From DEPARTMENT OF MOLECULAR MEDICINE AND SURGERY
Karolinska Institutet, Stockholm, Sweden

EXPLORING THE GUT-BRAIN AXIS IN ATTENTION-DEFICIT HYPERACTIVITY DISORDER

Liu Yang

杨柳

Karolinska Institutet

Stockholm 2020

Cover image by Ina Schuppe Koistinen. **Connectedness.** 2019, Stockholm
All previously published papers were reproduced with permission from the publisher.
Published by Karolinska Institutet.
Printed by US-AB

ISBN 978-91-7831-994-7

Exploring the Gut-brain Axis in Attention-Deficit Hyperactivity Disorder

THESIS FOR DOCTORAL DEGREE (Ph.D.)

Publicly defended in L8:00 Lecture Hall, Center for Molecular Medicine, Visionsgatan 18, Karolinska University Hospital, Solna

Friday, December 4th, 2020 at 09:30

By

Liu Yang

Principal Supervisor:
Assoc. Prof. Catharina Lavebratt
Karolinska Institutet
Department of Molecular Medicine and Surgery

Co-supervisor(s):
Prof. Martin Schalling
Karolinska Institutet
Department of Molecular Medicine and Surgery

Dr. J. Carlos Villaescusa
Karolinska Institutet
Department of Molecular Medicine and Surgery

Prof. Yvonne Forsell
Karolinska Institutet
Department of Global Public Health

Opponent:
Prof. Nanda Lambregts-Rommelse
Radboud University
Department of Psychiatry

Examination Board:
Prof. Gilberto Fisone
Karolinska Institutet
Department of Neuroscience

Assoc. Prof. Velmurugesan Arulampalam
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology

Assoc. Prof. Peik Gustafsson
Lund University
Faculty of Medicine
Department of Clinical Sciences, Lund

To clinicians and researchers working on ADHD;

To all who contributed to this thesis;

To my beloved family and friends.

ABSTRACT

There is a bidirectional interaction between the gut and the brain, termed the gut-brain axis (GBA), involving e.g. the gut microbiota, and connecting the peripheral intestinal elements to the central nervous system (CNS). The GBA is increasingly recognized as a vital factor in the development and prognosis of neurological and psychiatric disorders. Of particular interest in this thesis is the signaling from gut microbiota to brain and back which is enabled through neural, endocrine, immune, and humoral pathways.

Studies in germ-free and antibiotic-treated animal models have created a significant body of knowledge on the GBA and its role in regulating behavior. There are fewer studies using human subjects or with human materials.

This thesis is focused on both clinical and biological aspects of GBA in attention-deficit hyperactivity disorder (ADHD). ADHD is a common childhood-onset psychiatric and neurodevelopmental disorder, which is characterized by having problems with paying attention and/or excessive activity and impulsivity, along with impairments on daily function and emotion regulation. ADHD is highly heritable and has high comorbidity with other psychiatric disorders and a higher-than-normal co-occurrence with some inflammatory disorders (e.g. asthma, eczema, rhinitis, celiac disease). However, ADHD is diagnosed primarily based on clinical observations of behaviors. Biomarkers for diagnosis and approaches for new therapy are lacking and the pathophysiology is poorly understood.

This thesis consists of five studies attempting to explore some aspects of the GBA in neurodevelopmental and psychiatric disorders, with a focus on ADHD, utilizing a nationwide population-based cohort, a case-control design, a randomized controlled trial (RCT) and an *in vitro* model. It covers different aspects of the GBA, including gut microbiota and related antibiotic exposure, probiotic and prebiotic intervention, derived metabolites, inflammatory mediators, as well as the associations with clinical observations.

Specifically, in **Study I**, we investigated whether exposure to antibiotic drugs, *in utero* and first two years after birth was associated with a risk for childhood-onset psychiatric disorders. Using Finnish nationwide registers, we found that antibiotic exposure was associated with a 10–50% increased risk for development of sleep disorders, ADHD, conduct disorders, mood disorders and anxiety disorders, which were accompanied by an increased risk for psychotropic drug use in childhood. The association with prenatal antibiotic exposure was neither explained completely by confounding factors related to family, nor by factors related to maternal infections.

In **Study II**, we used a case-control design to study plasma levels of four inflammatory mediators: C-reactive protein (CRP), serum amyloid A (SAA), soluble intercellular adhesion molecule 1 (sICAM-1) and soluble vascular cell adhesion molecule 1 (sVCAM-1) in ADHD and their associations with medication and clinical symptoms. We found that ADHD patients

had higher levels of sICAM-1 and sVCAM-1 than healthy controls, especially in children currently on ADHD medication. Among adult participants with ADHD, sICAM-1 levels were positively associated with comorbid autism symptoms, and CRP levels were associated with GI symptoms and emotion dysregulation.

In **Study III**, a double-blind RCT was conducted to determine whether a synbiotic (Synbiotic 2000), consisting of a mixture of three lactic acid bacteria and four fibers had effects on symptoms, daily functioning, and comorbid traits in ADHD patients. We found that Synbiotic 2000, compared to placebo, significantly reduced typical restricted, repetitive and stereotyped behaviors of autism symptoms in children, and alleviated problems with goal-directed behavior of emotion regulation in adults. The effects on autism symptoms and problems with emotion regulation were stronger in the subgroup with higher sVCAM-1 levels.

In **Study IV**, using data from the same RCT as in study III, we investigated the effects of Synbiotic 2000 on plasma levels of immune activity markers and short-chain fatty acids (SCFAs) in ADHD. Adults with ADHD had at baseline higher levels of pro-inflammatory vascular adhesion molecules (sICAM-1, sVCAM-1) and lower levels of the anti-inflammatory interleukin (IL)-10 compared to controls. Synbiotic 2000, compared to placebo treatment, was associated with reduced the levels of IL-12/IL-23p40 in children on ADHD medication, and suggestively associated with reduced sICAM-1 levels and increased propionic acid levels in children. Moreover, in adult patients we found lower baseline levels compared to controls of formic acid and propionic acid. No obvious effects of Synbiotic 2000 were found on plasma levels of SCFAs. However, we found IL-10 levels correlating positively with formic acid and acetic acid at baseline for both children and adults with ADHD. In child patients, sVCAM-1 correlated negatively with acetic acid and propionic acid while sICAM-1 correlated negatively with acetic acid. In adult patients, a negative correlation was observed between sVCAM-1 and formic acid at baseline.

In **Study V**, an *in vitro* model was used to explore the effects of three SCFAs: acetate, propionate and butyrate, on cell growth and cell death of early stage human neural progenitor cells (hNPCs). We found that acetate, propionate and butyrate at low µM levels, relevant to physiological levels, significantly increased the proliferation of hNPCs and induced more cells to undergo mitosis, while the SCFAs at high mM levels had toxic effects on hNPCs. In support of this, the SCFA exposure to hNPCs changed the expression of genes involved in neurogenesis, proliferation and apoptosis.

These studies provide support of a role of components influenced by the GBA, including gut microbiota, immune activity markers and bacterial metabolites in clinical and biological aspects of ADHD. Finally, these studies contribute to a better understanding of the GBA in ADHD.

LIST OF PUBLICATIONS

I. Lavebratt C, **Yang LL**, Giacobini M, Forsell Y, Schalling M, Partonen T, Gissler M, **Early exposure to antibiotic drugs and risk for psychiatric disorders: a population-based study**. *Translational Psychiatry, 2019 9:317*.

II. **Yang LL**, Stiernborg M, Skott E, Söderström Å, Giacobini M, Lavebratt C, **Proinflammatory mediators and their associations with medication and comorbid traits in children and adults with ADHD**. *European Neuropsychopharmacology, in press*.

III. Skott E*, **Yang LL***, Stiernborg M, Söderström Å, Rùegg J, Schalling M, Forsell Y, Giacobini M, Lavebratt C, **Effects of a synbiotic on symptoms, and daily functioning in attention deficit hyperactivity disorder – A double-blind randomized controlled trial**. *Brain, Behavior, and Immunity 2020; 89: 9-19*. *Equal contribution.

IV. **Yang LL**, Skott E, Stiernborg M, Landberg R, Millischer V, Schalling M, Giacobini M, Lavebratt C, **Effects of a synbiotic on plasma immune activity markers and short-chain fatty acids in children and adults with ADHD – a randomized controlled trial**. *Manuscript*.

V. **Yang LL**, Millischer V, Rodin S, MacFabe DF,Villaescusa JC, Lavebratt C, **Enteric short-chain fatty acids promote proliferation of human neural progenitor cells**. *Journal of Neurochemistry, 2019: p. e14928-e14928.*

LIST OF ADDITIONAL PUBLICATIONS

1. Efstathopoulos, P, Andersson F, Melas PA, **Yang LL**, Villaescusa JC, Rüegg J, Ekström TJ, Forsell Y, Galanti MR, Lavebratt C, ***NR3C1* hypermethylation in depressed and bullied adolescents**. *Translational Psychiatry, 2018; 8:121*.

2. Månsson KNT, Lindqvist D, **Yang LL**, Svanborg C, Isung J, Nilsonne G, Bergman-Nordgren L, El-Alaoi S, Hedman-Lagerlöf E, Kraepelien M, Högström J, Andersson G, Boraxbekk C, Fischer H, Lavebratt C, Wolkowitz OM, Furmark T, **Improvement in indices of cellular protection after psychological treatment for social anxiety disorder**. *Translational Psychiatry, 2019; 9:340*.

3. Rönne-Petersén L, Niemi M, Walach H, Lavebratt C, Millisher V, **Yang LL**, Gerdle B, Ghafouri B, Falkenberg T, **Telomere length correlates with emotional and spiritual well-being in chronic pain patients**. *Manuscript*.

CONTENTS

Appendix

LIST OF ABBREVIATIONS

ADHD	Attention-deficit hyperactivity disorder
AF	Amniotic fluid
AIC	Akaike information criterion
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
ASD	Autism spectrum disorders
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
BMI	Body mass index
CBT	Cognitive behavioral therapy
CI	Confidence interval
CNS	Central nervous system
CRP	C-reactive protein
CSF	Cerebral spinal fluid
CV	Coefficient of variation
DSM	Diagnostic and Statistical Manual of Mental Disorders
DPD	Drugs and Pregnancy Database
FDR	False discovery rate
FFAR	Free fatty acid receptor
GBA	Gut-brain axis
GPR	G-protein-coupled receptor
GI	Gastrointestinal
GRO	Growth-regulated oncogene
GWAS	Genome-wide association study
HDAC	Histone deacetylase
HPA	Hypothalamic pituitary adrenal
hNPCs	Human neural progenitor cells
HR	Hazard ratio

IBD	Inflammatory bowel disease
ICD	International Statistical Classification of Diseases and Related Health Problems
IFN	Interferon
IL	Interleukin
MBR	Medical Birth Register
MCT1	Monocarboxylate transporter 1
MCP-1	Monocyte chemoattractant protein 1
MGBA	Microbiota-gut-brain axis
MSD	Meso Scale Discovery
pHH3	Phospho-histone H3
SAA	Serum amyloid A
SCFAs	Short-chain fatty acids
sICAM-1	Soluble intercellular adhesion molecule 1
sVCAM-1	Soluble vascular cell adhesion protein 1
RCT	Randomized controlled trial
RRD	Register on Reimbursement Drugs
rRNA	ribosomal RNA
TNF	Tumor necrosis factor
TGF	Transforming growth factor
VEGF-A	Vascular endothelial growth factor A
WHO	World Health Organization

1 INTRODUCTION

Attention-deficit hyperactivity disorder (ADHD), was first described as a disorder in 1902 by the pediatrician George Frederic Still, saying "an abnormal defect of moral control in children" [1]. The children affected by the disorder behaved differently from a typical child and could not control themselves, while still having normal intelligence. ADHD was recognized as a hyperkinetic impulse disorder in the second edition of "Diagnostic and Statistical Manual of Mental Disorders" (DSM) published in 1968 by the American Psychological Association. DSM is a manual, which lists all of the defined mental disorders, including the descriptions of symptoms, diagnosis criteria, known causes, risk factors, and treatments, and continuously updated versions have been used by clinicians and researchers. Until the revised version of the third edition of the DSM (DSM-III) published in 1987, the description ADHD was redefined into a single term that combined three symptoms, including inattentiveness, impulsivity and hyperactivity. In the current DSM-V used today, ADHD is added to the neurodevelopmental disorders and classified into three subtypes: predominantly inattention, predominantly hyperactivity-impulsivity and combined manifestation [2]. At the same time, a global classification system by the World Health Organization (WHO), International Classification of Diseases (ICD) as analogous to DSM, is being used as the main classification system among healthcare professionals in e.g. Europe, with the 10th version (ICD-10) currently used for ADHD [2].

The number of ADHD cases started to increase remarkably in the 1990s. This can probably be explained by more awareness and reporting of symptoms by parents and school teachers, more efficient diagnosis, better access to healthcare and more children actually developing ADHD [3]. Scientific findings have pointed to strong roles of genetic factors and some roles of environmental factors in the development of ADHD [4]. Researchers and psychiatrists are dedicated to figure out the underlying causes as well as possible diagnostic biomarkers and treatment targets for the disorder.

In recent decades, research into the communication between the gut microbiota (microorganisms such as bacteria, fungi, viruses and archaea) and the brain, summarized as gut-brain axis (GBA) or microbiota-gut-brain axis (MGBA), has started to elucidate causal roles of the gut microbiota on brain function and behaviors as well as the underlying molecular interactions. Animal models, typically germ-free and antibiotic drug-treated mice, implied the microbiota for influencing the endocrine hypothalamic–pituitary–adrenal (HPA) axis, behavior

regulation and brain development [5-8]. More molecular evidence involving brain-derived neurotrophic factor (BDNF), neurotransmitters, microbial metabolites (e.g. tryptophan, short-chain fatty acids (SCFAs)) and immune activity markers, supported the gut microbiota–brain communication [7, 9, 10]. Research on the GBA is growing in the field of psychiatric, neurodevelopmental and neurodegenerative disorders [11].

The overall aim of this thesis was to explore certain aspects of the GBA in neurodevelopment with a focus on ADHD, and to explore a new adjuvant therapeutic strategy for ADHD. In this thesis, I will start by introducing the current knowledge and major background on ADHD and GBA.

1.1 ADHD

1.1.1 Clinical manifestations

ADHD is a common neurodevelopmental disorder among children and adolescents, with the core symptoms indicated by the name: inattention and/or hyperactivity-impulsivity. The disorder has generally an onset age before 12 years and may continue throughout life or not be diagnosed until adulthood [12]. At present, the widely used and standard diagnosis tool is the DSM-V or ICD-10 criteria, within categories 314.0X or F90.X [13]. To get a diagnosis according to the DMS-V, at least six inattentive or hyperactive-impulsive symptoms for children up to 16 years, five or more symptoms for individuals older than 16 years and adults, must be present for more than 6 months. Several symptoms must be present before 12 years of year and lead to impairments in at least two significant areas of functioning, such as at work, school, family and social settings [2]. Emotion dysregulation is listed as a characteristic feature of ADHD according to DSM-V, which can support the diagnosis. Emotion dysregulation refers to inability to manage negative emotions such as anger, sadness and fear. DERS is a widely-used scale to measure emotion regulation difficulties, which include five different domains: lack of emotional clarity (e.g. confused about the feeling), difficulties in goal-directed behavior (e.g. difficult to get work done), difficulties in impulse control (e.g. behaviour outbursts), limited access to effective emotional regulation strategies (e.g. unable to make yourself feeling better), non-acceptance of emotional responses (e.g. ashamed of feeling that way) [14]. From a clinical perspective, the predominant features of ADHD in adults are different from those in children, showing less obvious hyperactivity-impulsivity symptoms and more inattentive symptoms [15]. Similar to other complex psychiatric disorders, ADHD presents with marked heterogeneity. Variation among individuals with a diagnosis of ADHD is found on core symptom

combinations, levels of function impairments and comorbidities. ADHD has a high comorbidity with other neuropsychiatric and neurological/somatic conditions such as Tourette syndrome, oppositional defiant and conduct disorder, autism spectrum disorder (ASD), mood disorders, anxiety disorder, learning disability, intellectual disability and sleep disorder [16-19], as well as epilepsy, migraine, asthma, rhinitis, eczema, celiac disease and obesity [20-22]. About 15–25% of young individuals with ADHD demonstrate ASD traits and symptoms, and 12.4% of them have an ASD diagnosis [19]. Meanwhile, for children who have ASD the comorbidity rates for ADHD is 40–70% [19]. Individuals diagnosed with ASD show deficits in the aspects of social interaction and communication and/or have restricted and repetitive patterns of behavior, activities or interests, which can cause significant functional impairments [19].

1.1.2 Epidemiology

In 2015, the globally estimated number of individuals to be diagnosed with ADHD was about 51.1 million [23]. A meta-analysis on the prevalence of ADHD diagnosed by DSM-IV estimated 5.9-7.1% in children and adolescents and slightly lower 5% in adults [24]. While other epidemiological studies reported much lower prevalence for both children (3.4%) and adults (2.5%) [25, 26]. The variability for the estimated prevalence was mostly due to methodological procedures and diagnostic criterions, but not significantly associated with geographical locations [27, 28]. Over the last three decades, there has been no increase in the population rate of ADHD using the standard criteria [28]. A newly published article reported trends for an increasing rate of ADHD diagnosis among adults in United States [29], which was confirmed in Scandinavian counties [3, 30]. The global increase in prevalence of ADHD cannot be attributed to insurance coverage or health care systems in different countries [29]. However, the rate decreased with age, since the core features of ADHD tend to decline with age [15, 26]. Up to 65% of pediatric ADHD patients persist to have symptoms in adulthood [15]. In children, ADHD is 3-9 times more common in boys than girls, while in adult population the sex ratio is more balanced [15, 24, 26]. ADHD is often overlooked in girls, one reason of which is the different clinical features between girls and boys, similar as in ASD [31-33]. This may partially explain the sex difference in prevalence among children.

1.1.3 Pathophysiology

ADHD is a complex disorder and could have long-term outcomes. Although ADHD is one of most studied psychiatric disorders, the pathophysiology of ADHD is not very clear. Growing number of evidence has shown that genetic and environmental risk factors, as well as their interactions play important roles in the etiology of the disorder. A twin study demonstrated a heritability of 70–80% for ADHD in both children and adults [34]. A family-based study indicated

that siblings of individuals with ADHD have much higher risk to develop the disorder than siblings of non-ADHD individuals [35]. As with the heterogeneity and multifactorial nature, ADHD is primarily attributed to multiple common genetic variants (single nucleotide polymorphisms). Recently, a meta-analysis of genome-wide association studies (GWAS) found 304 genetic variants in 12 loci that reached significance in ADHD and some of the loci were in, or close to genes (e.g. DUSP6, SORCS3, FOXP2) involved in neurodevelopment [36]. Data from 10 GWAS in ADHD suggested the involvement of genes related to neurotransmission, neural growth, cell adhesion, neurotransmitter system and synaptic plasticity, which was in line with classical candidate gene studies [4]. Biological pathway and network analysis on 105 ADHD-candidate genes identified 14 genes that were highly involved in nitric oxide synthase pathway and α-1 adrenergic pathway and were actively expressed in the cerebellum and cortex [37]. In addition, more rare genetic variants (copy number variants) have been shown to be associated with ADHD [38]. ADHD-associated copy number variants span genes that encode the nicotinic α7 acetylcholine receptor subunit, neuropeptide Y and several glutamate receptor genes [39, 40]. The high heritability of ADHD is due to polygenic components and approximately 33% of the heritability is explained by the common genetic variants [38]. The polygenic architecture in ADHD is similar to other neuropsychiatric disorders such as ASD and schizophrenia [41, 42].

Reported environmental risk factors for ADHD include prenatal/perinatal exposures: maternal stress, smoking, alcohol use, psychopathology, obesity, infections and premature birth and low birth weight; postnatal exposures: anti-infective agents, environmental chemicals (e.g. lead and organophosphates, nicotine, alcohol), socioeconomic factors (e.g. low income, family adversity, and harsh parenting,) as well as dietary factors (e.g. mineral deficiency, Omega-3 fatty acid deficiency) [43-45]. The prenatal exposures, especially the first trimester (1-12 gestational week), probably have the largest impacts on neurodevelopment due to the more pronounced vulnerability when complex development is happening [46]. Since gestational week 5, the embryo starts to form the neural tube, early nervous system begins to develop and the major structures of the fetus is present by the end of week 11. The development of the neural system runs through almost all 3 trimesters and is complete several years after birth. The environmental risks and the outcomes can be determined by genetic properties of the disorder, and on the other hand, environmental factors can influence the genetically associated phenotypes via epigenetic modifications [4].

Moreover, employment of neuroimaging techniques (e.g. CT, MRI, fMRI) has showed brain structure and function abnormalities, which are involved in the pathophysiology of ADHD.

Alterations in brain regions, like the white matter, gray matter, basal ganglia, limbic areas and neural network for cognition, attention and executive functions were associated with ADHD [2].

1.1.4 Treatment

Currently, there are pharmacological and non-pharmacological treatments for the management of symptoms and function deficits in ADHD, but no therapeutic strategy to cure the disorder entirely. The stepwise treatment options according to specific guidelines vary a bit geographically. In Europe, pharmacotherapy is widely accepted as first line treatment for both children and adults, which include stimulants (methylphenidate, dexamphetamine, lisdexamfetamine and amphetamine) and nonstimulants (atomoxetine, guanfacine and clonidine) [47]. Stimulants that aim to increase extracellular levels of dopamine and norepinephrine by blocking their transporters and their reuptake into the presynaptic neurons have been proven to be effective with long-term benefits for ADHD. Non-stimulants are used as second-line pharmacological treatment. The non-pharmacotherapy for ADHD, namely behavior therapy includes parenting intervention, classroom intervention and cognitive behavioral therapy (CBT) [16]. Behavior therapy is an alternative option when considering the safety and efficiency for young children. The multi-modal treatment with CBT as an adjunct to medication seemed to have the best improvement for patients [48]. The vast majority of ADHD patients are on pharmacological treatments for a long time. A number of common side effects are observed from ADHD medication, such as loss of appetite, dry month, nausea, insomnia, cardiac problems etc. [2]. Dietary intervention has been proposed to be another non-pharmacological option that can ameliorate some symptoms of ADHD [49]. A recent published twin-registry study in adults showed high fat, sugar, protein consumptions and unhealthy dietary pattern were positively correlated with ADHD symptoms, while fruits, vegetable consumptions and a healthy dietary pattern were negatively correlated with ADHD symptoms [50]. Restriction on artificial food colorants, artificial sweeteners, sugar and supplements with amino acids, fatty acids, vitamins, minerals have shown some beneficial effects, but only restricted food colorant diet and omega-3 supplementation had consistent effects and are effective in reducing ADHD symptoms [49, 51]. A small randomized trial has proposed that early life probiotic supplementation reduced the risk for developing ADHD and Asperger syndrome later in childhood [52]. Furthermore, a newly published pilot study revealed an effect of probiotic on improving quality of life for young persons with ADHD [53]. However, data from the studies are not sufficient to recommend diet intervention as a standard ADHD treatment, more knowledge is in great need. Therefore, more research and investigation is required to substantively ascertain the link between the gut flora and the central nervous system (CNS).

