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INVESTIGATION INTO THE MECHANISMS UNDERLYING THE TRANSGENERATIONAL EFFECTS OF MATERNAL HIGH-FAT DIET-INDUCED DYSBIOSIS ON OFFSPRING BRAIN AND METABOLISM

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Perseverance.

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Undertaking this PhD was a truly life-changing experience for me. Moving to another continent, adjusting to a different culture and environment, experiencing all sorts of unforeseen events, and last but not least facing a pandemic, made my path towards the PhD particularly challenging, and required a lot of perseverance and determination that only the passion I have for this job could make possible. Yet, taking this journey has been one of the most significant experiences of my life, an experience that is destined to shape my career and my future.

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List of Abbreviations

3C – Three chambers

4E-BPs - eukaryotic initiation factor 4E (eIF4E)-binding proteins

5-HT - 5-hydroxytryptamine

5-HTR - 5-Hydroxytryptamine receptor 4

α-KG α-ketoglutarate

AA – Amino acid

ABA - Applied behavioral analysis

ABC - Aberrant behavior checklist

Ach – Acetylcholine

ACEs - Adverse childhood experiences

ACTH - Adrenocorticotropic hormone

AD – Alzheimer's disease

ADDM - Autism and Developmental Disabilities Monitoring Network

ADHD - Attention deficit hyperactivity disorder

AKT - Serine/threonine-protein kinase

AMPs - Antimicrobial peptides

AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AMPAR - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

ANA - Anti-nuclear antibody

ANS – Autonomic nervous system

APA - American Psychiatric Association

ArAAs - Aromatic amino acids

AS - Angelman Syndrome

ASD – Autism spectrum disorder

ASDEU - Autism Spectrum Disorders in the European Union

ATCC - American Type Culture Collection

ATIMA - Agile toolkit for incisive microbial analyses

ATP - Adenosine triphosphate

BAC - Bacterial artificial chromosome

BBB – Blood brain barrier

BCAA – Branched chain amino acid

BCATc- Cytosolic mitochondrial branched-chain aminotransferase

BCATm - Mitochondrial branched-chain aminotransferase

BCKDC - Branched-chain α-keto acid dehydrogenase enzyme complex

BDNF - Brain-derived neurotrophic factor

BH4 - Tetrahydrobiopterin

BMA – Body mass analysis

BMI – Body mass index

CACNA1C - Calcium Voltage-Gated Channel Subunit Alpha1 A

CAM - Cell-adhesion molecules

cAMP - Cyclic adenosine monophosphate

CAMKII - Calcium/calmodulin-dependent protein kinase type II

CBT - Cognitive behavioral therapy

CDC - Centers for Disease Control and Prevention

CDH10 - Cadherin 10

CDH9 - Cadherin 9

CFC - Contextual fear conditioning test

CFU - colony forming units

CHARGE - Childhood Autism Risks from Genetics and the Environment

cKO – Conditional knock-out

CMMR – Center for metagenomics and Microbiome Research

CNS – Central nervous system

CNTNAP - Contactin-associated protein

CNVs - Copy Number Variations

CR - Corticosteroid receptor

CREB - cAMP response element-binding protein

CRF - Corticotropin-releasing factor

CRH - Corticotrophin-releasing hormone

CRHR1 - Corticotropin-releasing hormone receptor 1

CS - Conditioned stimulus

DA - Dopamine

DCs - Dendritic cells

DIO - Diet-induced obesity

DLGAP2 - DLG Associated Protein 2

DOHaD - Developmental origins of health and disease

DSM - Diagnostic and statistical manual of mental disorders

DZ – Dizygotic

ED - Embryonic day

E/I – Excitation/inhibition

EECs - Enteroendocrine cells

eEF2 - eukaryotic elongation factor

eEF2K - eukaryotic elongation factor kinase

eIF2α - Alpha subunit of eukaryotic initiation factor 2

eIF4E - eukaryotic initiation factor 4E

eIF4F - Eukaryotic initiation factor 4F

eIF4G - Eukaryotic initiation factor 4G

EIBI - Early Intensive Behavioral Intervention

EMA - European Medicines Agency

ENS – Enteric nervous system

ERK - Extracellular signal-regulated kinases

ESDM - Early Start Denver Model

F/B - Firmicutes/Bacteroidetes

FDA – Food and drug administration

FFAR - Free fatty acid receptor

FMR1- Fragile X mental retardation 1

FMRP - Fragile X mental retardation protein

FMT - Fecal microbiota transplant

FOS - Fructo-oligosaccharides

FRAA - Folate receptor α autoantibody

FXS - Fragile X syndrome

GABA - Gamma aminobutyric acid

GCN2 - General control non-derepressible 2

GD – Gestational day

GDH - Glutamate dehydrogenase

GF – Germ free

GI - Gastrointestinal

GLA - Glutaminase

Gln - Glutamine

GLP1 - Glucagon-like peptide 1

Glu - Glutamate

GOS - Galacto-oligosaccharides

GPCR – G protein coupled receptor

GR – Glucocorticoid receptor

GS - Glutamine synthase

GTP – Guanosine triphosphate

GWAS – Genome wide association study

HCD - High-carbohydrate diet

HDP - Host defense peptides

HF – High fat

HFD – High fat diet

HM – Human microbiota

HMT - Histone methyltransferase

HN - Hypothalamic–neurohypophyseal axis

HPA - Hypothalamic-pituitary-adrenal axis

HPG - Hypothalamic–pituitary–gonadal axis

HPT - Hypothalamic–pituitary–adrenal axis

HRSA - Health resources and services administration

IBS - Irritable bowel syndrome

ID – Intellectual disability

IFN-γ – Interferon gamma

IL-1β – Interleukin-1 beta

IL-6 – Interleukin 6

IL-10 – Interleukin 10

IL-18 – Interleukin-18

IL-17 – interleukin 17

IL-22 – Interleukin 22

In-del - Insertion-deletion

IP - Imidazole propionate

IPGTT - Intraperitoneal glucose tolerance test

IPITT - Intraperitoneal insulin tolerance test

iPSC - Induced pluripotent stem cells

IR – Insulin resistance

IRES - Internal ribosome entry site

IRS - Insulin receptor substrate

IU - International unit

IUGR - Intrauterine growth restriction

KEGG - Kyoto Encyclopedia of Genes and Genomes

KIC - α-ketoisocaproate

KO – Knock-out

LAT - Large amino acid transporter

LBP - Lipopolysaccharides binding protein

LD – Learning disability

LGA - Large-for-gestational-age

LM-PCR – Ligation mediated PCR

LMW – Low molecular weight

lncRNAs - long non-coding RNAs

LPS - Lipopolysaccharides

LRRTM - leucine-rich repeat transmembrane protein

LTD – Long-term depression

LTM – Long-term memory

L-LTM – Late-long term memory

LTP – Long-term potentiation

MACs – Microbiota accessible carbohydrates

MAG - Myelin associated glycoprotein

MBP - Myelin basic protein

MCTs - Monocarboxylate transporters

MECP2 - Methyl-CpG binding protein 2

metaHIT - Metagenomics of the human intestinal tract

Met. – Metformin

MGBA - Microbiota-gut-brain axis

mGluR – Metabotropic glutamate receptor

MHCI - Major histocompatibility complex I

MHFD – Maternal high fat diet

MIA – Maternal immune activation

miRNAs - MicroRNAs

MRC - Medical research council (vitamin study)

