (blue), plotted by reactive aggression scores.

2.3.3 Reactive Aggression – Composition: correction for ADHD Diagnosis

Table 4. Correction for ADHD Diagnosis

	Estimate	Std. Error	z-value / t-value	*p*-value
Reactive Aggression ~ genus + age + sex + bmi + ADHD Diagnosis				
Lactobacillus	0.23	0.10	2.23	2.7E-02
Slackia	0.23	0.08	2.95	3.6E-03
Eubacterium xylanophilum group	-0.15	0.11	-1.49	1.4E-01
Analysis in individuals with ADHD and without ADHD separately				
Eubacterium xylanophilum group ADHD	-0.09	0.04	-1.87	6.5E-02
Eubacterium xylanophilum group No ADHD	-0.06	0.05	-1.21	2.2E-01

Results of significant associations with reactive aggression, corrected for ADHD diagnosis. Eubacterium xylanophilum group was significantly associated with ADHD Diagnosis, the association with reactive aggression was not significant after correction for ADHD Diagnosis. Within-group analysis shows sub-threshold associations of Eubacterium xylanophilum group with reactive aggression in the ADHD group.

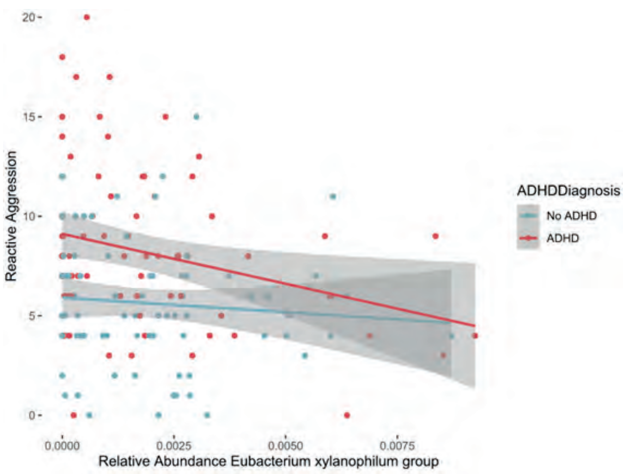

Figure 7. Relative abundance of Eubacterium xylanophilum group in participants with (red) and without ADHD (blue), plotted by reactive aggression scores.

2.3.4 ADHD diagnosis – Composition: relative abundance plots of significant genera

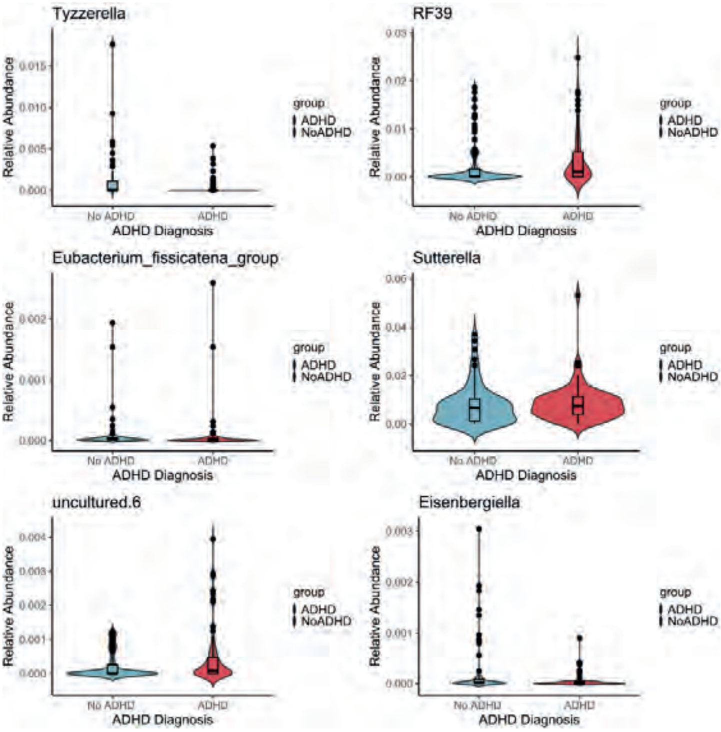

Figure 8. Relative abundance plots of the genera that were significantly different between adults with and without ADHD across logistic regression and ALDEx2.

Table 5. Medication effects in participants with ADHD

	Estimate	Std. Error	z-value	p-value
ADHD medication ~ genus + age + sex + bmi + smoking				
Tyzzerella	-0.02	0.07	-0.32	7.48E-01
Uncultured.6	0.14	0.10	1.38	1.67E-01
Eubacterium fissicatena group	-0.08	0.16	-0.45	6.50E-01
RF39	0.08	0.08	1.03	3.05E-01
Eisenbergiella	0.01	0.11	-0.06	9.47E-01
Sutterella	-0.04	0.08	-0.45	6.50E-01

Associations of the group-different genera with current usage of ADHD medication in participants with ADHD diagnosis only.

2.4 Mediation analysis

Table 6. Selection of potential mediators

	High-energy Diet Factor		***Reactive Aggression***	
Genus	Rho	*p-value*	Rho	*p-value*
Eubacterium nodatum group	-0.15	5.66 E-02	-0.13	9.25 E-02
Lachnospiraceae UCG 010	-0.17	3.65 E-02	-0.16	4.51 E-02

P-values and rho from the identification step as potential mediator. Potential mediators are identified by p< .1 for both, independent variable (high-energy diet factor) and dependent variable (Reactive Aggression) (default setting).

Table 7. Results of the mediation analysis

Mediation	**Estimate**	**95% CI lower**	**95% CI upper**	***p*-value**
Eubacterium_nodatum_group				
ACME	0.07	-0.01	0.15	6.6 E-2
ADE	0.74	0.25	1.15	4.0 E-3
Total effect	0.81	0.34	1.15	4.0 E-3
Proportion Mediated	0.08	-0.02	0.30	NA
Lachnospiraceae_UCG_010				
ACME (average)	0.03	-0.03	0.13	3.3 E-1
ADE (average)	0.84	0.23	1.24	1.2 E-2
Total effect	0.86	0.39	1.28	8.0 E-3
Proportion Mediated	0.034	-0.06	0.22	NA

Results of the mediation analyses of Eubacterium nodatum group (top) and Lachnospiraceae UCG 010 (bottom) on the association of the high-energy diet factor with reactive aggression scores. Estimates, p values, lower and upper boundaries of the confidence intervals for the Average Causal Mediated Effect (ACME), Average Direct Effect (ADE), total effect, and the proportion of the effect that was mediated are presented.

3 STORMS checklist

Table 8. STORMS guidelines for reporting microbiome research

#	Item	Recommendation	Item Source
Abstract			
1.0	Structured or Unstructured Abstract	Abstract should include information on background, methods, results, and conclusions in structured or unstructured format.	STORMS
1.1	Study Design	State study design in abstract.	STORMS
1.2	Sequencing methods	State the strategy used for metagenomic classification.	STORMS
1.3	Specimens	Describe body site(s) studied.	STORMS
Introduction			
2.0	Background and Rationale	Summarize the underlying background, scientific evidence, or theory driving the current hypothesis as well as the study objectives.	STORMS
2.1	Hypotheses	State the pre-specified hypothesis. If the study is exploratory, state any pre-specified study objectives.	STORMS
Methods			
3.0	Study Design	Describe the study design.	STORMS

Additional Guidance	Yes/No/NA	Comments or location in manuscript
	Yes	
See 3.0 for additional information on study design.	Yes	
For example, targeted 16S by qPCR or sequencing, shotgun metagenomics, metatranscriptomics, etc.	Yes	
	Yes	
	Yes	
	Yes	Exploratory
Observational (Case-Control, Cohort, Cross-sectional survey, etc.) or Experimental (Randomized controlled trial, Non-randomized controlled trial, etc.). For a brief description of common study designs see: DOI: 10.11613/BM.2014.022 If applicable, describe any blinding (e.g. single or double-blinding) used in the course of the study.	Yes	

Table 8. Continued

#	Item	Recommendation	Item Source
3.1	Participants	State what the population of interest is, and the method by which participants are sampled from that population. Include relevant information on physiological state of the subjects or stage in the life history of disease under study when participants were sampled.	STORMS
Methods			
3.2	Geographic location	State the geographic region(s) where participants were sampled from.	MIxS: geographic location (country and/or sea,region)
3.3	Relevant Dates	State the start and end dates for recruitment, follow-up, and data collection.	STORMS
3.4	Eligibility criteria	List any criteria for inclusion and exclusion of recruited participants.	Modified STROBE
3.5	Antibiotics Usage	List what is known about antibiotics usage before or during sample collection.	STORMS

Additional Guidance	Yes/No/NA	Comments or location in manuscript
Examples of the population of interest could be: adults with no chronic health conditions, adults with type II diabetes, newborns, etc. This is the total population to whom the study is hoped to be generalizable to. The sampling method describes how potential participants were selected from that population. If the participants are from a substudy of a larger study, provide a brief description of that study and cite that study. Clearly state how cases and controls are defined. An example of relevant physiological state might be pre/post menopausal for a vaginal microbiome study; examples of stage in the life history of disease could be whether specimens were collected during active or dormant disease, or before or after treatment.	Yes	Methods and Materials
Geographic coordinates can be reported to prevent potential ambiguities if necessary.	Yes	Methods and Materials
Recruitment is the period in which participants are recruited for the study. In longitudinal studies, follow-up is the date range in which participants are asked to complete a specific assessment. Finally, data collection is the total period in which data is being collected from participants including during initial recruitment through all follow-ups.	Yes	Methods and Materials
Among potential recruited participants, how were some chosen and others not? This could include criteria such as sex, diet, age, health status, or BMI. If there is a primary and validation sample, describe inclusion/exclusion criteria for each.	Yes	Methods and Materials
If participants were excluded due to current or recent antibiotics usage, state this here. Other factors (e.g. proton pump inhibitors, probiotics, etc.) that may influence the microbiome should also be described as well.	Partly	Methods and Materials

4

Table 8. Continued

#	Item	Recommendation	Item Source
3.6	Analytic sample size	Explain how the final analytic sample size was calculated, including the number of cases and controls if relevant, and reasons for dropout at each stage of the study. This should include the number of individuals in whom microbiome sequencing was attempted and the number in whom microbiome sequencing was successful.	STORMS
3.7	Longitudinal Studies	For longitudinal studies, state how many follow-ups were conducted, describe sample size at follow-up by group or condition, and discuss any loss to follow-up.	STORMS
3.8	Matching	For matched studies, give matching criteria.	Modified STROBE
3.9	Ethics	State the name of the institutional review board that approved the study and protocols, protocol number and date of approval, and procedures for obtaining informed consent from participants.	STORMS
Methods			
4.0	Laboratory methods	State the laboratory/center where laboratory work was done.	STORMS
4.1	Specimen collection	State the body site(s) sampled from and how specimens were collected.	MIxS: sample collection device or method; host body site
4.2	Shipping	Describe how samples were stored and shipped to the laboratory.	STORMS

Additional Guidance	Yes/No/NA	Comments or location in manuscript
Consider use of a flow diagram (see template at https://stormsmicrobiome.org/figures). Also state sample size in abstract. If power analysis was used to calculate sample size, describe those calculations.	Partly	Methods and Materials
If there is loss to follow-up, discuss the likelihood that drop-out is associated with exposures, treatments, or outcomes of interest.	NA	
"Matched" refers to matching between comparable study participants as cases and controls or exposed / unexposed. Indicate whether participants were individual or frequency matched and in what ratio were they matched (e.g. 1 case to 1 control).	NA	
	Partly	Methods and Materials
Provide a reference to complete lab protocols if previously published elsewhere such as on protocols.io. Note any modifications of lab protocols and the reason for protocol modifications.	Yes	Supplementary Methods 2
Use terms from the Uber-anatomy Ontology (https://www.ebi.ac.uk/ols/ontologies/uberon) to describe body sites in a standardized format.	Yes	Methods and Materials, Supplementary Methods 2
Include length of time from collection to receipt by the lab and if temperature control was used during shipping.	Yes	Supplementary Methods 2

Table 8. Continued

#	Item	Recommendation	Item Source
4.3	Storage	Describe how the laboratory stored samples, including time between collection and storage and any preservation buffers or refrigeration used.	STORMS
4.4	DNA extraction	Provide DNA extraction method, including kit and version if relevant.	MIxS: nucleic acid extraction
4.5	Human DNA sequence depletion or microbial DNA enrichment	Describe whether human DNA sequence depletion or enrichment of microbial or viral DNA was performed.	STORMS
4.6	Primer selection	Provide primer selection and DNA amplification methods as well as variable region sequenced (if applicable).	MIxS: pcr primers
4.7	Positive Controls	Describe any positive controls (mock communities) if used.	STORMS
4.8	Negative Controls	Describe any negative controls if used.	STORMS
4.9	Contaminant mitigation and identification	Provide any laboratory or computational methods used to control for or identify microbiome contamination from the environment, reagents, or laboratory.	STORMS
4.10	Replication	Describe any biological or technical replicates included in the sequencing, including which steps were replicated between them.	STORMS
4.11	Sequencing strategy	Major divisions of strategy, such as shotgun or amplicon sequencing.	MIxS: sequencing method
4.12	Sequencing methods	State whether experimental quantification was used (QMP/cell count based, spike-in based) or whether relative abundance methods were applied.	STORMS
Methods			
4.13	Batch effects	Detail any blocking or randomization used in study design to avoid confounding of batches with exposures or outcomes. Discuss any likely sources of batch effects, if known.	STORMS

Additional Guidance	Yes/No/NA	Comments or location in manuscript
State where each procedure or lot of samples was done if not all in the same place. Include reagent/lot/catalogue #s for storage buffers.	Yes	Baseclear, Supplementary Methods 2
If any DNA quantification methods were used prior to DNA amplification or at the pooling step of library preparation, state so here.	Yes	Baseclear, Supplementary Methods 2
	NA	
	Yes	Supplementary Methods 2
If used, should be deposited under guidance provided in the 8.X items.	Yes	Supplementary Methods 2
If used, should be deposited under guidance provided in the 8.X items.	Yes	Supplementary Methods 2
Includes filtering of reagents and other steps to minimize contamination. It is relevant to state whether the specimens of interest have low microbial load, which makes contamination especially relevant.	NA	Baseclear inclusion of negative water control and sterile lab environment
Replication may be biological (redundant biological specimens) or technical (aliquots taken at different stages of analysis) and used in extraction, sequencing, preprocessing, and/or data analysis.	NA	
For amplicon sequencing (for example, 16S variable region), state the region selected. State the model of sequencer used.	Yes	Methods and Materials
These include read length, sequencing depth per sample (average and minimum), whether reads are paired, and other parameters.	NA	
Sources of batch effects include sample collection, storage, library preparation, and sequencing and are commonly unavoidable in all but the smallest of studies.	NA	No randomization, batches are filled with samples according to the date they were received IMpACT and other studies, mixing control subjects and participants with ADHD

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Table 8. Continued

#	Item	Recommendation	Item Source
4.14	Metatranscriptomics	Detail whether any mRNA enrichment was performed and whether/how retrotranscription was performed prior to sequencing. Provide size range of isolated transcripts. Describe whether the sequencing library was stranded or not. Provide details on sequencing methods and platforms.	STORMS
4.15	Metaproteomics	Detail which protease was used for digestion. Provide details on proteomic methods and platforms (e.g. LC-MS/MS, instrument type, column type, mass range, resolution, scan speed, maximum injection time, isolation window, normalised collision energy, and resolution).	STORMS
4.16	Metabolomics	Specify the analytic method used (such as nuclear magnetic resonance spectroscopy or mass spectrometry). For mass spectrometry, detail which fractions were obtained (polar and/or non-polar) and how these were analyzed. Provide details on metabolomics methods and platforms (e.g. derivatization, instrument type, injection type, column type and instrument settings).	STORMS
5.0	Data sources/ measurement	For each non-microbiome variable, including the health condition, intervention, or other variable of interest, state how it was defined, how it was measured or collected, and any transformations applied to the variable prior to analysis.	MIxS: host disease status
Methods			

Additional Guidance	Yes/No/NA	Comments or location in manuscript
Provide details on any internal standards which may have been used as well as parameters and versions of any software or databases used.	NA	
Provide details on any internal standards which may have been used as well as parameters and versions of any software or databases used.	NA	
Provide details on any internal standards which may have been used as well as parameters and versions of any software or databases used.	NA	
State any sources of potential bias in measurements, for example multiple interviewers or measurement instruments, and whether these potential biases were assessed or accounted for in study design. Use terms from a standardized ontology such as the Experimental Factor Ontology (https://www.ebi.ac.uk/efo/) to describe variables of interest in a standardized format.	Yes	Methods and Materials, Supplementary Methods