1.2 GUT-BRAIN AXIS

GBA, also known as MGBA is the bidirectional signaling pathway between the gastrointestinal (GI) tract and the CNS. The signaling pathways in the axis is via the neural system (spinal cord, vagus nerve, enteric nerve), the immune system (immune cells and inflammatory mediators), the neuroendocrine system (HPA axis), gut microbiota and the derived metabolites (e.g. SCFAs, peptidoglycan, tryptophan and gamma-aminobutyric acid) that can pass the barrier paths (intestinal mucosal barrier and blood-brain barrier (BBB)) [54]. In the past two decades, the GBA is gaining more and more attention in fields of psychiatric, neurodevelopmental, and neurological disorders, regarding the biology, physiology and pathology [55, 56].

1.2.1 Gut microbiota, synbiotics and antibiotics

Microbiome was originally defined as "a characteristic microbial community occupying a reasonably well-defined habitat which has distinct physio-chemical properties. The term thus not only refers to the microorganisms involved but also encompasses their theatre of activity". However, many current researchers have described the term microbiome simply as "the collective genomes of microorganisms inhabiting a particular environment and especially the human body". Microbiota are ecological communities of microorganisms (bacteria, virus, fungi and archaea) and gut microbiota is the community that lives in the GI tract. It is one of the principal components in GBA. Gut bacteria have the great numbers and biodiversity, 90% of which are anaerobes. The gut microbiota is an ecosystem that is dynamic throughout the lifespan. Gut microbiota has become an important player for host physiology in both health and disease and for maintaining organismal homeostasis, which is of the utmost importance [56]. Studies have highlighted the interactions between the gut bacterial microbiota and the brain, showing altered gut bacterial profiles in patients with depression, anxiety, ASD and schizophrenia [57-59]. Regarding ADHD, there are only two studies that have revealed difference in the gut bacterial profiles from controls: one showing less abundance of *Faecalibacterium* in children with ADHD (n=51) and the abundance was negatively associated with symptom severity; the other one showing increased abundance of *Bifidobacterium* in adolescents and adults with ADHD (n=19) [60, 61]. More supportive results from germ-free or antibiotic-treated animal models have indicated that the absence or depletion of gut bacterial microbiota affected the brain activities and behaviors (depression, anxiety, hyperactivity sociability and cognition), particularly in early life [8, 55, 62-65]. Possible roles of gut microbiota on neural pathways and endocrine and immune mechanisms have been studied, including maturation and activation of microglia cells, oligodendrocyte differentiation, mitochondrial metabolism, synaptogenesis, permeability of the BBB, neurotransmitter metabolism, neurotrophy and neurogenesis [62-64, 66,

[67]. Animals administrated with specific bacterial strains showed changes in behaviors [68, 69]. Although these behavioral and mechanistic studies are difficult to translate into humans, transferring fecal bacteria from autism, depression or schizophrenia patients to the germ-free or microbiota-deficient rodent intestine did induce behaviors resembling these disorders and influence biochemical modulations involved in the disorders [70-72].

Synbiotics are dietary supplements with a combination of probiotics (generally, live beneficial bacteria that normally inhabit the human intestinal tract) and prebiotics (non-digestible fibres promoting the survival of beneficial bacteria in the intestinal tract) [73, 74]. These ingredients are generally regarded as safe, and have health benefits for the host by restoring the ecosystem of microbiota in the GI tract. Previous studies have revealed some positive effects from certain prebiotics or probiotics on stimulating the immune activities, regulating vitamin synthesis and modulating brain functions [75-78]. Some open-label interventions with probiotics/prebiotics in pediatric autism patients reduced GI symptoms and psychiatric symptoms [79-81]. Notably, a recent randomized controlled trial (RCT) in Finland showed that oral administration of the probiotic strain *Lactobacillus rhamnosus GG* during the first 6 months of life reduced the development and presence of ADHD and Asperger syndrome when at 13 years of age [52]. Giving the same strain (*Lactobacillus rhamnosus GG)* to young persons with ADHD, improved the self-reported quality of life and changed cytokine levels in serum [53].

Many other factors can have influence on gut microbiota composition (abundance and biodiversity) especially in early life, such as infection, use of antibiotic drugs, mode of birth delivery, environmental stressors. Among them, antibiotics generally can disrupt the bacterial homeostasis in the gut and intestinal barrier, which can open up for further opportunistic infections. To date, population-based registry studies in Denmark have recently reported that infections and antibiotic drug exposures before adulthood increased the risks for mental disorders, including ADHD and ASD [45, 82]. However, other studies found no associations between the antibiotic exposure during the first two postnatal years for ASD or ADHD [83, 84]. Further, a cohort study conducted in New Zealand reported that the first year antibiotic exposure associated with behavioral difficulties and mood symptoms [85]. Although, results from current studies are a bit inconsistent, the possible associations between antibiotic drug exposure and psychiatric and neurodevelopmental disorders might not only reflect the link between the gut microbiota and brain, but also targeted infections or immune activation.

1.2.2 Immune activation, inflammatory mediators

The immune system constitutes an important part of GBA, with the circulating immune activity mediators (e.g. cytokines, chemokines, acute-phase proteins, adhesion molecules) being the key molecules. They are small proteins or peptides secreted from immune cells (e.g. lymphocytes, macrophages, mast cells, microglia) and other cell types (e.g. fibroblasts, endothelial cells, adipocytes), which are important for the cell signaling in the immune system. There is a growing number of studies from both humoral and cellular pathways demonstrating a bidirectional immune-brain interactions in psychiatric disorders [56, 86, 87]. The immune system is being recognized for its importance in neurodevelopment with specific interest in schizophrenia, ASD and ADHD [56, 87]. Dysregulations of the immuno-inflammatory system have been implicated in mood disorders, schizophrenia and ASD, and have been proposed to be implicated also in e.g. ADHD [88-92]. The prevalence of inflammation in depression, anxiety disorders and schizophrenia was 21-42% [93]. Alterations in peripheral inflammatory marker levels have consistently been reported overrepresented in psychiatric disorders. The commonly studied markers in cohort studies include interleukins (e.g. interleukin (IL)-1β, IL-2 IL-6, IL-10, IL-12, IL-13, IL-17, IL-23), chemokines (e.g. monocyte chemoattractant protein 1 (MCP1), Eotaxin1, growth-regulated oncogene α (GRO-α)), interferons (e.g. interferon (IFN)-γ), tumor necrosis factors (e.g. tumor necrosis factor α (TNFα)), growth factors (e.g. transforming growth factor β (TGF-β), vascular endothelial growth factor A (VEGF-A)), acute-phase proteins (e.g. C-reactive protein (CRP), serum amyloid A (SAA)), soluble cytokine receptors or receptor antagonists (e.g. sIL-2R, sTNFR and IL-1RA) , adhesion molecules (e.g. intercellular adhesion molecule 1(ICMA-1) and vascular cell adhesion protein 1 (VCAM-1)) and immunoglobulins (e.g. IgG, IgM) [87, 94, 95].

Among them, IL-1β, IL-6 and CRP are the most commonly investigated peripheral inflammatory markers and most promising markers showing consistent results to predict clinical outcomes [93, 96]. CRP is liver-derived acute phase protein that responds rapidly to infection, and clinically the level at between 2 to 10 mg/L are considered as metabolic inflammation states or noninfectious inflammatory conditions [97]. IL-12 subunit p40 (IL-12/IL-23p40) is a subunit shared with IL-23. Its plasma level was found to be elevated in patients with schizophrenia and ASD [98, 99]. In addition, IL-12/IL-23p40 was also reported to involve in the pathology in inflammatory bowel disease (IBD) and is a novel therapeutic target for the disease [100, 101]. The marker associated with both brain and gut, which suggested its important roles in the GBA. ICMA-1 have been recognized in psychiatric disorders because of its role in neuroinflammation and BBB function and the soluble isoform was increased in major

depression, bipolar disorder, and dementia as well as ADHD in a small case-control study [95, 102]. ICAM-1 is expressed in endothelia cells and immune cells (leukocytes, microglial cells and astrocytes), which are involved in both the peripheral and CNS immune system. VCAM-1 is only expressed on endothelial cells and is located on large and small blood vessels when stimulated by cytokines [103]. Both ICAM-1 and VCAM-1 are cell surface binding proteins that can mediate the immune-endothelial cell adhesion and signal transduction. They are commonly recognized as important biomarkers for inflammatory processes in cardiovascular diseases, particularly in atherosclerosis [103]. Only a few small studies found significant difference at levels, showing higher soluble ICAM-1 (sICAM-1), IL-6 and IL-10 in children with ADHD [102, 104, 105]. Studies on immuno-inflammation is much less in ADHD than in schizophrenia, mood disorders and ASD, especially in the adult population [87, 106-108]. The levels of IL-6 and TNF-α were correlated with hyperactivity/impulsivity scores in children and adolescents with obesity [109] and CRP levels correlated with child behavior checklist of attention problems [110]. Identification of inflammation state as well as the immune activities in ADHD would have valuable implications in pathophysiology of the disorder. Future studies of the molecular and cellular processes for inflammatory biomarkers underlying the GBA should be encouraged. The emerging field of "immuno-psychiatry" will possibly lead to the discovery of more effective personalized treatment strategies.

1.2.3 Short-chain fatty acids

SCFAs are a group of fatty acids with less than six carbon atoms, which have been proposed to be one of the key messengers in the GBA [11]. They are predominantly produced by anaerobic colonic microbiota when fermenting dietary fibres, such as beta-glucans and resistant starch, but are also naturally present or added as additives in food [111-115]. The shorter members, with 2-4 carbon atoms, acetic acid, propionic acid and butyric acid, are the common ones with great research interests [11]. SCFAs are weak organic acids that can be taken up and utilized as energy source by colon cells. SCFAs are able to pass into the blood vessels in the wall of the intestine through either passive diffusion or active transportation via the monocarboxylate transporter 1 (MCT1) or sodium-coupled MCT1, thereby enabling their systemic access and opportunity to reach the brain by passing the BBB [11, 113, 116]. SCFAs, notably acetate, propionate and butyrate, exert central effects via binding to specific G-protein-coupled receptors, also called free fatty acid receptors (mainly GPR41/FFAR3, GPR43/FFAR2) [117, 118], which are widely expressed on a variety of cell types, including the intestinal epithelial cells, adipose cells, immune cells, pancreatic β cells, brain endothelial cells and neural cells in ganglia [117, 119]. The metabolic and molecular pathways of SCFAs are complex. There are various studies on the different functions

of these molecules in both health and disease, including maintenance of gut function, immune regulation, metabolism homeostasis and associations with IBD, obesity, diabetes, autoimmune diseases, neurological disorders and psychiatric disorders[113, 120, 121].

Data from animal models have shown that SCFAs are implicated in broad physiological activities of the nervous system, which include the calcium-dependent neurotransmitter release, electrophysiology, neuroinflammation, microglial maturation and activation, as well as gap junction gating in the BBB [10, 66, 122, 123]. Furthermore, butyrate was shown to elevate *BDNF* expression, to promote neurogenesis and neural proliferation in rodents and to facilitate long-term memory consolidation [124-127]. Emerging evidence shows that SCFAs, such as propionate and butyrate, play important roles in brain activities and behavioral development. An acute dose of butyrate enhanced learning and memory, increased sociability, and decreased depressive-like and perseverative behaviors in an ASD mouse model [128]. In contrast, administration of high-doses of butyrate or propionate induced reversible stress-like, anxiety-like and autism-like behavior in rodents [113, 129, 130]. The different observations of SCFAs on behaviors may likely depend on the dose applied. It is worth noting that physiologically circulating levels of SCFAs are relative low, in the μM range and the only detectable SCFA in cerebral spinal fluid (CSF) is acetate (36 μM) in human [111, 131]. Importantly, in an *in vitro* model, propionate at as low as 1 μM can influence the BBB function [132], which further indicates that physiologically relevant SCFAs could be crucial in regulating brain activities. Lower fecal acetic acid, butyric acid and higher valeric acid levels were found in children with ASD and the lower levels were associated with less emotional problems in healthy children [133, 134].

SCFAs are also detectable in human amniotic fluid (AF) [135]. A few studies found live microbes/microbial DNA in human AF, placenta, meconium, as well as umbilical cord blood [135-143]. However, a recently published paper found no evidence for the existence of microbial DNA in placenta of healthy full-term pregnancies [144]. Currently, there is no consensus of the existence of microbes in AF due to the limitation of contamination and cultivation. Normally, the exposure of SCFAs to fetus *in utero* is thought to originate primarily from the maternal gut-produced SCFAs via the blood passing the placenta and possibly from amniotic microbiota [135]. In rodents, maternal diet had impacts on gut microbiota, which further influenced the social behaviors and synaptic activity in the offspring [145]. Maternal oral supplementation of SCFAs increased number of fetuses, reduced abortion rate [146], and ameliorated type 1 diabetes in offspring of a rat model via downregulation of inflammation [147]. Subcutaneous injection of propionate into pregnant rat, or later to offspring induced more anxious and antisocial behaviors in female offspring [148]. All these findings suggested that the fetus can be exposed to the SCFAs

produced by the maternal gut bacteria through systemic routes, and that this can influence the neurodevelopment in a very early time window.

Anti-inflammatory actions of SCFAs are known to be exerted by modulating immune cell differentiation, chemotaxis, reactive oxygen species release and cytokine release [120]. Butyrate elicited anti-inflammatory effects by inhibition of pro-inflammatory molecules IL-12, IL-1β, TNFα, nitric oxide production and upregulation of anti-inflammatory cytokine IL-10 production [120]. Moreover, SCFAs are important for modulating both intestinal barrier and brain barrier. SCFAs can stimulate mucus production and tight junction assembly that affect the intestinal mucosal and epithelial barrier [149, 150]. A recent *in vitro* study showed that propionate has an anti-inflammatory role and protects BBB functions via several pathways [132]. Some of the above functions of SCFAs are possibly mediated by their specific receptors (FFAR2/FFAR3). Other functions, in particularly for propionate and butyrate, are likely mediated through their histone deacetylase (HDAC) inhibitory role, which is important for epigenetic regulation of gene expression [124, 151-153].

Taken together, SCFAs seem to be of great importance in GBA. It is worth exploring the roles and mechanism in ADHD as well as interactions with other players in GBA, especially the immune mediators.

"Behavior isn't something someone has. Rather, it emerges from the interaction of a person's biology, past experiences, and immediate context."

---- L. Todd Rose

2 AIMS

The overall aim of this thesis was to explore certain aspects of the GBA in neurodevelopment, with a focus on ADHD, and to explore a new adjuvant therapeutic strategy for ADHD. **Study I** was mainly focusing on early life exposure of antibiotics for developing ADHD and other psychiatric disorders later in childhood, while **Study II**, was designed to investigate the vascular immune activity in ADHD. **Studies III and IV** aimed to study the effects of an intervention on the gut microbiota in regard to clinical outcomes, immune activity and microbiota metabolites. **Study V** investigated the effect of SCFAs on human neural progenitor cells *in vitro* model.

The specific aims of each study were listed as follows:

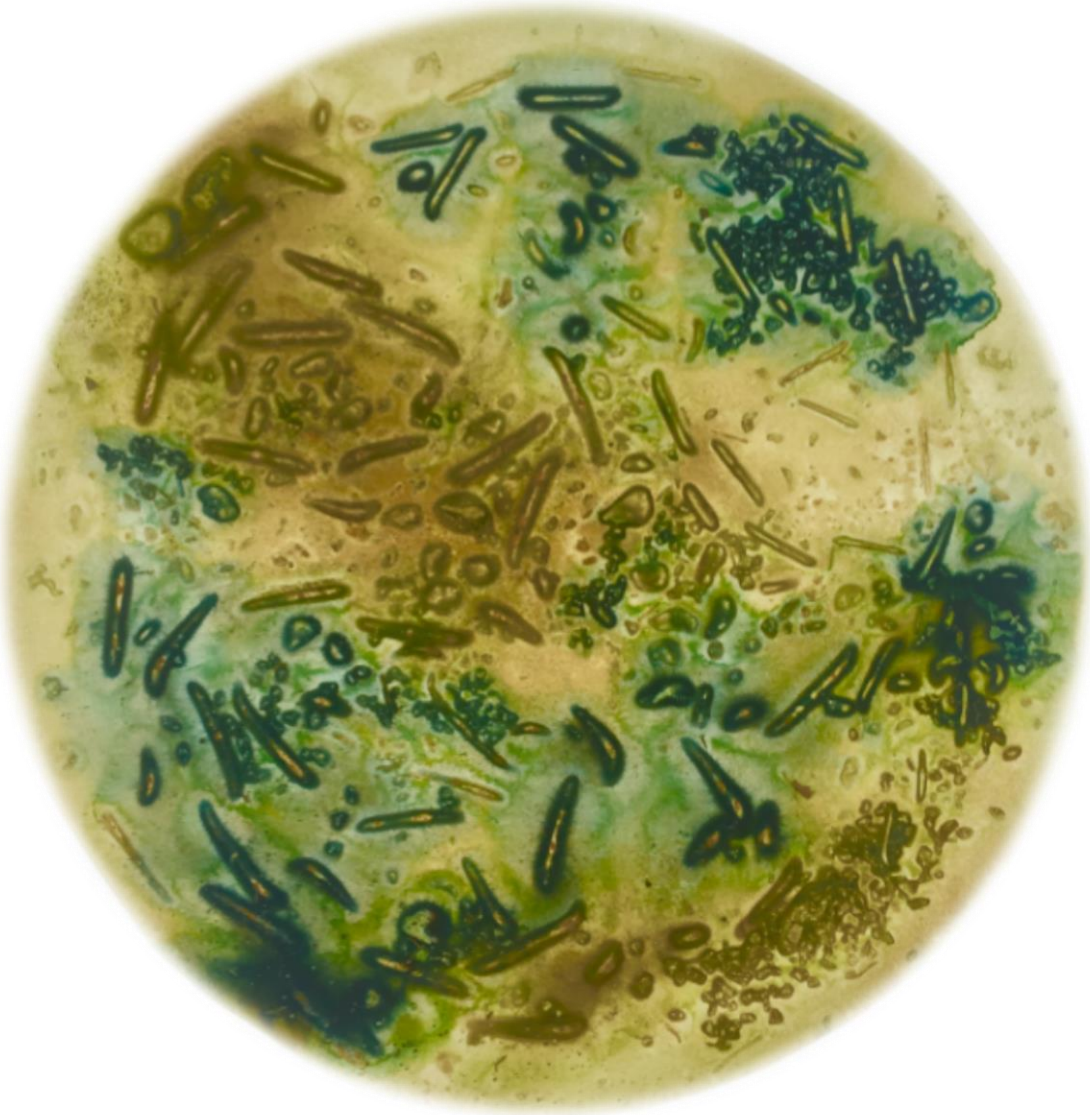
Study I: To investigate if exposure to antibiotics, prenatally and in the first 2 years of life, influences the risk for a wide spectrum of psychiatric disorder up to 18 years of age using Finnish nationwide registers.

Study II: To explore levels of vascular inflammation in ADHD and their associations with psychiatric features in a case-control setting.

Study III: To determine if Synbiotic 2000 composed of three anti-inflammatory lactic acid bacteria and four anti-inflammatory fibres has an effect on ADHD, comorbid autistic symptoms and daily functioning in patients with an ADHD diagnosis using a placebo-controlled randomized trial.

Study IV: To investigate the effects of Synbiotic 2000, a combination of dietary fibres and lactic acid bacteria, on the plasma levels of immune activity markers and SCFAs in children and adults with ADHD.

Study V: To explore if SCFAs affect the growth of early stage neural cells, using human neural progenitor cells as an *in vitro* model.



Microbiome by Ina Schuppe Koistinen (http://www.inasakvareller.se/)

3 MATERIALS AND METHODS

This section briefly describes the study populations and cell resource, as well as basic explanations of experimental and statistical methods used in the studies for the thesis. More details regarding materials, methodologies and statistics can be found in the Materials and Methods section of each individual study.

3.1 STUDY POPULATIONS

3.1.1 Finnish nation-wide registers (Study I)

All live births (N=990,098) in Finland between 1996 and 2012 registered in the Drugs and Pregnancy Database (DPD) were included in **Study I**. The DPD is derived from the Medical Birth Register (MBR), the Register on Induced Abortions (supplemented by birth and death certificates, Cause-of-Death Register and maternity hospital records) and the Register of Congenital Malformations. The majority of the contents in MBR were well supported and validated in hospital record data.

Information on exposure of antibiotic drug prescriptions were collected prenatally (mother during pregnancy) and postnatally (child at first 2 years of life) from the Finnish Register on Reimbursement Drugs (RRD), which registers all reimbursed drug purchases at pharmacies between 1996 and 2012. The DPD includes all RRD records of maternal purchases during pregnancy and the offspring purchases as well.

The outcome of the study was the psychiatric disorders for the offspring, primary or secondary diagnoses from birth to 2014. Information was obtained from the Finnish Care Registers for Health Care according to the WHO ICD-10 system. Additional outcome was the prescription of psychotropic drugs for offspring, which was obtained from the RRD using the Anatomical Therapeutic Chemical classification system and included N05 (antipsychotics, anxiolytics, hypnotics and sedatives), N06A (antidepressants) and N06B (psychostimulants and nootropics).

Demographic information on offspring birth year, sex, perinatal problems (prematurity and small birth size), number of fetuses, mode of delivery, maternal age at delivery, parity, the mother's country of birth and marital status, and maternal smoking were available in the DPD.

All the data was kept and obtained via our collaborator at the Finnish National Institute for Health and Welfare. The study was approved by the Regional Ethical Review Board in Stockholm (THL/1662/5.05.00/2015 and THL/1853/5.05.00/2016).

3.1.2 Randomized controlled trial (Study II, III, IV)

Our group set up an RCT (ISRCTN57795429) for individuals with ADHD (named BAMBA) at three psychiatric out-patient clinics in Stockholm from January 2016. Participants were recruited until June 2018, also via local newspapers. Patients, who had a confirmed ADHD-diagnosis with ICD-10 or DSM-V, were 5–55 years old, on stable pharmacological treatment (no change during the last 4 weeks), able to read Swedish, but not had an autism diagnosis, GI-disorder diagnosis (except irritable bowel syndrome), celiac disease, diabetes and antibiotic use (during the last 6 weeks) were included and randomly allocated to interventions (N=248). The active invention, Synbiotic 2000 (Synbiotics AB, Sweden), was a composition of 4×10^{11} CFU per dose of three lactic acid bacteria *Pediococcus pentosaceus* 5–33:3/16:1 (Strain deposit number: LMG P20608), *Lactobacillus casei ssp paracasei* F19 (LMG P-17806), *Lactobacillus plantarum* 2362 (LMG P-20606), and 2.5 g of each of the fermentable fibers betaglucan, inulin, pectin and resistant starch. Previous RCTs with Synbiotic 2000 have shown anti-inflammatory and anti-infection effects in patients after surgery (liver transplantation, multiple trauma, pancreas resection) [154-156]. Placebo was maltodextrin, an oligosaccharide without prebiotic effect. Each intervention lasted for nine weeks with assessments at baseline (the day before treatment start) and post-treatment (within 2 weeks after last treatment intake). A number of participants dropped out during the intervention in both placebo (N=21) and positive treatment (N=43) arms. 182 effective completers were included in the data analysis. Information on psychiatric symptoms and GI symptoms as well as biological samples (blood, urine and feces) from before and after intervention were collected.