MRD – Maternal regular diet

MRI - Magnetic resonance imaging

MS - Maternal separation

MS – Metabolic syndrome

MSUD - Maple syrup urine disease

mTOR - mammalian target of rapamycin

mTORC1 - mammalian target of rapamycin complex 1

mTORC2 - mammalian target of rapamycin complex 2

MTT - Microbiota Transfer Therapy

MZ – Monozygotic

NDD – Neurodevelopmental disorders

NF-kB - Nuclear factor-κB

NGS – Next generation sequencing

NIH - National Institutes of Health

NLGN - Neuroligin

NLR - Nucleotide-binding oligomerization domain-like receptors

NMDA - N-Methyl-D-aspartate

NMDAR - N-Methyl-D-aspartate receptor

NMR - Nuclear magnetic resonance

NOD - Nucleotide-binding oligomerization domain

NPY - Neuropeptide Y

NRXN - Neurexin

NTD - Neural tube defects

OCD - obsessive-compulsive disorders

OF – Open field test

OT - Oxytocin

OUTs- Operational taxonomic units

OXTR – Oxytocin receptor

PCoA – Principal component analysis

PCOS - polycystic ovary syndrome

PERK - PKR-like endoplasmic reticulum kinase

PGN - Peptidoglycan

PHE – Phenylalanine

PI3K - Phosphoinositide 3-kinases

PKC – Protein kinase C

PKR - Protein kinase R

poly(I:C) - Polyinosinic-polycytidylic acid

PMS - Phelan McDermid syndrome

PNS – Peripheral nervous system

PP1 - Protein phosphatase 1

PRR - Pattern recognition receptors

PSD – Post synaptic density

PSNS - Parasympathetic nervous system

PTEN - Phosphatase and tensin homolog

PVN – Paraventricular nuclei

PWS - Prader-Willi Syndrome

PYY - Peptide YY

RasGap - RasGTPase-activating protein

RD – Regular diet

RELN - Reelin

ROI - Regions of interest

RORγ - RAR-related orphan receptor gamma

RRR - Relative recurrence risk

RS – Reciprocal social

RTT - Rett syndrome

S6K - S6 kinases

SAPAP - Guanylate kinase-associated protein

SCAF - Short-chain fatty acids

SCN1A - Sodium Voltage-Gated Channel Alpha Subunit 1

SD – Standard deviation

SEM - Standard error of the mean

SFB - Segmented filamentous bacteria

SHANK - SH3 And Multiple Ankyrin Repeat Domains

SK – Skeletal muscle

SLC6A4 - Solute Carrier Family 6 Member 4

sncRNAs - Small non-coding RNAs

SN – Social Novelty

SNP - Single-nucleotide polymorphism

SNS - Sympathetic nervous system

SNV - Single nucleotide variant

SSRI - Selective serotonin reuptake inhibitors

T2DM – Type 2 diabetes mellitus

TD - Typically developing children

TGF-β - Transforming growth factor beta

TH1- Type 1 helper T cell

TH2 - Type 2 helper T cell

TH17 - Type 17 T helper

TLR - Toll-like receptor

TMAO - Trimethylamine N-oxide

TNF-α – Tumor necrosis factor alpha

Treg - Regulatory T cells

TRP – Tryptophan

TSC - Tuberous sclerosis complex

TSC1 - Hamartin

TSC2 - Tuberin

TYR – Tyrosine

US - Unconditioned stimulus

US – United States

UBE3A - Ubiquitin-protein ligase

Veh. - Vehicle

VN - Vagus nerve

VTA – Ventral tegmental area

WES - Whole exome sequencing

WGS – Whole genome sequencing

WGS - Whole genome shotgun

WPD – Western pattern diet

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Abstract

Genetic and environmental factors, and their interactions, contribute to the etiology and pathophysiology of neurodevelopmental disorders (NDDs), such as autism spectrum disorder (ASD). The clinical heterogeneity and phenotypic variability in patients with NDDs have made identification of causal mechanisms contributing to their onset difficult. Yet, unraveling the underlying causes of NDDs is essential to the development of appropriate preventive/therapeutic strategies. Maternal obesity is considered one of the main nongenetic risk factors for NDDs in progeny. We and others have found that high-fat (HF) obesogenic diets in both humans and animal models induce significant modifications in maternal gut microbiome composition (dysbiosis), which are causally related to detrimental health outcomes in offspring, including ASD-like social deficits. Given the increasing prevalence of obesity and overweight in women of childbearing age, further investigation into the mechanisms by which maternal obesity impacts offspring health is needed. Here, working in a mouse model, we aimed to determine in whether vertical transmission of maternal high-fat diet (MHFD)-induced obese-type gut microbiota could serve as the primary driver of cognitive and metabolic dysfunction in offspring, identify the underlying mechanisms, and test the efficacy of prenatal modulation of the maternal gut microbiome on preventing behavioral and metabolic dysfunction in offspring. In a second study, we tested the hypothesis that MHFD-induced dysbiosis of the gut microbiome and related social dysfunction persists across generations and are therefore propagated in generations beyond the first, even in the absence of direct exposure to MHFD. We anticipate that our studies have the potential to revolutionize antenatal care for women of overweight and obese status and could lead to the development of innovative preventative and therapeutic strategies, such as prenatal probiotic administration, to improve the health of children affected by maternal diet-induced obesity *in utero*.

Chapter I – General Introduction

Part I – Neurodevelopmental Disorders, Focus on Autism Spectrum Disorder.

1.1 Neurodevelopmental Disorders (NDDs): DSM V Classification.

According to the *Diagnostic and Statistical Manual of Mental Disorders,* fifth edition (DSM-V)[1], the taxonomic and diagnostic tool published by the American Psychiatric Association (APA), Neurodevelopmental disorders (NDDs) are identified as a group of conditions with onset in early development, typically before entry in grade school. They are characterized by developmental deficits, which are responsible for impairments of social, personal, or occupational functioning. Such deficits range from specific limitations of learning and memory, language and speech, behavior, or motor skills, to more general impairment of social skills and intelligence. The most recent classification of NDDs includes intellectual disability (Intellectual Developmental Disorder), communication disorders, autism spectrum disorder (ASD), attention deficit hyperactivity disorder (ADHD), specific learning disorder, and motor disorders (**Table 1**).