Table 8. Continued

#	Item	Recommendation	Item Source
6.0	Research design for causal inference	Discuss any potential for confounding by variables that may influence both the outcome and exposure of interest. State any variables controlled for and the rationale for controlling for them.	STORMS
6.1	Selection bias	Discuss potential for selection or survival bias.	STORMS
7.0	Bioinformatic and Statistical Methods	Describe any transformations to quantitative variables used in analyses (e.g. use of percentages instead of counts, normalization, rarefaction, categorization).	STORMS
7.1	Quality Control	Describe any methods to identify or filter low quality reads or samples.	MIxS: sequence quality check
7.2	Sequence analysis	Describe any taxonomic, functional profiling, or other sequence analysis performed.	MIxS: feature prediction; similarity search method
Methods			

Additional Guidance	Yes/No/NA	Comments or location in manuscript
For causal inference, this item refers to describing the assumptions that would be required to draw causal inferences from observational data. See Vujkovic-Cvijin, I., Sklar, J., Jiang, L. et al. Host variables confound gut microbiota studies of human disease. Nature 587, 448–454 (2020). https://doi.org/10.1038/s41586-020-2881-9 for more details on confounding in observational microbiome studies. For example, hypothesized confounders may be controlled for by multivariable adjustment. Consider using a directed acyclic graph (DAG) to describe your causal model and justify any variables controlled for. DAGs can be made using www.dagitty.net.	NA	
Selection bias can occur when some members of the target study population are more likely to be included in the study/final analytic sample than others. Some examples include survival bias (where part of the target study population is more likely to die before they can be studied), convenience sampling (where members of the target study population are not selected at random), and loss to follow-up (when probability of dropping out is related to one of the things being studied).	NA	Feature selection was applied, while bias is introduced underestimating low prevalent features, the data-driven selection instead of hypothesis driven might on the other hand reduce bias
If a variable is analyzed using different transformations, state rationale for the transformation and for each analyses which version of the variable is used. In case of any complex or multistep transformations, give enumerated instructions for reproducing those transformations.	Yes	Methods and Materials, Supplementary Methods 2
If samples were excluded based on quality or read depth, list the criteria used, the number of samples excluded, and the final sample size after quality control.	Yes	Supplementary Methods 2
	Yes	Supplementary Methods 2

Table 8. Continued

#	Item	Recommendation	Item Source
7.3	Statistical methods	Describe all statistical methods.	Modified STROBE
7.4	Longitudinal analysis	If the study is longitudinal, include a section that explicitly states what analysis methods were used (if any) to account for grouping of measurements by individual or patterns over time.	STORMS
7.5	Subgroup analysis	Describe any methods used to examine subgroups and interactions.	STROBE
7.6	Missing data	Explain how missing data were addressed.	STROBE
7.7	Sensitivity analyses	Describe any sensitivity analyses.	STROBE
7.8	Findings	State criteria used to select findings for reporting.	STORMS
7.9	Software	Cite all software (including read mapping software) and databases (including any used for taxonomic reference or annotating amplicons, if applicable) used. Include version numbers.	Modified STREGA
Methods			

Additional Guidance	Yes/No/NA	Comments or location in manuscript
Describe any statistical tests used, exploratory data analysis performed, dimension reduction methods/unsupervised analysis, alpha/beta metrics, and/or methods for adjusting for measurement bias. If multiple statistical methods are possible, discuss why the methods used were selected. If a multiple hypothesis testing correction method was used, describe the type of correction used. State which taxonomic levels are analyzed.	Yes	Methods and Materials, Supplementary Methods
	NA	
	NA	
"Missing data" refers to participant measurements such as covariates, exposures, outcomes, or time points that should have been collected but were not, not to zeros in taxonomic abundance tables or data points not applicable to that observation.	Yes	missing data was excluded
	NA	
For example, false discovery rate with total number of tests, effect size threshold, significance threshold, microbes of interest.	Yes	Convergence across tools, FDR-corrected
Installed packages, add-ons or libraries should be stated and cited in addition to the software used. All parameters employed that differ from the default of that software/version should be provided. This is in addition to, not a replacement for, publishing of code as outlined in the section Reproducible Research.	Yes	Methods and Materials, Supplementary Methods

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Table 8. Continued

#	Item	Recommendation	Item Source
8.0	Reproducible research	Make a statement about whether and how others can reproduce the reported analysis.	STORMS
8.1	Raw data access	State where raw data may be accessed including demultiplexing information.	STORMS
8.2	Processed data access	State where processed data may be accessed.	STORMS

Additional Guidance	Yes/No/NA	Comments or location in manuscript
Any protected information that has been excluded or provided under controlled access should be listed along with any relevant data access procedures. "On request from authors" is not sufficiently detailed; formal data access procedures and conditions should be defined. If data are unavailable, state so clearly. Consider using a specialized rubric for reproducible research (such as:https://mbio.asm.org/content/9/3/e00525-18.short). Consider preregistering the study protocol (such as on osf.io orhttps://plos.org/open-science/preregistration/).	NA	Only open-source tools were applied and applications and settings deviating from default settings were described. Applied tools are cited in place.
Robust, long-term databases such as those hosted by NCBI and EBI are preferred. If using a private repository, provide rationale.	NA	Raw sequences and demultiplexing information are stored on local servers, behavioral information is deposited in the dans (https://doi.org/10.17026/dans-xs2-nvp8). All data is available upon motivated request and must be dedicated to the research of ADHD. This is due to the informed consent form stating that collected data from the IMpACT2-NL study will only be used for the research of ADHD.
Unfiltered data should be provided. Robust, long-term databases such as those hosted by NCBI and EBI-EMBL are preferred. Repositories like zenodo (https://zenodo.org/) or publisso (https://www.publisso.de/en/working-for-you/doi-service/) can be used to provide a DOI and long-term storage for processed datasets, even those which cannot be published openly.	NA	

Table 8. Continued

#	Item	Recommendation	Item Source
8.3	Participant data access	State where individual participant data such as demographics and other covariates may be accessed, and how they can be matched to the microbiome data.	STORMS
Methods			
8.4	Source code access	State where code may be accessed.	STORMS
8.5	Full results	Provide full results of all analyses, in computer-readable format, in supplementary materials.	STORMS
Results			
9.0	Descriptive data	Give characteristics of study participants (e.g. dietary, demographic, clinical, social) and information on exposures and potential confounders.	STROBE
10.0	Microbiome data	Report descriptive findings for microbiome analyses with all applicable outcomes and covariates.	STORMS
10.1	Taxonomy	Identify taxonomy using standardized taxon classifications that are sufficient to uniquely identify taxa.	STORMS

Additional Guidance	Yes/No/NA	Comments or location in manuscript
If re-categorized, transformed, or otherwise derived variables were used in the analysis, these variables or code for deriving them should be provided. Examples of how participant data can be matched to microbiome data are: using the same set of anonymized identifiers, or using different anonymized identifiers but providing a map. Provided data should be sufficient to independently replicate the current analysis.	NA	
If a standard or formalized workflow was employed, reference it here.	Yes	Individual packages are all open access and cited in text
For example, any fold-changes, p-values, or FDR values calculated, provided as a spreadsheet. Use a machine-readable, plain-text format such as csv or tsv.	Yes	Results, Supplementary sesults
Typically reported in a table included in the paper or as a supplementary table. Indicate number of participants with missing data for each variable of interest. This includes environmental and lifestyle factors that may affect the relationship between the microbiome and the condition of interest. Participant diet and medication use should be summarized, if known. At minimum, age and sex of all participants should be summarized.	Yes	Results
This includes measures of diversity as well as relative abundances. These descriptive findings should be reported both for the sample overall and for individual groups.	Yes	Results, Supplementary Methods
If not using full taxonomic hierarchy, make sure it is clear whether names stated are species, genera, family, etc. Italicize genus/species pairs. Consult journal guidelines or standardized references on taxonomic nomenclature. For instance,https://wwwnc.cdc.gov/eid/page/scientific-nomenclature	Yes	Genus only

Table 8. Continued

#	Item	Recommendation	Item Source
10.2	Differential abundance	Report results of differential abundance analysis by the variable of interest and (if applicable) by time, clearly indicating the direction of change and total number of taxa tested.	STORMS
10.3	Other data types	Report other data analyzed--e.g. metabolic function, functional potential, MAG assembly, and RNAseq.	STORMS
Results			
10.4	Other statistical analysis	Report any statistical data analysis not covered above.	STORMS
Discussion			
11.0	Key results	Summarise key results with reference to study objectives	STROBE
12.0	Interpretation	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence.	STROBE
13.0	Limitations	Discuss limitations of the study, taking into account sources of potential bias or imprecision.	STROBE
13.1	Bias	Discuss any potential for bias to influence study findings.	STORMS
13.2	Generalizability	Discuss the generalisability (external validity) of the study results	STROBE

Additional Guidance	Yes/No/NA	Comments or location in manuscript
If there are more than two groups, include omnibus (multigroup) test results if applicable to the research question. If applicable, reported effect sizes should include a measure of uncertainty such as the confidence interval.	Yes	Results, Supplementary Results
	NA	
This could include subgroup analysis, sensitivity analyses, and cluster analysis. Visualizations should be easily interpretable and colorblind-friendly. The caption and/or main text should provide a detailed description of visualizations for visually-impaired readers.	Yes	Results, Supplementary Results
	Yes	Discussion
Define or clarify any subjective terms such as "dominant," "dysbiosis," and similar words used in interpretation of results. When interpreting the findings, consider how the interpretation of the findings may be summarized or quoted for the general public such as in press releases or news articles. If causal language is used in the interpretation (such as "alters," "affects," "results in," "causes," or "impacts"), assumptions made for causal inference should be explicitly stated as part of 6.0 and 13.0. Distinguish between function potential (ie inferred from metagenomics) and observed activity (ie metatranscriptomic, metabolomic, proteomic) if discussing microbial function.	Yes	Discussion
Also consider limitations resulting from the methods (especially novel methods), the study design, and the sample size.	Yes	Discussion
May include sampling method, representativeness of study participants, or potential confounding.	Yes	Discussion
To what populations or other settings do you expect the conclusions to generalize?	NA	

Table 8. Continued

#	Item	Recommendation	Item Source
14.0	Ongoing/future work	Describe potential future research or ongoing research based on the study's findings.	STORMS
Other information			
15.0	Funding	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	STROBE
15.1	Acknowledgements	Include acknowledgements of those who contributed to the research but did not meet critera for authorship.	STORMS
15.2	Conflicts of Interest	Include a conflicts of interest statement.	STORMS
16.0	Supplements	Indicate where supplements may be accessed and what materials they contain.	STORMS
17.0	Supplementary data	Provide supplementary data files of results with for all taxa and all outcome variables analyzed. Indicate the taxonomic level of all taxa.	STORMS

Additional Guidance	Yes/No/NA	Comments or location in manuscript
	Yes	Discussion
	Yes	Acknowledgements
For general guidelines on authorship, see http://www.icmje.org and https://www.elsevier.com/authors/journal-authors/policies-and-ethics/credit-author-statement	NA	
	Yes	Conflict of Interest/Disclosure
	Yes	In text, where applicable
Depending on the analysis performed, examples of the supplemental results included could be mean relative abundance, differential abundance, raw p-value, multiple hypothesis testing-adjusted p-values, and standard error. All discussed taxa should include the taxonomic level (e.g. class, order, genus).	Yes	Supplementary Methods & results in supplementary material file

4

Chapter 5

General Discussion

In this thesis, I aimed to gain a better understanding of adult reactive aggression and ADHD. In three empirical association studies, I therefore investigated their potentially shared etiological mechanisms such as 1) altered brain functioning during emotion processing, 2) altered gut-microbial profiles, and 3) the role of diet for reactive aggression and adult ADHD. In this last chapter, I first summarize the results of each individual study and discuss them in the context of the current literature. I furthermore consider challenges of current research on the gut-brain axis and diet, elaborate considerations and good practices for future studies, and speculate on potential clinical and social implications of my work.

SUMMARY

For the primary analyses in this thesis, we collected the IMpACT2-NL cohort containing measures of brain functioning, the gut-microbiome, and relevant phenotypic information. The IMpACT2-NL dataset contains 173 inclusions and 153 participants with complete data. In the scientific chapters, subsets with the relevant data were included.

Chapter 2 of this thesis was focused on brain functioning associated with reactive aggression in adult ADHD. To identify brain functional alterations relevant for reactive aggressive behavior, displayed in many adults with persistent ADHD, we applied functional MRI during an emotional face processing task in 78 adults with and 78 adults without ADHD (IMpACT2-NL). We studied associations of reactive aggression with clinical expression of ADHD, whole-brain activity in association with ADHD and reactive aggression scores, as well as their interaction. Hyperactivity/impulsivity symptoms and impairments in social domains were associated with higher reactive aggression scores. While adults with and without ADHD showed similar neural activation patterns when processing emotional faces, we identified an interaction effect of ADHD with reactive aggression scores in several brain regions (e.g. *precentral, postcentral and lingual gyri, superior frontal and middle temporal areas,* as well as the *caudate nucleus*). Focusing the analysis on individuals with ADHD, we found that brain activity in several regions was particularly increased in people with ADHD if they had higher reactive aggression scores. This activation pattern included small clusters within the *insula, limbic system* and *middle and superior frontal areas*. Notably, social and self-referential impairment, and hyperactivity/impulsivity (but not inattention) symptoms were associated with brain functioning relevant for reactive aggression. With this work, we provide evidence for partly altered brain functional emotion processing in adults with ADHD compared to neurotypical individuals. Hyper-reactivity in the salience network as much as differential engagement of higher-order control areas might contribute to reactive aggressive behavior, displayed in many individuals with persistent ADHD. This altered brain functioning might be part of the shared etiology between reactive aggression and ADHD symptoms in a specific group of individuals.

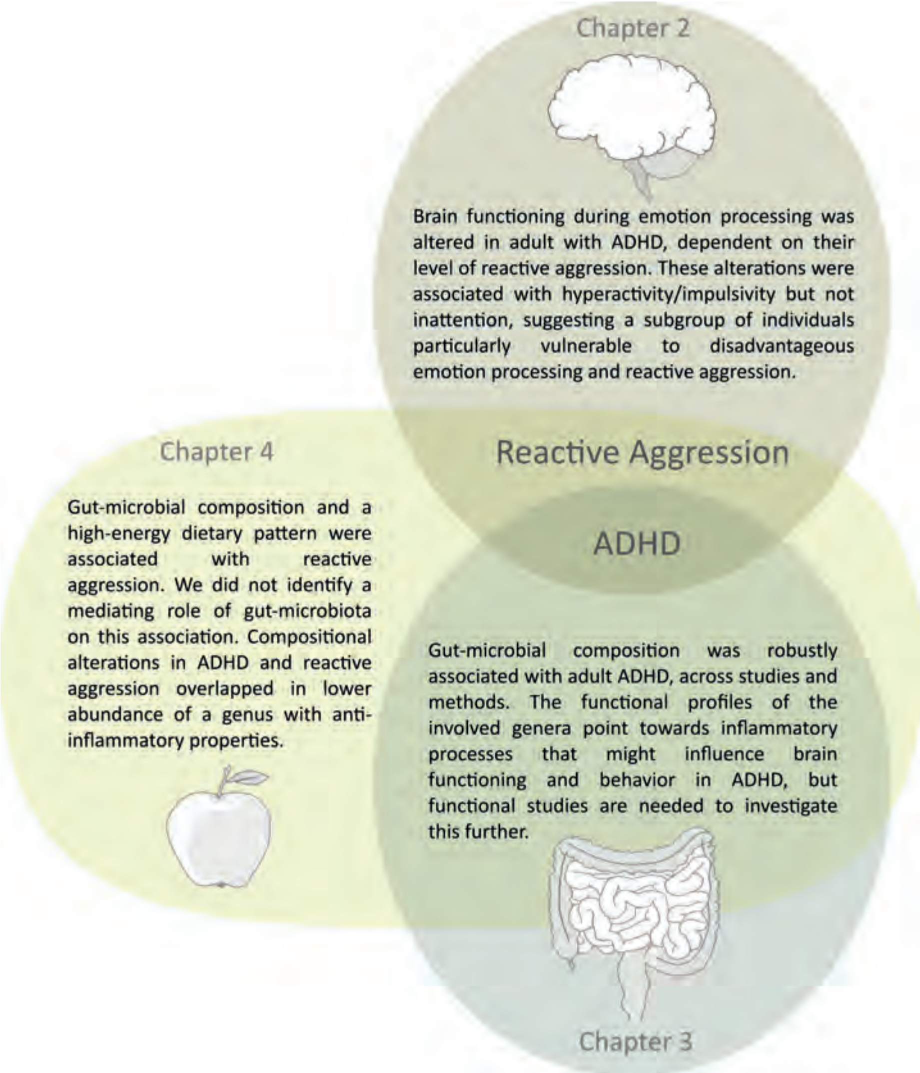

Figure 1. Summary of the results from the three chapters.