In parallel, healthy controls (N=61) fulfilling the same criteria, but without ADHD diagnosis were recruited as well. Information and biological samples were collected at one timepoint, and was applied to the **Study II** for the case-control design.

The studies were approved by the Regional Ethical Review Board in Stockholm (2015/884-31/1 and 2017/91-31,).

3.2 IN VITRO EXPERIMENTATION

3.2.1 Cell culture (Study V)

The human neural progenitor cells (hNPCs) used in **Study V** were generated from the human embryonic stem cell line (HS980) kindly provided by Professor Outi Hovatta at Karolinska Institutet as follows. HS980 were cultured in NutriStem hESC XF medium on laminin-521 (LN-521, 30 μg/ml) coated plate, in a way similar to the protocol by *Rodin et al.* [157]. Following the previously described method by *Falk, Koch, & Kesavan* [158], HS980 cells were dissociated into single cells using TrypLE Select and plated on non-adhesive plastic in new medium of DMEM/F12 supplemented with 1% N2, SMAD inhibitors, SB-431542 and LDN-193189. 50% of the culture medium was replace with fresh medium every day until the 6-day-old floating aggregates were seeded into tissue culture plates coated with 0.002% poly-L-ornithine and 20

μg/ml murine laminin. About 2–3 days after plating, it started to form the neural rosette structures. On day 4 after plating, neural rosettes were manually picked up and transferred to a new coated well at high density in DMEM/F12 medium supplemented with 10 ng/ml FGF2, 10 ng/ml EGF, 1% N2 and 0.1% B27. There, the hNPCs were generated and could be cultured to at least the 25th passage in our laboratory.

Cultivation and *in vitro* experimentation of HS980 and hNPCs were approved by the Swedish ethical review board in Stockholm (2017/1079-31/1).

3.2.2 IncuCyte live imaging

Cells were seeded into 48-well plates and treated with SCFAs and incubated for 12 hr. Thereafter, 300 μl of fresh cell culture medium, SCFAs and 20 μg/ml propidium iodide (a common red-fluorescent nuclear dye used to stain apoptotic cells) were added into each well. The plate was placed into the IncuCyte ZOOM system incubator. Every second hour, four live images were taken from each well of the plate and the confluency percentage data and red staining count of each image were recorded at the same time. Data from the triplicates' mean of the four images per well were treated as one biological replicate for each condition. Cell growth rate was model with the confluency data applying a nonlinear least square model, which was fitted to the data by finding the best coefficients. The maximum growth rate was calculated and reported for cell proliferation. Apoptosis rate from the red-stained cell count data was calculated as change in number of red cells over two hours.

3.2.3 Immunofluorescence

After exposure, cells were harvested inside each well of the plate. Immunofluorescence staining with pHH3 (phospho-histone H3, a marker for mitosis) was performed as follow: cells were washed twice with phosphate-buffered saline and fixed with 4% formaldehyde on ice for 30 min. Cells were washed twice more with PBT buffer (PBS + 0.5% Tween-20) and blocked with PBTA buffer (PBS + 0.3% TritonX-100 + 0.1% bovine serum albumin + 5% donkey serum) at 22°C for 40 min. Cells were incubated with pHH3 primary antibody in PBTA at 4°C overnight, followed by washing twice with PBT and incubating with secondary antibody at 22°C for 45 min. After staining with DAPI at 22°C for 5 min, cells were imaged by an automated high-content imaging system, Cell Observer. After loading the layout information of plate and setting parameters of the system, images were taken automatically. Automatic cell counting for all images was carried out by ImageJ, which recorded the total number of cells and intensity of the fluorescence of each cell. Percentage of pHH3 positive cells was calculated and reported (mean of triplicates per condition per plate) corresponding to a predefined intensity threshold for pHH3 positivity, which was done by a human analyst.

3.2.4 Quantitative reverse transcriptase PCR

Total RNA was extracted from the cells using using Direct-zol™ RNA MinpRrep Plus Kit according to the standard manufacturer's instructions. RNA concentration and purity (A260/A280) were measure by NanoDrop™ 2000 Spectrophotometers, with concentration at

50 ~ 120 ng/l and A260/A280 at 1.8 ~ 2.1 for all samples. cDNA was synthesized directly after RNA extraction using SuperScript® III First-Strand Synthesis System for quantitative reverse transcriptase PCR (qRT-PCR) according to the standard protocol for cDNA synthesis. qRT-PCR was carried out in a QuantStudio™ 6 Flex Real-Time PCR system using SYBR Green kit following the standard settings. All reactions were run in triplicates. The relative mRNA gene expression was presented based on Ct value of the triplicates that was normalized by reference genes, β-actin (ACTB) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

3.3 IMMUNE ACTIVITY MARKER MEASUREMENTS

Plasma levels of inflammatory cytokines, chemokines and acute phase proteins were measured using the Meso Scale Discovery (MSD) platform. It is a sandwich immunoassay technology that allows both pre-designed and custom-designed multiplex assays for the measurement of a combination of molecules in biological samples. The biotinylated capture antibody is coupled to a linker that specifically self-assemble to the unique spot in each well in the multiplex plate. The molecule of interest in the sample binds to the captured antibody-linker complex, and can be detected by another antibody conjugated with an electrochemiluminescent label of SULGO-TAG that can bind to the molecule as well. The MSD instrument read the light signal emitted from the captured label, and the intensity corresponds to the quantity of the molecule in the sample.

The self-designed inflammatory profile of 24 markers were measured with VPLEX, 3-spot, 7-spot and 10-spot UPLEX assays in our lab according to the manufacturers. Four markers measured with VPLEX were included in **Study II and III**, and all 24 markers were investigated in **Study IV**. Five markers were excluded from **Study IV** because the signals were substantially (>25 % total measures) below the lower limit of detection. All samples were measured in five 96-well plates for each assay. The median (range) of intra- and inter-assay coefficients of variation (CVs) were below 2.5 (1.1%- 5.3%) and 9.9% (4.6%-16.9%), respectively.

3.4 SHORT-CHAIN FATTY ACID MEASUREMENTS

The plasma SCFA profile included nine fatty acids (formic, acetic, propionic, butyric, isobutyric, succinic, valeric, isovaleric and caproic acid) and were measured by liquid chromatography–mass spectrometry (LC-MS) according to a protocol published by *Han J et al. 2015* with some modifications, at the Department of Biology and Biological Engineering, Chalmers University of Technology, Gothenburg [159]. All samples were measured in two rounds (four months in between), being six 96-well plates for the first round and five 96-well plates for the second round. Twenty-two samples were selected to run in both rounds in order to control for batch effects. The levels of five SCFAs (formic, acetic, propionic, succinic and isovaleric acid) showed high consistency between the two rounds. Therefore, data from these five SCFAs were included in the analysis. The median (range) of intra-assay CV was 9% (5%-

11%) for the same two quality controls run on each plate. The inter-assay variation was controlled by normalization with the two quality controls. All plasma samples had undergone the same sample preparation steps and two freeze/thaw cycles for the analysis.

3.5 STATISTICAL ANALYSIS

This section covers main statistical methods used in the five studies, including the Cox proportional hazards model used in **Study I**, correlation and linear regression used in **Study II, III and IV**, analysis of covariance (ANCOVA) used in **Study III and IV** as well as corrections for multiple testing in **Study II, IV and V**. A number of commonly used simple tests (**Study II-V**), including *t*-tests, Mann–Whitney *U* test and analysis of variance (ANOVA) will not be further discussed in the thesis. All data analysis were performed in R programming software.

3.5.1 Cox proportional hazards model

The Cox proportional hazards model is an advanced regression model that is commonly used in medical research, to study the association between a specific event happening (e.g. diagnosis, death) at a time point and one or more predictor variable(s) (e.g. smoking, infection). The model evaluates the effect of the predictors for outcome over time and simultaneously reports the hazard rate. In study design, the predictors always have more than one conditions, and one of them was selected as the reference group. Hazard ratio (HR) is the ratio of the hazard rates corresponding to the reference group.

In medical research, there are many situations or factors, also known as covariates, which can potentially affect patient prognosis. Here, the Cox model allows us to analyze survival or outcome with respect to several factors simultaneously and provides the effect size for each factor at the same time.

In **Study I**, we studied the effects of predictor variables, exposed or not exposed to antibiotic drugs in early life, on the development of psychiatric disorders in childhood. For each individual born between 1996 and 2012 and followed up until 2014, the onset time of a psychiatric disorder is different, therefore we selected Cox model to study the hazard ratios representing instantaneous risk with less bias on the endpoints of the study.

3.5.2 Correlation

Correlation, also known as dependence is a statistical method mainly to study to what extent two quantitative variables are related. The most common Pearson correlation test is applied to assess the linear relationship between normally distributed variables. There are also more robust methods for non-normally distributed data, such as Spearman's rank correlation test and Kendall's rank correlation test, to measure the effect as one variable increases, while the other variable tends to increase or decrease. A high correlation coefficient indicates that the two variables have a strong relationship with each other.

In **Study II** and **IV**, the relationship between two marker levels as well as between marker levels and clinical symptom scores were assessed by Spearman's rank correlation. The coefficients (*r*) and *p* values were reported in the results.

3.5.3 Classical linear modeling

Linear regression is a statistical model to study the relationship between an outcome variable (dependent variable) and one or more predictors (independent variables). The parameters of a model are estimated from the given set of data. To fit a linear model, it is possible to increase the likelihood by adding parameters, but may result in overfitting. Least squares approach is often used to fit a linear regression models. There are also other ways for model fitting, such as Akaike information criterion (AIC). It is a useful criterion for model selection, which attempt to resolve the overfitting and underfitting problems by estimating the amount of information lost by a model. Thereby, the values from AIC estimate the relative quality of statistical models, and the lowest values are preferred for a fitted model.

In **Study II**, we modelled the relationship between the inflammatory marker levels and the clinical variables collected, we performed stepwise backward elimination regression and used AIC to select the best model with certain predictive variables for each marker.

ANCOVA, as the name indicates, is an extended statistical model of ANOVA, which introduces regression into the model. It is a general linear model to evaluate whether the means of a dependent variable are different among several independent groups within a categorical variable (e.g. treatment), meanwhile statistically controlling for the effects of other variables, known as covariates that are not of primary interest but have possible influence on a dependent variable. We always interpret the model as the difference between groups if it is explained only by independent variable, since the model has adjusted for the variance explained by the covariates.

In **Study III** and **IV**, the treatment effect between placebo and Synbiotic 2000 on symptoms, immune marker levels and SCFA levels were analyzed by ANCOVA, adjusted by age, sex and baseline measurements. Statistically, for pretest-posttest control group design, the treatment effects can be tested by either using (1) ANOVA with the change from baseline (post-treatment – pre-treatment) or (2) ANCOVA with the post-treatment timepoint as outcome and pre-treatment timepoint as covariate. For **Study III and IV**, ANCOVA has been chosen based on its unbiasedness and increase in power in randomized studies compared to the power of the ANOVA with change [160]. We reported the 95% and/or 99% confidence interval (CI) instead of *p* value, which is more informative especially for clinically relevant studies. A *p*-value gives information on if the null hypothesis should be rejected or not. The rejection of the null hypothesis can either be true (there is actually no difference between the groups) or false (there is a difference between the groups, but it could not be detected due to small effect sizes, large within-population variation or too small sample size). However, a CI reports the significance and the width of a CI also indicates the likelihood of a zero effect being true or not [161].

3.5.4 Correction for multiple testing

Multiple correction is a statistical procedure used to control multiple testing problems, which happen when a statistical analysis involves multiple simultaneous tests of the same dataset or dependent datasets and each of the tests has a potential to produce a "discovery". Bonferroni correction is one of commonly used methods for correction for multiple testing in order to not obtaining false positive results. Bonferroni correction compensates the hypothesis testing by setting the desired α level to the former α level divided by the number of tests performed. Thus, Bonferroni correction offers a great protection against the type I error (false positive), but is often seen as overprotective and lacks power [162, 163] . The false discovery rate (FDR) is another, less stringent, method for correction and focuses on the proportion of false positives [164]. In **Study II**, we used Bonferroni correction for the four markers investigated in the study by the α level at 0.025 (2 independent tests), because the four markers were highly correlated into two groups. In **Study IV**, we corrected multiple testing of nineteen markers or five SCFAs with FDR and Bonferroni (ANCOVA for treatment effect with α level at 0.01) methods. In **Study V**, we corrected multiple testing of SCFA exposures with the Holm–Bonferroni method.

BAMBA project logo designed by Miranda Stiernborg.

4 RESULTS AND DISCUSSION

This section summarizes the main findings and provides a brief discussion of the studies included in the thesis. More details for each study can be found in the end of the thesis, which lists individual full-length paper or manuscript.

4.1 STUDY I

Antibiotic exposure and childhood psychiatric disorders

Previous studies have reported that certain infections or anti-infective drug exposures were associated with increased risks for psychiatric disorders, such as psychotic disorders and affective disorders [45, 82]. Moreover, peripheral immune activation is common in children with psychiatric disorders and recognized as an important factor in neurodevelopment [56, 87]. Early life, especially the prenatal period is an important time window for neurodevelopment. There are a few cohort studies of effects of prenatal exposure to antibiotic drugs on psychiatric disorders, but without considering potential confounders, like family factors and socioeconomic factors. The hypothesis of this study was that antibiotic drug exposure during the fetal period and the first two postnatal years is associated with risks for later development of psychiatric disorders in children.

4.1.1 Results

In this cohort study, we used Finnish nationwide registers that included 1 million births to investigate the associations between exposure to antibiotics in utero and the first two years after birth and the risk for a group of childhood-onset psychiatric disorders. We found that antibiotic exposure during pregnancy or in the first two years after birth associated with modestly elevated risks for developing Mood disorders (ICD-10, F20–39, F92), Anxiety disorders (F40–43, F93), Non-organic sleeping disorders (F51), ADHD and Conduct disorders (F90–F91) and Other behavioral and emotional disorders (F98) (**Figure 1**). These findings were also supported by the results from purchases of psychotropic drugs (N05: antipsychotics, anxiolytics, hypnotics and sedatives; N06A: antidepressants; N06B: psychostimulants and nootropics) to offspring (**Figure 1**). A dose-dependent effect was found with the number of antibiotic purchases during pregnancy and any offspring psychiatric diagnosis. Moreover, antibiotic drug purchase by mother during pregnancy had a higher risk than purchase three months before or three months after pregnancy for any psychiatric diagnosis in the offspring. Sibling pair analysis suggested that the effects detected were not fully explained by familial confounding. Moreover, the increased risk of offspring psychiatric disorder after prenatal exposure did not change after adjusting for maternal infection during pregnancy, which implied an antibiotic effect on psychiatric disorders independent of maternal infection. Stratification analysis with the type of antibiotics (airway antibiotics, urinary tract antibiotics and combined) showed no

clear difference in either prenatal or postnatal exposure, but it suggested that the broad-spectrum antibiotics had a slightly higher risk for psychiatric disorders than narrow spectrum antibiotics.

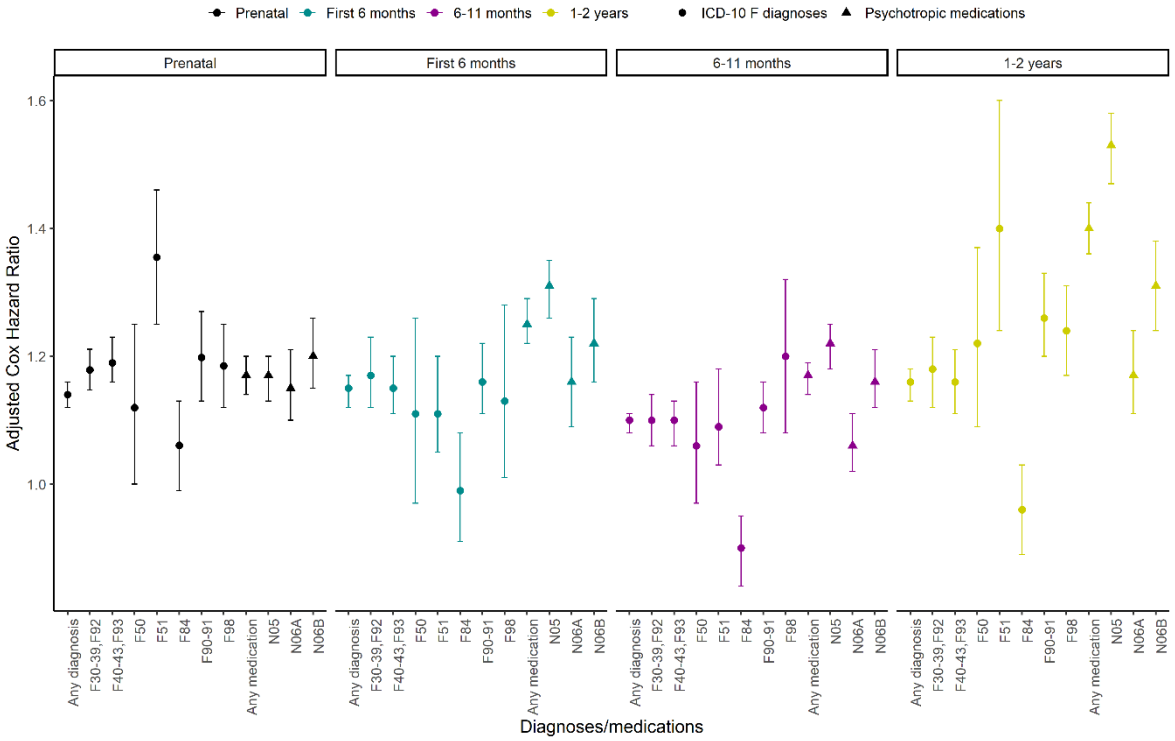


Figure 1. Overview of adjusted Cox Hazard Ratios for (i) psychiatric diagnoses (ICD-10 F diagnoses), and (ii) psychotropic medication, in relation to exposure to any antibiotic drug prenatally (trimester 1–3) or in the first 2 years of life, among 990,098 births (1996–2012). Figure from Lavebtratt C, Yang LL et al., 2019, *Translational Psychiatry*.

4.1.2 Discussion

This was a large longitudinal study (1996-2014) with 1 million participants and detected modest risks of early antibiotic exposure on offspring psychiatric diagnosis. Some previous nation-wide register studies on infection or antibiotic drugs exposures found increased risk for later ADHD, but some did not. Our study found that antibiotic exposure increased risks for ADHD. A dose-dependent effect of antibiotic purchase during pregnancy, and the higher effect size of the association with maternal antibiotic use during pregnancy compared to outside pregnancy, supported the vulnerability to antibiotic drug exposure in fetal period. The associations between antibiotic drug exposure and later psychiatric disorders might reflect direct effects of the targeted infections or antibiotic-induced bacterial dysbiosis that can further facilitate the downstream opportunistic infections and influence GBA signaling. A number of confounding factors, including maternal parameters, and birth-related parameters were adjusted for in the model, but we lack the genetic and environmental factors from the paternal side. The associations observed were modest, with the strongest being with sleeping disorder

(HR: 1.09-1.40), but, notably, there was no association with autism. This may partially be explained by the final sample size for each psychiatric disorder. However, the proportion of complete cohort for each disorder was not exactly in line with the onset age. All participants were followed up until 2014, with age 2-18 years. We did not control for the effects of antibiotic drug use after the first 2 years. The validity of psychiatric diagnoses, other than autism, in the registries used has not been reported and the magnitudes of the associations between diagnoses are considerably overlapping. This is an association study of epidemiological design that cannot infer causality.

4.2 STUDY II

Pro-inflammatory mediators in children and adults with ADHD

Altered levels of inflammatory markers are gradually being discovered in psychiatric disorders, like ASD, schizophrenia and mood disorders [87, 94]. However, there were not many studies on ADHD, especially among adults. Neurobiologically, ADHD as a neurodevelopmental disorder is quite similar to ASD and the comorbidity of each other is 15-25% for ADHD and 40-70% for ASD, respectively [19]. Patients with ADHD often have problems on emotion regulation and GI disturbance that is closely associated with gut microbiota and immune activation. Acute phase protein, CRP and SAA are commonly investigated inflammatory marker in psychiatry. Adhesion molecules sICAM-1 and sVCAM-1 play important roles in endothelial function that facilitates the inflammation responses. These four markers share the same role in vascular inflammation and related cardiovascular diseases. We hypothesized that CRP, SAA, sICAM-1, sVCAM-1 levels would be elevated in ADHD compared to controls and that higher levels would associate with severity of ADHD symptoms, functioning and medication with stimulants. Further, we hypothesized that the ADHD patients would manifest more GI symptoms and autistic traits, which would associate with elevated levels of the inflammatory markers.

4.2.1 Results

In this study, we measured the plasma levels of a panel of four markers in both ADHD and healthy controls. Levels of pro-inflammatory sICAM-1 and sVCAM-1 were significantly higher in ADHD patients than in healthy controls, also after adjusted for sex and age (**Figure 2**). These four markers were highly correlated with each other. As expected, more comorbid autism symptoms and GI symptoms were found in ADHD than in controls. Among children with ADHD, those with ADHD medication had higher levels of sICAM-1 and sVCAM-1 than those without. The differences were significant for almost all types of ADHD drugs, especially for those children who were currently on medication. CRP levels were positively associated with GI symptoms and emotional regulation problems, and sICAM-1 were positively correlated with autistic symptoms in adults with ADHD. However, the associations were not found in children with ADHD.

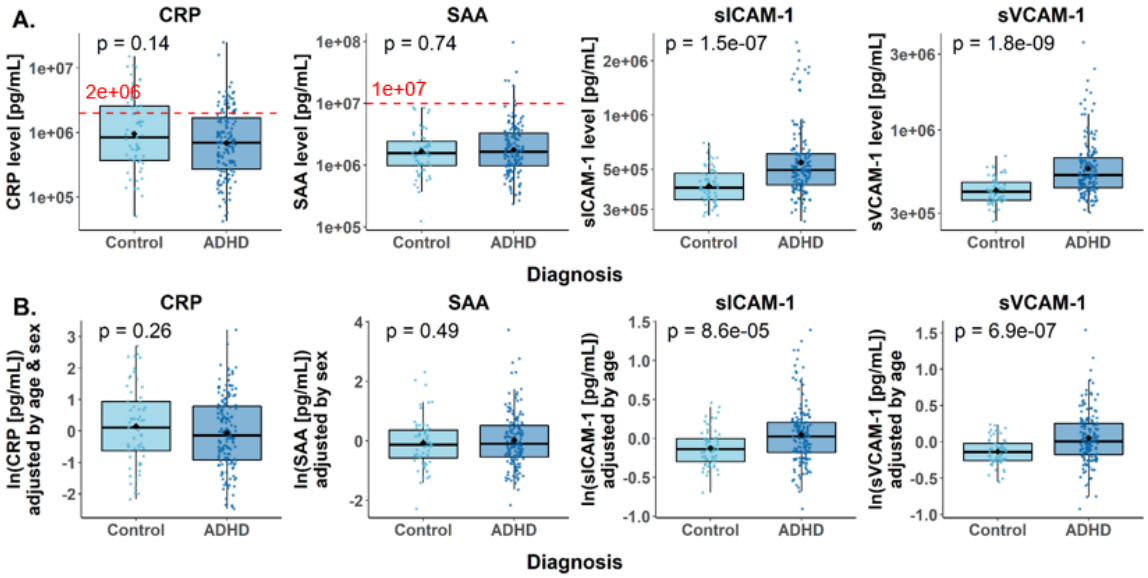


Figure 2. Levels of CRP, SAA, sICAM-1 and sVCAM-1 in patients with ADHD and healthy controls. Figure from Yang LL et al., 2020, *European Neuropsychopharmacology*, in press.