NDDs often co-occur; most children are affected by more than one disorder.[2] The symptoms and specific features of neurodevelopmental disabilities tend to change with growth. However, most of these are lifelong conditions and are characterized, with specific differences between the various disorders, by a higher risk of impact by adverse psychological and psychosocial outcomes, such as a lower quality of life, unemployment, poor social and romantic function, social isolation, physical and sexual abuse, violent and non-violent offenses, and suicide. The tendency for symptoms and deficits to mount in a cumulative fashion also impacts physical health, including various conditions such as epilepsy or seizures, asthma, allergies, overweight and obesity, sleep disorders, and gastrointestinal disorders.

Intellectual Disabilities	***Communication Disorders***
Intellectual Disability (Intellectual Developmental Disorder)	Language Disorder
Global Developmental Delay	Speech Sound Disorder (previously Phonological Disorder)
Unspecified Intellectual Disability (Intellectual Developmental Disorder)	Childhood-onset Fluency Disorder (Shuttering)
	Social (Pragmatic) Communication Disorder
	Unspecified Communication Disorder
Attention-Deficit/Hyperactivity Disorder	***Motor Disorders***
Attention-Deficit/Hyperactivity Disorder	Developmental Coordination Disorder
Other Specified Attention-Deficit/Hyperactivity Disorder	Stereotypic Movement Disorder
	Tic Disorder
Unspecified Attention-Deficit/Hyperactivity Disorder	Tourette's Syndrome
	Persistent (Chronic) Motor or Vocal Tic Disorder
	Provisional Tic Disorder
	Other Specified Tic Disorder
	Unspecified Tic Disorder
Autism Spectrum Disorder	***Specific Learning Disorder***
Autism Spectrum Disorder	Specific Learning Disorder
Other Neurodevelopmental Disorders	
Other Specified Neurodevelopmental Disorder	
Unspecified Neurodevelopmental Disorder	

Table 1. **Classification of Neurodevelopmental disorders.** In the DSM-V, the classification of mental disorders for children and adolescents has been revised. The category 'neurodevelopmental disorders' includes seven main groups of disorders: *(1)* disorders of intellectual development (intellectual disabilities), *(2)* developmental speech or language disorders (communication disorders) *(3)* autism spectrum disorders, *(4)* attention deficit hyperactivity disorder, *(5)* specific learning disorders, *(6)* motor disorders, *(7)* a remainder category labeled "other neurodevelopmental disorders".

1.2 Focus on Autism Spectrum Disorder (ASD).

Among NDDs, the prevalence of autism spectrum disorder (ASD) has increased dramatically in recent decades[2]. The definition of autism was first introduced by Eugen Bleuler, a Swiss psychiatrist, in 1911 to illustrate a symptom of schizophrenia. In his text, Dementia Praecox or the Group of Schizophrenias, Bleuler used this concept to indicate a condition of extreme insulation from reality, with a marked "*detachment from reality, together with the relative and absolute predominance of the inner life*".[3] As schizophrenia was considered to be an adult disorder, autism was used to describe the equivalent in children. This definition was broadly shared and used by psychologists, psychoanalysts, and psychiatrists for many decades. Over time, the scientific community started to differentiate autism from schizophrenia, refusing the concept that the former was a pediatric version of the latter. In this regard, in 1943 Leo Kanner, an Austrian-American psychiatrist referred to as the "father of child psychiatry", provided a significant contribution to the description of "early infantile autism" as a distinct clinical syndrome on the basis of his observation of 11 children who exhibited similar behavior. He described autism as a disorder characterized by subjects' inability to relate themselves to others, extreme aloneness, and obsessive desire to maintain sameness in their environment.[4] Terms like 'childhood schizophrenia' and 'childhood psychosis' fully fell out of use with the 3rd edition of the Diagnostic and Statistical Manual of Mental Disorders, DSM III (1980), which introduced new terms such as 'pervasive developmental disorders' and its sub-groups, 'infantile autism', 'childhood onset pervasive developmental disorder', 'residual autism,' and an atypical form of autism.[5] The introduction and application of statistical methods and epidemiological parameters strongly influenced the definition of autism, expanding the description of the associated behavioral, cognitive, and

communicative features, thus detaching autism from its canonical association with insanity, and redefining diagnostic criteria and classification. ASD is categorized as syndromic or non-syndromic[6]. Syndromic autism occurs in subjects with other neurological conditions, such as Rett Syndrome, Fragile X Mental Syndrome (FXS), or Tuberous Sclerosis, and it is determined by a mutation in a specific gene or group of genes. On the other hand, non-syndromic autism is not linked to other defined conditions and cannot be traced to mutations in a single gene or specific chromosomic aberrations. A more detailed classification of the two main categories of ASD is provided in the following chapters.

1.2.1 ASD: Clinical Presentation.

Clinical manifestation of ASD differs based on the severity of the disorder, developmental stage, and age of the subjects. Therefore, the term *spectrum* is used to include a broad range of disorders previously identified as high-functioning autism, Asperger's disorder, pervasive developmental disorders not otherwise specified, atypical autism, and others. The main characteristics of ASD are: (1) "*persistent deficits in social communication and social interaction across multiple contexts*", such as deficits in nonverbal communications, including eye contact, body language and face expressions, and deficits in developing social interactions and lack of interest in peers; (2) *"restricted, repetitive patterns of behavior, interests, or activities",* such as stereotyped and repetitive movements, inability to adapt to changes in routines, extremely restricted interests, abnormal reactions to environmental stimuli like sounds, smells, etc.; (3) "*symptoms must be present in the early developmental period"* and *"symptoms cause clinically significant impairment in social, occupational, or other important areas of current functioning."*

These core features of ASD often appear in early development, but the specific age at which a diagnosis is made may vary in relation to the subjects and their environment. In most cases, ASD is not diagnosed until after age three; however, early detection and intervention are associated with better outcomes.[7] The first signs of ASD are usually related to delayed language development, with absence of interest for social interactions and uncommon patterns of communication. These features become more apparent after the second year of life. With some exceptions, ASD symptoms usually improve during adolescence; however, only a restricted number of affected individuals can work and live independently in adult life, depending on the severity of the disorder.