In **Chapter 3**, we focused on the identification of robust associations between adult ADHD and the gut-microbiome across four cohorts (IMpACT2-NL, MIND-Set, NeuroIMAGE, and Mental-Cat, N=617). We harmonized bioinformatic pipelines for raw 16S sequencing data, and analyzed group differences of diversity and composition in the individual studies across methods, where converging results were regarded more likely to reflect true findings. We applied feature selection prior to differential abundance testing, mitigating the multiple testing burden, and meta-analyzed across the individual study results. Despite harmonized data processing, we also observed pronounced between-cohort differences in beta diversity, potentially reflecting different wet-lab procedures. We also identified differences in beta diversity between the groups. Differential abundance analysis revealed robust associations of several genera with ADHD, including *Eubacterium xylanophilum*, which was less abundant in adults with ADHD, and *Ruminococcus torques*, which was more abundant in adults with ADHD compared to neurotypical peers. *Ruminococcus torques* abundance was further associated with more symptoms of hyperactivity/impulsivity, and the genus *Eisenbergiella* with more inattention and hyperactivity/impulsivity symptoms. The literature suggests a potential role of these genera in inflammatory processes, which might be risk-conferring or beneficial, but functional studies are needed to address these properties further. Next to identifying robust associations of particular genera with adult ADHD, this study also highlighted the potential influence of wet-lab choices on gut-microbial outcome measures and stressed the importance of harmonization and convergence across statistical approaches to produce robust results.

.**Chapter 4** of this thesis was focused on the role of diet and gut-microbial alterations related to reactive aggression and adult ADHD. To identify dietary patterns and gut-microbial patterns associated with ADHD and reactive aggression, we applied exploratory factor analysis on a dietary questionnaire and estimated diversity and composition of 16S-sequenced fecal microbiome samples in 77 adults with and 76 adults without ADHD (IMpACT2-NL). We further explored the potential mediating effects of bacterial genus abundance on significant diet-behavior associations. We identified three dietary patterns, characterized by high-energy, high-alcohol and high-fiber consumption. While the high-energy factor was associated with reactive aggression, neither factor was associated with ADHD diagnosis. Several genera were associated with reactive aggression or ADHD diagnosis. While there was no significant overlap between genera associated with ADHD and reactive aggression in this study, we found that lower levels of *Eubacterium xylanophilum group* were associated with low reactive aggression scores – this genus was less abundant in adults with ADHD in the meta-analysis of **Chapter 3**. No mediation was detected of the selected genera on the association between reactive aggression and the high-energy diet. With this chapter, we have identified disadvantageous dietary patterns associated with reactive aggression, as well as gut-microbial alterations in reactive aggression and/or ADHD that might be shared, but the way diet and the gut-microbiome interact has to be studied further.

Figure 1 summarizes the results of the three individual chapters. These results may be linked to shared etiological processes or environmental influences.

INTERPRETATION OF FINDINGS IN THE CONTEXT OF EXISTING LITERATURE

2.1 Clinical representation of reactive aggression and adult ADHD

In this thesis, we studied reactive aggression in adults with persistent ADHD. In the IMpACT2-NL cohort, reactive aggression scores were higher in adults with ADHD compared to neurotypical individuals, associated with persistent **hyperactivity/impulsivity symptoms** and impairments in **relationships and family** and **self-image**. This finding is in line with reported emotion dysregulation and reactive aggressive behaviors in ADHD across the lifespan and the developmental coupling of hyperactivity/impulsivity symptoms with (reactive) aggression across childhood and adolescence [14, 15]. It also supports the benefits of identifying subgroups or individuals affected by emotion dysregulation, which may help tailor treatment approaches accounting for the heterogeneity of symptoms and impairments at hand. Notably, a recent clustering approach identified the subgroup with the combined type scoring highest in emotion dysregulation questionnaires [16].

2.2 Brain systems and associated stages of emotion processing

In **Chapter 2**, we aimed to identify potential alterations in brain functioning, that may be relevant for emotion regulation problems like reactive aggression in the context of adult ADHD. **Brain functioning during emotion processing was generally comparable across adults with and without ADHD**. Previous studies in children and adolescents with ADHD had reported partially altered activity and connectivity profiles of e.g., the *amygdala/limbic system*, *insula*, *(anterior) cingulate cortex (ACC)*, *(ventral) striatum, prefrontal (PFC)*, and *orbitofrontal cortex (OFC)* during emotion processing, with varying results across studies; for a review see [19]. [19]. The few published studies including adults focused on (partially) remitted males, reporting either no group differences or differences in activity or connectivity of e.g. the *inferior parietal lobule (IPL)*, the *temporo-parietal junction (TPJ)*, the *amygdala, cingulate cortex*, occipital, parietal and prefrontal areas during emotion processing [20-23]. Notably, some of the reported differences were only shown in medication-naïve adults with ADHD: pharmacological treatment for ADHD was reported to moderate brain activity during emotion processing, potentially driving a normalization of brain functioning (e.g. increased *ACC* and *PFC* activity in children; increased insula and decreased amygdala activity in adults) [21, 22]. In our study, however, pharmacological ADHD treatment at the time of participation was not associated with reactive aggression scores, ADHD symptoms, impairments or measures of brain functioning. These findings contribute to a rather scattered picture of brain functional alterations during emotion processing in ADHD.

We did, however, identify brain functional alterations in ADHD, that were dependent on the level of reactive aggression - **an interaction effect of ADHD and reactive aggression during emotion processing** in the brain. This effect may be related to the considerable variability in the presentation and severity of reactive aggression among individuals with ADHD. A recent clustering approach, for example, has identified a subgroup within the adult ADHD population with particular vulnerability to emotional dysregulation [16]. The regions displaying altered activity profiles in individuals with high reactive aggression scores and ADHD overlap with regions generally implicated in emotion regulation and reactive aggression. Instead of pointing towards one particular psychological process, the findings of this study support a potential role of 1) bottom-up **emotional reactivity,** 2) **cognitive control** for top-down emotion regulation, as well as 3) **social** and **self-referential** processes for high reactive aggression scores in individuals with ADHD. However, some findings often reported in relation to ADHD, emotions, and aggression (e.g. striatum, amygdala, ventral and orbitofrontal *PCF, ACC*, see [19]) were not associated with either measure in our sample.

Emotional hyperreactivity

Recent meta-analyses link the *amygdala/limbic system* and *insula* to emotional (hyper)reactivity [29-31], and of the *caudate nucleus* with reactivity to frustration or threat in reactive aggression [31, 32]. Finding altered activity profiles of these regions in our study suggests that emotional hyperreactivity may contribute to elevated reactive aggression scores in adults with ADHD. In line with our findings, emotional hyperreactivity has shown the strongest associations with ADHD and impairment, compared to e.g. regulation deficits [33].

The *insula* is linked to the subjective experience of emotions and plays a role in processing bodily sensations associated with emotional states, while the *caudate* *nucleus* is described as an interface for emotional and cognitive processing [34, 35]. Both regions have shown altered activity patterns in children with ADHD and reactive aggression in a previous study during an aggression task [36]. Considering the *limbic system*, several studies have reported altered reactivity or connectivity of the *amygdala* [21, 23, 37, 38]. While these results stem from tasks or analyses targeting the amygdala, whole-brain studies and meta-analyses in ADHD and aggression suggest a less prominent role of the *amygdala* [21, 31, 39]. In line with this, we did not find clearly delineated associations with *amygdala* activity (or connectivity when analyzing *amygdala*-whole-brain psychophysiological interactions). In our study, a small cluster in the *hippocampus*, including voxels of the *amygdala*, was associated with high reactive aggression scores in adults with ADHD. The hippocampus is associated with emotional memory and learning and regulates amygdala reactivity [40]; Influences of emotional experiences may elicit hippocampal activity particularly in adults, as observed in our study. Except during fear extinction learning, hippocampal involvement is not frequently reported in emotion processing studies in ADHD [41].

Cognitive control and inhibition

Activity in *middle* and *superior frontal areas* was associated with elevated reactive aggression in adults with ADHD in our study. As part of the frontal cortex, these regions are involved in response inhibition and cognitive control, the most prominent impairment across ADHD and aggression studies [19, 31, 32]. The regions have also consistently been reported to increase activity during explicit emotion regulation tasks [29, 30]. In addition to these regions, studies have consistently reported higher activity in the *PFC* or *OFC* during emotion processing in ADHD as well as associations of *PFC, ACC*, and *inferior frontal gyrus (IFG)* activity with explicit emotion regulation and emotional lability in ADHD [19, 23, 29, 30, 42-44], which were not observed in our study. Differences between ours and other studies may be influenced by the task, e.g. implicit (passive viewing of emotions) or explicit (instruction to regulate emotional response), the use of different emotion regulation strategies, and the emotional valence. Recent research suggests that implicit, but not explicit emotion regulation may be impaired in adult ADHD [45, 46]. If not instructed, adults with ADHD more often use maladaptive strategies, like expressive suppression, which may result in activation of cognitive control areas independent of instructions [47]. Negative emotions in particular elicited activity in these regions in previous studies, the passive viewing of all emotional valences in our study may require less activity in cognitive control areas.

Social and self-referential cognition

Social and self-referential processes are often associated with activity in *middle temporal areas*, and have been consistently reported in neuroimaging studies about emotion regulation and ADHD [29, 30, 43]. Also in studies of reactive aggression, provocation has shown to elicit activity in regions of social cognition reflected in middle temporal or temporo-parietal areas, but also visual (*occipital cortex*) and sensorimotor processing [31]. Among other functions, middle temporal (or temporo-parietal) areas are relevant for social cognition, e.g. for the reasoning about other people's thoughts and intentions or semantic integration [48]. In children with ADHD and comorbid disruptive behavior disorder, activity in temporo-parietal regions were associated with low reactive aggression, while children without ADHD showed an inverse effect [36]. In line with these findings, we identified an interaction effect of ADHD diagnosis and reactive aggression scores in *middle temporal, precentral* and *lingual* areas in adults with persistent ADHD. Activity in these regions was higher in individuals with ADHD with higher reactive aggression scores, and lower in participants without ADHD and lower reactive aggression scores. Associations of these regions with social/self-referential cognition, visual and sensorimotor processing suggest that social cognition and perception may be relevant for reactive aggression in adult ADHD, while these processes contribute to well adapted behavior in unaffected individuals.

Note: Functional profiles of activity clusters cannot be clearly separated between regions – while several regions can be linked to the same function, one region is implicated across different contexts. E.g., while the role of insular and hippocampal activity can be described as part of emotional reactivity, their influence on amygdala reactivity may be considered a top-down control function as well. Individual studies identify different sets of brain regions, often only discussed in part or summarized. The depiction of our findings in the context of the literature above should be considered a brief summary of reoccurring findings in the literature, drawn predominantly from review articles and meta-analyses. Potential bias related to this issue is discussed further in section 4.1.1. Challenges and future directions.

2.3 The gut-microbiota in adult ADHD and reactive aggression

ADHD

In **Chapter 3** of this thesis, we aimed to identify **robust gut-microbiota alterations** in adult ADHD. Several systematic reviews had previously been published touching this topic [49-51]. While some of the few original association studies had reported alterations of gut-microbial diversity and/or composition in ADHD. The results were inconsistent and partially conflicting, potentially due to small sample sizes, and strong methodological limitations of the individual studies. We minimized these limitations by comparing diversity and composition across several indices and state of the art methods, where converging results are more likely reflect true findings, harmonizing bioinformatic pipelines and statistical analyses across four big cohorts and meta-analyzing the results.

Alpha Diversity

Several studies compared alpha diversity measures between individuals with and without ADHD to identify general alterations in the gut-microbial community (e.g. enrichment/depletion, evenness, or phylogenic relatedness) as high diversity is generally associated with positive health outcomes [7, 52]. While alterations of richness and phylogenic diversity were not reported across studies, the results on evenness were conflicting [49, 53]. In our meta-analysis, **no differences in richness, evenness, or phylogenetic diversity** were observed. This suggests, that not depletion, but potent compositional changes of the gut-microbiota may be related to ADHD pathophysiology. The few published positive findings on alpha diversity may be related to the limitations discussed in Chapter 3, however, they might as well be relevant for the young male-dominated study population of the individual studies [54, 55]. Interestingly, concerning the richness, one study found that, before antibacterial intervention, children with ADHD had a significantly lower number of live bacteria in their fecal samples compared to unaffected children; the distinction between live and dead cells may play a role for the assessment of alpha diversity, as well as the functional interpretability of differential abundance results [56].

Beta Diversity

Similar to alpha diversity, studies investigating beta diversity report inconsistent results across measures and studies [49, 51, 57, 58]. In our study, three out of four individual cohorts showed **significant beta dissimilarity** of the gut-microbiota community between people with and without ADHD. Our mega-analysis across cohorts confirmed a significant difference between individuals with and without ADHD, again suggesting general compositional differences that have to be examined further. The direction of the detected effect however is uninterpretable. In theory, more similarity of the gut-microbiota among people with ADHD could be related to predominant groups of features across people with ADHD, that are functionally relevant for risk to develop symptoms (e.g. neurotransmitter synthesis, inflammatory pathways). Significantly less similarity of the gut-microbial ecosystem in ADHD compared to unaffected individuals, on the other hand, may be related to the high behavioral heterogeneity in ADHD, in particular concerning the influences of planning, energy expenditure and inattention on lifestyle, which may differ more between two affected than between two unaffected individuals. More detailed analyses of the composition and lifestyle factors, as described in this thesis, but also functional pathways and causal intervention studies are needed to make sense of differences in beta diversity.

Differential Abundance

The few differential abundance results overlapping between previously published studies reported lower abundance of *Faecalibacterium* (two Chinese studies, comparing children with and without ADHD [59, 60]) and altered abundance of *Dialister* [60, 61] (yet with conflicting direction of effects). Further, altered abundances of *Odoribacter* [59, 61, 62], *Prevotella* [55, 58], and *Bifidobacterium* [57, 60] have been mentioned across studies, yet, below the significance threshold of the individual studies or in non-peer reviewed reports. None of these genera were associated with ADHD in our meta-analysis. In fact, only the genus *Dialister* was included in our analyses after feature selection, potentially due to the fact, that one study reporting enriched Dialister abundance in ADHD was included in this meta-analysis [61]. Notably, even studies based on the same cohort (NeuroIMAGE) showed no overlap with each other or our meta-analysis [57, 58], likely due to influences of 1) the different bioinformatic pipelines and statistical approaches, 2) different individuals in the control group in [57], and 3) the inclusion of sub-threshold ADHD and mixed developmental stages of [58]. Recently, another study attempting to identify significant signals across studies has been published. The authors extracted relative abundances of fourteen genera from supplementary materials and relative abundance plots of original research articles, transformed into standardized mean differences, and attempted meta-analysis of the pairs of studies including the same genus [53]. No differential abundance results of the individual studies were replicated, but the meta-analysis of non-significant results of three studies suggested higher abundance of the genus *Blautia* in individuals with ADHD [55, 56, 60]. This approach neglects e.g. the compositionality of this data, important confounders, and reporting biases from the individual studies, potentially contributing to the scattered picture of potential gut-microbiota alterations in ADHD. In our meta-analysis in **Chapter 3**, we reduced the risk of bias from confounders (age, sex, cohort, diet, and medication), false discovery rates (feature selection, bigger sample size), and influences of bioinformatic pipelines (harmonized quality assessment, processing and appropriate statistical analysis across tools). Next to **higher abundance of** *Ruminococcus_torques* and **lower abundance of** *Eubacterium xylanophilum* in adults with ADHD, we identified **associations of** *Ruminococcus_torques* **with hyperactivity/**

impulsivity symptoms, and *Eisenbergiella* **with hyperactivity/impulsivity and inattention**, across all participants and tools. The few differential abundance results overlapping between previously published studies reported lower abundance of *Faecalibacterium* (two Chinese studies, comparing children with and without ADHD [59, 60]), and altered abundance of *Dialister* [60, 61] (yet with conflicting direction of effects). Further, altered abundances of *Odoribacter* [59, 61, 62], *Prevotella* [55, 58], and *Bifidobacterium* [57, 60] have been mentioned across studies, yet, below the significance threshold of the individual studies or in non-peer reviewed reports. None of these genera were associated with ADHD in our meta-analysis. In fact, only the genus *Dialister* was included in our analyses after feature selection, potentially due to the fact, that one study reporting enriched Dialister abundance in ADHD was included in this meta-analysis [61].