4.2.2 Discussion

This was a case-control study that investigated four inflammatory mediators and their associations with ADHD medication, psychiatry symptoms and comorbid traits in children and adults ADHD. There is a lack of published studies on immune activity markers in adults with ADHD. Previous reports on cardiometabolic risk for ADHD suggested vascular inflammation in the disorder [165]. Higher sICAM-1 levels as well as other inflammatory markers (e.g. IL-6, IL-10) were found in ADHD, which supported immune activation in ADHD [102, 104]. Our study found significant higher levels of sICAM-1 and sVCAM-1 in ADHD, which indicated vascular inflammation in ADHD. Moreover, a common side effect of ADHD medication is increased heart rate and blood pressure, and sICAM-1 and sVCAM-1 are associated with hypertension and atherosclerosis. The higher levels of sICAM-1 and sVCAM-1 found in children with ADHD medication in our study implied a possible cardiovascular risk later for the group of patients. Both ICAM-1 and VCAM-1 are closely related BBB permeability, which is an important guard for neuroinflammation[166, 167]. The higher levels of sICAM-1 correlated with more autism symptoms in adult patients of our study.

In the study, we analyzed four markers at a time with the pre-designed VPLEX, which is a robust assay with >99% specificity, high sensitivity and no cross-reactivity. For statistical analysis, we corrected for two independent tests for the four markers, because of the high correlations of sICAM-1/sVCAM-1 and CRP/SAA. At the same time, we also controlled for the potential co-variables (age, sex, body mass index (BMI) and ADHD medication) when analyzing the data. However, there were some limitations for the study. Firstly, the sample size of children was small (n=53) in this study. Secondly, only four children controls were recruited and the controls for adults consisted of both family members and unrelated people without

ADHD diagnosis. There was thus a certain bias regarding the genetics and environment for the two control subgroups, however we did not find any difference in marker levels between them. Thirdly, the BMI was not available for children. Fourthly, it was a study focus only on peripheral inflammation, not considering neuroinflammation. Replications with larger sample size will be necessary.

4.3 STUDY III

Effects of Synbiotic 2000 on clinical outcomes in ADHD

In **Study II**, we have shown that GI symptoms were overrepresented in patients with ADHD and increased levels of vascular inflammation were in adults associated with more severe symptoms. Other studies also showed that patients who had more psychiatric symptoms, in parallel had more GI symptoms [168, 169]. Growing evidence exists that the gut microbiota, a main component of GBA, influences the gut-brain interactions. Gut microbiota has shown to influence the systemic immune activities, permeability of the BBB, maturation and activation of microglia cells, synaptogenesis and motor control [56]. Altered gut microbiota has been reported in patients with ADHD. Oral administration of certain bacteria (*L. rhamnosus GG*) the first 6 months of life reduced risk for ADHD and a few RCTs of prebiotics or probiotics suggested some positive effects on improving psychiatric symptoms [52, 61, 79, 170]. Dietary supplementations, such as prebiotics/probiotics/synbiotic and omega-3 fatty acids have been proposed as possible options as adjuvant treatments to ameliorate ADHD symptoms. However, there is no randomized placebo-controlled trials in patients with ADHD. Our hypothesis was that Synbiotic 2000, a combination with three anti-inflammatory lactic acid bacteria and four anti-inflammatory fibres would reduce psychiatric symptoms and improve daily functioning.

4.3.1 Results

In this RCT, a total number of 182 ADHD patients completed a 9-week intervention with either placebo or Synbiotic 2000, which was combination of prebiotic (fibres) and probiotic (lactic acid bacteria) content (**Figure 3**). After the interventions, total ADHD symptoms and inattention or hyperactivity-impulsivity symptoms were significantly reduced for all participants regardless of treatment types. The reduction was similar in placebo and Synbiotic 2000 and not different between the two groups. Likewise, there was no difference between Synbiotic 2000 and placebo on changing daily functioning. For autism symptoms, Synbiotic 2000 treatment significantly reduced the restricted, repetitive and stereotyped behaviors as compared to placebo. The effect was pronounced for children with elevated inflammation levels (sVCAM-1) in plasma and for children currently on ADHD drugs such that also total autism scores was improved. Among adults, Synbiotic 2000 treatment resulted in better effects than placebo on emotion regulation in difficulties in engaging goal-directed behavior. This was also pronounced in those with higher sVCAM-1 levels such that function in four subdomains (difficulties in engaging goal-directed behavior, lack of emotional clarity, limited access to

effective emotion regulation strategies and nonacceptance of emotional responses) were improved.

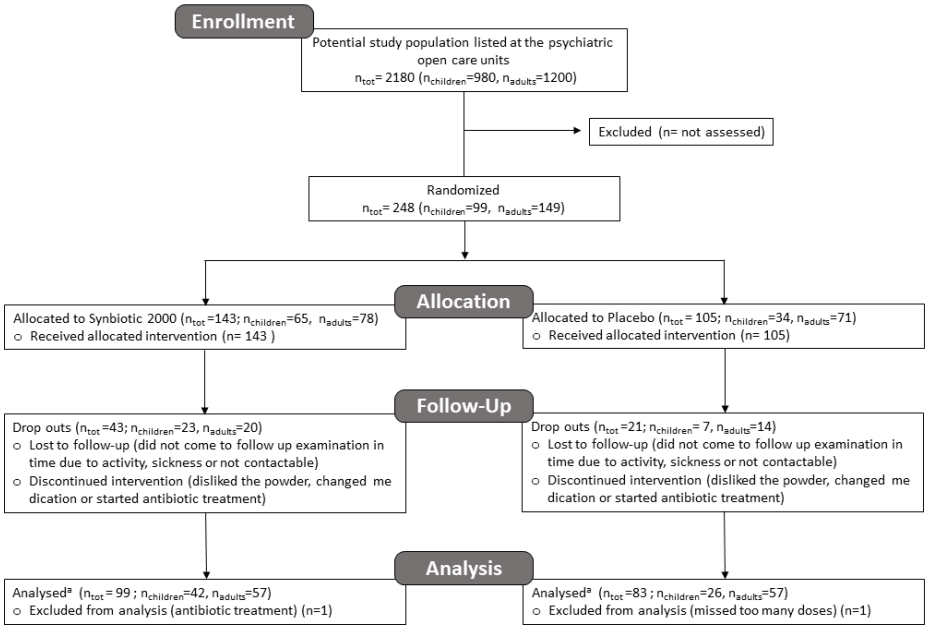


Figure 3. Flow chart of randomized controlled trail. Figure form Skott E*, Yang LL* et al., 2020, *Brain, Behavior, and Immunity*.

4.3.2 Discussion

This is the first completed placebo-controlled RCT exploring the effects of a synbiotic on psychiatric symptoms and functioning in ADHD. Previous studies, mainly open-label design, have shown some positive effects of prebiotics and probiotics on autism, a common comorbidity with ADHD [81]. In our study, we did not find any effect of Synbiotic 2000 on ADHD symptoms, but a significant effect on restricted, repetitive and stereotyped behavior of autism in children with ADHD. Altered gut microbiota and peripheral immune activities have been found in individuals with ADHD [60, 61 102, 104, 105] and the interactions between the microbiota, immune and nervous systems are becoming important for psychiatric disorders [56]. Our ADHD patients manifested elevated sVCAM-1 levels at baseline (**Study II**) and among the child patients with higher sVCAM-1 levels at baseline, an effect of Synbiotic 2000 on total autism symptoms and on the restricted, repetitive and stereotyped behaviors was observed. Likewise, for emotion dysregulation in adults with ADHD, we found that Synbiotic 2000 improved difficulties in engaging in goal-directed behavior and among those with elevated sVCAM-1 levels at baseline there were effects both on total scale and on four out of five subdomains. These findings may reflect anti-inflammatory properties of Synbiotic 2000.

The design of the study is a double-blind and placebo-controlled trial that reduced or eliminated certain experimental biases. The total sample size of the trial was large and the intervention time was long in the context of probiotic or prebiotic interventions in neuropsychiatric

disorders. The baseline clinical characteristics were similar the completers in the placebo and Synbiotic 2000 groups. In this study, we did not find significantly better synbiotic effects on the main primary outcomes of ADHD, but indeed for subdiagnostic autism traits and emotion regulation. A placebo effect reducing ADHD symptoms was observed and this was similar to that of Synbiotic 2000 effect. Placebo effects are however common in RCTs in psychiatry. In the analysis, we controlled for age and sex and stratified by an inflammation marker and ADHD medication. However, we did not control for drugs that have been reported to affect the gut microbiota, which may consequently influence the outcomes of Synbiotic 2000. Dietary variables, such as nutrients and dietary patterns, which recently were reported to be associated with ADHD symptoms in adulthood [50], are also important to consider. Unfortunately, we only had information at the times before and after treatment, which showed no changes over time except for beta-carotene. Our suggestive findings are preliminary and need to be confirmed using larger cohorts.

4.4 STUDY IV

Effects of Synbiotic 2000 on immune activity markers and SCFAs

The effects of Synbiotic 2000 that we found on autism symptoms and emotion dysregulation in **Study III**, may act through lowering the inflammatory state in ADHD patients. SCFAs are fatty acids with less than six carbons, and they have been proposed to be important players or mediators in the bidirectional GBA. They are predominantly produced from dietary fibres by anaerobic microbiota in the gut, but they can pass the intestinal barrier, go into the blood circulation and reach the entire body. Some SCFAs have in *in vitro* studies been reported to have anti-inflammatory effects [120]. We hypothesized that Synbiotic 2000 would influence plasma levels of inflammatory markers and SCFAs and that there would be associations between immune activity markers and SCFAs.

4.4.1 Results

Adult ADHD patients had a higher inflammation state than healthy controls at baseline, with elevated plasma levels of pro-inflammatory sICAM-1 and sVCAM-1 and reduced anti-inflammatory IL-10 levels. Meanwhile, lower baseline plasma levels of formic acid and propionic acid were found in adults with ADHD. Synbiotic 2000, compared to placebo, reduced the levels of IL-12/IL-23p40 in children on ADHD medication and suggestively increased the levels of propionic acid in child patients. IL-10 levels negatively correlated with formic acid and acetic acid levels at baseline in both children and adults with ADHD. Further, at baseline sVCAM-1 negatively correlated with acetic acid and propionic acid, and sICAM-1 with acetic acid in children with ADHD, while sVCAM-1 negatively correlated with formic acid in adults with ADHD. These correlations were not observed in ADHD patients after treatment.

4.4.2 Discussion

With the same RCT as **Study III**, we explored the effects of Synbiotic 2000 on plasma immune activity markers and SCFAs. Results from **Study III**, showed that Synbiotic 2000 effects were more detectable in higher inflammatory state. In this study, we detected a higher inflammatory state in adult ADHD by increased pro-inflammatory sICAM-1 and sVCAM-1, and decreased anti-inflammatory IL-10 levels at baseline. However, we only found a significant effect of Synbiotic 2000 on IL-12/IL-23p40 level in children on ADHD medication, who had elevated vascular inflammation as reported in **Study II**. IL-12/IL-23p40 is a pro-inflammatory cytokine involved in the pathology of IBD [101] and has been reported to be increased in CSF from schizophrenia patients [98]. On the other hand, the ingredients of Synbiotic 2000 include four fibres, which are a major source of SCFAs. The production of gut SCFAs are dependent not only on bacterial species but also on substrates. Anti-inflammatory and immune-modulatory roles of SCFAs were reported in previous studies [171]. Here, the adult ADHD patients had lower formic acid and propionic acid levels at baseline. Although, there was only a suggestive Synbiotic 2000-specific treatment effect on SCFA levels, correlations between formic/acetic/propionic acid and sICAM-1/sVCAM-1/IL-10 were found at baseline. This suggested immune-SCFA interactions or GBA signaling in ADHD. Notably, the correlations were not seen neither for controls nor after Synbiotic 2000 treatment.

In this study, we measured 24 immune activity markers in plasma with four independent assays (VPLEX, 3-spot, 7-spot and 10-spot UPLEX) from MSD. The VPLEX assay is a robust pre-designed panel from MSD that measures 4 vascular inflammation markers (CRP, SAA, sICAM-1, sVCAM-1). The assay has been validated critically for sensitivity, specificity, accuracy, and precision. The other 20 markers were custom-designed and measured with three UPLEX assays and MSD platform have carefully controlled for cross-reactivity between markers. All the assays had generated standard curves with correlation coefficients >0.99. 19 immune activity markers and 5 SCFAs had detectable values with acceptable precision and were included in the data analysis. In the analysis, we corrected for multiple testing using FDR. However, for the treatment effects, we adjusted for 5 independent tests through Bonferroni and reported 95% CI and 99% CI from ANCOVA models, to simplify result visualization. The most interesting findings were the correlations between the plasma immune activity markers and SCFAs, which were found to be altered in ADHD patients compared to healthy controls. Associations between SCFAs and membrane-bound endothelial adhesion molecules are still unknown. The small control sample size (n=4) made it impossible to detect any case-control difference for children. Replication in larger cohort is warranted.

4.5 STUDY V

Effects of SCFAs on neurodevelopment with *in vitro* model

SCFAs derived from gut microbiota are found in the CSF and the AF [131, 135]. SCFAs regulate several aspects of the neural system, e.g. neurotransmitter synthesis and release, microglia activation, growth of neurospheres and differentiation of embryonic stem cells [10, 152, 172].

Prenatal exposure to propionate revealed associations with later development of ASD, in both animal models and cell lines [113]. Our hypothesis was that SCFAs would influence proliferation and apoptosis of hNPCs.

4.5.1 Results

In this study, early stage hNPCs generated from Human embryonic stem cell line HS980 were exposed to three main SCFAs, acetate, propionate and butyrate at different levels (**Figure 4**). We found that SCFAs at high doses (mM) had obvious toxic effects on hNPCs. However, at low doses (μM) that were close to physiological levels in human body fluids, the proliferation of hNPCs was increased and the apoptosis was not affected. A dose-dependent pattern was seen for all three SCFAs on proliferation (**Figure 5**). Furthermore, we found that low-dose SCFAs regulated the expression of genes involved in cell cycle, apoptosis and neurogenesis.

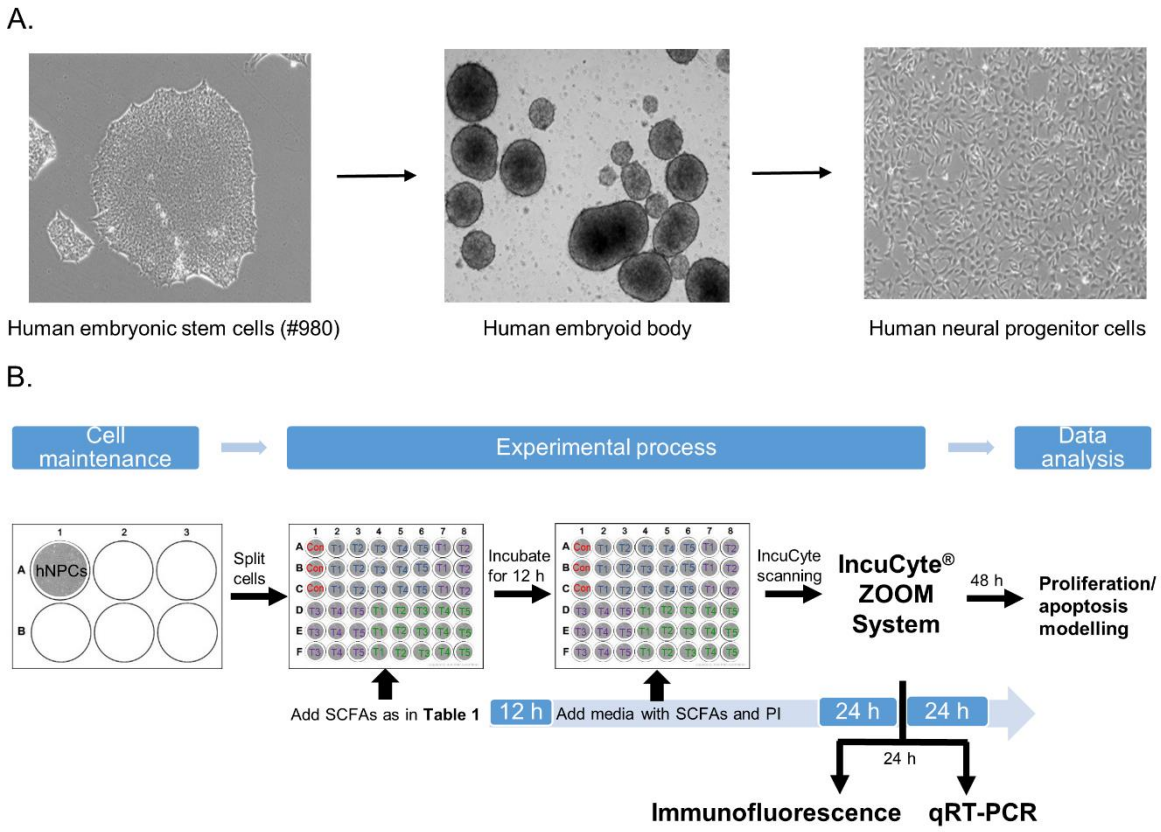


Figure 4. Workflow for the (A) generation and (B) short-chain fatty acid (SCFA) exposure of human neural progenitor cells (hNPCs). Figure from Yang LL et al., 2019, *Journal of Neurochemistry*.

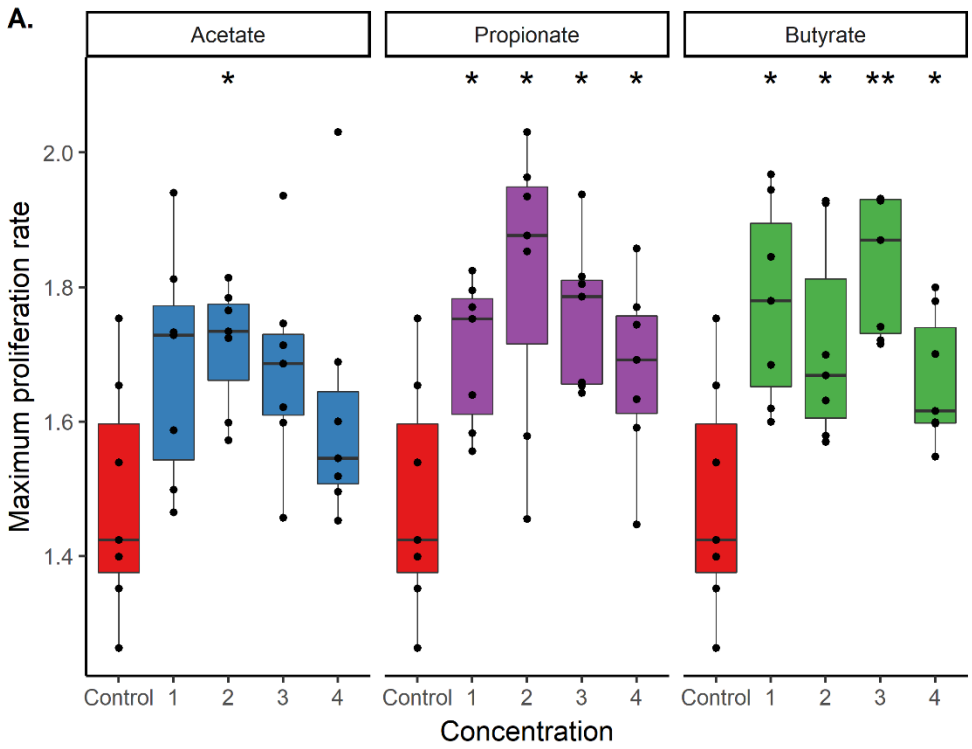


Figure 5. Maximum proliferation rate estimated from the growth curves for SCFA treatments on human neural progenitor cells monitored using IncuCyte live imaging. Figure from Yang LL et al., 2019, *Journal of Neurochemistry*.

4.5.2 Discussion

To our current knowledge, this is the first *in vitro* study that explored the effects on hNPCs by SCFAs at physiologically relevant levels. The three SCFAs, acetate, propionate and butyrate that we have examined are among the most common and abundant ones, and propionate and butyrate seemed to have the stronger effects than acetate on hNPCs. SCFAs are present in AF and are thought to be primarily from maternal gut microbiota. Our hNPCs are early neural cells generated from a human embryonic stem cell line, which to certain degree probably mimics cells starting to appear *in utero* during the fourth week. Our findings of SCFAs stimulating the proliferation of hNPCs might reflect a putative 'maternal gut-fetal brain-axis'. Furthermore, the pronounced association between prenatal antibiotic exposure and risk for developing ADHD found in **Study I** could possibly in part be explained by the 'maternal gut-fetal brain-axis'. The theory could be that antibiotic use during pregnancy affect maternal gut microbiota and SCFA production, which in turn influences the fetal neurodevelopment. Obviously, more studies are needed to test this speculation.

In this study, the relative concentrations of the three SCFAs were designed according to physiological levels, but we did not investigate the exposures of the combination of the three. It is unknown whether the combination would have synergistic effects or toxic/acidic effects for the cells. SCFA effects are remarkably tissue and dose specific. The exposure time of

SCFAs in our study was 24 h and 48 h due to the technical limit for cultivation of the cells. Long-term effects can be addressed by exposures to not only hNPCs but also later stage neurons and 3D-organoids for longer time. Since SCFAs are molecules with multiple functions, e.g. FFAR2/3 activation, acidification, and HDAC inhibition, etc., more studies about the underlying mechanisms of effects observed are needed.

"The road to health is paved with good intestines!"

— Sherry A. Rogers

5 CONCLUSIONS AND FUTURE PERSPECTIVES

5.1 CONCLUSIONS

This section lists a brief summary of conclusions for each study and ends with a short concluding remark.

Study I: Early life exposure to antibiotic drugs modestly increased the risk for developing a broad spectrum of psychiatric disorders (e.g. ADHD) later in childhood, which might reflect direct effects of the antibiotic-targeted infections, as well as indirect effects from antibiotic-induced alteration of microbiota or a perturbed barrier. This may lead to downstream opportunistic infections and modified GBA signaling. This study is of value for public health, given the high frequency of early-life antibiotic drug use and the high prevalence of psychopathology in childhood and adolescence.