1.2.2 ASD: Comorbidities.

The majority of individuals affected by ASD have other comorbid disorders. A growing number of studies support the idea that many symptoms usually considered additional features of ASD are in fact related to other disorders which should be diagnosed as comorbid conditions. The presence of comorbidities generally leads to more pronounced behavioral and cognitive deficits.[8]The general prevalence of ASD-associated comorbidity varies depending on the specific method of clinical assessment and the heterogeneity of the groups being studied. Neuropsychiatric disorders are often associated with ASD.[9,10] The prevalence of anxiety disorders in individuals affected by ASD ranges between 1.47% and 54% across studies[11,12]; depressive disorders are present in 2.5% - 47.1%[11,13], and bipolar and mood disorders have been found to have a prevalence of 4.4% to 37%[13,14] across ASD samples. Furthermore, the incidence of schizophrenia spectrum and other psychotic disorders[15] ranged from 4% to 67%, whereas obsessive-compulsive disorders (OCD) co-occurring in ASD ranged from 9% to 22%[11], and suicidal ideation and attempts from 10.9% to 66%.[16] Several studies have reported a significant frequency of sleep disturbances in around 80%

of both adults and children affected by ASD[17]. ASD is often co-diagnosed with other NDDs. Attention-deficit/hyperactivity disorder (ADHD) prevalence ranges from 25.7% to 65%[13,18]. Intellectual disability (ID) is frequently diagnosed in children with ASD, but the prevalence of ID in ASD individuals is strongly debated[19], with some studies indicating that as many as 70% of ASD cases showed co-occurring ID[20], while other epidemiological reports suggest that the association was found only in 30% of individuals.[21] Epilepsy is another condition with a considerable prevalence in ASD individuals, about 25%.[22] Interestingly, a strong risk factor for epilepsy in ASD is represented by the co-occurrence of ID.[23] Less expectedly, however, gastrointestinal (GI) dysfunction is often found in children with ASD[24], but the reported prevalence varies from 9% to 91%.[25] ASD subjects are four times more likely to display GI disorders, compared to non-ASD subjects, with diarrhea, constipation and abdominal pain the most frequent ones.[26] The presence of GI disturbances correlates with more severe ASD.[27] Additionally, GI dysfunction is commonly cited in parental reports.[28]

1.3 NDD Prevalence.

The prevalence of NDDs varies globally. The estimated prevalence of any developmental disorder in the United States increased from 12.84% in 1997–1999 to 17.76% in 2015–2017 in children between ages three to 17 (**Fig. 1a**), with ADHD and learning disabilities the most represented.[2] During the period 2015-2017, diagnoses increased for attention-deficit/hyperactivity disorder (ADHD) (8.5% to 9.5%), autism spectrum disorder (ASD) (1.1% to 2.5%), and intellectual disabilities (ID) (0.9% to 1.2%) The reasons behind the increasing trend in NDDs are controversial. Possible explanations include both etiological and nonetiological factors, with the latter including

refined diagnostic criteria, modifications in reporting methods, and higher public awareness.[29,30] In a recent study[2] based on a National Health Interview Survey (an annual, cross-sectional survey intended to provide nationally representative estimates on a wide range of health issues), scientists from the Centers for Disease Control and Prevention (CDC) and the Health Resources and Services Administration (HRSA) reported differences in prevalence based on age, sex, ethnicity, socio-economic conditions, etc. Children aged between 12-17 had higher chances to be diagnosed with ADHD, learning disability (LD), and ID. A similar trend was observed when boys and girls were compared, with boys displaying a higher prevalence. As for ethnic groups, non-Hispanic white and non-Hispanic black children were more likely to be diagnosed with NDDs compared to Hispanic children or non-Hispanic children. Health insurance also represents a crucial factor in determining the prevalence of NDDs, with children benefiting from public health insurance more likely to receive a diagnosis of any developmental disability compared with children supported by only private health insurance and uninsured children. Sociodemographic factors, such as maternal level of education, rural or urban residence, and economic status, also influenced the prevalence of NDDs. In fact, children with mothers with less than a high school education, children living in a household below 200% of the federal poverty line, and children living in rural areas had a higher risk of developing an NDD.

1.4 ASD Prevalence.

Data from epidemiological studies conducted since 2000 in various geographical areas across the globe, ASD global prevalence is around 62 in 10.000 children, without remarkable differences with regard to cultural and socioeconomical factors or specific geographic regions.[31] It must be

noted that the potential lack of accuracy of the available data sets, especially in low-income regions, strongly influence the accuracy of predicted global ASD prevalence. Indeed, accurate national estimates of prevalence are critically needed to better calculate ASD geographical prevalence and financial cost, which would enable more effective government intervention in the management and distribution of resources to public health and social services. In order to do so, standardized and consistent use of diagnostic criteria and disorder classification, as well as the ability to ensure an accurate and prompt diagnosis to the affected individuals is crucial. However, NDDs are often under-diagnosed and/or mis-diagnosed, especially in the case of co-occurring NDDs. The most recent report from the CDC's *Autism and Developmental Disabilities Monitoring* (ADDM)[21,32] network estimates that, in 2016, about 1 in 54 children (18.5 per 1,000 children) aged eight had been identified with ASD (**Fig. 1b**). This is an increase of 10% from the previous 2014 prevalence estimate (16.8). Compared to the first ADDM Network estimates in 2000, there is an almost threefold increase in diagnosis. These variations might be the result of different diagnostic procedures or changes in the pool of data available to the surveillance system.

Interestingly, ASD is 4.3 times more frequently diagnosed in boys compared to girls. The prevalence was identical for non-Hispanic white, non-Hispanic black, and Asian/Pacific Islander children (18.5, 18.3, and 17.9, respectively) but lower for Hispanic children (15.4). Overall, boys were more likely than girls to have ID (40% versus 32%), with a higher percentage in black and Hispanic children than white children (47%, 36%, and 27%, respectively). The disparate frequency of ASD diagnosis in male children is an area of active investigation, spawning various working hypotheses including "male fragility," which posits that fewer genetic mutations and/or environmental insults are required to drive phenotypic manifestation in males *versus* females[33]. I provide further discussion on sex differences in the following section of this chapter. These data

are not uniform across the ADDM network sites, and there is a positive association between prevalence and socioeconomic status, suggesting that ASD might be better identified in areas with better services and of higher income and access. *Autism Spectrum Disorders in Europe* (ASDEU) was a three-year (2015-2018) program funded by the European Parliament to research autism prevalence throughout Europe.[34] The program included 631,619 children, with an average estimated prevalence of 12.2 per 1,000 (one in 89) children aged 7-9 years. Overall ASD prevalence estimates varied among European countries, from 4.4 - 19.7 (percentiles 10 and 90) per 1,000 aged 7-9 years. In Italy, a population-based study conducted in 2016 reported that the prevalence of ASD in children aged 7-9 was of about one in 87.[35]