Reactive aggression

In **Chapter 4**, we identified associations of the bacterial genera *Eubacterium_xylanophilum*, *Lactobacillus*, and *Slackia* with reactive aggression robust across statistical approaches. No associations with the diversity measures were found. In other species (e.g. dogs, rodents, and Drosophila [63-65]) and in the context of other psychiatric disorders, aggression-related phenotypes have been associated with reduced diversity and differential abundance in several genera: In schizophrenia, lower abundance of *Enterococcus, Candidatus_Saccharimonas*, and *Bifidobacterium* was associated with violent behavior [66]; lower alpha-diversity, lower abundance of *Bacteroides*, *Faecalibacterium*, *Blautia*, *Bifidobacterium*, *Collinsella,* and *Eubacterium_coprostanoligenes*, and increased abundance of *Prevotella* were associated with aggressive behavior [67]. Reduced diversity, enriched pro-inflammatory bacteria (e.g. *Bacteroides* and *Eggerthella*) and depletion of anti-inflammatory bacteria (e.g. *Blautia, Faecalibacterium, Coprococcus,* and *Sutterella*) have consistently associated with depression [68], and one study identified associations of important factors for emotion regulation (rumination, reappraisal, and catastrophizing) with bacterial genera (*Subdoligranulum, Alistipes, Faecalibacterium*, and *Odoribacter* for rumination, *Weissella* for reappraisal, and *Dialister* for catastrophizing, respectively) [69]. Altered gut-microbiota composition has been associated with emotional well-being [70, 71], was discussed as a risk factor for the development of emotion dysregulation in infants [72] and in alcohol misuse [73], and it may even modulate the effects of antidepressant medication on mood and emotions [74]. While there appears to be partial overlap among these studies, the results of our study on reactive aggression have yet to be replicated. However, other, larger human studies covering gut-microbiota in reactive aggression are lacking (see [17, 75] for recent systematic reviews). Most of the results covering reactive aggression (and related behaviors) are summarized across small individual studies, often applying methods that are particularly biased towards common features [76] (e.g. *Prevotella* , *Bacteroides, Faecalibacterium, Ruminococcus, Blautia*, and *Clostridium* [77]), rendering these results likely to reflect broad commonalities among psychiatric participants rather than phenotype-specific relevance.

Notably, *Eubacterium_xylanophilum*, associated with low reactive aggression scores, was also less abundant in adults with ADHD in the meta-analysis of Chapter 3. The latter association was only detected with greater power of the meta-analysis, and not in the individual study of Chapter 4. As described in more detail in the General Introduction of this thesis, the frequent co-occurrence of ADHD with reactive aggression may be ascribed to shared underlying biological pathways resulting in altered brain functioning. One other study has recently addressed the potential role of the gut-microbiota in emotional problems in children with ADHD, reporting associations of withdrawal and depression symptoms with *Agathobacter*, and rule-breaking behavior with *Ruminococcus_gnavus*, which were both more abundant in children with ADHD compared to their neurotypical control group [78]. Positive associations like these might be related to shared consequences, but may also be considered risk-conferring to the co-occurrence of emotional problems with ADHD and could be relevant targets for treatment support for emotional problems in ADHD.

Note: The results of our study are limited to European adults.

- Age: The gut-microbiome develops throughout the lifespan of an individual, with critical developmental periods. Lifespan perspectives on the gut-microbiota in ADHD are promising: In a recent birth cohort study, diversity at 6 months of age, as well as the composition at 1 month and 6 months of age have been associated with ADHD in preadolescence [1]. The authors suggest that altered gut-microbiome maturation, as well as differential abundance of several bacterial features within the first year of age (including families, genera and species like *Ruminococcus* and *Eubacterium*, but also *Dialister, Prevotella, Blautia* and *Bifidobacterium*), point towards a mechanistic factor in ADHD, altering brain structure and function already early in life [1-3].
- Origin: Geography and ethnicity are important environmental and host-genetic factors influencing the composition of the gut-microbiota [7]. Symptoms of ADHD are observed in populations all over the world (with different culturally determined clinical relevance), underlining the need for diverse study populations [8]. While the identification of coherent patterns across samples from different origins may be difficult based on taxonomy from 16S sequencing data, the functional profiles of altered gut-microbiota may be more promising.

2.4 Dietary patterns in adult ADHD and reactive aggression

ADHD

Despite recent meta-analysis, showing that that children and adolescents with ADHD consume less healthy foods, more sweets, and more highly processed "fast-foods" compared to neurotypical peers [79-81], **we did not identify altered eating patterns in adults with ADHD** compared to neurotypical peers in three diet factors high-energy, high-fiber, or high-alcohol (Chapter 4). Besides our study, the literature on eating behavior in adults with ADHD is scarce (see [82, 83]). Two population-based studies found associations of ADHD symptoms with an unhealthy dietary pattern (food with high proportions of refined sugar and saturated fat), unhealthy lifestyles, and poor diets with high consumption of sweets [84, 85]. Yet, the studies showed small correlation coefficients (inattention r = 0.11, hyperactivity r = 0.09), and were based on superficial self-reports (e.g. portions of sweets, portions of vegetables, self-reported "healthy diet"). In contrast, another recent study with a more thorough assessment of eating behavior reported no significant alterations in eating patterns or diet between adults with and without ADHD [86]. In fact, there is evidence that older age as well as higher health literacy influence adequate eating behavior among adults with ADHD, potentially driving converging eating behavior with unaffected adults - Instead of a mechanistic role of diet - dietary intake and food choices may be driven by factors like low health literacy or impulsivity, reflected in high rates of emotional and night time eating in adults with ADHD [87, 88].

Reactive aggression

Even fewer studies have assessed the link between dietary patterns and reactive aggression or related phenotypes. Associations of **reactive aggression scores with the high-energy diet** factor, characterized by for example high intake of sweetened beverages, and low intake of vegetables, in our study are partly similar to dietary patterns previously associated with (emotional) self-regulation difficulties and negative emotions (increased consumption of sweets, and lower consumption of fruits and vegetables) [89-92]. In addition to scarce literature of association studies, dietary or supplementary intervention studies could be relevant for the identification of shared mechanisms or potential targets for treatment support. Recent reviews on interventions in ADHD and aggression have reported potential positive effects on ADHD symptoms, emotional symptoms and impairments [75, 82].

5

CHALLENGES AND FUTURE DIRECTIONS

4.1 Robust and reproducible research

Overarching challenges across the research domains described in this thesis are related to the lack of reproducibility, replicability, and robustness in the literature. Important factors, discussed in the following section, include the role of statistical power, the development of standardized assessment and analysis tools, and reporting. These factors may contribute to the scattered and conflicting findings in the literature, could waste of up to 85% of biomedical research efforts, and hamper the interpretability of the findings of the three scientific chapters [93].

Study design

Statistical power is a strong limitation of task-based fMRI and gut-microbiota sequencing studies, with an average sample size N ~ 50 in published task-based fMRI studies (2019) [94] and N ~ 90 (N_{ADHD} ~40) in the gut-microbiota sequencing studies in ADHD (summarized from recent systematic reviews [49, 50]). Sample size is the largest driver of replicability in neuroimaging studies, followed by peak activation by the task and interindividual variability. Universal power calculations neglect these factors, and assume a known biologically relevant difference, being less useful for exploratory studies and new research fields like that of gut-microbiota studies.

To conduct the studies reported in this thesis, we collected a new cohort (IMpACT2-NL) combining all relevant measures, where we initially aimed to include 400 participants. This number was drastically reduced, as the obligatory closing of the labs from the Donders Centre for Cognitive Neuroimaging halted data acquisition during the COVID-19 pandemic in 2020. When the labs reopened, we had lost previously screened participants, and additional restrictive rules and reduced access to the labs hampered the acquisition. The IMpACT2-NL dataset contains 173 inclusions and 153 participants with complete data. In the following chapters, subsets with the relevant data were included. The continuation with reduced protocols, including online questionnaires and phone instructions for sample collection (as implemented in IMpACT-light), is a promising way to avoid complete standstill during problematic phases of such research projects in the future.

While the sample size of the presented scientific chapters exceeds most previous research (**Chapter 2**, N_{fMRI} = 156, **Chapter 4**, $N_{Diet+Microbiome}$ = 163), and includes a meta-analytic approach, where we reached out to the authors of 11 studies (**Chapter 3**, gut-microbiome, $N_{AllCohortsMicrobiome}$ = 617) to improve individual study sample size limitations, it remains unclear if this data suffices to detect presumably small effects in heterogeneous populations like ADHD. Evidently, the most promising approach to overcome power limitations in heterogeneous disorders like ADHD is based on large-scale studies, such as population-based cohorts. However, smaller individual studies may still be of use, e.g. through data sharing with meta-analyses/consortia (e.g. we shared neuroimaging data from the IMpACT2-NL study with big consortia [95], uploaded relevant data of **Chapters 2 and 3** to public data repositories and applied meta-analysis in **Chapter 2**), the analyses of secondary features for hypothesis-generating purposes, or through the use of methods that respect the interindividual differences (e.g. normative modeling) [94, 96, 97]. Additionally, the high number of variables in both domains, with hundreds of voxels and features to compare between groups or associate with phenotypic data, increases false discovery rates of the classic statistical tests. Data-driven approaches, including the use of machine learning to select informative features (as applied in **Chapter 3** of this thesis) have found application across research domains. While these approaches may, combined with appropriate statistical approaches, reduce statistical testing burden, and reduce the risk bias compared to hypothesis-driven research, the results often are harder to interpret, and appropriate learning of patterns within the data requires large amounts of data.

Measurement Error

Differences in assessment tools and analytical approaches represent a large source of variability introduced across research fields. Differences in diagnostic or dietary assessment, collection and storing of fecal samples, and fMRI recording have shown to produce different results [98, 99]. Data quality control, preprocessing and analysis methods have similar influence on the reliability of results, particularly in gut-microbiome and neuroimaging research [76, 98, 100]. Recent studies summarizing best-practices underline the importance of the development of flexible open-source pipelines, providing standardized data collection and quality control protocols, as well as the development of reliability-enhancing methods [93, 98, 99]. Also preregistration of studies, the use of reporting guidelines available for neuroimaging and microbiota studies, or documentation of the methods (e.g. notebooks/markdowns, GitHub) can improve the reproducibility of results, help the detection of limitations or flaws, and reduce bias and replicability issues [94, 101]. In **Chapters 3 and 4** of this thesis, we have applied a standardized preprocessing pipeline, analyzed data across statistical approaches (interpreting converging results), and reported microbiota findings according to recent guidelines, to overcome some of these limitations and uploaded the QIIME2 scripts and R markdowns used to generate the results to public data repositories.

4.1.1 Functional neuroimaging in ADHD and reactive aggression

The clinical value of functional neuroimaging for ADHD and reactive aggression is currently limited. Task-based fMRI is often used to assess brain functioning in different cognitive domains relevant for externalizing behaviors (e.g. inhibition, emotion processing). While the resulting activation patterns are interpretable, given the task and the context of the literature, task-based studies are limited to a fraction of a cognitive domain (e.g. the dynamic facial expression task in **Chapter 2** of this thesis is used to interpret emotion regulation) and is biased by previously reported findings (e.g. regions like the amygdala are overrepresented in the literature due to region of interest and seed-based approaches in task-based fMRI). Furthermore, task-based neuroimaging results are barely reproducible [98, 102]. Not only small sample sizes, heterogeneous tasks and performances of individuals with ADHD are limiting factors, recent large-scale efforts have shown very low reliability and stability of task-elicited activity within subject even in large samples with standardized protocols across tasks [103]. In contrast, rsfMRI studies show somewhat higher reliability and stability of connectivity maps (resting-state networks) [104]. They are not limited to a cognitive domain, have reduced variability introduced by different protocols, data-driven independent component analyses avoid literature bias, and rsfMRI data is available in many neuroimaging cohorts. While data-driven rsfMRI analyses are promising tools, the resulting independent components can be difficult to interpret, are more stable in large samples, and (similar to task-based fMRI) arbitrary choices have to be made, e.g. the number of components to extract. Preprocessing, different nomenclature and delineation across atlases and research groups makes reproducible results even across data-driven studies difficult. While the field is slowly moving away from previously mentioned underpowered individual studies towards data-driven approaches and meta-analytic consortia research like Enhancing Neuroimaging Genetics through Meta-Analysis (ENIGMA) [105] (to which we contributed neuroimaging data from the IMpACT2-NL cohort [95]) and large population-based dimensional studies like the Adolescent Brain Cognitive Development study (ABCD) [106], the focus still lies on isolated features of brain functioning. Instead of investigating isolated features, a recent study has developed the so-called polyneuro risk score for ADHD, in which cumulative effects across neuroimaging domains are combined - similar to polygenetic risk scores, this method may have improved reproducibility and clinical value [107, 108].

4.1.2 Gut-microbiome research in ADHD and reactive aggression

Previously discussed challenges concerning the robustness and reproducibility of individual studies and low-resolution sequencing demand for large-scale metagenomic studies. To produce reliable results, harmonized protocols are needed, not only for the processing and analyses, but also for the study setup, including wet-lab choices, as was shown in **Chapter 3** by the prominent differences between cohorts with different wet-lab procedures, where processing and analysis were harmonized. One challenge that has particularly shown in gut-microbiota studies of ADHD is the risk of bias, for example in relation to age (mixed results from children, adolescents, and adults, with skewed age distributions and younger ADHD populations) and sex (all studies except one [61] were biased towards male participants with two or three times as many males compared to females in the ADHD group). We investigated adults with a balanced distribution of male and female participants (52% male participants across all four studies, 55% in the ADHD groups). Several research groups have made an effort to investigate the validity of analysis tools and develop best-practices to improve reliability of gut-microbiome studies if followed [76, 99, 100]. Also related to potential confounding variables, future research of ADHD should include the potential interaction of gut-microbiota with the medical treatment of ADHD.

4.1.3 Research of dietary patterns in ADHD and reactive aggression

Observational studies of dietary behavior usually assess food intake by the use of food-frequency questionnaires (FFQ). These food frequency questionnaires are often designed for specific countries or regions and compromise between precision (self-report measures are potentially skewed, particularly in adults), comprehensiveness, and practicality, resulting in a variety of applied tools [109]. Food intake is dependent on personal preferences, modified by cultural, social, and economic factors. Low resolution and outdated food item lists (due to rapidly changing assortment of available processed foods), missing information on food processing and nutritional value further complicate the classification as health promoting (healthy) or unhealthy [109]. Technological advances can improve practicality, comprehensiveness, and precision: the use of food tracking apps with QR-/and barcode scanning features, for example, enable extracting nutritional information from comprehensive macro-nutrient databases created and updated by AI models, reducing the effort of food intake tracking, at least for products available in the supermarket. In combination with the repeated assessment of ADHD symptoms and/or other behaviors, similar to ecological momentary assessment [86], these studies may not only evaluate general associations, but they could also provide information about short-term effects at the macronutrient level. Another important challenge of association studies of dietary habits with e.g. ADHD, is the inseparability of cause and consequence – food intake may be influenced by - or have an influence on ADHD symptoms. Dietary intervention studies, reporting ameliorating influences of some dietary restrictions or supplementation on ADHD symptoms, may suggest a causal role of diet. However, the composition of this diet is not necessarily the driving factor affecting ADHD symptoms and behavior. A recent study, comparing two dietary interventions (a healthy diet and a restrictive elimination diet) with care as usual, has shown that already the adherence to a healthy diet, as advised by Dutch governmental guidelines, improves ADHD symptoms and emotion dysregulation comparable to care as usual [TRACE]. This result could reflect improved nutritional value of the healthy diet, but it may also indicate the beneficial effect that regularization of behavior and routines can have on ADHD symptoms. Including control-interventions introducing similarly structured routines may overcome this limitation. However, next to diet, other lifestyle measures, such as sleep and exercise can have an important influence on the metabolism and the availability of nutrients in our body and should be considered in the context of observational studies.

4.2 Functional interpretations

Brain systems of emotion processing, gut-microbiota, and dietary habits may point towards biological pathways shared between ADHD and reactive aggression. The following section describes and discusses potential evidence for common pathways, see Box 1, evaluates the results of the three scientific chapters of this thesis in this context and proposes future directions.

Disclaimer

The pathways discussed in this section are selected for their potential association with the role of the gut-microbiome-brain axis in ADHD and reactive aggression. The discussed pathways are **highly unspecific to the investigated phenotypes**, as they reflect broad natural biological functions. Equally unspecific and of low-resolution are the dietary patterns, gut-microbial alterations, and brain functional measures, rendering all interpretations in the context of potential mechanisms speculative. The association studies of this thesis cannot disentangle **cause or consequence** or provide functional information. Immune, endocrine, and neurotransmitter **pathways interact** with each other and other factors. Likely, externalizing behavior results from the combination and interaction of etiological factors and biological pathways.