Study II: Plasma sICAM-1 and sVCAM-1 levels were higher in patients with ADHD than in healthy controls, indicating vascular inflammation or endothelia dysfunction in ADHD. It would be of interest to examine the vascular biology both as it pertains to ADHD and current medications. Pro-inflammatory markers levels were related to current ADHD medication in children and certain comorbid autistic symptoms and difficulties in emotion regulation in adults. This may have diagnostic and therapeutic implications.

Study III: Synbiotic 2000, as compared to placebo, did not have an effect on overall ADHD symptoms but it had beneficial effects on ADHD patients on reducing the restricted, repetitive and stereotyped behaviors in children and difficulties in regulating goal-directed behaviors in adults. In those with sVCAM-1 levels above the median these beneficial effects were found also on autism traits in general among children as well as on four of five domains of emotion dysregulation in adults. Currently, there is no effective pharmacological treatment specific for core autistic symptoms. If safe and easy dietary supplements, such as synbiotics could help to ameliorate symptoms or improve functioning in persons with ADHD or ASD, that would be beneficial for many patients. It would be of interest to further explore synbiotic treatment either during antibiotic treatment and/or for longer periods in ADHD patients.

Study IV: Synbiotic 2000, as compared to placebo, significantly reduced the pro-inflammatory IL-12/IL-23p40 levels in children with ADHD on ADHD medication, suggestively reduced sICAM-1 levels, and suggestively increased propionic acid levels in children with ADHD. Baseline levels of immune markers (sICAM-1, sVCAM-1 and IL-10) and SCFAs (formic acid and acetic acid) in adult ADHD patients were different from those in healthy adults, indicating a higher inflammation level in patients. These immune marker levels were correlated with formic acid, acetic acid or propionic acid levels at baseline. The clinical effects of Synbiotic 2000 observed in **Study III** may be through restoring enteric metabolites, in turn alleviating inflammation. The study proposes possible underlying mechanisms for the Synbiotic 2000

effects in individuals with ADHD, which warrants further exploration of immune activity and gut bacterial metabolites in ADHD.

Study V: SCFAs, at human physiological levels, increased the proliferation and mitosis but did not affect apoptosis of human early neural progenitor cells. These findings lead to the proposal that may be a role for dietary and enteric SCFAs in early neurodevelopment. Considering the prenatal antibiotic drug exposure findings from **Study I**, these effects of SCFAs on hNPCs suggest that maternal SCFAs/gut microbiota may have a role in fetal brain development via a putative 'maternal gut-fetal brain-axis' in health and disease.

The emerging understanding of the communications between gut and brain in ADHD adds to the classical pathophysiological theories focusing only on the brain. Expanding the studies into immune activation and inflammation, may help to discover early biomarkers of the disorder. Interests in diet, gut microbiota and derived metabolites will open the mind for possible prenatal risk factors and novel therapies without adverse effects. More studies on the underlying mechanisms of GBA and components in ADHD are in great need.

To summarize, the studies included in this thesis provide support for a putative role of immune activation and the GBA, and may in the future lead to the identification of therapeutic targets for certain individuals with ADHD (**Figure 6**).

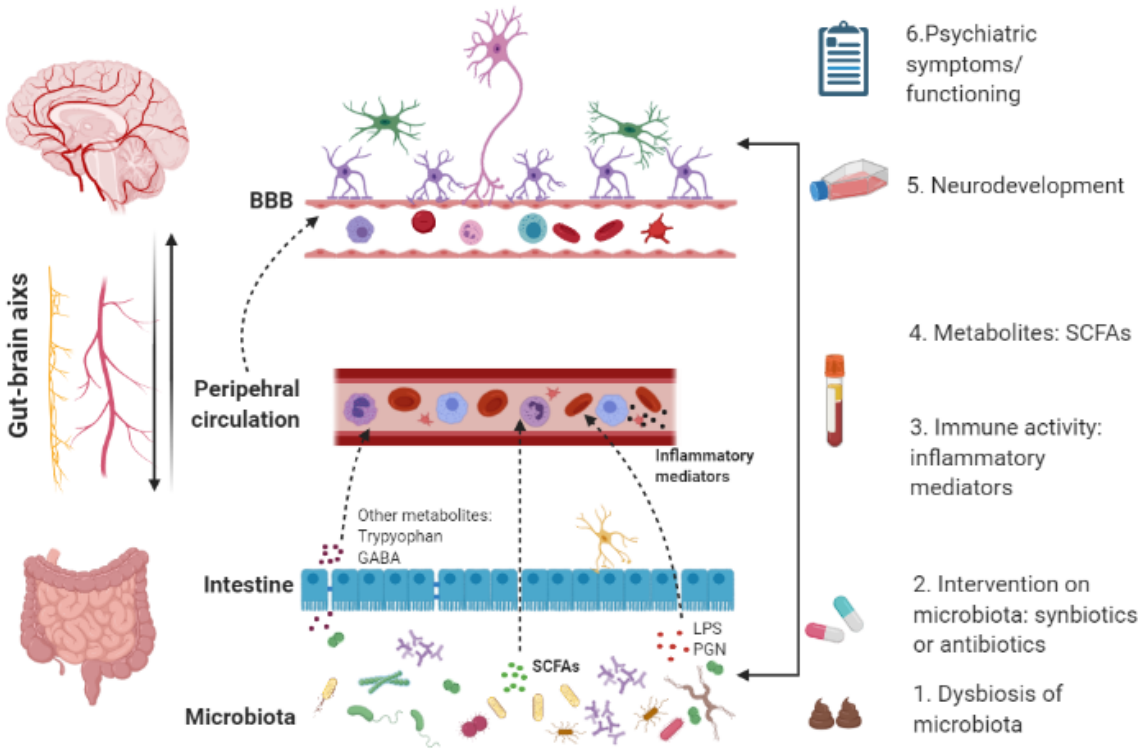


Figure 6. Schematic representation of the five studies in the thesis.

5.2 FUTURE PERSPECTIVES

The importance of the GBA is increasing in neuropsychiatry and neurology. Yet, there is overall less focus on ADHD when compared to other psychiatric disorders such as ASD. The results presented in this thesis needs to be replicated, and several questions related to GBA in ADHD remain to be answered in the future.

As it was suggested in **Study II**, endothelial dysfunction may be overrepresented in ADHD. This is important for barrier functions in both intestine and brain, and can further associate with neuroinflammation. Markers for permeability of intestinal barrier and BBB, such as Occludine, Claudines, JAMs, ZO-1, Zonulin, S100B can be measured in blood samples. Due to the limitation of a sample direct resource from brain (e.g. CSF, brain tissue), animal model or *in vitro* model is a feasible alternative. To confirm the effects of Synbiotic 2000 found in **Study III**, we hope to collaborate with a similar RCT that is running in three sites in Europe (Germany, Spain and Hungary). Followed by **Study IV** of inflammation and SCFA profiles in ADHD, gut metabolomics will be conducted to explore other interesting microbiota metabolites, like bile acids. Another plan is to profile the gut microbiota with fecal samples, evaluate the biodiversity and abundance of microbes and detect the bacterial genes and functions. Shallow shotgun metagenomic sequencing was employed to obtain the whole genome of the organisms in the samples, data analyses are ongoing. To further study the role of microbiota, fecal transplantation from patients to germ-free animal model and oral administration of certain bacteria stain can be performed. Further, the neuroendocrine component (HPA axis) of GBA has not been explored in this thesis, but would be an interesting path to take on.

Generally, in ADHD and the broader psychiatric field, diagnostic tools and treatments are limited. I hope our work will contribute a little to the understanding of the GBA in ADHD and provide a small step towards new clinical applications.

6 ACKNOWLEDGEMENTS

This is a great opportunity for me to deliver my sincere thanks to all people who supported me during the past four years, since I might have missed my chance to say thank you to a person at the right point. Personally, I think this section is the most beautiful part of the thesis. You may agree with me because it is one of the most read sections of a thesis. Now pieces of beautiful memories are flowing into my mind. ☺

First, I would like to start with the most important persons that made the thesis possible. Great thanks to my main supervisor **Catharina Lavebratt**, for having me as a PhD student in your group. You are not only a good supervisor but also an excellent scientist. You have taken great responsibility to teach me research step by step from the beginning and gradually guide me to become an independent researcher. You are always patient and caring for the students in both work and life. I like your smiles and lovely nickname (Cattis), which always make me feel relaxed, comfortable and close. You never pushed me and gave me a lot of freedom to make plans according to my time. Whenever I had questions or problems, you were there to help. I still remembered the first time I gave a presentation on the group meeting and I felt very sad and ashamed because of my poor English. You noticed my emotion and encouraged me to talk more and not shut myself down. Since then, I progress little by little on speaking and I gained more confidence in myself. You have invited me to your place several times to experience Swedish food, games and culture. I also want to thank my co-supervisor **Martin Schalling**. You can always think out of the box and come up with brilliant ideas. You have guided me with your 30-year experience in research. You are not only a scientist, but also a representative of innovation. **Carlos Villaescusa**, my co-supervisor, thanks for setting up my interesting project with the neural stem cells. You have brought the valuable resource of stem cell model and techniques to me and given advice, feedback and comments. **Yvonne Forsell**, thanks for the supervision and support for discussion and clinical inputs whenever needed.

Even the names of the collaborators have already been listed in the publications, it is important to mention them again. Because without their contributions, the work can't be done. Thanks to **MaiBritt Giacobini**, a clinician and expert in child and adult psychiatry, for the great contributions from the clinical aspect of the RCT projects; **Elin Skott** and **Åsa Söderström**, research nurses, for helping to recruit patients and collect the clinical data and biological samples for the projects as well as their knowledge from clinical experience; additionally, Elin for helping to write part of a manuscript; **Mika Gissler,** for all his knowledge of epidemiology design, statistical support and the access to the Finnish registry data; **Timo Partonen, Joëlle Rùegg**, **Sergey Rodin** and **Derrick F MacFabe**, for valuable inputs and comments for the projects and manuscripts.

Many thanks to my mentor, **Anna Fogdell-Hahn**, for the talks and discussions on my PhD progress as well as any interesting topics over lunch or Friday breakfast. Your thoughts, advice and knowledge inspired me a lot. A special thanks to my previous supervisor during master,

Dawei Xu. Thank you for introducing me to Catharina and the wonderful Translational Psychiatry group here in Sweden and also your care and support when I was sick. In the following, I want to thank to both my previous and present group members, who have joined a wonderful journey with me in my PhD life: **Tomas Ekström** for the broad of knowledge, thoughts, ideas, inputs appearing at the meeting or over lunch break that always inspire me, and the help with English for this thesis; **Ida Nilsson**, for organizing the lovely Christmas dinners at your place and sharing your experience and knowledge of animal work; **Miranda Stiernborg**, for all your contributions to the BAMBA projects and your talent of translating Swedish words for me; **Vincent Millischer**, for help with the statistical models and introducing R program to me; We had a lot of fun from learning R together and I can't imagine how my PhD life would be, if I did not learn R; **Linghua Kong**, for being warm-hearted and dedicated whenever I met problems and an example of hard work; **Yabin Wei**, for encouraging me to talk to people in English and ask for help when I newly arrived to Sweden; **Philippe Melas**, for always being patient and sharing the experience about Swedish culture; **Martin Lundberg**, for the open-mind and kindness; **Annika Eriksson**, for keeping the unique L8:00 Friday breakfast culture going on; **Malin Alvehus**, for your help with experimental reagent and material ordering; **Katarina Gell,** for checking our working environment and lab safety; **Parvin Kumar,** for teaching me how to do mesoscale, improving my English and your in-depth knowledge of Chinese culture and the lucky money in the red bag; **Paschalis Efstathopoulos**, for being patient to talk to me when I came with poor English and the collaboration with you; Thank you for all your company and supports!

To my friends and colleague knowing from lab, **Laura Baqué Vidal**, for social events you organized, especially the trip to your hometown, which made my spare time a colorful life; **Jakob Schuy**, for pushing me to join the R club and learn more statistics and your positive attitude and happiness and beautiful German word "*schmetterling*" (butterfly); **Iris Garcia Alcantarilla**, for the enthusiasm and the amazing dance talent; **Alan Kavsek** and **Edgar Fabricio Santos Rincon** for the fun we had on trips for summer vacations; the **Hematology group**, for being the best neighbor in the corridor; I would also like to thank all the other people I have met at **L8:00 CMM**, **Anna Matsson**, **Mikael Ringh**, **Sahl Khalid Bedri**, **Xinxia Chen**, **Nastya Kharlamova, Pauline Schaal**, **Iina-Lotta Eleonoora Korkala, Judit Ozsvár**, **Elizabeta Zaplatic**, **Elin Engdahl**, **Christina Hermanrud**, **Malin Almgren**, **Louise Sjöholm**, **Annika Lindblom**, **Anna-Lee Köstinger**, **Selim Sengul**, **Paulina Łuków** and **Claire Thume** for creating such a pleasant working environment personally and scientifically that was always happy to stay all the time.

Many thanks to the **Kristoffer Månsson**, **B Torkel Falkenberg** and **Linn Rönne-Petersen**. Thank you for let me participate in your projects with the interest of telomere length and telomerase activity, which is an extension of my master background. I have learnt a lot theoretically and practically from these.

Thank you to all members of the **MMK administration** and **CMM administration** and **IT-department**, in particular to **Ann-Britt Wikström** for the support that made my entire doctoral

education run as planned, and **Annika Hederby** for all the help with organizing seminars, arranging facilities and sharing the important information at CMM that made my research run smoothly. Thanks also the **Chinese Scholarship Council** for the founding that supported me to study here.

To my Chinese friends, **Xiaotian Yuan** for teaching me the method of analyzing telomere length and telomerase activity, you and **Lizhou Fan** for inviting me to your wedding and your place to have traditional Chinese food and barbecue; **Jingya Yu**, my best company here, for sharing a lot of weekends and holidays with me, teaching me to swim as well as the fantastic and memorable travels we have done together; **Xiangling Xing** and **Yujiao Wu**, for wonderful girls' time of exploring new restaurants, shops and taking photos of precious moments; **Yunhan Zhao,** for taking me to my living place and working place and showing me the surroundings after I arrived here; **Chuanyou Xia,** for your numerous humor and male's power; **Xia Hao**, for the company being the only roommate at the beginning of my life abroad; **Xiuming Liang**, **Qing Wang**, **Min Guo, Jiwei Gao, MailinZhou, Mingmei Shang, Wenyu Li, Chenfei He, Chikai Zhou, Shuijie Li, Jinming Han, Yang Wang, Qingyang Xiao, Meng Yu, Huazhen Wang, Yanan Zong, Yun Du, Weiwei Bian, Weiwei Cai** and **other friends I made here**, for the fun time we have together, when we celebrated Chinese festivals and birthdays or join after-work activities and parties; I feel so lucky to meet you all here, which made me seldom feel lonely and homesick. Great thanks also to my friends in China, **Xue Zhang**, **Manfei Si, Yuan Ren** and **Xiaolin Yin**, for the continuous help and distant supports from China.

I would like to deliver my special thanks to the researcher and artist **Ina Schuppe Koistinen** for providing the amazing watercolor paintings that was included in this thesis (http://www.inasakvareller.se/).

Last but not the least! Huge amount of thank you to my beloved family. To **my parents**, who have given me all their best love, care and freedom, thank you for the trust, understanding and support. I am happy to grow up and become a person as I am in the lovely family. I hope that I could become your pride. To **my brother** and **little nephew**, for always cheering me up me and bringing me courage and confidence. Also to all **my relatives**, thank you for always being there for me. I love you.

感恩所有，继续前行！❤

PS: Thousand words more than the representative four photos for the wonderful four years (2017-2020) of my Ph.D..

"If you can't fly then run, if you can't run then walk, if you can't walk then crawl, but whatever you do you have to keep moving forward."

---- Martin Luther King

7 REFERENCES

1. Wolraich ML, Chan E, Froehlich T, Lynch RL, Bax A, Redwine ST, *et al*. ADHD Diagnosis and Treatment Guidelines: A Historical Perspective. *Pediatrics* 2019; **144**(4).

2. Thapar A, Cooper M. Attention deficit hyperactivity disorder. *Lancet (London, England)* 2016; **387**(10024)**:** 1240-1250.

3. Rydell M, Lundström S, Gillberg C, Lichtenstein P, Larsson H. Has the attention deficit hyperactivity disorder phenotype become more common in children between 2004 and 2014? Trends over 10 years from a Swedish general population sample. *Journal of child psychology and psychiatry, and allied disciplines* 2018; **59**(8)**:** 863-871.

4. Palladino VS, McNeill R, Reif A, Kittel-Schneider S. Genetic risk factors and gene-environment interactions in adult and childhood attention-deficit/hyperactivity disorder. *Psychiatric genetics* 2019; **29**(3)**:** 63-78.

5. Sudo N, Chida Y, Aiba Y, Sonoda J, Oyama N, Yu XN, *et al*. Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. *The Journal of physiology* 2004; **558**(Pt 1)**:** 263-275.

6. Hsiao EY, McBride SW, Hsien S, Sharon G, Hyde ER, McCue T, *et al*. Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. *Cell* 2013; **155**(7)**:** 1451-1463.

7. Clarke G, Grenham S, Scully P, Fitzgerald P, Moloney RD, Shanahan F, *et al*. The microbiome-gut-brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner. *Mol Psychiatry* 2013; **18**(6)**:** 666-673.

8. Diaz Heijtz R, Wang S, Anuar F, Qian Y, Bjorkholm B, Samuelsson A, *et al*. Normal gut microbiota modulates brain development and behavior. *Proc Natl Acad Sci U S A* 2011; **108**(7)**:** 3047-3052.

9. Kim S, Kim H, Yim YS, Ha S, Atarashi K, Tan TG, *et al*. Maternal gut bacteria promote neurodevelopmental abnormalities in mouse offspring. *Nature* 2017; **549**(7673)**:** 528-532.

10. Erny D, Hrabe de Angelis AL, Jaitin D, Wieghofer P, Staszewski O, David E, *et al*. Host microbiota constantly control maturation and function of microglia in the CNS. *Nat Neurosci* 2015; **18**(7)**:** 965-977.

11. Cryan JF, O'Riordan KJ, Cowan CSM, Sandhu KV, Bastiaanssen TFS, Boehme M, *et al*. The Microbiota-Gut-Brain Axis. *Physiological reviews* 2019; **99**(4)**:** 1877-2013.

12. Kooij JJS, Bijlenga D, Salerno L, Jaeschke R, Bitter I, Balazs J, *et al*. Updated European Consensus Statement on diagnosis and treatment of adult ADHD. *European psychiatry : the journal of the Association of European Psychiatrists* 2019; **56:** 14-34.

13. Doernberg E, Hollander E. Neurodevelopmental Disorders (ASD and ADHD): DSM-5, ICD-10, and ICD-11. *CNS spectrums* 2016; **21**(4)**:** 295-299.

14. Bjureberg J, Ljotsson B, Tull MT, Hedman E, Sahlin H, Lundh LG, *et al*. Development and Validation of a Brief Version of the Difficulties in Emotion Regulation Scale: The DERS-16. *Journal of psychopathology and behavioral assessment* 2016; **38**(2)**:** 284-296.

15. Faraone SV, Biederman J, Mick E. The age-dependent decline of attention deficit hyperactivity disorder: a meta-analysis of follow-up studies. *Psychological medicine* 2006; **36**(2)**:** 159-165.

16. Tarver J, Daley D, Sayal K. Attention-deficit hyperactivity disorder (ADHD): an updated review of the essential facts. *Child Care Health Dev* 2014; **40**(6)**:** 762-774.

17. Jensen CM, Steinhausen HC. Comorbid mental disorders in children and adolescents with attention-deficit/hyperactivity disorder in a large nationwide study. *Attention deficit and hyperactivity disorders* 2015; **7**(1)**:** 27-38.

18. Reale L, Bartoli B, Cartabia M, Zanetti M, Costantino MA, Canevini MP, *et al*. Comorbidity prevalence and treatment outcome in children and adolescents with ADHD. *Eur Child Adolesc Psychiatry* 2017; **26**(12)**:** 1443-1457.

19. Antshel KM, Zhang-James Y, Wagner KE, Ledesma A, Faraone SV. An update on the comorbidity of ADHD and ASD: a focus on clinical management. *Expert review of neurotherapeutics* 2016; **16**(3)**:** 279-293.

20. Instanes JT, Klungsøyr K, Halmøy A, Fasmer OB, Haavik J. Adult ADHD and Comorbid Somatic Disease: A Systematic Literature Review. *Journal of attention disorders* 2018; **22**(3)**:** 203-228.

21. Downs J, Giust J, Dunn DW. Considerations for ADHD in the child with epilepsy and the child with migraine. *Expert review of neurotherapeutics* 2017; **17**(9)**:** 861-869.

22. Schans JV, Çiçek R, de Vries TW, Hak E, Hoekstra PJ. Association of atopic diseases and attention-deficit/hyperactivity disorder: A systematic review and meta-analyses. *Neuroscience and biobehavioral reviews* 2017; **74**(Pt A)**:** 139-148.

23. Global, regional, and national incidence, prevalence, and years lived with disability for 310 diseases and injuries, 1990-2015: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet (London, England)* 2016; **388**(10053)**:** 1545-1602.

24. Willcutt EG. The prevalence of DSM-IV attention-deficit/hyperactivity disorder: a meta-analytic review. *Neurotherapeutics : the journal of the American Society for Experimental NeuroTherapeutics* 2012; **9**(3)**:** 490-499.

25. Polanczyk GV, Salum GA, Sugaya LS, Caye A, Rohde LA. Annual research review: A meta-analysis of the worldwide prevalence of mental disorders in children and adolescents. *Journal of child psychology and psychiatry, and allied disciplines* 2015; **56**(3)**:** 345-365.

26. Simon V, Czobor P, Balint S, Meszaros A, Bitter I. Prevalence and correlates of adult attention-deficit hyperactivity disorder: meta-analysis. *The British journal of psychiatry : the journal of mental science* 2009; **194**(3)**:** 204-211.

27. Polanczyk G, de Lima MS, Horta BL, Biederman J, Rohde LA. The worldwide prevalence of ADHD: a systematic review and metaregression analysis. *The American journal of psychiatry* 2007; **164**(6)**:** 942-948.

28. Polanczyk GV, Willcutt EG, Salum GA, Kieling C, Rohde LA. ADHD prevalence estimates across three decades: an updated systematic review and meta-regression analysis. *International journal of epidemiology* 2014; **43**(2)**:** 434-442.

29. Chung W, Jiang SF, Paksarian D, Nikolaidis A, Castellanos FX, Merikangas KR, *et al*. Trends in the Prevalence and Incidence of Attention-Deficit/Hyperactivity Disorder Among Adults and Children of Different Racial and Ethnic Groups. *JAMA network open* 2019; **2**(11)**:** e1914344.

30. Mohr-Jensen C, Müller Bisgaard C, Boldsen SK, Steinhausen HC. Attention-Deficit/Hyperactivity Disorder in Childhood and Adolescence and the Risk of Crime in Young Adulthood in a Danish Nationwide Study. *Journal of the American Academy of Child and Adolescent Psychiatry* 2019; **58**(4)**:** 443-452.

31. Munoz-Suazo MD, Navarro-Munoz J, Diaz-Roman A, Porcel-Galvez AM, Gil-Garcia E. Sex differences in neuropsychological functioning among children with attention-deficit/hyperactivity disorder. *Psychiatry research* 2019; **278:** 289-293.