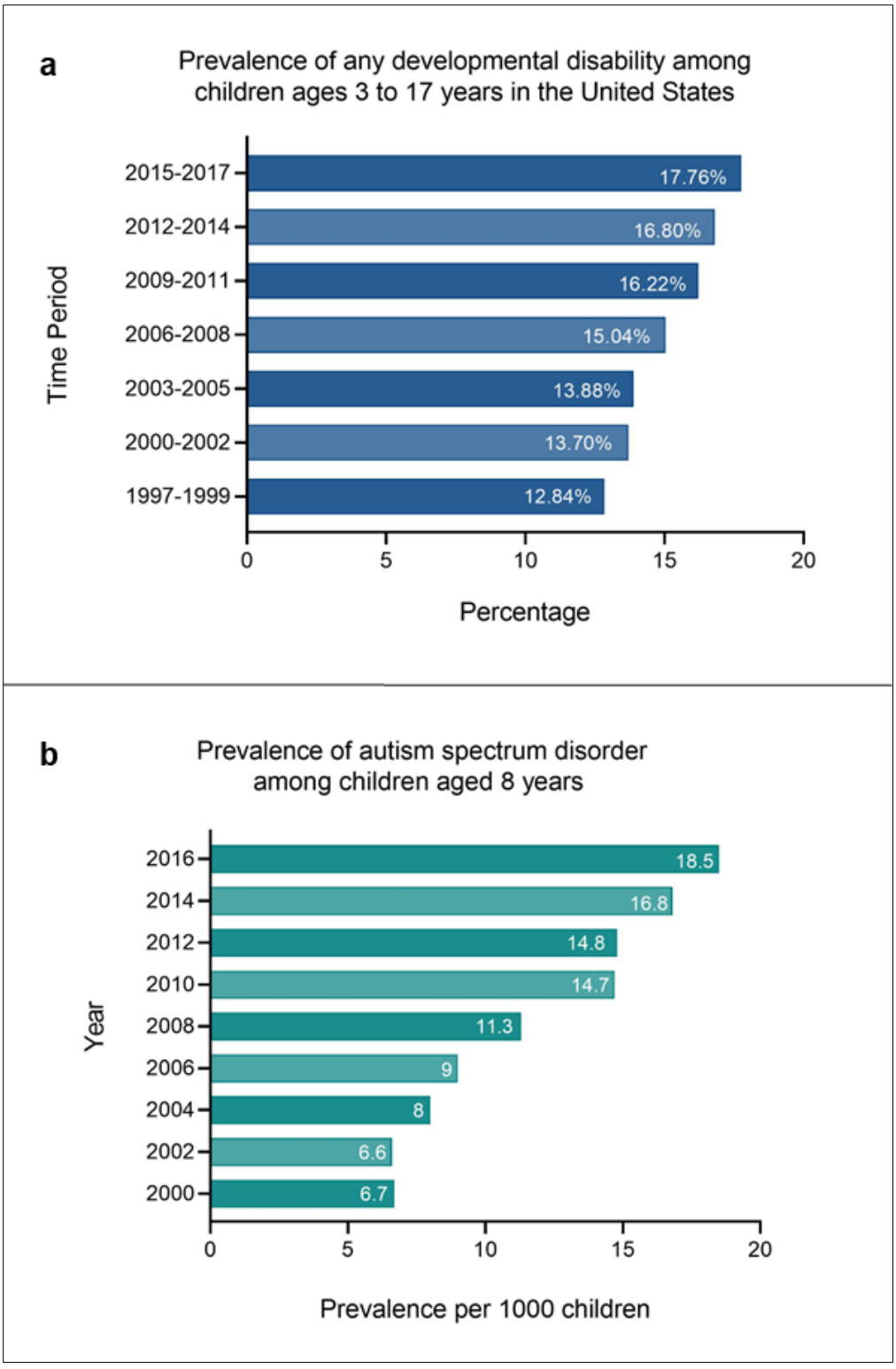
a
Prevalence of any developmental disability among children ages 3 to 17 years in the United States
Time Period
2015-2017
2012-2014
2009-2011
2006-2008
2003-2005
2000-2002
1997-1999
17.76%
16.80%
16.22%
15.04%
13.88%
13.70%
12.84%
0
5
10
15
20
Percentage
b
Prevalence of autism spectrum disorder among children aged 8 years
Year
2016
2014
2012
2010
2008
2006
2004
2002
2000
18.5
16.8
14.8
14.7
11.3
9
8
6.6
6.7
0
5
10
15
20
Prevalence per 1000 children

Figure 1. Developmental disorder and ASD prevalence in the United States. (**a**) Prevalence of any developmental disability among children ages 3 to 17 years in the United States, 1997 to 2017 based on data from the 2009 to 2017 National Health Interview Survey.[2] (**b**) Autism and Developmental Disabilities Monitoring (ADDM) Network estimates for overall ASD prevalence in US over time based on data collected from health and special education records of children living in 11 communities across the United States during 2014.[32]

1.4.1 ASD Sex Ratio.

The DSM-V reports that "*autism spectrum disorder is diagnosed four times more often in males than in females*". This ratio is based on a 2009 review of 43 studies published since 1966 on the prevalence of ASD[36]. However, this average ratio does not consider the great variability across different epidemiological reports, including study design, case-ascertainment method and sample size and age. It has been proposed that this ratio might actually be as low as 3:1.[37] Regarding the reasons underlying this different proportion among males and females, it is possible that girls affected by ASD are misdiagnosed[38] or late-identified[39]; this might be due to the fact that the female autism phenotype diverges from the established diagnostic criteria for ASD[40,41]. Nonetheless, biological factors such sex hormones and sex-based genetic risk, areas of active research investigation, might be responsible for the apparent male vulnerability to ASD.[42-44]

1.5 Food and Drug Administration (FDA)-Approved Treatments for ASD.

Currently, no therapy has been shown to cure ASD, but multiple interventions have been developed to improve cognitive, social, and communication skills of the affected subjects. ASD encompasses a range of neurodevelopmental conditions, and the severity of different symptoms varies between individuals. Treatment plans are therefore multidisciplinary and are targeted to the child's specific needs. Current interventions are grouped as pharmacological therapy, dietary interventions, and cognitive behavioral and communication therapy. However, due to the limitations of current therapeutic options, which are aimed to relieve behavioral symptoms of

autism in younger populations, but do not target the social and communication deficits at the core of ASD, further research is needed to provide new therapeutic and preventative strategies for ASD.

1.5.1 Pharmacological Treatments.

Risperidone[45,46] is a second-generation antipsychotic used to treat schizophrenia and bipolar disorder by inhibiting several 5-HT (serotonin) receptor subtypes. In 2006, as a result of a an eight-week, randomized, double-blind, placebo-controlled trial of risperidone children 5 to 17 years of age it became the first drug approved by the Food and Drug Administration (FDA) for the treatment of irritability and maladaptive behaviors in children 5 years of age and older affected by ASD. In 2009, the FDA approved aripiprazole, an atypical antipsychotic, to treat irritability in children with ASD[47]. The safety and efficacy of both drugs is similar in children with ASD.