Evidence concerning brain functioning and future directions

Serotonin pathways project from the so-called raphe nuclei in the brainstem to the entire cortex, the hippocampus, and the thalamus. They are involved in emotional processing, with decreased serotonergic activity resulting in decreased cognition and an emotional bias towards negative stimuli [110]. The mesocorticolimbic pathway of dopamine signaling, originating in the ventral tegmental area and passing through the amygdala, nucleus accumbens, and hippocampus to the prefrontal cortex, has previously been reported in association with emotional dysregulation and emotional symptoms in ADHD [111]. Higher activity in the **hippocampus** and **middle and superior frontal areas** in association with elevated reactive aggression in ADHD in **Chapter 2** may be linked to altered dopamine and serotonin signaling, but to investigate functional pathways, large-scale PET studies investigating emotion processing in ADHD are needed to further examine the roles of these and other neurotransmitters across the brain [112]. To investigate inflammation or immune activation in the brain, PET scans (targeting microglial activation or receptors associated with inflammation, such as translocator protein), or diffusion tensor imaging (e.g. alterations in fractional anisotropy (FA) reflecting demyelination in consequence of neuroinflammatory processes) may be useful. There is evidence for altered FA in people with ADHD across lifespan, although potentially influenced by uncorrected increased head movement in the ADHD groups of individual studies [113].

Evidence concerning gut-microbiota and future directions

Gut-microbiota can influence the availability of different neurotransmitters and fulfills various immune regulating functions. [114] [52]. Fecal transplant studies in animals have shown correlations of the abundance of several features with altered levels of blood and/or brain tryptophan, serotonin, norepinephrine, and dopamine, alongside increased aggressive behavior [115]. Also in children with ADHD, metabolic pathway analysis revealed significant differences in neurotransmitter systems (e.g. serotonin and dopamine) [59], and metabolic flux analyses suggest influences of several bacterial species on differences in the import and export of glutamate (neurotransmitter and precursor of GABA), tryptophan (precursor of serotonin), and tyrosine (precursor of dopamine) between individuals with and without ADHD [116]. Many gut-microbiota that are associated with ADHD have been suggested to be involved in the SCFA metabolism (among others, the genera ***Lactobacillus*** and ***Eubacterium xylanophilum*** in reactive aggression and adult ADHD in **Chapters 3** and **4**) [49, 51, 53]. SCFAs can inhibit the production of pro-inflammatory cytokines/mediators, such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and factor-kappa B (NF-κB), and can influence the differentiation and function of immune cells; for a review see [117, 118]. Also indirect effects on neurotransmitter pathways are possible [114]. Metabolic flux analysis has shown that the SCFA im- and export differs between individuals with and without ADHD, based on the composition of their gut-microbiota [67].

Neurotransmission systems

Several neurotransmitter systems are implicated in ADHD, including dopamine, norepinephrine, and serotonin. These neurotransmitters play crucial roles in regulating attention, motivation, and mood, or the body's "fight or flight" response. They are common targets of pharmacological interventions in ADHD: Stimulant medications (e.g., methylphenidate and amphetamine) increase the availability of dopamine, non-stimulant medications like atomoxetine increase the levels of norepinephrine, and certain antidepressants, sometimes prescribed in conjunction with other ADHD treatment, increase the serotonin availability in the brain. Recent studies have further suggested a role of glutamate and Gamma-Aminobutyric Acid (GABA) in ADHD; for recent reviews see [5, 6]. Similar neurotransmitter disbalances are suggested to play a role in reactive aggression. Low levels of serotonin have been associated with increased impulsivity and aggression, and selective serotonin reuptake inhibitors (SSRIs) can be prescribed to reduce aggressive behavior. Dysregulations of norepinephrine may contribute to heightened states of arousal and reactivity, dysregulation of inhibitory signaling through GABA may lead to decreased inhibition and increased likelihood of aggressive behaviors, and also glutamate and dopamine have been associated with aggression phenotypes; for recent reviews see [9, 10].

The immune system

Immune system activation has been proposed as a potential mechanistic factor for neuropsychiatric disorders. Inflammation is a natural response of the immune system to challenges such as infections or injuries. Neuroinflammation and long-term activation of the immune system are suggested to impact brain structure and function and may also be relevant for the development or exacerbation of ADHD symptoms. Next to genetic factors, environmental factors, such as prenatal exposure to infections or early-life stress, may contribute to inflammation and increase the risk for ADHD and reactive aggression. Elevated levels of inflammatory markers have been associated with ADHD. However, due to different choices of inflammatory markers across studies, there is no consensus on the particular markers involved. Additionally, high comorbidity with immune disorders and microglial activation have been reported in ADHD, pointing towards inflammatory processes in people with ADHD; for a recent review see [11]. In reactive aggression, cortisol stress responses resulting from perceived provocation or threat are discussed to influence long-term low-grade inflammation. Similar to ADHD, altered levels of cortisol and proinflammatory blood markers have been observed in the context of aggressive behavior; for a recent review see [12, 13].

Other potential pathways

Several other biological pathways may be relevant for the co-occurrence of reactive aggression and ADHD. One example is the endocrine system. Hormones frequently investigated in the context of aggression are testosterone, due to frequently observed sex-biases in aggressive behaviors, and cortisol, associated with stress. Also in Chapter 2 of this thesis, sex differences in aggression and aggression-associated brain functioning were observed. Reactive aggression has been linked to high levels of testosterone and low levels of basal cortisol. Notably, the results of some studies differed between sexes, suggesting associations of estradiol with high impulsivity and reactive aggression in women, and were dependent on co-morbid disorders; for a review see [17, 18]. Also in ADHD, alterations of sex hormones and lower basal cortisol levels have been reported [24, 25]. Oxytocin, often associated with social bonding, and oxytocin-related genes have received attention in aggression and ADHD research. Some studies suggest oxytocin involvement in altered neurodevelopment, lower serum levels of oxytocin in ADHD and reactive aggression, and beneficial effects of oxytocin interventions on reactive aggressive behavior in men [18, 26-28]. Despite the complexity of the neuroendocrine system, its interactions with the immune system, diet, and the gut-brain axis, neuroendocrinology may play a role in ADHD and aggression.

Box 1. Potential pathways involved in ADHD and reactive aggression.

Additionally, ***Ruminococcus_torques,*** found in association with ADHD in **Chapter 3**, has also been implicated in immune-related disorders, such as inflammatory gut diseases and autism spectrum disorder, and influenced gut-barrier integrity – another important immune-regulating factor maintained by gut-microbiota [119-123]. Few studies have targeted the gut-microbiota in aggression, but in the context of schizophrenia, alterations in the gut-microbiota composition were associated with plasma cytokines and aggressive behavior, suggesting ongoing inflammatory processes to play a role [67]. The genus-level results of our 16S sequencing studies do not provide information about the functional potential of the bacteria. Functional studies, investigating fecal metabolites, together with plasma-levels of inflammatory markers, may help clarify the role of the microbiota in chronic systemic inflammation, especially longitudinal studies and birth cohorts are promising sources to investigate the role of inflammation for altered neurodevelopment in ADHD. Also functional metagenomics and culturing studies investigating the influence of bacterial strains belonging to these genera are needed, and should be combined with metabolic analyses in the feces, the bloodstream, and positron emission tomography (PET) to further examine the potential influence of these gut-microbiota on neurotransmitter levels in the brain.

Evidence concerning food intake

Next to evident influences on energy homeostasis, consumption of various food groups containing neurotransmitters and/or their precursors may influence their availability in the system (even though the effects of food intake on neurotransmitter availability in the brain have yet to be proven) [124]. In turn, neurotransmitters like GABA, serotonin, and dopamine can regulate appetite and food intake, e.g. through activation of the reward system [125, 126]. Diet may also influence immune activation. Dietary patterns characterized by the consumption of processed foods, sugar, and certain fats may have proinflammatory influences. Conversely, diets rich in polyunsaturated fatty acids, antioxidants, and nutrients from vegetables, legumes, grains, and fruit may have anti-inflammatory properties; for a review see [127]. In children with ADHD, some studies reported higher intake of high processed and high caloric foods (rich in sugar and saturated fats) and less intake of nutrients like fiber, poly-unsaturated fatty acids, and minerals [81]. Nutritional or dietary interventions, reducing foods with pro-inflammatory potential or introducing anti-inflammatory supplements, have shown beneficial effects on ADHD symptomatology in some studies [79, 128]. In a population-based study, inflammatory markers mediated the association of diet with disinhibition, a behavior associated with ADHD [129]. The dietary pattern associated with reactive aggression of **Chapter 4** was characterized by high consumption of meat, dairy, and sweetened beverages and low consumption of vegetables. These broad descriptions of food groups do not implicate neurotransmitter (or precursor) content, and do not allow for a general interpretation, but the consumption of sweet foods/drinks, for example, has been associated with increased activation of the reward system [126]. While influences of dopamine signaling may contribute to sweet drink choices in reactive aggression, these interpretations are highly speculative and isolated from other components of the associated dietary pattern. The high-energy diet pattern may contain pro-inflammatory foods, as indicated by frequent consumption of sweet beverages, and is characterized by low consumption of the vegetables generally assigned high content of fiber (needed to produce SCFA) and anti-inflammatory phytochemicals. Some research suggests that the inflammatory properties of food may be brought about by gut-microbiota metabolism. Interestingly, the mediating role of gut-microbiota in the association of reactive aggression with the high-energy diet remains unclear, as mediation analyses of selected gut-microbiota did not yield significant results in our interventional study. Thorough dietary assessment, including nutritional information, and integration with observational and interventional diet studies, as well as studies of gut-microbiota composition, metabolites, and inflammatory markers, is needed to investigate the role of diet in inflammation and reactive aggression. More detailed dietary information, in combination with metabolic analyses and PET studies concerning food choices may complement this information.

4.3 Integration of findings

The view of psychiatric disorders as disorders of the brain has evolved. As an organ of the body, the brain is constantly interacting with other bodily systems including, but not restricted to, mental health. In fact, a recent paper found that a score based on health measures for seven other (i.e. somatic) body systems predicting mental health better [130]. The interactions of the brain with the gut (microbiome) have recently received more attention in relation to mental health. One of the biggest challenges concerning human research of the gut-brain axis is the integration of research findings from distant body sites and across disciplines. Despite speculations on the impact of gut-microbiota alterations on brain functioning in particular phenotypes, tracking these associations from the gut to the brain in humans is proving difficult. The analysis of biological pathways and the measurement of pathway markers at different body sites may provide evidence for the theoretical gut-brain influences, but the number of potential markers, different body sites, and the complexity of interactions in the human body render biological approaches to summarize gut-brain interactions likely unsuccessful. In contrast, some studies have attempted to investigate statistical associations between gut-microbiota and brain functioning. To this end, most authors selected significant findings or targets of interest from each domain and introduced them to associations; for a recent systematic review see [131]. Considering the lack of reliability and reproducibility in both research fields, the small sample sizes and vast amounts of variables to consider, this approach is highly biased by the selection steps and potentially produces high false discovery rates. Most studies used either correlations or multiple regressions, assuming independence of either the gut-microbiota, or the introduced functional connectivity measures. Data-driven selection in each domain, like feature selection applied in Chapter 3, or data reduction like clustering approaches may help to reduce the selection bias. More promising attempts are multivariate approaches like ordination (co-inertia) or generalized canonical correlation analysis, or more data-driven methods like linked independent component analysis [131, 132]. Co-inertia analysis simultaneously finds trends across datasets, being suitable for studies with more variables than samples [133]. In linked independent component analysis (ICA), developed to assess co-variation patterns across neuroimaging modalities, the four-dimensional fMRI images per subject and the abundance table can theoretically be introduced directly without further selection, and - linked in a mixing matrix - allow for the simultaneous spatial decomposition of the functional scans together with the gut-microbial abundances [134].However, the interpretability of both, canonical correlation analysis and the components resulting from linked ICA can be difficult; a trade-off between the selection of relevant phenotypes and simultaneous decomposition, as applied by [135] may improve the interpretability of results.

Note: Future directions in the conceptualization of ADHD

ADHD is characterized by a diagnostic framework with clear cutoffs – a strict number of symptoms that must have been present during childhood and is accompanied by impairment related to these symptoms - either you have it, or you don't [4, 5]. While these somewhat arbitrary borders have been defined to justify the access to specialized care for individuals in need, they might not fulfill this objective in the light of more recent research. Accumulating research points towards a continuous distribution of ADHD symptoms in the general population, and comorbidities across psychiatric disorders have made it clear that biological processes underlie clusters of behaviors rather than diagnoses [4, 5]. Research on clinically diagnosed ADHD compares the extremes of the distribution, the biggest possible difference. However, these categorical approaches tend to group together individuals with highly heterogeneous presentation of symptoms, impairment, and potential etiology. This might not only hamper the detection of mechanisms involved, it can increase the stigma experienced by affected individuals, and it may prevent people with high risk for ADHD or sub-threshold symptoms from receiving treatment. Also in our studies, results across microbiota and brain functioning show individual patterns associated with the symptomatic subdomains of ADHD. Studying ADHD symptoms dimensionally in large (population-based or cross-disorder) samples as well as using normative modelling are promising approaches to study biological aspects of ADHD in the future.

CLINICAL AND SOCIAL IMPLICATIONS

Little is known about the factors underlying the frequent co-occurrence of adult ADHD with reactive aggression. While aggressive behavior may be clinically and criminologically relevant, and shared etiological mechanisms are proposed, current treatment approaches for ADHD do not sufficiently ameliorate aggression and have to be adapted to co-occurring aggressive behavior [136]. While the direct translation of findings presented in this thesis to the clinical context is limited, the individual association studies may contain indirect implications, and together with evidence from other studies can inform treatment and policy.

In **Chapter 2**, reactive aggression was associated with hyperactive-impulsive (but not inattention) symptoms and social impairment. A particular vulnerability for reactive aggression in adults with high hyperactivity/impulsivity symptoms, combined with the missing pharmacological treatment effects on reactive aggression, stress the importance to investigate alternative treatment approaches. This subgroup may particularly benefit from interventions targeting aggressive behavior and resulting social problems. Across all studies, different associations between aggression and the subdomains of ADHD symptoms have been found, supporting the heterogeneous dimensional view on ADHD, where different individuals are affected by different expressions of symptoms and behaviors. Clustering based on such associated behaviors and symptoms bears the potential for more personalized treatment approaches.

While brain functional alterations - in theory and if they were robust - could potentially serve as predictive or diagnostic biomarkers to help prevention or early intervention of unwanted behavior, this clinical relevance is currently strongly limited. Carefully interpreted, results like the partially altered brain functioning observed in **Chapter 2,** that points towards involved psychological processes, may be considered in the development of behavioral interventions targeting reactive aggression and other emotional symptoms in ADHD. Instead of task based functioning, a complex combination of structural and functional alterations more reflects biological markers.

Similarly, links between the gut-microbiome and externalizing behavior, not yet suitable as biomarkers, may support the development of novel interventions, but also rehabilitation programs to reduce adverse outcomes associated with ADHD and aggressive tendencies. Research on the gut-microbiota brain axis has stirred public attention, and media coverage of gut microbiome studies has reached newspapers and television, beyond scientific journals [137]. Due to the media attention, companies, as well as fitness and lifestyle influencers (including e.g. Neuroscientist Andrew Huberman, and self-proclaimed “microbiome-influencer” Norbert Bomba) pick up on the topic, simplifying presented information into recommendations (so-called “bio-hacks”), or for commercial purposes such as the marketing and production of pre- and probiotica supposedly improving gut-health. Recently, the field of the so-called psychobiotics has grown on the basis of hypothetical beneficial effects of microbiota interventions targeting mental health [138]. The commercial interest in this topic has seemingly resulted in the publication of more probiotic intervention studies than basic research identifying robust targets. Next to the commercialization of microbiome-related interventions, dietary interventions, so-called psychobiotic diets, containing foods influencing beneficial gut-microbiota feeding and growth, have been proposed to support a healthy gut-flora and propagate mental health. Observational study results, gut-microbiota, diet, and behavior associations may be misinterpreted and their causal relevance overestimated through media-coverage and the oversimplification of findings.

Lastly, also potential hints towards relevant biological pathways from microbiota and diet studies, like inflammation for reactive aggression, may have implications for therapeutic interventions. Approaches that target stress reduction, inflammation modulation, or both could be explored in the management of aggression.

CONCLUSION

In conclusion, in this thesis, I described studies of brain functioning, gut-microbiota, and diet in ADHD and reactive aggression. I identified a partially different brain functional mechanism of emotion processing in ADHD linked to reactive aggression, involving cortical and subcortical structures of emotional hyperreactivity and cognitive control. I further detected robust associations of gut-microbiota composition with reactive aggression and ADHD, overlapping signatures like decreased abundance of the genus Eubacterium_xylanophilum in both behaviors, and a link between a high-energy diet and reactive aggression. Next to the identification of these associations, with the work of this thesis, I underline the importance of robust results, the required harmonization, and generalization of results across cohorts and methods. To this end, future studies should focus on large dimensional samples, apply standardized assessment and analysis tools, and integrate findings across research domains and extend this research to functional studies and biological pathways.