32. Mowlem FD, Rosenqvist MA, Martin J, Lichtenstein P, Asherson P, Larsson H. Sex differences in predicting ADHD clinical diagnosis and pharmacological treatment. *Eur Child Adolesc Psychiatry* 2019; **28**(4)**:** 481-489.

33. Strang JF, Kenworthy L, Dominska A, Sokoloff J, Kenealy LE, Berl M, *et al*. Increased gender variance in autism spectrum disorders and attention deficit hyperactivity disorder. *Archives of sexual behavior* 2014; **43**(8)**:** 1525-1533.

34. Larsson H, Chang Z, D'Onofrio BM, Lichtenstein P. The heritability of clinically diagnosed attention deficit hyperactivity disorder across the lifespan. *Psychological medicine* 2014; **44**(10)**:** 2223-2229.

35. Faraone SV, Perlis RH, Doyle AE, Smoller JW, Goralnick JJ, Holmgren MA, *et al*. Molecular genetics of attention-deficit/hyperactivity disorder. *Biological psychiatry* 2005; **57**(11)**:** 1313-1323.

36. Demontis D, Walters RK, Martin J, Mattheisen M, Als TD, Agerbo E, *et al*. Discovery of the first genome-wide significant risk loci for attention deficit/hyperactivity disorder. *Nature genetics* 2019; **51**(1)**:** 63-75.

37. Hayman V, Fernandez TV. Genetic Insights Into ADHD Biology. *Front Psychiatry* 2018; **9:** 251.

38. Faraone SV, Larsson H. Genetics of attention deficit hyperactivity disorder. *Mol Psychiatry* 2019; **24**(4)**:** 562-575.

39. Akutagava-Martins GC, Salatino-Oliveira A, Genro JP, Contini V, Polanczyk G, Zeni C, *et al*. Glutamatergic copy number variants and their role in attention-deficit/hyperactivity disorder. *American journal of medical genetics Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics* 2014; **165b**(6)**:** 502-509.

40. Grimm O, Kittel-Schneider S, Reif A. Recent developments in the genetics of attention-deficit hyperactivity disorder. *Psychiatry Clin Neurosci* 2018; **72**(9)**:** 654-672.

41. Hawi Z, Cummins TD, Tong J, Johnson B, Lau R, Samarrai W, *et al*. The molecular genetic architecture of attention deficit hyperactivity disorder. *Mol Psychiatry* 2015; **20**(3)**:** 289-297.

42. Identification of risk loci with shared effects on five major psychiatric disorders: a genome-wide analysis. *Lancet (London, England)* 2013; **381**(9875)**:** 1371-1379.

43. Froehlich TE, Anixt JS, Loe IM, Chirdkiatgumchai V, Kuan L, Gilman RC. Update on environmental risk factors for attention-deficit/hyperactivity disorder. *Current psychiatry reports* 2011; **13**(5)**:** 333-344.

44. Sciberras E, Mulraney M, Silva D, Coghill D. Prenatal Risk Factors and the Etiology of ADHD-Review of Existing Evidence. *Current psychiatry reports* 2017; **19**(1)**:** 1.

45. Kohler-Forsberg O, Petersen L, Gasse C, Mortensen PB, Dalsgaard S, Yolken RH, *et al*. A Nationwide Study in Denmark of the Association Between Treated Infections and the Subsequent Risk of Treated Mental Disorders in Children and Adolescents. *JAMA psychiatry* 2018.

46. Rice D, Barone S, Jr. Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. *Environmental health perspectives* 2000; **108 Suppl 3:** 511-533.

47. Taylor E, Döpfner M, Sergeant J, Asherson P, Banaschewski T, Buitelaar J, *et al*. European clinical guidelines for hyperkinetic disorder -- first upgrade. *Eur Child Adolesc Psychiatry* 2004; **13 Suppl 1:** I7-30.

48. Arnold LE, Hodgkins P, Caci H, Kahle J, Young S. Effect of treatment modality on long-term outcomes in attention-deficit/hyperactivity disorder: a systematic review. *PloS one* 2015; **10**(2)**:** e0116407.

49. Heilskov Rytter MJ, Andersen LB, Houmann T, Bilenberg N, Hvolby A, Molgaard C, *et al*. Diet in the treatment of ADHD in children - a systematic review of the literature. *Nordic journal of psychiatry* 2015; **69**(1)**:** 1-18.

50. Li L, Taylor MJ, Bälter K, Kuja-Halkola R, Chen Q, Hegvik TA, *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 : the official publication of the International Society of Psychiatric Genetics* 2020.

51. Chang JP, Su KP, Mondelli V, Pariante CM. Omega-3 Polyunsaturated Fatty Acids in Youths with Attention Deficit Hyperactivity Disorder: a Systematic Review and Meta-Analysis of Clinical Trials and Biological Studies. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* 2018; **43**(3)**:** 534-545.

52. Partty A, Kalliomaki M, Wacklin P, Salminen S, Isolauri E. A possible link between early probiotic intervention and the risk of neuropsychiatric disorders later in childhood: a randomized trial. *Pediatr Res* 2015; **77**(6)**:** 823-828.

53. Kumperscak HG, Gricar A, Ülen I, Micetic-Turk D. A Pilot Randomized Control Trial With the Probiotic Strain Lactobacillus rhamnosus GG (LGG) in ADHD: Children and Adolescents Report Better Health-Related Quality of Life. *Front Psychiatry* 2020; **11:** 181.

54. Bienenstock J, Kunze W, Forsythe P. Microbiota and the gut-brain axis. *Nutr Rev* 2015; **73 Suppl 1:** 28-31.

55. Arneth BM. Gut-brain axis biochemical signalling from the gastrointestinal tract to the central nervous system: gut dysbiosis and altered brain function. *Postgraduate medical journal* 2018; **94**(1114)**:** 446-452.

56. Fung TC, Olson CA, Hsiao EY. Interactions between the microbiota, immune and nervous systems in health and disease. *Nat Neurosci* 2017; **20**(2)**:** 145-155.

57. Sandhu KV, Sherwin E, Schellekens H, Stanton C, Dinan TG, Cryan JF. Feeding the microbiota-gut-brain axis: diet, microbiome, and neuropsychiatry. *Transl Res* 2017; **179:** 223-244.

58. Cenit MC, Sanz Y, Codoner-Franch P. Influence of gut microbiota on neuropsychiatric disorders. *World J Gastroenterol* 2017; **23**(30)**:** 5486-5498.

59. Wang L, Christophersen CT, Sorich MJ, Gerber JP, Angley MT, Conlon MA. Increased abundance of Sutterella spp. and Ruminococcus torques in feces of children with autism spectrum disorder. *Mol Autism* 2013; **4**(1)**:** 42.

60. Jiang HY, Zhou YY, Zhou GL, Li YC, Yuan J, Li XH, *et al*. Gut microbiota profiles in treatment-naive children with attention deficit hyperactivity disorder. *Behavioural brain research* 2018; **347:** 408-413.

61. Aarts E, Ederveen THA, Naaijen J, Zwiers MP, Boekhorst J, Timmerman HM, *et al*. Gut microbiome in ADHD and its relation to neural reward anticipation. *PloS one* 2017; **12**(9)**:** e0183509.

62. Luczynski P, McVey Neufeld KA, Oriach CS, Clarke G, Dinan TG, Cryan JF. Growing up in a Bubble: Using Germ-Free Animals to Assess the Influence of the Gut Microbiota on Brain and Behavior. *The international journal of neuropsychopharmacology* 2016; **19**(8).

63. Desbonnet L, Clarke G, Traplin A, O'Sullivan O, Crispie F, Moloney RD, *et al*. Gut microbiota depletion from early adolescence in mice: Implications for brain and behaviour. *Brain, behavior, and immunity* 2015; **48:** 165-173.

64. Guida F, Turco F, Iannotta M, De Gregorio D, Palumbo I, Sarnelli G, *et al*. Antibiotic-induced microbiota perturbation causes gut endocannabinoidome changes, hippocampal neuroglial reorganization and depression in mice. *Brain, behavior, and immunity* 2018; **67:** 230-245.

65. Diaz Heijtz R. Fetal, neonatal, and infant microbiome: Perturbations and subsequent effects on brain development and behavior. *Semin Fetal Neonatal Med* 2016; **21**(6)**:** 410-417.

66. Braniste V, Al-Asmakh M, Kowal C, Anuar F, Abbaspour A, Toth M, *et al*. The gut microbiota influences blood-brain barrier permeability in mice. *Sci Transl Med* 2014; **6**(263)**:** 263ra158.

67. Ntranos A, Casaccia P. The Microbiome-Gut-Behavior Axis: Crosstalk Between the Gut Microbiome and Oligodendrocytes Modulates Behavioral Responses. *Neurotherapeutics : the journal of the American Society for Experimental NeuroTherapeutics* 2018; **15**(1)**:** 31-35.

68. Bravo JA, Forsythe P, Chew MV, Escaravage E, Savignac HM, Dinan TG, *et al*. Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. *Proc Natl Acad Sci U S A* 2011; **108**(38)**:** 16050-16055.

69. McKernan DP, Fitzgerald P, Dinan TG, Cryan JF. The probiotic Bifidobacterium infantis 35624 displays visceral antinociceptive effects in the rat. *Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society* 2010; **22**(9)**:** 1029-1035, e1268.

70. Kelly JR, Borre Y, C OB, Patterson E, El Aidy S, Deane J, *et al*. Transferring the blues: Depression-associated gut microbiota induces neurobehavioural changes in the rat. *Journal of psychiatric research* 2016; **82:** 109-118.

71. Zheng P, Zeng B, Liu M, Chen J, Pan J, Han Y, *et al*. The gut microbiome from patients with schizophrenia modulates the glutamate-glutamine-GABA cycle and schizophrenia-relevant behaviors in mice. *Science advances* 2019; **5**(2)**:** eaau8317.

72. Sharon G, Cruz NJ, Kang DW, Gandal MJ, Wang B, Kim YM, *et al*. Human Gut Microbiota from Autism Spectrum Disorder Promote Behavioral Symptoms in Mice. *Cell* 2019; **177**(6)**:** 1600-1618.e1617.

73. Gibson GR, Roberfroid MB. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *The Journal of nutrition* 1995; **125**(6)**:** 1401-1412.

74. Williams NT. Probiotics. *American journal of health-system pharmacy : AJHP : official journal of the American Society of Health-System Pharmacists* 2010; **67**(6)**:** 449-458.

75. Frei R, Akdis M, O'Mahony L. Prebiotics, probiotics, synbiotics, and the immune system: experimental data and clinical evidence. *Current opinion in gastroenterology* 2015; **31**(2)**:** 153-158.

76. LeBlanc JG, Chain F, Martin R, Bermudez-Humaran LG, Courau S, Langella P. Beneficial effects on host energy metabolism of short-chain fatty acids and vitamins

produced by commensal and probiotic bacteria. *Microbial cell factories* 2017; **16**(1)**:** 79.

77. Seifert S, Watzl B. Inulin and oligofructose: review of experimental data on immune modulation. *The Journal of nutrition* 2007; **137**(11 Suppl)**:** 2563s-2567s.

78. Tillisch K, Labus J, Kilpatrick L, Jiang Z, Stains J, Ebrat B, *et al*. Consumption of fermented milk product with probiotic modulates brain activity. *Gastroenterology* 2013; **144**(7)**:** 1394-1401, 1401.e1391-1394.

79. Slykerman RF, Kang J, Van Zyl N, Barthow C, Wickens K, Stanley T, *et al*. Effect of early probiotic supplementation on childhood cognition, behaviour and mood a randomised, placebo-controlled trial. *Acta paediatrica (Oslo, Norway : 1992)* 2018; **107**(12)**:** 2172-2178.

80. Steenbergen L, Sellaro R, van Hemert S, Bosch JA, Colzato LS. A randomized controlled trial to test the effect of multispecies probiotics on cognitive reactivity to sad mood. *Brain, behavior, and immunity* 2015; **48:** 258-264.

81. Ng QX, Loke W, Venkatanarayanan N, Lim DY, Soh AYS, Yeo WS. A Systematic Review of the Role of Prebiotics and Probiotics in Autism Spectrum Disorders. *Medicina (Kaunas, Lithuania)* 2019; **55**(5).

82. Kohler O, Petersen L, Mors O, Mortensen PB, Yolken RH, Gasse C, *et al*. Infections and exposure to anti-infective agents and the risk of severe mental disorders: a nationwide study. *Acta psychiatrica Scandinavica* 2017; **135(**2**):** 97-105.

83. Hamad AF, Alessi-Severini S, Mahmud SM, Brownell M, Kuo IF. Early childhood antibiotics use and autism spectrum disorders: a population-based cohort study. *International journal of epidemiology* 2018.

84. Axelsson PB, Clausen TD, Petersen AH, Hageman I, Pinborg A, Kessing LV, *et al*. Investigating the effects of cesarean delivery and antibiotic use in early childhood on risk of later attention deficit hyperactivity disorder. *Journal of child psychology and psychiatry, and allied disciplines* 2019; **60**(2)**:** 151-159.

85. Slykerman RF, Thompson J, Waldie KE, Murphy R, Wall C, Mitchell EA. Antibiotics in the first year of life and subsequent neurocognitive outcomes. *Acta paediatrica (Oslo, Norway : 1992)* 2017; **106**(1)**:** 87-94.

86. Savitz J, Harrison NA. Interoception and Inflammation in Psychiatric Disorders. *Biological psychiatry Cognitive neuroscience and neuroimaging* 2018; **3**(6)**:** 514-524.

87. Mitchell RH, Goldstein BI. Inflammation in children and adolescents with neuropsychiatric disorders: a systematic review. *Journal of the American Academy of Child and Adolescent Psychiatry* 2014; **53**(3)**:** 274-296.

88. Khandaker GM, Cousins L, Deakin J, Lennox BR, Yolken R, Jones PB. Inflammation and immunity in schizophrenia: implications for pathophysiology and treatment. *The lancet Psychiatry* 2015; **2**(3)**:** 258-270.

89. Rosenblat JD, Cha DS, Mansur RB, McIntyre RS. Inflamed moods: a review of the interactions between inflammation and mood disorders. *Progress in neuro-psychopharmacology & biological psychiatry* 2014; **53:** 23-34.

90. Furtado M, Van Lieshout RJ, Van Ameringen M, Green SM, Frey BN. Biological and psychosocial predictors of anxiety worsening in the postpartum period: A longitudinal study. *Journal of affective disorders* 2019; **250:** 218-225.

91. Siniscalco D, Schultz S, Brigida AL, Antonucci N. Inflammation and Neuro-Immune Dysregulations in Autism Spectrum Disorders. *Pharmaceuticals (Basel, Switzerland)* 2018; **11**(2).

92. Leffa DT, Torres ILS, Rohde LA. A Review on the Role of Inflammation in Attention-Deficit/Hyperactivity Disorder. *Neuroimmunomodulation* 2018; **25**(5-6)**:** 328-333.

93. Dubois T, Reynaert C, Jacques D, Lepiece B, Patigny P, Zdanowicz N. Immunity and psychiatric disorders: variabilities of immunity biomarkers are they specific? *Psychiatria Danubina* 2018; **30**(Suppl 7)**:** 447-451.

94. Yuan N, Chen Y, Xia Y, Dai J, Liu C. Inflammation-related biomarkers in major psychiatric disorders: a cross-disorder assessment of reproducibility and specificity in 43 meta-analyses. *Translational psychiatry* 2019; **9**(1)**:** 233.

95. Müller N. The Role of Intercellular Adhesion Molecule-1 in the Pathogenesis of Psychiatric Disorders. *Front Pharmacol* 2019; **10:** 1251-1251.

96. Yang C, Wardenaar KJ, Bosker FJ, Li J, Schoevers RA. Inflammatory markers and treatment outcome in treatment resistant depression: A systematic review. *Journal of affective disorders* 2019; **257:** 640-649.

97. Markanday A. Acute Phase Reactants in Infections: Evidence-Based Review and a Guide for Clinicians. *Open forum infectious diseases* 2015; **2**(3)**:** ofv098.

98. Bedrossian N, Haidar M, Fares J, Kobeissy FH, Fares Y. Inflammation and Elevation of Interleukin-12p40 in Patients with Schizophrenia. *Front Mol Neurosci* 2016; **9:** 16-16.

99. Inga Jácome MC, Morales Chacòn LM, Vera Cuesta H, Maragoto Rizo C, Whilby Santiesteban M, Ramos Hernandez L, *et al*. Peripheral Inflammatory Markers Contributing to Comorbidities in Autism. *Behavioral sciences (Basel, Switzerland)* 2016; **6**(4).

100. Moschen AR, Tilg H, Raine T. IL-12, IL-23 and IL-17 in IBD: immunobiology and therapeutic targeting. *Nat Rev Gastroenterol Hepatol* 2019; **16**(3)**:** 185-196.

101. Neurath MF. IL-23 in inflammatory bowel diseases and colon cancer. *Cytokine & growth factor reviews* 2019; **45:** 1-8.

102. Alasehirli B, Oguz E, Gokcen C, Erbagci AB, Orkmez M, Demiryurek AT. Relationship between soluble intercellular adhesion molecules and attention-deficit/hyperactivity disorder. *International journal of psychiatry in medicine* 2015; **50**(2)**:** 238-247.

103. Blankenberg S, Barbaux S, Tiret L. Adhesion molecules and atherosclerosis. *Atherosclerosis* 2003; **170**(2)**:** 191-203.

104. Darwish AH, Elgohary TM, Nosair NA. Serum Interleukin-6 Level in Children With Attention-Deficit Hyperactivity Disorder (ADHD). *Journal of child neurology* 2019; **34**(2)**:** 61-67.

105. Donfrancesco R, Nativio P, Di Benedetto A, Villa MP, Andriola E, Melegari MG, *et al*. Anti-Yo Antibodies in Children With ADHD: First Results About Serum Cytokines. *Journal of attention disorders* 2016.

106. Corominas-Roso M, Armario A, Palomar G, Corrales M, Carrasco J, Richarte V, *et al*. IL-6 and TNF-α in unmedicated adults with ADHD: Relationship to cortisol awakening response. *Psychoneuroendocrinology* 2017; **79:** 67-73.

107. Anand D, Colpo GD, Zeni G, Zeni CP, Teixeira AL. Attention-Deficit/Hyperactivity Disorder And Inflammation: What Does Current Knowledge Tell Us? A Systematic Review. *Front Psychiatry* 2017; **8:** 228.

108. Verlaet AAJ, Breynaert A, Ceulemans B, De Bruyne T, Fransen E, Pieters L, *et al*. Oxidative stress and immune aberrancies in attention-deficit/hyperactivity disorder (ADHD): a case-control comparison. *European child & adolescent psychiatry* 2019; **28**(5)**:** 719-729.

109. Cortese S, Angriman M, Comencini E, Vincenzi B, Maffeis C. Association between inflammatory cytokines and ADHD symptoms in children and adolescents with obesity: A pilot study. *Psychiatry research* 2019; **278:** 7-11.

110. Holtmann M, Poustka L, Zepf FD, Banaschewski T, Priller J, Bolte S, *et al*. Severe affective and behavioral dysregulation in youths is associated with a proinflammatory state. *Zeitschrift fur Kinder- und Jugendpsychiatrie und Psychotherapie* 2013; **41**(6)**:** 393-399.

111. Cummings JH, Pomare EW, Branch WJ, Naylor CP, Macfarlane GT. Short chain fatty acids in human large intestine, portal, hepatic and venous blood. *Gut* 1987; **28**(10)**:** 1221-1227.

112. Kasubuchi M, Hasegawa S, Hiramatsu T, Ichimura A, Kimura I. Dietary gut microbial metabolites, short-chain fatty acids, and host metabolic regulation. *Nutrients* 2015; **7**(4)**:** 2839-2849.

113. MacFabe DF. Enteric short-chain fatty acids: microbial messengers of metabolism, mitochondria, and mind: implications in autism spectrum disorders. *Microbial ecology in health and disease* 2015; **26:** 28177.

114. MacFabe DF, Cain DP, Rodriguez-Capote K, Franklin AE, Hoffman JE, Boon F, *et al*. Neurobiological effects of intraventricular propionic acid in rats: possible role of short chain fatty acids on the pathogenesis and characteristics of autism spectrum disorders. *Behavioural brain research* 2007; **176**(1)**:** 149-169.

115. Al-Lahham SH, Peppelenbosch MP, Roelofsen H, Vonk RJ, Venema K. Biological effects of propionic acid in humans; metabolism, potential applications and underlying mechanisms. *Biochimica et biophysica acta* 2010; **1801**(11)**:** 1175-1183.

116. Tsuji A. Small molecular drug transfer across the blood-brain barrier via carrier-mediated transport systems. *NeuroRx : the journal of the American Society for Experimental NeuroTherapeutics* 2005; **2**(1)**:** 54-62.

117. Miyamoto J, Hasegawa S, Kasubuchi M, Ichimura A, Nakajima A, Kimura I. Nutritional Signaling via Free Fatty Acid Receptors. *Int J Mol Sci* 2016; **17**(4)**:** 450.

118. Hara T, Kimura I, Inoue D, Ichimura A, Hirasawa A. Free fatty acid receptors and their role in regulation of energy metabolism. *Rev Physiol Biochem Pharmacol* 2013; **164:** 77-116.

119. Cryan JF, Dinan TG. Gut microbiota: Microbiota and neuroimmune signalling-Metchnikoff to microglia. *Nat Rev Gastroenterol Hepatol* 2015; **12**(9)**:** 494-496.

120. Tan J, McKenzie C, Potamitis M, Thorburn AN, Mackay CR, Macia L. The role of short-chain fatty acids in health and disease. *Advances in immunology* 2014; **121:** 91-119.

121. Hu J, Lin S, Zheng B, Cheung PCK. Short-chain fatty acids in control of energy metabolism. *Critical reviews in food science and nutrition* 2018; **58**(8)**:** 1243-1249.

122. DeCastro M, Nankova BB, Shah P, Patel P, Mally PV, Mishra R, *et al*. Short chain fatty acids regulate tyrosine hydroxylase gene expression through a cAMP-dependent signaling pathway. *Brain research Molecular brain research* 2005; **142**(1)**:** 28-38.

123. Severson CA, Wang W, Pieribone VA, Dohle CI, Richerson GB. Midbrain serotonergic neurons are central pH chemoreceptors. *Nat Neurosci* 2003; **6**(11)**:** 1139-1140.

124. Kim HJ, Leeds P, Chuang D-M. The HDAC inhibitor, sodium butyrate, stimulates neurogenesis in the ischemic brain. *Journal of Neurochemistry* 2009; **110**(4)**:** 1226-1240.

125. Yoo DY, Kim W, Nam SM, Kim DW, Chung JY, Choi SY, *et al*. Synergistic effects of sodium butyrate, a histone deacetylase inhibitor, on increase of neurogenesis induced by pyridoxine and increase of neural proliferation in the mouse dentate gyrus. *Neurochemical research* 2011; **36**(10)**:** 1850-1857.

126. Wei Y, Melas PA, Wegener G, Mathe AA, Lavebratt C. Antidepressant-like effect of sodium butyrate is associated with an increase in TET1 and in 5-hydroxymethylation levels in the Bdnf gene. *The international journal of neuropsychopharmacology* 2014; **18**(2).