In fact, a decrease in the aberrant behavior checklist (ABC) subscales, including stereotypic behavior, hyperactivity and irritability, was observed for both risperidone and aripiprazole treatment.[48] Recent experimental evidence has suggested the use of oxytocin (OT) as a treatment for behavioral deficits in disorders of social dysfunction[49], including ASD, generating great excitement. This has led to an increase in the number of clinical trials involving intranasal OT therapy, but results have been inconsistent[50]–some trials showed no benefits[51], while others demonstrated that OT treatment could potentially improve social skills in affected individuals[52]. Improved social and cognitive skills have been reported as a result of the treatment with sapropterin, a synthetic form of tetrahydrobiopterin (BH_4). BH_4 is an essential cofactor for many important metabolic processes dysregulated in ASD, such as nitric oxide metabolism. Both controlled[53,54] and open-label trials reported beneficial effects of sapropterin administration to

children with ASD. However, since the specific pathway targeted by this drug is still under investigation, there is no FDA approved indication for ASD, and further studies are needed. In addition to pharmacotherapy, dietary interventions have shown efficacy in some ASD subjects, suggesting GI-targeted treatments as a novel therapeutic strategy in ASD populations.[55] Several dietary approaches, including nutritional supplementation[56,57] and restricted diet[58], have been developed to target ASD symptoms. However, there is lack of strong evidence supporting the effectiveness of these interventions, and most of the studies do not consider the influence of concomitant therapies. Moreover, small sample sizes, heterogenous populations, and missing long-term evaluation of effects are strong limiting factors of these studies.[59]

Several studies report that ASD subjects have substantial differences in the composition of the gut microbiome compared to neurotypical individuals.[60-62] Animal studies showed that gut microorganisms produce metabolites that can have a strong impact on behavior through the gut-brain-axis.[63-65] Therefore, modulating the gut microbiome composition might represent a novel strategy to treat ASD. An open-label study of Microbiota Transfer Therapy (MTT)[66] investigated the effects of fecal microbiota transplant (FMT) to children with ASD and chronic gastrointestinal disturbances, and a follow-up study[67], conducted two years after the treatment stopped, assessed the long-term effects of this treatment. Results obtained showed marked improvements for all of the 18 participants, with a reduction in both behavioral and GI symptomatology, which persisted at two years. Despite promising results, FMT is not yet officially approved by the FDA, which expressed concerns regarding the potential presence of drug-resistant bacteria in the fecal matter.

1.5.2 Behavioral Interventions.

Since both social and communication skills are usually impaired in individual affected by ASD, behavioral and speech language therapy typically comprise the basis of a treatment plan. The most generally successful approach for children with autism is behavioral therapy, which includes different kinds of interventions. One of these is applied behavior analysis (ABA), often considered the gold-standard treatment for children with ASD or other developmental conditions. There are different types of ABA, such as the Early Start Denver Model (ESDM)[68], for children with ASD between the ages of 12-48 months, and Early Intensive Behavioral Intervention (EIBI)[69], for children younger than five years old. ABA is the usual starting point for children with more severe symptoms and is aimed to reinforce wanted behavior and reduce unwanted behavior. Instead, cognitive Behavioral Therapy (CBT)[70] is usually recommended for children with milder symptoms of autism, who are often considered high-functioning ASD patients. CBT aims to define the triggers of particular behaviors, so that a child starts to recognize those moments himself and is used to address a variety of symptoms of ASD, such as social behavior deficits and anxiety.

1.6 Estimating the Burden of ASD.

ASD is a significant global public health concern[71], given that it affects >3% of children worldwide and its prevalence is increasing. The lifetime cost of ASD alone for U.S. cases identified between 1990 and 2019 is estimated to be $7 trillion, an amount expected to double in the next ten years.[72] The detrimental effects of ASD also impact the family of affected individuals, with highest burdens placed on parents, in particular. Recent studies have shown that parents, both mothers and fathers,

of children with ASD experience a level of parenting stress significantly higher than parents of typically developing children (TD), manifested as increased anxiety, discomfort, and lack of confidence in parenting.[73,74] Therefore, parents of children with ASD should be supported with specific interventions aimed to reduce stress and improve both their parental skills and quality of life.

The attempt to estimate the burden of disease of ASD, measured by financial cost, mortality, morbidity, or other indicators, is a challenging task due to fact that the literature is characterized by both inconsistency and inaccuracy. This has consequences on the ability to compare the global impact of ASD, either between disorders, between geographical areas, between racial groups and/or over time. Despite lack of clarity in the literature, evidence suggests that ASD has a significant financial impact on families and schools. Families of children with ASD have increased health care-related expenses and are more likely to have reduced working hours, and consequently wages. In particular, in the US, maternal earnings of children with ASD are substantially decreased compared to those of non-ASD children.[75] A big portion of ASD-associated costs is represented by special school services through an Individualized Education Program, used by 73-91% of children with ASD in relation to the severity of the disease.[76] Differences in terms of diagnosis, prevalence, and treatment between different countries are particularly evident when comparing countries with primarily public *versus* primarily private healthcare. In the United States, for example, private insurance plans do not include coverage for most intensive long-term treatments required by NDDs, and children with private insurance may not benefit from appropriate insurance coverage, thus increasing the out-of-pocket costs for families and reducing the chances to receive proper medical and educational services.[77]

1.6.1 Demographics Influence Availability and Outcome.

Quality of life in individuals affected by ASD is significantly impaired. Indeed, on average ASD patients have lower life expectancy and heightened morbidity compared to those without ASD. In addition, the assessment of disparities[78,79] in access to diagnostic tools and therapeutic intervention for ASD highlight the fact that people affected by ASD who also experience disadvantages in regard to race/ethnicity, financial status, geographical locations, etc. are more likely to receive lowered level of care, and therefore see their life expectancy further reduced. For these reason, early childhood interventions, such as pharmacological or behavioral therapy, might be out of reach for some ASD patients, thus determining worse health outcomes. The inequality in health care for ASD, prompts the necessity to develop new and more accessible prevention strategies, such as those targeting maternal health status during pregnancy, which could reduce the severity of ASD-related deficits and therefore, have a greater impact across sociodemographic disparities.

1.6.2 Value of Identifying and Developing Preventative Treatments.

As ASD prevalence increases worldwide, so do the costs associated with the lifelong treatments required to improve the quality of life of the affected individuals. A crucial way to reduce ASD-related social and financial burden is to identify the modifiable risk factors, and thus lessen global prevalence. In this regard, the compelling story of how folic acid supplementation during pregnancy has become a crucial intervention to prevent the development of major birth defects such as spina bifida and anencephaly that are either fatal or have serious, long-term consequences for the child is worthy of consideration.

In 1965, Richard Smithells and Elizabeth Hibbard published a paper[80] reporting anomalies in formiminoglutamic acid tests performed in women who gave birth to babies with serious birth defects, notably neural tube defects (NTDs). This finding suggested that impaired folate status might be connected to the development of these defects. Women who had previously given birth to infants with NTDs were recruited into a trial[81] of periconceptional vitamin supplementations, and the results showed a four-fold reduction of NTD recurrence risks. The subsequent 'MRC Vitamin Study' trial[82] clarified that, out of the eight vitamins present in the multivitamin complex of the previous trial, folic acid alone was providing the protective effect against NTDs. This study demonstrated that 80% of NTDs could be prevented by daily administration of 4mg of folic acid immediately before and throughout pregnancy.