REFERENCES

1. Cassidy-Bushrow, A.E., et al., *Early-life gut microbiota and attention deficit hyperactivity disorder in preadolescents.* Pediatric research, 2023. **93**(7): p. 2051-2060.
2. Gao, W., et al., *Gut microbiome and brain functional connectivity in infants-a preliminary study focusing on the amygdala.* Psychopharmacology, 2019. **236**: p. 1641-1651.
3. Carlson, A.L., et al., *Infant gut microbiome associated with cognitive development.* Biological psychiatry, 2018. **83**(2): p. 148-159.
4. Agnew-Blais, J. and G. Michelini, *Taking stock of the present and looking to the future of ADHD research: a commentary on Sonuga-Barke et al.(2023).* Journal of Child Psychology and Psychiatry, 2023. **64**(4): p. 533-536.
5. Sonuga-Barke, E.J., et al., *Annual Research Review: Perspectives on progress in ADHD science–from characterization to cause.* Journal of Child Psychology and Psychiatry, 2023. **64**(4): p. 506-532.
6. Mehta, T.R., et al., *Neurobiology of ADHD: a review.* Current Developmental Disorders Reports, 2019. **6**: p. 235-240.
7. Shanahan, F., T.S. Ghosh, and P.W. O'Toole, *The healthy microbiome—what is the definition of a healthy gut microbiome?* Gastroenterology, 2021. **160**(2): p. 483-494.
8. Faraone, S.V., et al., *The worldwide prevalence of ADHD: is it an American condition?* World psychiatry, 2003. **2**(2): p. 104.
9. Narvaes, R. and R.M. Martins de Almeida, *Aggressive behavior and three neurotransmitters: dopamine, GABA, and serotonin—A review of the last 10 years.* Psychology & Neuroscience, 2014. **7**(4): p. 601.
10. Sturmey, P., *Biological Evolution of Violence and Aggression. II: Brains, Neurotransmitters, and Hormones*, in *Violence and Aggression: Integrating Theory, Research, and Practice*. 2022, Springer. p. 121-143.
11. Leffa, D.T., I.L. Torres, and L.A. Rohde, *A review on the role of inflammation in attention-deficit/hyperactivity disorder.* Neuroimmunomodulation, 2019. **25**(5-6): p. 328-333.
12. Takahashi, A., et al., *Aggression, social stress, and the immune system in humans and animal models.* Frontiers in Behavioral Neuroscience, 2018. **12**: p. 56.
13. Petruso, F., et al., *Inflammation and emotion regulation: a narrative review of evidence and mechanisms in emotion dysregulation disorders.* Neuronal Signaling, 2023. **7**(4): p. NS20220077.
14. Murray, A.L., et al., *Developmental relations between ADHD symptoms and reactive versus proactive aggression across childhood and adolescence.* Journal of attention disorders, 2020. **24**(12): p. 1701-1710.
15. McKAY, K.E. and J.M. Halperin, *ADHD, aggression, and antisocial behavior across the lifespan: Interactions with neurochemical and cognitive function.* Annals of the New York Academy of Sciences, 2001. **931**(1): p. 84-96.
16. Hirsch, O., M.L. Chavanon, and H. Christiansen, *Emotional dysregulation subgroups in patients with adult Attention-Deficit/Hyperactivity Disorder (ADHD): a cluster analytic approach.* Scientific Reports, 2019. **9**(1): p. 5639.
17. Gulledge, L., D. Oyebode, and J.R. Donaldson, *The influence of the microbiome on aggressive behavior: an insight into age-related aggression.* FEMS microbiology letters, 2023. **370**: p. fnac114.
18. Romero-Martinez, A., C. Sarrate-Costa, and L. Moya-Albiol, *Reactive vs proactive aggression: A differential psychobiological profile? Conclusions derived from a systematic review.* Neuroscience & Biobehavioral Reviews, 2022. **136**: p. 104626.

19. Rubia, K., *Cognitive neuroscience of attention deficit hyperactivity disorder (ADHD) and its clinical translation.* Frontiers in human neuroscience, 2018. **12**: p. 100.
20. Lindholm, P., et al., *Brain response to facial expressions in adults with adolescent ADHD.* Psychiatry Research: Neuroimaging, 2019. **292**: p. 54-61.
21. Bottelier, M.A., et al., *Age-dependent effects of acute methylphenidate on amygdala reactivity in stimulant treatment-naive patients with attention deficit/hyperactivity disorder.* Psychiatry Research: Neuroimaging, 2017. **269**: p. 36-42.
22. Schlochtermeier, L., et al., *Childhood methylphenidate treatment of ADHD and response to affective stimuli.* European Neuropsychopharmacology, 2011. **21**(8): p. 646-654.
23. Schulz, K.P., et al., *Emotional bias of cognitive control in adults with childhood attention-deficit/hyperactivity disorder.* NeuroImage: Clinical, 2014. **5**: p. 1-9.
24. Chang, J.P.-C., et al., *Cortisol and inflammatory biomarker levels in youths with attention deficit hyperactivity disorder (ADHD): evidence from a systematic review with meta-analysis.* Translational psychiatry, 2021. **11**(1): p. 430.
25. Camara, B., C. Padoin, and B. Bolea, *Relationship between sex hormones, reproductive stages and ADHD: a systematic review.* Archives of women's mental health, 2021: p. 1-8.
26. Sarkar, A. and R.W. Wrangham, *Evolutionary and neuroendocrine foundations of human aggression.* Trends in cognitive sciences, 2023.
27. Lønfeldt, N.N., et al., *Assessing risk of neurodevelopmental disorders after birth with oxytocin: a systematic review and meta-analysis.* Psychological medicine, 2019. **49**(6): p. 881-890.
28. Zhu, R., et al., *Intranasal oxytocin reduces reactive aggression in men but not in women: A computational approach.* Psychoneuroendocrinology, 2019. **108**: p. 172-181.
29. Morawetz, C., et al., *Multiple large-scale neural networks underlying emotion regulation.* Neuroscience & Biobehavioral Reviews, 2020. **116**: p. 382-395.
30. Frank, D., et al., *Emotion regulation: quantitative meta-analysis of functional activation and deactivation.* Neuroscience & Biobehavioral Reviews, 2014. **45**: p. 202-211.
31. Fanning, J.R., et al., *Neural correlates of aggressive behavior in real time: A review of fMRI studies of laboratory reactive aggression.* Current behavioral neuroscience reports, 2017. **4**: p. 138-150.
32. Bertsch, K., J. Florange, and S.C. Herpertz, *Understanding brain mechanisms of reactive aggression.* Current psychiatry reports, 2020. **22**: p. 1-16.
33. Graziano, P.A. and A. Garcia, *Attention-deficit hyperactivity disorder and children's emotion dysregulation: A meta-analysis.* Clinical psychology review, 2016. **46**: p. 106-123.
34. Gasquoine, P.G., *Contributions of the insula to cognition and emotion.* Neuropsychology review, 2014. **24**: p. 77-87.
35. Graff-Radford, J., et al., *Caudate nucleus as a component of networks controlling behavior.* Neurology, 2017. **89**(21): p. 2192-2197.
36. Bubenzer-Busch, S., et al., *Neural correlates of reactive aggression in children with attention-deficit/hyperactivity disorder and comorbid disruptive behaviour disorders.* Acta Psychiatrica Scandinavica, 2016. **133**(4): p. 310-323.
37. Brotman, M.A., et al., *Amygdala activation during emotion processing of neutral faces in children with severe mood dysregulation versus ADHD or bipolar disorder.* American Journal of Psychiatry, 2010. **167**(1): p. 61-69.

38. Posner, J., et al., *The attenuation of dysfunctional emotional processing with stimulant medication: an fMRI study of adolescents with ADHD.* Psychiatry Research: Neuroimaging, 2011. **193**(3): p. 151-160.
39. Cortese, S., et al., *Toward systems neuroscience of ADHD: a meta-analysis of 55 fMRI studies.* American Journal of Psychiatry, 2012. **169**(10): p. 1038-1055.
40. Phelps, E.A., *Human emotion and memory: interactions of the amygdala and hippocampal complex.* Current opinion in neurobiology, 2004. **14**(2): p. 198-202.
41. Spencer, A.E., et al., *Abnormal fear circuitry in attention deficit hyperactivity disorder: a controlled magnetic resonance imaging study.* Psychiatry Research: Neuroimaging, 2017. **262**: p. 55-62.
42. Baweja, R. and J.G. Waxmonsky, *Updates in pharmacologic strategies for emotional dysregulation in attention deficit hyperactivity disorder.* Child and Adolescent Psychiatric Clinics, 2022. **31**(3): p. 479-498.
43. Kohn, N., et al., *Neural network of cognitive emotion regulation—an ALE meta-analysis and MACM analysis.* Neuroimage, 2014. **87**: p. 345-355.
44. Pozzi, E., et al., *Neural correlates of emotion regulation in adolescents and emerging adults: A meta-analytic study.* Biological Psychiatry, 2021. **89**(2): p. 194-204.
45. Kiani, B., et al., *Examining emotion regulation using a distraction and reappraisal task in children and adolescents with and without ADHD.* Current Psychology, 2023: p. 1-9.
46. Materna, L., et al., *Adult patients with ADHD differ from healthy controls in implicit, but not explicit, emotion regulation.* Journal of Psychiatry and Neuroscience, 2019. **44**(5): p. 340-349.
47. Liu, Q., et al., *Emotion dysregulation in adults with ADHD: The role of cognitive reappraisal and expressive suppression.* Journal of affective disorders, 2022. **319**: p. 267-276.
48. Patel, G.H., C. Sestieri, and M. Corbetta, *The evolution of the temporoparietal junction and posterior superior temporal sulcus.* Cortex, 2019. **118**: p. 38-50.
49. Shirvani-Rad, S., et al., *The role of gut microbiota-brain axis in pathophysiology of ADHD: a systematic review.* Journal of Attention Disorders, 2022. **26**(13): p. 1698-1710.
50. Gkougka, D., et al., *Gut microbiome and attention deficit/hyperactivity disorder: a systematic review.* Pediatric Research, 2022. **92**(6): p. 1507-1519.
51. Sukmajaya, A.C., et al., *Systematic review of gut microbiota and attention-deficit hyperactivity disorder (ADHD).* Annals of general psychiatry, 2021. **20**: p. 1-12.
52. Warner, B.B., *The contribution of the gut microbiome to neurodevelopment and neuropsychiatric disorders.* Pediatric Research, 2019. **85**(2): p. 216-224.
53. Wang, N., et al., *Composition of the gut microbiota in attention deficit hyperactivity disorder: a systematic review and meta-analysis.* Frontiers in Endocrinology, 2022. **13**: p. 838941.
54. Wang, L.-J., et al., *Gut microbiota and dietary patterns in children with attention-deficit/hyperactivity disorder.* European child & adolescent psychiatry, 2020. **29**: p. 287-297.
55. Prehn-Kristensen, A., et al., *Reduced microbiome alpha diversity in young patients with ADHD.* PloS one, 2018. **13**(7): p. e0200728.
56. Zhou, G., et al., *Biosynthesis and Characterization of Zinc Oxide Nanoparticles and Their Impact on the Composition of Gut Microbiota in Healthy and Attention-Deficit Hyperactivity Disorder Children.* Frontiers in Microbiology, 2021. **12**: p. 700707.
57. Aarts, E., et al., *Gut microbiome in ADHD and its relation to neural reward anticipation.* PloS one, 2017. **12**(9): p. e0183509.

58. Szopinska-Tokov, J., et al., *Investigating the gut microbiota composition of individuals with attention-deficit/hyperactivity disorder and association with symptoms.* Microorganisms, 2020. **8**(3): p. 406.
59. Wan, L., et al., *Case-control study of the effects of gut microbiota composition on neurotransmitter metabolic pathways in children with attention deficit hyperactivity disorder.* Frontiers in Neuroscience, 2020. **14**: p. 127.
60. Jiang, H.-y., et al., *Gut microbiota profiles in treatment-naïve children with attention deficit hyperactivity disorder.* Behavioural Brain Research, 2018. **347**: p. 408-413.
61. Richarte, V., et al., *Gut microbiota signature in treatment-naïve attention-deficit/hyperactivity disorder.* Translational psychiatry, 2021. **11**(1): p. 382.
62. Akram, H., *Characterizing a link between gut microbiome and attention deficit hyperactive disorder.* 2017.
63. Kirchoff, N.S., M.A. Udell, and T.J. Sharpton, *The gut microbiome correlates with conspecific aggression in a small population of rescued dogs (Canis familiaris).* PeerJ, 2019. **7**: p. e6103.
64. Jia, Y., et al., *Gut microbiome modulates Drosophila aggression through octopamine signaling.* Nature communications, 2021. **12**(1): p. 2698.
65. Ren, C.C., et al., *Photoperiod modulates the gut microbiome and aggressive behavior in Siberian hamsters.* Journal of Experimental Biology, 2020. **223**(3): p. jeb212548.
66. Chen, X., et al., *Profiling the differences of gut microbial structure between schizophrenia patients with and without violent behaviors based on 16S rRNA gene sequencing.* International journal of legal medicine, 2021. **135**: p. 131-141.
67. Deng, H., et al., *Altered gut microbiota and its metabolites correlate with plasma cytokines in schizophrenia inpatients with aggression.* BMC psychiatry, 2022. **22**(1): p. 1-12.
68. Liu, L., et al., *Gut microbiota and its metabolites in depression: from pathogenesis to treatment.* EBioMedicine, 2023. **90**.
69. WU, Y., et al., *Relationship between Intestinal Flora and Cognitive Emotion Regulation in Patients with First-episode Depression.* Chinese General Practice, 2020. **23**(18): p. 2259.
70. Lee, S.-H., et al., *Emotional well-being and gut microbiome profiles by enterotype.* Scientific Reports, 2020. **10**(1): p. 20736.
71. Ke, S., et al., *Gut feelings: associations of emotions and emotion regulation with the gut microbiome in women.* Psychological Medicine, 2023: p. 1-10.
72. Fox, M., et al., *Development of the infant gut microbiome predicts temperament across the first year of life.* Development and psychopathology, 2022. **34**(5): p. 1914-1925.
73. Carbia, C., et al., *A biological framework for emotional dysregulation in alcohol misuse: from gut to brain.* Molecular Psychiatry, 2021. **26**(4): p. 1098-1118.
74. Brown, L.C., et al., *Pharmacomicrobiomics of antidepressants in depression: A systematic review.* Journal of Personalized Medicine, 2023. **13**(7): p. 1086.
75. Tcherni-Buzzeo, M., *Dietary interventions, the gut microbiome, and aggressive behavior: Review of research evidence and potential next steps.* Aggressive behavior, 2023. **49**(1): p. 15-32.
76. Nearing, J.T., et al., *Microbiome differential abundance methods produce different results across 38 datasets.* Nature Communications, 2022. **13**(1): p. 342.
77. Piquer-Esteban, S., et al., *Exploring the universal healthy human gut microbiota around the World.* Computational and Structural Biotechnology Journal, 2022. **20**: p. 421-433.