127. Levenson JM, O'Riordan KJ, Brown KD, Trinh MA, Molfese DL, Sweatt JD. Regulation of histone acetylation during memory formation in the hippocampus. *The Journal of biological chemistry* 2004; **279**(39)**:** 40545-40559.

128. Stilling RM, van de Wouw M, Clarke G, Stanton C, Dinan TG, Cryan JF. The neuropharmacology of butyrate: The bread and butter of the microbiota-gut-brain axis? *Neurochem Int* 2016; **99:** 110-132.

129. Gagliano H, Delgado-Morales R, Sanz-Garcia A, Armario A. High doses of the histone deacetylase inhibitor sodium butyrate trigger a stress-like response. *Neuropharmacology* 2014; **79:** 75-82.

130. Gundersen BB, Blendy JA. Effects of the histone deacetylase inhibitor sodium butyrate in models of depression and anxiety. *Neuropharmacology* 2009; **57**(1)**:** 67-74.

131. Nagashima H, Morio Y, Meshitsuka S, Yamane K, Nanjo Y, Teshima R. High-resolution nuclear magnetic resonance spectroscopic study of metabolites in the cerebrospinal fluid of patients with cervical myelopathy and lumbar radiculopathy. *European spine journal : official publication of the European Spine Society, the European Spinal Deformity Society, and the European Section of the Cervical Spine Research Society* 2010; **19**(8)**:** 1363-1368.

132. Hoyles L, Snelling T, Umlai UK, Nicholson JK, Carding SR, Glen RC, *et al*. Microbiome-host systems interactions: protective effects of propionate upon the blood-brain barrier. *Microbiome* 2018; **6**(1)**:** 55.

133. Liu S, Li E, Sun Z, Fu D, Duan G, Jiang M, *et al*. Altered gut microbiota and short chain fatty acids in Chinese children with autism spectrum disorder. *Sci Rep* 2019; **9**(1)**:** 287.

134. Michels N, Van de Wiele T, De Henauw S. Chronic Psychosocial Stress and Gut Health in Children: Associations With Calprotectin and Fecal Short-Chain Fatty Acids. *Psychosomatic medicine* 2017; **79**(8)**:** 927-935.

135. Orczyk-Pawilowicz M, Jawien E, Deja S, Hirnle L, Zabek A, Mlynarz P. Metabolomics of Human Amniotic Fluid and Maternal Plasma during Normal Pregnancy. *PloS one* 2016; **11**(4)**:** e0152740.

136. Ardissone AN, de la Cruz DM, Davis-Richardson AG, Rechcigl KT, Li N, Drew JC, *et al*. Meconium microbiome analysis identifies bacteria correlated with premature birth. *PLoS One* 2014; **9**(3)**:** e90784.

137. Jimenez E, Marin ML, Martin R, Odriozola JM, Olivares M, Xaus J, *et al*. Is meconium from healthy newborns actually sterile? *Research in microbiology* 2008; **159**(3)**:** 187-193.

138. Aagaard K, Ma J, Antony KM, Ganu R, Petrosino J, Versalovic J. The placenta harbors a unique microbiome. *Sci Transl Med* 2014; **6**(237)**:** 237ra265.

139. Zheng J, Xiao X, Zhang Q, Mao L, Yu M, Xu J. The Placental Microbiome Varies in Association with Low Birth Weight in Full-Term Neonates. *Nutrients* 2015; **7**(8)**:** 6924-6937.

140. DiGiulio DB. Diversity of microbes in amniotic fluid. *Semin Fetal Neonatal Med* 2012; **17**(1)**:** 2-11.

141. DiGiulio DB, Romero R, Amogan HP, Kusanovic JP, Bik EM, Gotsch F, *et al*. Microbial prevalence, diversity and abundance in amniotic fluid during preterm labor: a molecular and culture-based investigation. *PloS one* 2008; **3**(8)**:** e3056.

142. Stinson LF, Boyce MC, Payne MS, Keelan JA. The Not-so-Sterile Womb: Evidence That the Human Fetus Is Exposed to Bacteria Prior to Birth. *Frontiers in microbiology* 2019; **10:** 1124.

143. Jimenez E, Fernandez L, Marin ML, Martin R, Odriozola JM, Nueno-Palop C, *et al*. Isolation of commensal bacteria from umbilical cord blood of healthy neonates born by cesarean section. *Current microbiology* 2005; **51**(4)**:** 270-274.

144. Sterpu I, Fransson E, Hugerth LW, Du J, Pereira M, Cheng L, *et al*. No evidence for a placental microbiome in human pregnancies at term. *American journal of obstetrics and gynecology* 2020.

145. Buffington SA, Di Prisco GV, Auchtung TA, Ajami NJ, Petrosino JF, Costa-Mattioli M. Microbial Reconstitution Reverses Maternal Diet-Induced Social and Synaptic Deficits in Offspring. *Cell* 2016; **165**(7)**:** 1762-1775.

146. Lin Y, Fang ZF, Che LQ, Xu SY, Wu D, Wu CM, *et al*. Use of sodium butyrate as an alternative to dietary fiber: effects on the embryonic development and anti-oxidative capacity of rats. *PloS one* 2014; **9**(5)**:** e97838.

147. Needell JC, Ir D, Robertson CE, Kroehl ME, Frank DN, Zipris D. Maternal treatment with short-chain fatty acids modulates the intestinal microbiota and immunity and ameliorates type 1 diabetes in the offspring. *PloS one* 2017; **12**(9)**:** e0183786.

148. Foley KA, Ossenkopp KP, Kavaliers M, Macfabe DF. Pre- and neonatal exposure to lipopolysaccharide or the enteric metabolite, propionic acid, alters development and behavior in adolescent rats in a sexually dimorphic manner. *PloS one* 2014; **9**(1)**:** e87072.

149. Jung TH, Park JH, Jeon WM, Han KS. Butyrate modulates bacterial adherence on LS174T human colorectal cells by stimulating mucin secretion and MAPK signaling pathway. *Nutrition research and practice* 2015; **9**(4)**:** 343-349.

150. Wang HB, Wang PY, Wang X, Wan YL, Liu YC. Butyrate enhances intestinal epithelial barrier function via up-regulation of tight junction protein Claudin-1 transcription. *Dig Dis Sci* 2012; **57**(12)**:** 3126-3135.

151. Patnala R, Arumugam TV, Gupta N, Dheen ST. HDAC Inhibitor Sodium Butyrate-Mediated Epigenetic Regulation Enhances Neuroprotective Function of Microglia During Ischemic Stroke. *Mol Neurobiol* 2016.

152. Yao X, Zhang JR, Huang HR, Dai LC, Liu QJ, Zhang M. Histone deacetylase inhibitor promotes differentiation of embryonic stem cells into neural cells in adherent monoculture. *Chinese medical journal* 2010; **123**(6)**:** 734-738.

153. Nankova BB, Agarwal R, MacFabe DF, La Gamma EF. Enteric bacterial metabolites propionic and butyric acid modulate gene expression, including CREB-dependent catecholaminergic neurotransmission, in PC12 cells--possible relevance to autism spectrum disorders. *PloS one* 2014; **9**(8)**:** e103740.

154. Kotzampassi K, Giamarellos-Bourboulis EJ, Voudouris A, Kazamias P, Eleftheriadis E. Benefits of a synbiotic formula (Synbiotic 2000Forte) in critically

Ill trauma patients: early results of a randomized controlled trial. *World journal of surgery* 2006; **30**(10)**:** 1848-1855.

155. Rayes N, Seehofer D, Hansen S, Boucsein K, Muller AR, Serke S, *et al*. Early enteral supply of lactobacillus and fiber versus selective bowel decontamination: a controlled trial in liver transplant recipients. *Transplantation* 2002; **74**(1)**:** 123-127.

156. Rayes N, Seehofer D, Theruvath T, Mogl M, Langrehr JM, Nussler NC, *et al*. Effect of enteral nutrition and synbiotics on bacterial infection rates after pylorus-preserving pancreatoduodenectomy: a randomized, double-blind trial. *Annals of surgery* 2007; **246**(1)**:** 36-41.

157. Rodin S, Antonsson L, Niaudet C, Simonson OE, Salmela E, Hansson EM, *et al*. Clonal culturing of human embryonic stem cells on laminin-521/E-cadherin matrix in defined and xeno-free environment. *Nature communications* 2014; **5:** 3195.

158. Falk A, Koch P, Kesavan J, Takashima Y, Ladewig J, Alexander M, *et al*. Capture of neuroepithelial-like stem cells from pluripotent stem cells provides a versatile system for in vitro production of human neurons. *PloS one* 2012; **7**(1)**:** e29597.

159. Han J, Lin K, Sequeira C, Borchers CH. An isotope-labeled chemical derivatization method for the quantitation of short-chain fatty acids in human feces by liquid chromatography-tandem mass spectrometry. *Analytica chimica acta* 2015; **854:** 86-94.

160. Van Breukelen GJ. ANCOVA versus change from baseline: more power in randomized studies, more bias in nonrandomized studies [corrected]. *Journal of clinical epidemiology* 2006; **59**(9)**:** 920-925.

161. Colegrave N, Ruxton GD. Confidence intervals are a more useful complement to nonsignificant tests than are power calculations. *Behavioral Ecology* 2003; **14**(3)**:** 446-447.

162. Ludbrook J. On making multiple comparisons in clinical and experimental pharmacology and physiology. *Clinical and experimental pharmacology & physiology* 1991; **18**(6)**:** 379-392.

163. Nichols TE. Multiple testing corrections, nonparametric methods, and random field theory. *NeuroImage* 2012; **62**(2)**:** 811-815.

164. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society Series B (Methodological)* 1995; **57**(1)**:** 289-300.

165. Chen H-J, Lee Y-J, Yeh GC, Lin H-C. Association of attention-deficit/hyperactivity disorder with diabetes: a population-based study. *Pediatric research* 2013; **73**(4 Pt 1)**:** 492-496.

166. Müller N. Inflammation in Schizophrenia: Pathogenetic Aspects and Therapeutic Considerations. *Schizophrenia bulletin* 2018; **44**(5)**:** 973-982.

167. Haarmann A, Nowak E, Deiß A, van der Pol S, Monoranu C-M, Kooij G, *et al*. Soluble VCAM-1 impairs human brain endothelial barrier integrity via integrin α-4-transduced outside-in signalling. *Acta Neuropathol* 2015; **129**(5)**:** 639-652.

168. McKeown C, Hisle-Gorman E, Eide M, Gorman GH, Nylund CM. Association of constipation and fecal incontinence with attention-deficit/hyperactivity disorder. *Pediatrics* 2013; **132**(5)**:** e1210-1215.

169. Kang V, Wagner GC, Ming X. Gastrointestinal dysfunction in children with autism spectrum disorders. *Autism research : official journal of the International Society for Autism Research* 2014; **7**(4)**:** 501-506.

170. Liu YW, Liong MT, Chung YE, Huang HY, Peng WS, Cheng YF, *et al*. Effects of Lactobacillus plantarum PS128 on Children with Autism Spectrum Disorder in Taiwan: A Randomized, Double-Blind, Placebo-Controlled Trial. *Nutrients* 2019; **11**(4).

171. Sivaprakasam S, Prasad PD, Singh N. Benefits of short-chain fatty acids and their receptors in inflammation and carcinogenesis. *Pharmacol Ther* 2016; **164:** 144-151.

172. Abdelli LS, Samsam A, Naser SA. Propionic Acid Induces Gliosis and Neuro-inflammation through Modulation of PTEN/AKT Pathway in Autism Spectrum Disorder. *Sci Rep* 2019; **9**(1)**:** 8824.

SNAP-IV 26-Item Teacher and Parent Rating Scale

James M. Swanson, Ph.D., University of California, Irvine, CA 92715

Patient/Client Name: _____

Date of birth: _____ Gender: _____

Grade: _____ Type of class: _____ Class size: _____

Completed by: _____ Date: _____

Physician Name: _____

For each item, check the column which best describes this child/adolescent:

	Not at all	Just a little	Quite a bit	Very much
1. Often fails to give close attention to details or makes careless mistakes in schoolwork or tasks				
2. Often has difficulty sustaining attention in tasks or play activities				
3. Often does not seem to listen when spoken to directly				
4. Often does not follow through on instructions and fails to finish schoolwork, chores, or duties				
5. Often has difficulty organizing tasks and activities				
6. Often avoids, dislikes, or reluctantly engages in tasks requiring sustained mental effort				
7. Often loses things necessary for activities (e.g., toys, school assignments, pencils or books				
8. Often is distracted by extraneous stimuli				
9. Often is forgetful in daily activities				
10. Often fidgets with hands or feet or squirms in seat				
11. Often leaves seat in classroom or in other situations in which remaining seated is expected				
12. Often runs about or climbs excessively in situations in which it is inappropriate				
13. Often has difficulty playing or engaging in leisure activities quietly				
14. Often is “on the go” or often acts as if “driven by a motor”				
15. Often talks excessively				
16. Often blurts out answers before questions have been completed				
17. Often has difficulty awaiting turn				
18. Often interrupts or intrudes on others (e.g., butts into conversations/ games				
19. Often loses temper				
20. Often argues with adults				
21. Often actively defies or refuses adult requests or rules				
22. Often deliberately does things that annoy other people				
23. Often blames others for his or her mistakes or misbehaviour				
24. Often is touchy or easily annoyed by others				
25. Often is angry and resentful				
26. Often is spiteful or vindictive				

Scoring guide for SNAP-IV 26-Item Teacher and Parent Rating Scale

The SNAP-IV 26-item scale is an abbreviated version of the Swanson, Nolan, and Pelham (SNAP) Questionnaire (Swanson, 1992; Swanson et al., 1983). Items from the *DSM-IV* criteria for attention-deficit/hyperactivity disorder (ADHD) are included for the two subsets of symptoms: Inattention (items 1–9) and Hyperactivity/Impulsivity (items 10–18). Also, items from the *DSM-IV* criteria for oppositional defiant disorder (ODD) are included (items 19–26) because ODD is often present in children with ADHD.

Symptom severity is rated on a 4-point scale. Responses are scored as follows:

Not at all = 0
Just a little = 1
Quite a bit = 2
Very much = 3

The scores in each of the three subsets (inattention, hyperactivity/impulsivity, and opposition/defiance) are totalled. A suggested scoring guideline is below:

Questions 1 – 9: Inattention Subset
< 13/27 = Symptoms not clinically significant
13 – 17 = Mild symptoms
18 – 22 = Moderate symptoms
23 – 27 = Severe symptoms

Questions 10 – 18: Hyperactivity/Impulsivity Subset
<13/27 = Symptoms not clinically significant
13 – 17 = Mild symptoms
18 – 22 = Moderate symptoms
23 – 27 = Severe symptoms

Questions 19 – 26: Opposition/Defiance Subset
< 8/24 = Symptoms not clinically significant
8 – 13 = Mild symptoms
14 – 18 = Moderate symptoms
19 – 24 = Severe symptoms

Suggested Targets:
<13/27 for inattention
<13/27 for hyperactivity/impulsivity
<8/24 for oppositional defiant disorder

If desired, the average rating for each subset can be calculated by totalling the scores for the items in the subset and dividing by the number of items. The average can be compared with cut-off scores suggestive of ADHD reported in the literature.

CADDRA
CANADIAN ADHD RESOURCE ALLIANCE

ADULT ADHD SELF-REPORT SCALE (ASRS-V1.1) SYMPTOM CHECKLIST INSTRUCTIONS

Description:

The Symptom Checklist is an instrument consisting of the 18 DSM-IV-TR criteria. Six of the 18 questions were found to be the most predictive of symptoms consistent with ADHD. These six questions are the basis for the ASRS-V1.1 screener and are also Part A of the Symptom Checklist. Part B of the Symptom Checklist contains the remaining 12 questions.

Instructions:

Symptoms

1. Ask the patient to complete both Part A and Part B of the Symptom Checklist by marking an X in the box that most closely represents the frequency of occurrence of each of the symptoms.
2. Score Part A. If four or more marks appear under Often/Very Often then the patient has symptoms highly consistent with ADHD in adults and further investigation is warranted.
3. The frequency scores on Part B provide additional cues and can serve as further probes into the patient's symptoms. Pay particular attention to marks appearing under Often/Very Often. The frequency-based response is more sensitive with certain questions. No total score or diagnostic likelihood is utilized for the 12 questions. It has been found that the six questions in Part A are the most predictive of the disorder and are best for use as a screening instrument.

Impairments

1. Review the entire Symptom Checklist with your patients and evaluate the level of impairment associated with the symptom.
2. Consider work/school, social and family settings.
3. Symptom frequency is often associated with symptom severity, therefore the Symptom Checklist may also aid in the assessment of impairments. If your patients have frequent symptoms, you may want to ask them to describe how these problems have affected the ability to work, take care of things at home, or get along with other people such as their spouse/significant other.

History

1. Assess the presence of these symptoms or similar symptoms in childhood. Adults who have ADHD need not have been formally diagnosed in childhood. In evaluating a patient's history, look for evidence of early-appearing and long-standing problems with attention or self-control. Some significant symptoms should have been present in childhood, but full symptomology is not necessary.

References:
1. Schweitzer JB et al. Med Clin North Am. 2001;85(3),10-11:757-777.
2. Barkley RA. Attention Deficit Hyperactivity Disorder: A Handbook for Diagnosis and Treatment. 2nd ed. 1998.
3. Biederman J, et al. Am J Psychiatry. 1993;150:1792-1798.
4. American Psychiatric Association: Diagnostic and Statistical Manual of Mental Disorders.Fourth Edition, Text Revision. Washington, DC, American Psychiatric Association. 2000:85-93.

CADDRA
CANADIAN ADHD RESOURCE ALLIANCE

Patient Name:
Date of Birth: MRN/File No:
Physician Name: Date:

ADULT ADHD SELF-REPORT SCALE (ASRS-V1.1) SYMPTOM CHECKLIST

Please answer the questions below, rating yourself on each of the criteria shown using the scale on the right side of the page. As you answer each question, place an X in the box that best describes how you have felt and conducted yourself over the past 6 months. Please give this completed checklist to your healthcare professional to discuss during your appointment	Never	Rarely	Sometimes	Often	Very often
PART A					
1. How often do you have trouble wrapping up the final details of a project, once the challenging parts have been done?					
2. How often do you have difficulty getting things in order when you have to do a task that requires organization?					
3. How often do you have problems remembering appointments or obligations?					
4. When you have a task that requires a lot of thought, how often do you avoid or delay getting started?					
5. How often do you fidget or squirm with your hands or feet when you have to sit down for a long time?					
6. How often do you feel overly active and compelled to do things, like you were driven by a motor?					
PART B					
7. How often do you make careless mistakes when you have to work on a boring or difficult project?					
8. How often do you have difficulty keeping your attention when you are doing boring or repetitive work?					
9. How often do you have difficulty concentrating on what people say to you, even when they are speaking to you directly?					
10. How often do you misplace or have difficulty finding things at home or at work?					
11. How often are you distracted by activity or noise around you?					
12. How often do you leave your seat in meetings or in other situations in which you are expected to stay seated?					
13. How often do you feel restless or fidgety?					
14. How often do you have difficulty unwinding and relaxing when you have time to yourself?					
15. How often do you find yourself talking too much when you are in social situations?					
16. When you're in a conversation, how often do you find yourself finishing the sentences of the people you are talking to, before they can finish it themselves?					
17. How often do you have difficulty waiting your turn in situations when turn taking is required?					
18. How often do you interrupt others when they are busy?					

WEISS FUNCTIONAL IMPAIRMENT RATING SCALE (WFIRS) INSTRUCTIONS

Purpose

- ADHD symptoms and actual impairment overlap but are distinct concepts. It is important to measure both since some patients are highly symptomatic but not impaired or vice versa
- This scale contains those items that are most likely to represent the patient's target of treatment. Therefore, the use of the scale before and after treatment can allow the clinician to determine not only if the ADHD has improved, but if the patient's functional difficulties are also better.
- This instrument has been translated into 18 languages. It has been used in many studies and is psychometrically validated. This is the only measure of functional impairment that looks at specific domains and has been validated in the ADHD population.

Design and Validation Information

Scoring The instrument uses a Likert scale such that any item rating 2 or 3 is clinically impaired. The scale can be scored by looking at the total score or by creating a mean score for the total score/number items for each domain, omitting those rated not applicable. For clinical purposes, when defining impairment for DSM-IV, clinicians can consider that any domain with at least two items scored 2, one item scored 3 or a mean score >1.5 is impaired.

Validation The scale has been psychometrically validated with an internal consistency >.8 for each domain and for the scale as a whole. It has moderate convergent validity (0.6) with other measures of functioning (i.e. Columbia Impairment Scale and the Global Assessment of Functioning (GAF). It has moderate discriminating validity (0.4) from symptoms pre-treatment (i.e. ADHD-Rating Scale) and quality of life (CHIP). The domains have been confirmed by factor analysis, although the domain of school functioning separates into learning and behaviour. The scale is highly sensitive to change with treatment and, in particular, significantly correlated to change in ADHD symptoms (40% change) and overall psychopathology. Each anchor point on the Likert scale represents approximately one standard deviation(SD). A total score change of 13 would be considered a significant improvement or about half a SD. The change obtained in treatment is typically one full SD. The mean score for risky behaviour in children is 0.5 but increases with age. For adolescents the mean score is 1.

Copyright Information

CADDRA
CANADIAN ADHD RESOURCE ALLIANCE

Patient Name:
Date of Birth: MRN/File No:
Physician Name: Date:

WEISS FUNCTIONAL IMPAIRMENT RATING SCALE – SELF REPORT (WFIRS-S)

Work: ☐ Full time ☐ Part time ☐ Other _____ School: ☐ Full time ☐ Part time

Circle the number for the rating that best describes how your emotional or behavioural problems have affected each item in the last month.