In acknowledgment of the significant benefit to society, in 1998, the FDA introduced mandatory supplementation of flour with folic acid to aid the prevention of NTDs. The success story of folate fortification in the periconceptional period is a shining example of how an easily implementable preventive measure could lead to significant reduction in the risk of some birth defects in offspring. A better understanding of the mechanism by which maternal conditions contribute to the risk for ASD might lead to the development of similar interventions, which might have a substantial impact on the prevalence of the disease.

In the studies presented herein, I provide data in support of the hypothesis that interventions that target maternal gut microbiome composition during pregnancy and lactation can improve long-term health outcomes, including cognitive and behavioral health, in subsequent generations.

Part II – Genetic and Epigenetic Etiology of ASD.

2.1 The Multifactorial Etiology of NDDs.

Multiple studies highlight the multifactorial etiology of neurodevelopmental disorders (NDDs), which is determined by the interaction of genetic, epigenetic, and/or environmental factors.[83,84] This complex and diversified etiology is reflected in the clinical heterogeneity and phenotypic variability within these disorders and has made identification of mechanisms contributing to their onset difficult. Yet, unraveling the underlying causes of NDDs is essential to development of appropriate preventive/therapeutic strategies. Interestingly, the co-occurrence of distinct NDDs in the same individual has been reported by many studies, thus suggesting the idea that shared underlying biological/cellular mechanisms may exist. On the other hand, the investigation of phenotype–genotype correlation reveals that patients with overlapping genetic etiology display a great variety of clinical signs, both in terms of numbers and severity. Together with missing heritability, this phenotypic variability suggests a multifactorial and/or polygenic nature of NDDs.

2.2 Investigation of Genetic Factors in ASD.

ASD has heterogeneous phenotypic features. Similarly, the genetic basis of ASDs is complex and is far from being fully determined–indeed, previous studies have been unable to consistently report associations between genetic variants and increased risk of ASD. However, despite this significant genetic heterogeneity, a growing body of evidence suggests that there is a converging pathophysiology in autism spectrum disorder, which will be discussed at the end of this chapter. Many cases of ASD show a highly complex genetic architecture[85]. Progress has been made towards unraveling this intricate network of genetic alterations, thanks to the significant technological

advances in the investigation of the genetic of human diseases brought about by the Human Genome Sequencing Project executed in the early 2000s.

Genetic variations can be classified into several different groups, such as common and rare variations, which have a frequency greater or lower of 1%, respectively, in the population; transmitted and *de novo* variation, which are passed from the parents to the offspring, or spontaneous mutation arising in either the sperm or egg, respectively; single-nucleotide polymorphism (SNP) and single nucleotide variant (SNV), which are variations in the DNA sequence common or rare, respectively, in the population; copy number variation and Insertion-deletion (in-del): variation in chromosomal structure greater that 1000 nucleotides or involving a small number of nucleotides, respectively; coding and non-coding variants, which affect DNA sequences encoding for proteins, or DNA sequence not encoding for proteins (introns, regulatory elements, etc.).[86]

The genetics of ASD includes all the aforementioned classes of mutations. Some genes associated with diseases are considered highly vulnerable genes due to the fact that disruptive mutations in these genes are linked to high disease risk. These alterations, responsible for monogenic forms of ASD, are rare in the population and make a small contribution to inherited risk, since they are less likely to be passed onto the next generation. Interestingly, other genes associated with ASD display low sensitivity to disruptive mutations and confer low disease risk. Such variants include SNPs, and while frequent in the general population, do not cause the disease *per se*. However, they increase ASD susceptibility when combined with other low risk variants. In this case, both the sum of the effects of each mutation and the interaction between the mutations (i.e., epistasis), are the mechanisms responsible for the increased risk to develop clinical presentation of the disease.[87]

Different combinations of rare high-risk mutations with many low-risk variants also influence the severity of the symptoms: deleterious low-risk alleles may exacerbate the effect of the rare high-risk alleles. On the other hand, protective variants may mitigate the influence of the rare variants[88]. An important distinction in the clinical presentation of ASD is that between syndromic and non-syndromic autism.[89] The term "syndromic" is relative to a medical condition in which ASD is manifested together with other phenotypes or features. The etiology of these conditions is usually defined by and is associated with single-gene mutations, chromosomal aberrations, or CNVs. Examples of syndromic autism are tuberous sclerosis complex (TSC)[90], Rett syndrome (RTT)[91], fragile X syndrome (FXS)[92], and PTEN macrocephaly syndrome.[93] The identification of the genetic basis of syndromic ASD represented a milestone in the investigation of the genetics of ASD. However, these monogenic disorders are very rare and, taken together, only account for 5% of all ASD cases. Syndromic ASDs will be described in detail in the following paragraphs.

The term "non-syndromic" indicates forms of ASD without additional symptoms. The etiology of these cases is not defined, and, therefore, the definition "idiopathic autism" is widely used. The genetic basis of idiopathic forms of ASD is far from being fully understood, however, since the conceptualization of ASD by Leo Kanner, various studies have attempted to unravel the genetic mechanisms underlying the disorder by means of different approaches. Twin studies and family studies represented the first generalized approach in field of ASD genetics. These studies showed that ASD aggregates in families and established the concept of the strong heritability of ASD. However, subsequent studies on larger samples questioned the reliability of early estimates of ASD heritability[94-96]. A recent study[97] conducted on a Swedish population, aimed to calculate the relative recurrence risk (RRR) to measure familial aggregation of ASD, reported an estimated

heritability of ASD of approximately 50%. Other studies were based on genetic linkage analysis designed to identify chromosomal portions co-inherited within families. This approach did not produce many significant and reproducible results, with the exception of a couple of loci with a strong genome-wide significance: one located on chromosome 7q35 and the other on chromosome 20p13.[98]

An alternative approach to determine variants conferring increased susceptibility to ASD was based on candidate gene studies[99], which can be functional (based on the involvement in pathophysiological mechanisms) or positional (based on the specific location in the chromosomes). In the last 25 years, over 200 candidate genes have been investigated, however, very few genes with an evident involvement in ASD have been identified. This small group includes NLGN3[100] and NLGN4[101] genes which codify for neuroligin proteins (reviewed later in this chapter), SLC6A4[102] which codifies for the serotonin transporter, and RELN which codifies for reelin[103,104]. In most cases, findings provided by candidate gene studies have not been replicated by following studies.

2.3 Genome Wide Association Study (GWAS).

An important turning point in the investigation of the genetics of ASD occurred in 2005 with the introduction of new techniques which allowed for extensive sequencing of genomes of affected individuals. In contrast with candidate gene studies, genome wide association studies (GWAS) are hypothesis-free methods for the identification of associations between loci and traits[105], thus avoiding the necessity for an initial hypothesis for the main determinant of disease and resulting a more suitable approach for the investigation of complex trait such ASD.