78. Lee, M.-J., et al., *Association between gut microbiota and emotional-behavioral symptoms in children with attention-deficit/hyperactivity disorder.* Journal of Personalized Medicine, 2022. **12**(10): p. 1634.
79. Del-Ponte, B., et al., *Dietary patterns and attention deficit/hyperactivity disorder (ADHD): a systematic review and meta-analysis.* Journal of affective disorders, 2019. **252**: p. 160-173.
80. Shareghfarid, E., et al., *Empirically derived dietary patterns and food groups intake in relation with Attention Deficit/Hyperactivity Disorder (ADHD): A systematic review and meta-analysis.* Clinical nutrition ESPEN, 2020. **36**: p. 28-35.
81. Pinto, S., et al., *Eating Patterns and Dietary Interventions in ADHD: A Narrative Review.* Nutrients, 2022. **14**(20): p. 4332.
82. Breda, V., et al., *Is there a place for dietetic interventions in adult ADHD?* Progress in Neuro-Psychopharmacology and Biological Psychiatry, 2022: p. 110613.
83. Papanastasiou, G., A. Drigas, and P. Papanastasiou, *The association of diet quality and lifestyle factors in children and adults with ADHD: a systematic review and meta-analysis.* Scientific Electronic Archives, 2021. **14**(9).
84. Li, L., et al., *Attention-deficit/hyperactivity disorder symptoms and dietary habits in adulthood: A large population-based twin study in Sweden.* American Journal of Medical Genetics Part B: Neuropsychiatric Genetics, 2020. **183**(8): p. 475-485.
85. Weissenberger, S., et al., *ADHD symptoms in adults and time perspectives–findings from a Czech national sample.* Frontiers in psychology, 2020: p. 950.
86. Ruf, A., et al., *Microtemporal Dynamics of Dietary Intake, Physical Activity, and Impulsivity in Adult Attention-Deficit/Hyperactivity Disorder: Ecological Momentary Assessment Study Within Nutritional Psychiatry.* JMIR Mental Health, 2023. **10**(1): p. e46550.
87. Wolmar, A., *Associations between health literacy, ADHD, and eating behaviour*. 2023.
88. Gürbüzer, N., et al., *The Relationship Between Eating-Attitudes and Clinical Characteristics, Agouti-Related Peptide, and Other Biochemical Markers in Adult-Attention Deficit Hyperactivity Disorder.* Journal of Attention Disorders, 2023. **27**(4): p. 394-409.
89. Holt, M., *Association of Dietary Intake Patterns with Emotion Regulation*. 2013.
90. Lipsanen, J., et al., *Temperament profiles are associated with dietary behavior from childhood to adulthood.* Appetite, 2020. **151**: p. 104681.
91. Riggs, N.R., et al., *Executive cognitive function and food intake in children.* Journal of nutrition education and behavior, 2010. **42**(6): p. 398-403.
92. Vollrath, M.E., et al., *Associations between temperament at age 1.5 years and obesogenic diet at ages 3 and 7 years.* Journal of developmental and behavioral pediatrics: JDBP, 2012. **33**(9): p. 721.
93. Munafò, M.R., et al., *A manifesto for reproducible science.* Nature human behaviour, 2017. **1**(1): p. 1-9.
94. Acar, F., et al., *reporting practices for task fMRI studies.* Neuroinformatics, 2023. **21**(1): p. 221-242.
95. Patel, Y., et al., *Virtual ontogeny of cortical growth preceding mental illness.* Biological psychiatry, 2022. **92**(4): p. 299-313.
96. Turner, B.O., et al., *Small sample sizes reduce the replicability of task-based fMRI studies.* Communications Biology, 2018. **1**(1): p. 62.
97. Bossier, H., et al., *The empirical replicability of task-based fMRI as a function of sample size.* NeuroImage, 2020. **212**: p. 116601.
98. Botvinik-Nezer, R. and T.D. Wager, *Reproducibility in neuroimaging analysis: Challenges and solutions.* Biological Psychiatry: Cognitive Neuroscience and Neuroimaging, 2022.

99. Schloss, P.D., *Identifying and overcoming threats to reproducibility, replicability, robustness, and generalizability in microbiome research*. MBio, 2018. **9**(3): p. 10.1128/mbio. 00525-18.

100. Bharti, R. and D.G. Grimm, *Current challenges and best-practice protocols for microbiome analysis*. Briefings in bioinformatics, 2021. **22**(1): p. 178-193.

101. Mirzayi, C., et al., *Reporting guidelines for human microbiome research: the STORMS checklist*. Nature medicine, 2021. **27**(11): p. 1885-1892.

102. Constable, R.T., *Challenges in fMRI and its limitations*. Functional neuroradiology: principles and clinical applications, 2023: p. 497-510.

103. Kennedy, J.T., et al., *Reliability and stability challenges in ABCD task fMRI data*. Neuroimage, 2022. **252**: p. 119046.

104. Pannunzi, M., et al., *Resting-state fMRI correlations: From link-wise unreliability to whole brain stability*. Neuroimage, 2017. **157**: p. 250-262.

105. Thompson, P.M., et al., *The ENIGMA Consortium: large-scale collaborative analyses of neuroimaging and genetic data*. Brain imaging and behavior, 2014. **8**: p. 153-182.

106. Karcher, N.R. and D.M. Barch, *The ABCD study: understanding the development of risk for mental and physical health outcomes*. Neuropsychopharmacology, 2021. **46**(1): p. 131-142.

107. Byington, N., et al., *Polyneuro risk scores capture widely distributed connectivity patterns of cognition*. Developmental Cognitive Neuroscience, 2023. **60**: p. 101231.

108. Mooney, M.A., et al., *Cumulative effects of resting-state connectivity across all brain networks significantly correlate with ADHD symptoms*. MedRxiv, 2021: p. 2021.11. 16.21266121.

109. Cade, J.E., *Measuring diet in the 21st century: use of new technologies*. Proceedings of the Nutrition Society, 2017. **76**(3): p. 276-282.

110. Pourhamzeh, M., et al., *The roles of serotonin in neuropsychiatric disorders*. Cellular and molecular neurobiology, 2022. **42**(6): p. 1671-1692.

111. Mohamadian, M., et al., *Mood and behavior regulation: interaction of lithium and dopaminergic system*. Naunyn-Schmiedeberg's Archives of Pharmacology, 2023: p. 1-21.

112. Yamamoto, M. and T. Inada, *Positron emission tomography studies in adult patients with attention-deficit/hyperactivity disorder*. Japanese Journal of Radiology, 2023. **41**(4): p. 382-392.

113. Aoki, Y., S. Cortese, and F.X. Castellanos, *Research Review: Diffusion tensor imaging studies of attention-deficit/hyperactivity disorder: meta-analyses and reflections on head motion*. Journal of Child Psychology and Psychiatry, 2018. **59**(3): p. 193-202.

114. Dicks, L.M., *Gut bacteria and neurotransmitters*. Microorganisms, 2022. **10**(9): p. 1838.

115. Fu, Y., et al., *Cecal microbiota transplantation: unique influence of cecal microbiota from divergently selected inbred donor lines on cecal microbial profile, serotonergic activity, and aggressive behavior of recipient chickens*. Journal of Animal Science and Biotechnology, 2023. **14**(1): p. 1-16.

116. Taş, E. and K.O. Ülgen, *Understanding the ADHD-Gut Axis by Metabolic Network Analysis*. Metabolites, 2023. **13**(5): p. 592.

117. Liu, X.-f., et al., *Regulation of short-chain fatty acids in the immune system*. Frontiers in Immunology, 2023. **14**: p. 1186892.

118. Yao, Y., et al., *The role of short-chain fatty acids in immunity, inflammation and metabolism*. Critical reviews in food science and nutrition, 2022. **62**(1): p. 1-12.

119. Hoskins, L.C., et al., *Mucin degradation in human colon ecosystems. Isolation and properties of fecal strains that degrade ABH blood group antigens and oligosaccharides from mucin glycoproteins*. The Journal of clinical investigation, 1985. **75**(3): p. 944-953.

120. Malinen, E., et al., *Association of symptoms with gastrointestinal microbiota in irritable bowel syndrome.* World journal of gastroenterology: WJG, 2010. **16**(36): p. 4532.

121. Joossens, M., et al., *Dysbiosis of the faecal microbiota in patients with Crohn's disease and their unaffected relatives.* Gut, 2011. **60**(5): p. 631-637.

122. Iglesias-Vázquez, L., et al., *Composition of gut microbiota in children with autism spectrum disorder: a systematic review and meta-analysis.* Nutrients, 2020. **12**(3): p. 792.

123. Zhang, T., et al., *Perturbations of Gut Microbiota Compositions in Autism Spectrum Disorder: A Review and Meta-analysis.* 2023.

124. Gasmi, A., et al., *Neurotransmitters Regulation and Food Intake: The Role of Dietary Sources in Neurotransmission.* Molecules, 2022. **28**(1): p. 210.

125. Miller, G.D., *Appetite regulation: hormones, peptides, and neurotransmitters and their role in obesity.* American journal of lifestyle medicine, 2019. **13**(6): p. 586-601.

126. Kleinridders, A. and E.N. Pothos, *Impact of brain insulin signaling on dopamine function, food intake, reward, and emotional behavior.* Current nutrition reports, 2019. **8**: p. 83-91.

127. Galland, L., *Diet and inflammation*. Nutrition in Clinical Practice, 2010. **25**(6): p. 634-640.

128. Uldall Torp, N.M. and P.H. Thomsen, *The use of diet interventions to treat symptoms of ADHD in children and adolescents–a systematic review of randomized controlled trials.* Nordic Journal of Psychiatry, 2020. **74**(8): p. 558-568.

129. Shi, H., et al., *Low-grade inflammation as mediator between diet and behavioral disinhibition: A UK Biobank study.* Brain, Behavior, and Immunity, 2022. **106**: p. 100-110.

130. Tian, Y.E., et al., *Evaluation of Brain-Body Health in Individuals With Common Neuropsychiatric Disorders.* JAMA psychiatry, 2023. **80**(6): p. 567-576.

131. Mulder, D., et al., *A systematic review exploring the association between the human gut microbiota and brain connectivity in health and disease.* Molecular Psychiatry, 2023: p. 1-25.

132. Kissita, G., et al., *The Generalized Multiple CO-inertia Analysis (GMCOA).* Communications in Statistics-Theory and Methods, 2023. **52**(22): p. 7861-7885.

133. Dray, S., D. Chessel, and J. Thioulouse, *Co-inertia analysis and the linking of ecological data tables.* Ecology, 2003. **84**(11): p. 3078-3089.

134. Groves, A.R., et al., *Linked independent component analysis for multimodal data fusion.* Neuroimage, 2011. **54**(3): p. 2198-2217.

135. Kohn, N., et al., *Multivariate associative patterns between the gut microbiota and large-scale brain network connectivity.* Gut Microbes, 2021. **13**(1): p. 2006586.

136. Naguy, A. and B. Alamiri, *Attention-Deficit/Hyperactivity Disorder–Associated Impulsive Aggression Psychopharmacological Considerations.* American journal of therapeutics, 2023: p. 10.1097.

137. Prados-Bo, A. and G. Casino, *How have quality newspapers covered the microbiome? A content analysis of The New York Times, The Times, and El País.* Journalism, 2023. **24**(8): p. 1752-1771.

138. Dinan, T.G., C. Stanton, and J.F. Cryan, *Psychobiotics: a novel class of psychotropic.* Biological psychiatry, 2013. **74**(10): p. 720-726.

Appendices

Research data management
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RESEARCH DATA MANAGEMENT

This research followed the applicable laws and ethical guidelines. Research Data Management was conducted according to the FAIR principles. The analyses in this thesis are based on four human cohort studies. The data management is described in more detail below.

Ethics

All four studies were conducted in accordance with the principles of the Declaration of Helsinki. Relevant local ethics committees gave approval for the conducted studies, and all participants gave informed consent to collect their data. All necessary privacy measures have been taken to ensure anonymization of study participants. Chapters 2,3 and 4 utilize data of the IMpACT2-NL cohort. IMpACT2-NL is an amendment of the Dutch node of the IMpACT consortium (www.impactadhdgenomics.com). The study was approved by the regional ethics committee (Centrale Commissie Mensgebonden Onderzoek: CMO Regio Arnhem-Nijmegen; Dossiernummer ToetsingOnline: NL47721.091.14, protocol 2014-290). We additionally utilized data from the cohorts MIND-Set, NeuroIMAGE, and Mental-CAT in chapter 3. The MIND-Set study was approved by the local medical ethical committee (Commissie Mensgebonden Onderzoek Arnhem-Nijmegen) and the study design is published under doi: 10.2196/31269. The NeuroIMAGE study was approved by the regional ethics committee (Centrale Commissie Mensgebonden Onderzoek: CMO Regio Arnhem Nijmegen; 2008/163; ABR: NL23894.091.08) and the study design is published under https://doi.org/10.1007/s00787-014-0573-4. The Mental-Cat study was approved by the Clinical Research Ethics Committee (CREC) of Hospital Universitari Vall d'Hebron. The study is published under https://doi.org/10.1038/s41398-021-01504-6.

We acknowledge funding from the Netherlands Organization for Scientific Research (NWO), i.e. from the Veni Innovation Program (grant 016-196-115 to MH) and the Dutch National Science Agenda NeurolabNL project (grant 400-17-602). The work was also supported by funding from the European Community's Horizon 2020 Programme (H2020/2014 – 2020) under grant agreements n° 728018 (Eat2beNICE) and and n° 667302 (CoCA), and by the European College of Neuropsychopharmacology (ECNP) Network "ADHD Across the Lifespan". The NeuroIMAGE project was further supported by grants from National Institutes of Health (grant R01MH62873 to SV Faraone) for initial sample recruitment, and from NWO Large Investment (grant 1750102007010 to JK Buitelaar), NWO Brain & Cognition (grant 433-09-242 to JK Buitelaar), and grants from Radboud University

Medical Center, University Medical Center Groningen, Accare, and VU University Amsterdam for subsequent assessment waves. NeuroIMAGE also received funding from the European Community's Seventh Framework Programme (FP7/2007 – 2013) under grant agreements n° 602805 (Aggressotype), n° 278948 (TACTICS), and n° 602450 (IMAGEMEND), and from the European Community's Horizon 2020 Programme (H2020/2014 – 2020) under grant agreement n° 643051 (MiND). Concurrently, the research contributing to these results was also supported by funding from the European Community's Horizon 2020 CANDY project (grant agreement no. 847818) and the DISCOvERIE project (grant agreement no. 848228).

Findable, Accessible

The data of the IMpACT2-NL cohort is stored and archived as follows. The data of this cohort is only accessible to relevant members of the project. Further access to the data collections is restricted due to the informed consent of this study. Data can be made accessible upon reasonable motivated request. Informed consent was obtained on paper following the Centre procedure. All forms are archived in the central archive of the Radboudumc for 10 years after termination of the studies.

- The raw clinical, demographic, and neuropsychological data, as well as all questionnaires used for analyses across chapters 2, 3 and 4 of this thesis are located on secured servers at the Human Genetics department that undergo a regular back-up (H:\GR Theme groups\02 PI Group Barbara Franke\ADHD\ADHD IMpACT\IMpACT_II\Data) and the Data Archiving and Network Services (DANS) from the Dutch organization for scientific research (NWO) DOI: 10.17026/dansxs2-nvp8.
- The dietary questionnaire was used for the analyses of chapter 4. The R scripts for factor analyses of chapter 4 can be found at the same server In the folder T:\PIgroup-Alejandro-Arias-Vasquez\DietRAADHD.
- The raw neuroimaging data was stored at the Donders Centre for Cognitive Neuroimaging server and, according to policy of DCCN, automatically transferred to the Donders Repository (DCCN-initiated) Data Acquisition Collection. Individual scans can be found in this data acquisition collection or in the project folder (project/3017059.01/raw/), preprocessed data per subject can be found in the individual subject-directories within this folder (e.g. project/3017059.01/raw/subject/scan/). The python script (jupyter notebook) to the preprocessing pipeline can be found in the projectfolder under project/3017059.01/raw/preprocessing/jupyter_notebook/. Statistical analyses across subjects were done in FSL, the according analysis files are located in the folder project/3017059.01/Data_analyses/SecondLevelGLM/). All data and analyses files are further

uploaded to the Radboud Data Repository collection IMpACT2 under di.dccn.DAC_3017059.01_411. The results of this study are published under https://doi.org/10.3389/fpsyt.2022.840095.
- The 16S fecal sample sequencing data, Qiime2 pipeline scripts for first preprocessing and the generated biom-files can be found at a local server of the Human Genetics department (T:\PIgroup-Alejandro-Arias-Vasquez\Baseclear Shipments\Baseclear Shipment 3 -MindSetImpact, T:\PIgroup-Alejandro-Arias-Vasquez\MetaAnalysisADHD). This data is backed up on the Dutch National Supercomputer (snellius. surf.nl: /projects/0/gs/priscilla/IMpACT2/) and the Radboud Data Repository (https://doi.org/10.34973/nwfn-ms80). R scripts for preprocessing, quality control, and statistical analyses are located on the server at the Genetics Department of the Radboudumc in the folder T:\PIgroup-Alejandro-Arias-Vasquez\MetaAnalysisADHD.

Raw sequencing data, clinical and demographic data that have been shared with our group by the co-authors of chapter 3, and are stored as described in the following section.