		Never or not at all	Sometimes or somewhat	Often or much	Very often or very much	n/a
A	**FAMILY**					
1	Having problems with family	0	1	2	3	n/a
2	Having problems with spouse/partner	0	1	2	3	n/a
3	Relying on others to do things for you	0	1	2	3	n/a
4	Causing fighting in the family	0	1	2	3	n/a
5	Makes it hard for the family to have fun together	0	1	2	3	n/a
6	Problems taking care of your family	0	1	2	3	n/a
7	Problems balancing your needs against those of your family	0	1	2	3	n/
8	Problems losing control with family	0	1	2	3	n/a
B	**WORK**					
1	Problems performing required duties	0	1	2	3	n/a
2	Problems with getting your work done efficiently	0	1	2	3	n/a
3	Problems with your supervisor	0	1	2	3	n/a
4	Problems keeping a job	0	1	2	3	n/a
5	Getting fired from work	0	1	2	3	n/a
6	Problems working in a team	0	1	2	3	n/a
7	Problems with your attendance	0	1	2	3	n/a
8	Problems with being late	0	1	2	3	n/a
9	Problems taking on new tasks	0	1	2	3	n/a
10	Problems working to your potential	0	1	2	3	n/a
11	Poor performance evaluations	0	1	2	3	n/a
C	**SCHOOL**					
1	Problems taking notes	0	1	2	3	n/a
2	Problems completing assignments	0	1	2	3	n/a
3	Problems getting your work done efficiently	0	1	2	3	n/a
4	Problems with teachers	0	1	2	3	n/a
5	Problems with school administrators	0	1	2	3	n/a
6	Problems meeting minimum requirements to stay in school	0	1	2	3	n/a
7	Problems with attendance	0	1	2	3	n/a
8	Problems with being late	0	1	2	3	n/a
9	Problems with working to your potential	0	1	2	3	n/a
10	Problems with inconsistent grades	0	1	2	3	n/a
D	**LIFE SKILLS**					
1	Excessive or inappropriate use of internet, video games or TV	0	1	2	3	n/a
2	Problems keeping an acceptable appearance	0	1	2	3	n/a
3	Problems getting ready to leave the house	0	1	2	3	n/a
4	Problems getting to bed	0	1	2	3	n/a
5	Problems with nutrition	0	1	2	3	n/a

		Never or not at all	Sometimes or somewhat	Often or much	Very often or very much	n/a
6	Problems with sex	0	1	2	3	n/a
7	Problems with sleeping	0	1	2	3	n/a
8	Getting hurt or injured	0	1	2	3	n/a
9	Avoiding exercise	0	1	2	3	n/a
10	Problems keeping regular appointments with doctor/dentist	0	1	2	3	n/a
11	Problems keeping up with household chores	0	1	2	3	n/a
12	Problems managing money	0	1	2	3	n/a
E	**SELF-CONCEPT**					
1	Feeling bad about yourself	0	1	2	3	n/a
2	Feeling frustrated with yourself	0	1	2	3	n/a
3	Feeling discouraged	0	1	2	3	n/a
4	Not feeling happy with your life	0	1	2	3	n/a
5	Feeling incompetent	0	1	2	3	n/a
F	**SOCIAL**					
1	Getting into arguments	0	1	2	3	n/a
2	Trouble cooperating	0	1	2	3	n/a
3	Trouble getting along with people	0	1	2	3	n/a
4	Problems having fun with other people	0	1	2	3	n/a
5	Problems participating in hobbies	0	1	2	3	n/a
6	Problems making friends	0	1	2	3	n/a
7	Problems keeping friends	0	1	2	3	n/a
8	Saying inappropriate things	0	1	2	3	n/a
9	Complaints from neighbours	0	1	2	3	n/a
G	**RISK**					
1	Aggressive driving	0	1	2	3	n/a
2	Doing other things while driving	0	1	2	3	n/a
3	Road rage	0	1	2	3	n/a
4	Breaking or damaging things	0	1	2	3	n/a
5	Doing things that are illegal	0	1	2	3	n/a
6	Being involved with the police	0	1	2	3	n/a
7	Smoking cigarettes	0	1	2	3	n/a
8	Smoking marijuana	0	1	2	3	n/a
9	Drinking alcohol	0	1	2	3	n/a
10	Taking "street" drugs	0	1	2	3	n/a
11	Sex without protection (birth control, condom)	0	1	2	3	n/a
12	Sexually inappropriate behaviour	0	1	2	3	n/a
13	Being physically aggressive	0	1	2	3	n/a
14	Being verbally aggressive	0	1	2	3	n/a

SCORING:

1. Number of items scored 2 or 3
   or
2. Total score
   or
3. Mean score

DO NOT WRITE IN THIS AREA

A. Family _____
B. Work _____
C. School _____
D. Life skills _____
E. Self-concept _____
F. Social _____
G. Risk _____
Total _____

CADDRA
CANADIAN ADHD RESOURCE ALLIANCE

Patient Name:
Date of Birth: MRN/File No:
Physician Name: Date:

WEISS FUNCTIONAL IMPAIRMENT RATING SCALE – PARENT REPORT (WFIRS-P)

Your name: _____ Relationship to child: _____

Circle the number for the rating that best describes how your child's emotional or behavioural problems have affected each item in the last month.

		Never or not at all	Sometimes or somewhat	Often or much	Very often or very much	n/a
A	**FAMILY**					
1	Having problems with brothers & sisters	0	1	2	3	n/a
2	Causing problems between parents	0	1	2	3	n/a
3	Takes time away from family members' work or activities	0	1	2	3	n/a
4	Causing fighting in the family	0	1	2	3	n/a
5	Isolating the family from friends and social activities	0	1	2	3	n/a
6	Makes it hard for the family to have fun together	0	1	2	3	n/a
7	Makes parenting difficult	0	1	2	3	n/a
8	Makes it hard to give fair attention to all family members	0	1	2	3	n/a
9	Provokes others to hit or scream at him/her	0	1	2	3	n/a
10	Costs the family more money	0	1	2	3	n/a
B	**SCHOOL**					
	Learning					
1	Makes it difficult to keep up with schoolwork	0	1	2	3	n/a
2	Needs extra help at school	0	1	2	3	n/a
3	Needs tutoring	0	1	2	3	n/a
4	Receives grades that are not as good as his/her ability	0	1	2	3	n/a
	Behaviour					
1	Causes problems for the teacher in the classroom	0	1	2	3	n/a
2	Receives "time-out" or removal from the classroom	0	1	2	3	n/a
3	Having problems in the school yard	0	1	2	3	n/a
4	Receives detentions (during or after school)	0	1	2	3	n/a
5	Suspended or expelled from school	0	1	2	3	n/a
6	Misses classes or is late for school	0	1	2	3	n/a
C	**LIFE SKILLS**					
1	Excessive use of TV, computer, or video games	0	1	2	3	n/a
2	Keeping clean, brushing teeth, brushing hair, bathing, etc.	0	1	2	3	n/a
3	Problems getting ready for school	0	1	2	3	n/a

		Never or not at all	Sometimes or somewhat	Often or much	Very often or very much	n/a
4	Problems getting ready for bed	0	1	2	3	n/a
5	Problems with eating (picky eater, junk food)	0	1	2	3	n/a
6	Problems with sleeping	0	1	2	3	n/a
7	Gets hurt or injured	0	1	2	3	n/a
8	Avoids exercise	0	1	2	3	n/a
9	Needs more medical care	0	1	2	3	n/a
10	Has trouble taking medication, getting needles or visiting the doctor/dentist	0	1	2	3	n/a
D	**CHILD'S SELF-CONCEPT**					
1	My child feels bad about himself/herself	0	1	2	3	n/a
2	My child does not have enough fun	0	1	2	3	n/a
3	My child is not happy with his/her life	0	1	2	3	n/a
E	**SOCIAL ACTIVITIES**					
1	Being teased or bullied by other children	0	1	2	3	n/a
2	Teases or bullies other children	0	1	2	3	n/a
3	Problems getting along with other children	0	1	2	3	n/a
4	Problems participating in after-school activities (sports, music, clubs)	0	1	2	3	n/a
5	Problems making new friends	0	1	2	3	n/a
6	Problems keeping friends	0	1	2	3	n/a
7	Difficulty with parties (not invited, avoids them, misbehaves)	0	1	2	3	n/a
F	**RISKY ACTIVITIES**					
1	Easily led by other children (peer pressure)	0	1	2	3	n/a
2	Breaking or damaging things	0	1	2	3	n/a
3	Doing things that are illegal	0	1	2	3	n/a
4	Being involved with the police	0	1	2	3	n/a
5	Smoking cigarettes	0	1	2	3	n/a
6	Taking illegal drugs	0	1	2	3	n/a
7	Doing dangerous things	0	1	2	3	n/a
8	Causes injury to others	0	1	2	3	n/a
9	Says mean or inappropriate things	0	1	2	3	n/a
10	Sexually inappropriate behaviour	0	1	2	3	n/a

SCORING:

1. Number of items scored 2 or 3
   or
2. Total score
   or
3. Mean score

DO NOT WRITE IN THIS AREA

A. Family	
B. School	
Learning	
Behaviour	
C. Life skills	
D. Child's self-concept	
E. Social activities	
F. Risky activities	
Total	

Social Communication Questionnaire (SCQ) – Lifetime
PC Answer Sheet

Michael Rutter, M.D., F.R.S., Anthony Bailey, M.D., Sibel Kazak Berument, Ph.D.,
Catherine Lord, Ph.D., and Andrew Pickles, Ph.D.

Name of Subject: _____ D.O.B. _____ Interview Date _____ Age: ____

Gender: ○ F ○ M Name of Respondent: _____ Relation to Subject: _____

Directions: Thank you for taking the time to complete this questionnaire. Please answer each question by selecting *yes* or *no*. A few questions ask about several related types of behavior; please select *yes* if *any* of these behaviors were present during the past 3 months. Although you may be uncertain about whether some behaviors were present or not, please answer *yes* or *no* to every question on the basis of what you think.

Item	Yes	No
1. Is she/he now able to talk using short phrases or sentences? If *no*, skip to question 8.	○	○
2. Can you have a to and fro "conversation" with her/him that involves taking turns or building on what you have said?	○	○
3. Has she/he ever use odd phrases or say the same thing over and over in almost exactly the same way (either phases that she/he hears other people use or ones that she/he makes up?	○	○
4. Has she/he ever use socially inappropriate questions or statements? For example, has she/he ever regularly ask personal questions or make personal comments at awkward times?	○	○
5. Has she/he ever gotten his/her pronouns mixed up (e.g., saying *you* or *she/he* for *I*)?	○	○
6. Has she/he ever use words that she/he seems to have invented or made up her/himself; put things in odd, indirect ways; or use metaphorical ways of saying things (e.g., saying *hot rain* for *steam*)?	○	○
7. Has she/he ever say the same thing over and over in exactly the same way or insist that you say the same thing over and over again?	○	○
8. Has she/he have things that she/he seems to do in a very particular way or order or rituals that she/he insisted that you go through?	○	○
9. Has her/his facial expressions usually seemed appropriate to the particular situation, as far as you can tell?	○	○
10. Has she/he ever used your hand like a tool or as if it were part of his/her own body (e.g., pointing with your finger or putting your hand on a doorknob to get you to open the door)?	○	○
11. Has she/he ever have any interests that preoccupy her/him and might seem odd to other people (e.g., traffic lights, drainpipes, or timetables)?	○	○
12. Has she/he ever seemed to be more interested in parts of a toy or an object (e.g., spinning the wheels of a car), rather than in using the object as it was intended?	○	○
13. Has she/he ever have any special interests that are *unusual* in their intensity but otherwise appropriate for his/her age and peer group (e.g., trains or dinosaurs)?	○	○
14. Has she/he ever seemed to be *unusually* interested in the sight, feel, sound, taste, or smell of things or people?	○	○
15. Has she/he ever have any mannerisms or off ways of moving her/his hands or fingers, such as flapping or moving her/his fingers in front of her/his eyes?	○	○
16. Has she/he ever have any complicated movements of her/his whole body, such as spinning or repeatedly bouncing up and down?	○	○
17. Has she/he ever injured her/himself deliberately, such as by biting her/his arm or banging her/his head?	○	○
18. Has she/he ever have any objects (*other* than a soft toy or comfort blanket) that she/he *had* to carry around?	○	○
19. Does she/he have any particular friends or a best friend?	○	○

For the following behaviors, please focus on the time period between the child's fourth and fifth birthdays. You may find it easier to remember how things were at that time by focusing on key events, such as starting school, moving house, Christmastime, or other specific events that are particularly memorable for you as a family. If your child is not yet 4 years old, please consider her or his behavior in the past 12 months.

Item	Yes	No
20. When she/he was 4 to 5, did she/he ever talk with you just to be friendly (rather than to get something)?	○	○
21. When she/he was 4 to 5, did she/he ever *spontaneously* copy you (or other people) or what you are doing (such as vacuuming, gardening, or mending things)?	○	○
22. When she/he was 4 to 5, did she/he ever spontaneously point at things around her/him just to show you things (not because she/he wants them?	○	○
23. When she/he was 4 to 5, did she/he ever use gestures, other than pointing or pulling your hand, to let you know what she/he wants?	○	○
24. When she/he was 4 to 5, did she/he nod her/his head to mean *yes*?	○	○
25. When she/he was 4 to 5, did she/he shake her/his head to mean *no*?	○	○
26. When she/he was 4 to 5, did she/he usually look at you directly in the face when doing things with you or talking with you?	○	○
27. When she/he was 4 to 5, did she/he smile back if someone smiles at her/him?	○	○
28. When she/he was 4 to 5, did she/he ever show you things that interest her/him to engage your attention?	○	○
29. When she/he was 4 to 5, did she/he ever offer to share things other than food with you?	○	○
30. When she/he was 4 to 5, did she/he ever seem to want you to join in her/his enjoyment of something?	○	○
31. When she/he was 4 to 5, did she/he ever try to comfort you if you are sad or hurt?	○	○
32. When she/he was 4 to 5, when she/he wants something or wants help, did she/he look at you and use gestures with sounds or words to get your attention?	○	○
33. When she/he was 4 to 5, did she/he show a normal range of facial expressions?	○	○
34. When she/he was 4 to 5, did she/he ever spontaneously join in and try to copy the actions in social games, such as *The Mulberry Bush* or *London Bridges Is Falling Down*?	○	○
35. When she/he was 4 to 5, did she/he play any pretend or make-believe games?	○	○
36. When she/he was 4 to 5, did she/he seem interested in other children of approximately the same age whom she/he did not know?	○	○
37. When she/he was 4 to 5, did she/he respond positively when another child approached her/him?	○	○
38. When she/he was 4 to 5, if you come into a room and start talking to her/him without calling her/his name, did she/he usually look up and pay attention to you?	○	○
39. When she/he was 4 to 5, did she/he ever play imaginative games with another child in such a way that you can tell that each child understands what the other is pretending?	○	○
40. When she/he was 4 to 5, did she/he play cooperatively in games that need some form of joining in with a group of other children, such as hide-and-seek or ball games?	○	○

The Adult Autism Spectrum Quotient (AQ)

Ages 16+

SPECIMEN, FOR RESEARCH USE ONLY.

For full details, please see:

S. Baron-Cohen, S. Wheelwright, R. Skinner, J. Martin and E. Clubley, (2001)
The Autism Spectrum Quotient (AQ) : Evidence from Asperger Syndrome/High Functioning Autism, Males and Females, Scientists and Mathematicians
Journal of Autism and Developmental Disorders 31:5-17

Name:..... Sex:.....

Date of birth:..... Today's Date.....

How to fill out the questionnaire

Below are a list of statements. Please read each statement very carefully and rate how strongly you agree or disagree with it by circling your answer.

DO NOT MISS ANY STATEMENT OUT.

Examples

E1. I am willing to take risks.	definitely agree	slightly agree	**slightly disagree**	definitely disagree
E2. I like playing board games.	definitely agree	**slightly agree**	slightly disagree	definitely disagree
E3. I find learning to play musical instruments easy.	definitely agree	slightly agree	slightly disagree	**definitely disagree**
E4. I am fascinated by other cultures.	**definitely agree**	slightly agree	slightly disagree	definitely disagree

1. I prefer to do things with others rather than on my own.	definitely agree	slightly agree	slightly disagree	definitely disagree
2. I prefer to do things the same way over and over again.	definitely agree	slightly agree	slightly disagree	definitely disagree
3. If I try to imagine something, I find it very easy to create a picture in my mind.	definitely agree	slightly agree	slightly disagree	definitely disagree
4. I frequently get so strongly absorbed in one thing that I lose sight of other things.	definitely agree	slightly agree	slightly disagree	definitely disagree
5. I often notice small sounds when others do not.	definitely agree	slightly agree	slightly disagree	definitely disagree
6. I usually notice car number plates or similar strings of information.	definitely agree	slightly agree	slightly disagree	definitely disagree
7. Other people frequently tell me that what I've said is impolite, even though I think it is polite.	definitely agree	slightly agree	slightly disagree	definitely disagree
8. When I'm reading a story, I can easily imagine what the characters might look like.	definitely agree	slightly agree	slightly disagree	definitely disagree
9. I am fascinated by dates.	definitely agree	slightly agree	slightly disagree	definitely disagree
10. In a social group, I can easily keep track of several different people's conversations.	definitely agree	slightly agree	slightly disagree	definitely disagree
11. I find social situations easy.	definitely agree	slightly agree	slightly disagree	definitely disagree
12. I tend to notice details that others do not.	definitely agree	slightly agree	slightly disagree	definitely disagree
13. I would rather go to a library than a party.	definitely agree	slightly agree	slightly disagree	definitely disagree
14. I find making up stories easy.	definitely agree	slightly agree	slightly disagree	definitely disagree
15. I find myself drawn more strongly to people than to things.	definitely agree	slightly agree	slightly disagree	definitely disagree
16. I tend to have very strong interests which I get upset about if I can't pursue.	definitely agree	slightly agree	slightly disagree	definitely disagree
17. I enjoy social chit-chat.	definitely agree	slightly agree	slightly disagree	definitely disagree
18. When I talk, it isn't always easy for others to get a word in edgeways.	definitely agree	slightly agree	slightly disagree	definitely disagree

19. I am fascinated by numbers.	definitely agree	slightly agree	slightly disagree	definitely disagree
20. When I'm reading a story, I find it difficult to work out the characters' intentions.	definitely agree	slightly agree	slightly disagree	definitely disagree
21. I don't particularly enjoy reading fiction.	definitely agree	slightly agree	slightly disagree	definitely disagree
22. I find it hard to make new friends.	definitely agree	slightly agree	slightly disagree	definitely disagree
23. I notice patterns in things all the time.	definitely agree	slightly agree	slightly disagree	definitely disagree
24. I would rather go to the theatre than a museum.	definitely agree	slightly agree	slightly disagree	definitely disagree
25. It does not upset me if my daily routine is disturbed.	definitely agree	slightly agree	slightly disagree	definitely disagree
26. I frequently find that I don't know how to keep a conversation going.	definitely agree	slightly agree	slightly disagree	definitely disagree
27. I find it easy to "read between the lines" when someone is talking to me.	definitely agree	slightly agree	slightly disagree	definitely disagree
28. I usually concentrate more on the whole picture, rather than the small details.	definitely agree	slightly agree	slightly disagree	definitely disagree
29. I am not very good at remembering phone numbers.	definitely agree	slightly agree	slightly disagree	definitely disagree
30. I don't usually notice small changes in a situation, or a person's appearance.	definitely agree	slightly agree	slightly disagree	definitely disagree
31. I know how to tell if someone listening to me is getting bored.	definitely agree	slightly agree	slightly disagree	definitely disagree
32. I find it easy to do more than one thing at once.	definitely agree	slightly agree	slightly disagree	definitely disagree
33. When I talk on the phone, I'm not sure when it's my turn to speak.	definitely agree	slightly agree	slightly disagree	definitely disagree
34. I enjoy doing things spontaneously.	definitely agree	slightly agree	slightly disagree	definitely disagree
35. I am often the last to understand the point of a joke.	definitely agree	slightly agree	slightly disagree	definitely disagree
36. I find it easy to work out what someone is thinking or feeling just by looking at their face.	definitely agree	slightly agree	slightly disagree	definitely disagree
37. If there is an interruption, I can switch back to what I was doing very quickly.	definitely agree	slightly agree	slightly disagree	definitely disagree

38. I am good at social chit-chat.	definitely agree	slightly agree	slightly disagree	definitely disagree
39. People often tell me that I keep going on and on about the same thing.	definitely agree	slightly agree	slightly disagree	definitely disagree
40. When I was young, I used to enjoy playing games involving pretending with other children.	definitely agree	slightly agree	slightly disagree	definitely disagree
41. I like to collect information about categories of things (e.g. types of car, types of bird, types of train, types of plant, etc.).	definitely agree	slightly agree	slightly disagree	definitely disagree
42. I find it difficult to imagine what it would be like to be someone else.	definitely agree	slightly agree	slightly disagree	definitely disagree
43. I like to plan any activities I participate in carefully.	definitely agree	slightly agree	slightly disagree	definitely disagree
44. I enjoy social occasions.	definitely agree	slightly agree	slightly disagree	definitely disagree
45. I find it difficult to work out people's intentions.	definitely agree	slightly agree	slightly disagree	definitely disagree
46. New situations make me anxious.	definitely agree	slightly agree	slightly disagree	definitely disagree
47. I enjoy meeting new people.	definitely agree	slightly agree	slightly disagree	definitely disagree
48. I am a good diplomat.	definitely agree	slightly agree	slightly disagree	definitely disagree
49. I am not very good at remembering people's date of birth.	definitely agree	slightly agree	slightly disagree	definitely disagree
50. I find it very easy to play games with children that involve pretending.	definitely agree	slightly agree	slightly disagree	definitely disagree

Developed by:
The Autism Research Centre
University of Cambridge

"Totally correct" or "Pretty much right" to questions # 2, 4, 5, 6, 7, 9, 12, 13, 16, 18, 19, 20, 21, 22, 23, 26, 33, 35 , 39, 41, 42, 43, 45, 46 give 1 point.

"Does not match at all" or "Does not match" on question: 1, 3, 8, 10, 11, 14, 15, 17, 24, 25, 27, 28, 29, 30, 31, 32, 34, 36, 37 , 38, 40, 44, 47, 48, 49, 50 give 1 point.

Control group: 16.4 p. 80% of participants with AS had 32 p or more.

It comprises 50 questions, made up of 10 questions assessing 5 different areas:
social skill (items 1,11,13,15,22,36,44,45, 47,48);
attention switching (items 2,4,10,16,25,32,34, 37,43,46);
attention to detail (items 5,6,9,12,19,23,28, 29,30,49);
communication (items 7,17,18,26,27,31,33, 35,38,39);
imagination (items 3,8,14,20,21,24,40,41, 42,50).

DERS-16

Please indicate how often the following statements apply to you by writing the appropriate number from the scale above (1–5) on the line beside each item.

1	2	3	4	5
Almost never	Sometimes	About half the time	Most of the time	Almost always
0-10%	11-35%	36-65%	66-90%	91-100%

_____1) I have difficulty making sense out of my feelings

_____2) I am confused about how I feel.

_____3) When I am upset, I have difficulty getting work done.

_____4) When I am upset, I become out of control.

_____5) When I am upset, I believe that I will remain that way for a long time.

_____6) When I am upset, I believe that I'll end up feeling very depressed.

_____7) When I am upset, I have difficulty focusing on other things.

_____8) When I am upset, I feel out of control.

_____9) When I am upset, I feel ashamed with myself for feeling that way.

_____10) When I am upset, I feel like I am weak.

_____11) When I am upset, I have difficulty controlling my behaviors.

_____12) When I am upset, I believe that there is nothing I can do to make myself feel better.

_____13) When I am upset, I become irritated with myself for feeling that way.

_____14) When I am upset, I start to feel very bad about myself.

_____15) When I am upset, I have difficulty thinking about anything else.

_____16) When I am upset, my emotions feel overwhelming.

Citation: Bjureberg, J., Ljótsson, B., Tull, M. T., Hedman, E., Sahlin, H., Lundh, L.-G., L. G., Bjärehed,J., DiLillo, D., Messman-Moore, T., Gumpert, C., & Gratz, K. L. (2015). Development and Validation of a Brief Version of the Difficulties in Emotion Regulation Scale: The DERS-16. *Journal of Psychopathology and Behavioral Assessment*, 1–13. http://doi.org/10.1007/s10862-015-9514-x