- Original data, and analyses scripts of the studies analyzed in chapter 3 (except the Mental-Cat cohort, due to informed consent restricting data sharing in public repositories) are stored in the Radboud Data Repository under https://doi.org/10.34973/nwfn-ms80, and remain available for at least10 years after termination of the study. Non-identifiable variables can be shared upon motivated request. Preprocessed data, and metadata of all studies is additionally located at the MyDRE workspace 1259.
- Demographic, questionnaire, and clinical data of the IMpACT2-NL, NeuroIMAGE and Mental-Cat cohort are located on secured local servers of the Human Genetics department under T:\PIgroup-Alejandro-Arias-Vasquez\MetaAnalysisADHD. The 224 | Appendices Clinical information of MIND-Set is stored in the MIND-Set workspace in the MyDRE environment and Castor.
- 16S fecal sample sequencing data and Qiime2 pipeline scripts for first preprocessing can be found at a local server of the Human Genetics department MIND-Set: T:\PIgroup-Alejandro-Arias-Vasquez\Baseclear Shipments\Baseclear Shipment 3 -MindSetImpact, Mental-Cat: T:\PIgroup-Alejandro-Arias-Vasquez\SpainADHD_MartaRibases, NeuroIMAGE: T:\PIgroup-Alejandro-Arias-Vasquez\Joanna\fastq\NeuroIMAGE+Compulse+Humanization+B3_pilot1\) and are backed-up on the Dutch National Supercomputer (snellius. surf.nl: /projects/0/gs/priscilla/). R-scripts for preprocessing and analyses can be found at T:\PIgroup-Alejandro-Arias-Vasquez\MetaAnalysisADHD.

Interoperable, Reusable

For all storing, file formats have been used that ensure that data remains usable in the future (imaging: in .nii.gz, and DICOM, tables and information describing the datasets: .csv and .txt, microbiome data as .fastq , analyses scripts as .Rmd). The organization of the data is described in the readme files in the data collections. Results are reproducible by providing a description of the experimental setup, raw data, analysis scripts or pipelines in the folders of storage described above. The used software including version numbers is specified in the analyses scripts.

Privacy

The privacy of the participants in this study has been warranted. We acquired the data from the IMpACT2-NL cohort. Here, data acquisition was anonymized with participant code and personal information is stored and secured apart from data, and the links between participant codes and personal information were only accessible to members of the project who needed access to it because of their role within the project. The collected data of the IMpACT2 study will be stored 25 years after collection as agreed in the informed consent, and non-identifiable variables can be shared upon motivated request.

Due to the informed consent of this cohort, access to all collected anonymized data is restricted, and can only be made accessible upon reasonable request to the owners for the purpose to study topics relevant for ADHD. We have applied the same rules to the MIND-Set and the NeuroIMAGE cohort. For the Mental-Cat cohort, data sharing on repositories is not allowed due to the informed consent of those participants and the local ethical regulations.

NEDERLANDSE SAMENVATTING

Het was mijn doel met dit proefschrift om een beter begrip te krijgen van externaliserend gedrag bij volwassenen, zoals aandachtstekortstoornis met hyperactiviteit (ADHD) en reactieve agressie, door de darmmicrobioom-hersen-as te onderzoeken. Het begrijpen van de bijdrage van voeding en het darmmicrobioom aan de ontwikkeling en functie van de hersenen kan mogelijk de weg banen naar gerichte interventies voor bijvoorbeeld ADHD en het verminderen van ongunstige uitkomsten van de aandoening of mal-adaptief gedrag. In drie hoofdstukken heb ik veranderde hersenfunctie tijdens emotieverwerking, robuuste veranderingen in de samenstelling van het darmmicrobioom, en de complexe interactie van voeding en het darmmicrobioom bij volwassenen met ADHD en/of reactieve agressie.

In hoofdstuk 2 hebben we verschillen in functionele hersennetwerken geïdentificeerd tijdens emotieverwerking bij volwassenen met ADHD en in verband gebracht met scores voor reactieve agressie bij deze volwassenen. De hersengebieden met gewijzigde functie omvatten onder andere kleine clusters binnen de insula, het limbisch systeem en de middelste en bovenste frontale gebieden. Het was opmerkelijk dat sociaal en zelf-referentieel disfunctioneren, en symptomen van hyperactiviteit/impulsiviteit (maar niet onoplettendheid) geassocieerd waren met hersenfuncties relevant voor reactieve agressie. Dit werk wijst op een gedeeltelijk andere functionele hersenverwerking van emoties bij volwassenen met ADHD vergeleken met individuen zonder ADHD.

In hoofdstuk 3 hebben we het darmmicrobioom bij ADHD onderzocht, daarbij gebruik makend van een meta-analyse design over vier grote Europese cohorten. Hiervoor hebben we de bio-informatica-pipelines voor de analyse van de data van deze vier cohorten geharmoniseerd met behulp van state-of-the-art tools. Hoewel het darmmicrobioom van volwassenen met en zonder ADHD vergelijkbare alfa-diversiteit vertoonde, identificeerden we verschillen in bèta-diversiteit tussen de groepen. Bèta-diversiteit verschilde ook tussen cohorten, wat mogelijk wijst op invloeden van het laboratorium, zoals sequentiemethode en regio, op de meting van bèta-diversiteit. Differentiële overvloedanalyse onthulde robuuste associaties van verschillende bacteriën met ADHD, waaronder Eubacterium_xylanophilum, dat minder overvloedig was bij volwassenen met ADHD, en Ruminococcus torques, dat overvloediger was bij volwassenen met ADHD dan bij mensen zonder ADHD. De bacterie Ruminococcus_torques werd verder geassocieerd met meer symptomen van hyperactiviteit/impulsiviteit, en Eisenbergiella was geassocieerd met meer symptomen van onoplettendheid en hyperactiviteit/impulsiviteit. De literatuur suggereert een mogelijke rol van deze bacteriën in ontstekingsprocessen, die risicoverhogend of gunstig zouden kunnen zijn, maar functionele studies zijn nodig om deze eigenschappen verder te onderzoeken.

In hoofdstuk 4 hebben we het voedingspatroon en het darmmicrobioom van mensen met ADHD en verschillende levels van reactieve agressie geanalyseerd. We vonden drie voedingspatronen in onze dataset, gekenmerkt door respectievelijk een hoge consumptie van energie, alcohol of vezels. De factor van hoge energieconsumptie was geassocieerd met reactieve agressie, maar we vonden geen verbanden tussen de voedingspatronen en ADHD. Verschillende bacteriën werden geassocieerd met reactieve agressie of de diagnose ADHD. Er was geen significante overlap van darmmicrobiota-bevindingen tussen ADHD en reactieve agressie in deze studie. We ontdekten echter lagere niveaus van Eubacterium_xylanophilum in associatie met lage scores voor reactieve agressie – deze bacteriën waren ook minder overvloedig geweest bij volwassenen met ADHD in onze meta-analyse (hoofdstuk 3). Er kon geen samenhang worden gevonden tussen deze bacteriën, het dieet met hoge energieconsumptie en de reactieve agressie.

Samenvattend hebben we associaties geïdentificeerd tussen de samenstelling van het darmmicrobioom en reactieve agressie en ADHD, een gedeeltelijk verschillend hersenfunctioneel mechanisme van emotieverwerking bij ADHD met reactieve agressie, en een voedingspatroon gekenmerkt door hoge energieconsumptie geassocieerd met reactieve agressie. De resultaten van de beschreven associatiestudies dienen echter gerepliceerd te worden in grotere cohorten, bij voorkeur met meer gedetailleerde informatie over het darmmicrobiota en de voedingspatronen. Naast het identificeren van deze associaties benadruk ik met het werk in deze thesis het belang van robuuste resultaten en de vereiste harmonisatie en generalisatie van resultaten over cohorten en methoden. Voor dit doel moeten toekomstige studies zich richten op grote dimensionale steekproeven, gestandaardiseerde beoordelings- en analysehulpmiddelen toepassen, bevindingen integreren over onderzoeksgebieden en dit onderzoek uitbreiden naar functionele studies en biologische paden.

ENGLISH SUMMARY

My aim with this thesis was to gain a better understanding of adult externalizing behaviors, such as attention-deficit/hyperactivity disorder (ADHD) and reactive aggression, by investigating the gut-microbiome-brain axis. Understanding the contribution of diet and the gut microbiota to brain development and functioning may open up avenues to develop targeted interventions and reduce adverse outcomes in those affected with these conditions and maladaptive traits. In three chapters, I investigated the role of altered brain functioning during emotion processing, robust alterations in the gut-microbiome composition, and the complex interplay of diet and the gut-microbiota in adult ADHD and reactive aggression.

In chapter 2, we identified altered brain functional signatures during emotion processing in adults with ADHD in association with higher reactive aggression scores. Among the brain regions displaying altered functioning were small clusters within the *insula, limbic system,* and *middle and superior frontal areas*. Notably, social and self-referential impairment, and hyperactivity/impulsivity (but not inattention) symptoms were associated with brain functioning relevant for reactive aggression. With this work, we provide evidence for partly altered brain functional emotion processing in adults with ADHD compared to neurotypical individuals.

In chapter 3, we investigated the gut microbiome in ADHD, using a meta-analysis design across four large European cohorts. To this end, we harmonized the bioinformatics pipelines for analyzing the data from these four cohorts using state-of-the-art tools. While adults with and without ADHD showed similar alpha diversity, we identified differences in beta diversity between the groups. Beta diversity was further different between cohorts, potentially reflecting wet-lab influences like sequencing method and region on beta diversity. Differential abundance analysis revealed robust associations of several genera with ADHD, including *Eubacterium_xylanophilum*, which was less abundant in adults with ADHD, and *Ruminococcus torques*, which was more abundant in adults with ADHD. *Ruminococcus_torques* abundance was further associated with more symptoms of hyperactivity/impulsivity, and the genus *Eisenbergiella* with more inattention and hyperactivity/impulsivity symptoms. The literature suggests a potential role of these genera in inflammatory processes, which might be risk-conferring or beneficial, but functional studies are needed to address these properties further.

In chapter 4, we studied diet and the gut microbiome in ADHD and reactive aggression. We identified three dietary patterns in our dataset, characterized by

high-energy, high-alcohol, and high-fiber consumption. While the high-energy factor was associated with reactive aggression, we found no associations of dietary patterns with ADHD. Several genera were associated with reactive aggression or ADHD diagnosis. There was no significant overlap of gut-microbiota findings between ADHD and reactive aggression in this study. However, we found lower levels of *Eubacterium_xylanophilum* associated with low reactive aggression scores – we had earlier seen this genus also as less abundant in adults with ADHD in the meta-analysis of **Chapter 3**. No mediation was detected of the selected genera on the association between reactive aggression and the high-energy diet.

In conclusion, we identified associations of gut-microbiota composition with reactive aggression and ADHD, a partially different brain functional mechanism of emotion processing in ADHD linked to reactive aggression, and a high-energy diet associated with reactive aggression. However, the results obtained in the described association studies should be replicated in larger cohorts and with higher resolution of gut-microbiota and diet data. Next to the identification of these associations, with the work of this thesis, I emphasize the importance of robust results, the required harmonization, and generalization of results across cohorts and methods. To this end, future studies should focus on large dimensional samples, apply standardized assessment and analysis tools, integrate findings across research domains, and extend this research to functional studies and biological pathways.

ACKNOWLEDGEMENTS

This thesis was surely not the work of one person, but a reflects the people, talents and influences, that I was lucky enough to be surrounded by! Firstly, I would like to thank my supervision team: Alejandro, you were a great sparring partner, you gave me all autonomy and freedom to make my own decisions; Martine, for keeping an eye on finish lines, your reasonable calming personality and for bringing up opportunities outside of the daily business; Barbara for your empathic and helpful management style; and Han, who brought next to fun, also reason and good ideas to the evaluation meetings. I am grateful for the opportunities you gave me and your support. I would like to thank the MF group and the Microbiome group for interesting talks and discussions, but above all for a supportive and helpful work environment. A special thanks I would like to dedicate to my colleagues: Danique, Priscilla, Mandy, Sourena, and Yingjie for in-office as well as after-office fun, squash sessions, drinks and dancing; Jeanette and Mirjam for being great colleagues, superhelpful human beings and real rolemodels.

Also outside of work, many people contributed to this thesis, particularly to my sanity during the process. Besonders meiner Familie möchte ich an dieser Stelle danken. Mama, für deine endlose Geduld, und Papa, für deinen Tatendrang. Ganz abgesehen von eurer Liebe und Unterstützung hätte ich ohne diese Eigenschaften sicher niemals diese Thesis geschrieben. Euch beiden, sowie MJ, Jonas, Alex, Leni, Dani und Christina, tausend Dank für eure offenen Ohren, Arme, und Türen, und dass ihr mir -egal wo- ein Gefühl von zu Hause gebt. I'd like to thank Cookie and Lolli the dog - you two ground me, provide me with food, cuddles, and a healthy dose of nature and distractions. Every day with you two is awesome! Estoy eternamente agradecido por tener Marie & Lisa en mi vida - fantasticos artistas, los mejores companeros en la oficina valencia y los cantantes mas ruidosos y sinceros! Jasna, Michele, Mickey, Hanna, Benni, Anke, Kacki, Benfa, Matze, Steffen, und der Rest der Gurkentruppe - ohne euch wäre diese Thesis viel früher fertig gewesen! Danke für all die Jahre voller Feiern und Tanzen, für die genialen und besonders für die wirklich dummen Ideen wie wir unsere Zeit vertreiben könnten! Ook superbedankt aan Brianne, Inge, Joni en Elli - liefste huisgenootjes, geweldige meiden en mijn privédocenten voor Nederlandse taal en cultuur. And thank you Sorti Mavriks – the proof reader hero.

ABOUT THE AUTHOR

Babette Jakobi studied of Cognitive Science at Eberhard Karls University in Tübingen, Germany. Next to her studies, Babette worked as a research assistant at the department of Developmental Psychology. In a project under Prof. Dr. Claudia Friedrich and Dr. Ruth Kessler on the relationship of language processing with Theory of Mind abilities, she completed her bachelor thesis titled "*The development of idiomatic language processing and Theory of Mind in 10-year-old children: An ERP study*" with the grade of 14 points (magna cum laude). Orienting for an academic career, she did a voluntary internship at the Max Planck Institute for human cognitive and brain sciences in Leipzig, Germany. Under Dr. Jens Brauer she worked in a project about language processing and neurodevelopment. After the internship, she pursued her studies with a Master of Science in Cognitive and Integrative System's Neuroscience at Philipps-University Marburg, Germany. Next to her studies there, she worked as a research assistant at the psychiatric department of the University Clinic Giessen Marburg (UKGM) under Dr. Irina Falkenberg, and Dr. Florian Bitsch, and completed a voluntary internship at the Translational Neuroimaging Lab under Prof. Dr. Benjamin Straube, to learn more about neuroimaging in the context of mental health. She completed her Master thesis on "*Gesture-Speech-Integration in patients with Schizophrenia Spectrum Disorder. An EEG informed fMRI study integrating neural oscillatory changes and BOLD activity.*" with the grade of 15 points (summa cum laude) under Dr. Yifei He and Prof. Dr. Andreas Jansen. In 2019 she started her PhD trajectory under supervision of Prof. Dr. Barbara Franke, Prof. Dr. Han Brunner, Dr. Martine Hoogman, and Dr. Alejandro Arias-Vasquez at the department of Human Genetics at the Radboudumc and the Donders Institute of Medical Neuroscience. Her thesis focused on *the role of the gut-brain-axis in adult externalizing behaviors*, where she investigated associations of brain functioning, diet and gut-microbiome composition with ADHD and reactive aggression and was completed in 2024. During this period, Babette presented her work on multiple renowned international conferences and meetings in the research field, and published peer-reviewed papers.

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DONDERS GRADUATE SCHOOL FOR COGNITIVE NEUROSCIENCE

For a successful research Institute, it is vital to train the next generation of young scientists. To achieve this goal, the Donders Institute for Brain, Cognition and Behaviour established the Donders Graduate School for Cognitive Neuroscience (DGCN), which was officially recognised as a national graduate school in 2009. The Graduate School covers training at both Master's and PhD level and provides an excellent educational context fully aligned with the research programme of the Donders Institute.

The school successfully attracts highly talented national and international students in biology, physics, psycholinguistics, psychology, behavioral science, medicine and related disciplines. Selective admission and assessment centers guarantee the enrolment of the best and most motivated students.

The DGCN tracks the career of PhD graduates carefully. More than 50% of PhD alumni show a continuation in academia with postdoc positions at top institutes worldwide, e.g. Stanford University, University of Oxford, University of Cambridge, UCL London, MPI Leipzig, Hanyang University in South Korea, NTNU Norway, University of Illinois, North Western University, Northeastern University in Boston, ETH Zürich, University of Vienna etc.. Positions outside academia spread among the following sectors: specialists in a medical environment, mainly in genetics, geriatrics, psychiatry and neurology. Specialists in a psychological environment, e.g. as specialist in neuropsychology, psychological diagnostics or therapy. Positions in higher education as coordinators or lecturers. A smaller percentage enters business as research consultants, analysts or head of research and development. Fewer graduates stay in a research environment as lab coordinators, technical support or policy advisors. Upcoming possibilities are positions in the IT sector and management position in pharmaceutical industry. In general, the PhDs graduates almost invariably continue with high-quality positions that play an important role in our knowledge economy.

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