The aim of GWAS is to gather data to unravel common variants in two groups of subjects, affected and not affected by the disease. Variants predicted to be associated with the disease are found with greater frequency in the affected group compared to the non-affected controls. The introduction of such systematic, unbiased approaches has dramatically increased the identification of genetic alterations linked to ASD. Indeed, GWAS, by means of the investigation of large cohorts and dataset, unraveled a significant number of ASD-associated genes thus providing new targets for functional genomic studies with the intent to clarify the pathogenic mechanisms resulting from the reported mutations.

Notably, GWAS allowed for the identification of many SNPs potentially associated with ASD[106]. The first GWAS[107] compared cohorts of individual affected and control subjects with a European ancestry and revealed six SNPs in cadherin 10 (CDH10) and cadherin 9 (CDH9) genes, which encode for CNS-expressed cell-adhesion proteins. However, only one of these SNPs reached genome-wide significance. Unfortunately, GWAS findings have not been consistently replicated across studies and have often failed to identify causal mutations in ASD. The effect size of the common variants identified was quite small and, in a future perspective, these studies need to be reperformed on significantly larger cohorts.[86,105] An alternative molecular marker for GWAS is represented by copy number variations (CNVs), which consist of the duplication or deletion of DNA segments with size between 1,000 bp – 5 Mb.[108] Discovery of the contribution of CNVs to ASD incidence had a huge impact on the field ASD-related genetics, due to the fact that CNVs were found at significantly higher frequency among families with inherited ASD compared to controls[109,110] and involved, among others, genes with a strong association with ASD, such as SHANK3.[111] Interestingly, following studies provided insights about the clustering of some CNVs

in specific positions (recurrent CNVs), thus identifying at-risk regions on the genome.[112] About 7% of ASD individuals are estimated to carry these high-risk CNVs.[112-114]

In the last two decades, *de novo* mutations have emerged as an important component of the genetics of ASD. *De novo* mutations refer to variants which arise before or shortly after fertilization[115] in the proband and are not present in the parents. This phenomenon is particularly relevant in the case of CNVs[110]. Indeed, the majority of CNVs in ASD patients are classified as nonrecurrent and sporadic.[116,117] This class of genetic alterations is thought to confer high risk, but the extent to which they contribute to disease etiology remains largely unknown[118]. Interestingly, CNV mutations are found in protein-coding genes as well as in genetic regions responsible for transcription and splicing regulation[119].

2.4 Beyond GWAS.

The amount of *de novo* mutations in ASD has increased dramatically with the introduction of next generation sequencing (NGS) approach, such as whole exome sequencing (WES) and whole genome sequencing (WGS), with the latter revealing the importance of non-coding mutations in the development of ASD. WES and WGS allow for the large-scale screening of exome and genome, respectively, of affected individuals, and have led to the identifications of CNVs, in-dels, single-point mutations in coding[120,121] and non-coding[119,122] regions. In contrast to GWAS, WES and WGS approaches are targeted to direct identification of the disease-causing mutations, by means of heuristic filters.

A particularly successful application of these NGS-based approaches is represented by WES-trio, in which the genotypes of the proband and of the parents are sequenced and compared to identify both *de novo* mutations and inherited risk variants. This approach led to the discovery of several genes not previously associated with ASD and other NDDs.[123,124] WES- and WGS-based studies provided a great deal of new information regarding the genes potentially involved in the pathogenesis of ASD; however, these newly discovered genetic alterations may represent only the tip of the iceberg in terms of the great genetic heterogeneity of the spectrum, which might devalue a lot of significant findings. This scenario is further complicated by the fact that the majority of the genetic alterations associated with ASD show incomplete penetrance, meaning that the association with ASD does not occur in every individual who carries the specific variant, and variable expressivity, meaning that a genotype corresponds to different phenotypic **expression,** or both.[125]

Incomplete penetrance and variable expressivity might be the reason why studies aimed to draw a correlation between phenotype and genotype in ASD suggest that both the amount and the severity of the symptoms have a high degree of variability, even among subjects with overlapping genetic etiology[126]. On the other hand, this complexity leaves the majority of ASD cases without a precise genetic cause.[127,128] To overcome these challenges, one approach might include the extension of WES and WGS studies to larger cohorts of individuals. However, since it is established that epigenetic and environmental factors also play an important role in the etiology and clinical manifestations of ASD, looking at the genetic landscape alone might not reveal the full picture of the mechanisms underlying complex diseases such as ASD.

2.5 Mouse Models for ASD.

In recent decades, mouse models of ASD have been developed for mechanistic understanding and the discovery of novel therapeutics.[129] Many models reproduce one or more core behavioral features of ASD, including impaired social interactions, communication disorders, and stereotypy. They have been generated by means of either genetic engineering techniques or pre/post-natal exposure to environmental factors.[130] Again, the phenotypic and clinical heterogeneity of ASD makes the establishment of mouse models for the disease a difficult task. Due to the lack of robust pathological hallmarks in the ASD brain which reflect the underlying pathology and the fact that diagnostic criteria are solely based on behavioral features, mouse models for ASD recognized/accepted by the research field usually recapitulate one or more behavioral aberrations relevant to the diagnosis.[131]

The following section will focus on mouse models for ASD targeting candidate human genes, obtained by means of genetic engineering manipulations.[132,133] They can be divided in three categories: (1) mouse models of single gene mutations associated with syndromic ASD, (2) mouse models of single gene mutations associated with non-syndromic ASD, and (3) mouse models of copy number variations (CNVs) associated with ASD.

2.5.1 Mouse Models for Single Gene Mutations Associated with Syndromic ASD.

Mouse models for single gene mutations associated with syndromic ASD can be grouped by the cellular function of the disrupted protein: proteins involved in transcriptional regulation, proteins involved in post-transcriptional regulation, and proteins which constitute ion channels.

The first group include ***Mecp2* mutant mice**, which reproduce the genetic mutations associated with Rett syndrome (RTT), a neurodevelopmental disorder determined by mutation in the gene which encodes for methyl-CpG binding protein 2 (MECP2) located on the X chromosome. This protein has a crucial role in transcriptional regulation[134] and RNA splicing.[135] Affected individuals are almost exclusively females and show motor deficits, seizures, ASD-like behaviors, and problems in lung function.[136,137] *Mecp2* mutant mice include many models, mostly carrying the absence of the protein in a tissue-specific fashion and/or at different developmental stages.[138,139] In the majority of the cases, these mice show ASD-related[138,140] deficits, albeit with a certain degree of variability, likely due to the different mutations induced in the different models. However, the same variability is not observed for motor deficits and cognitive dysfunction, which are fairly consistent across the models.[141] Notably, complementing the relatively well-studied mouse model of Rett Syndrome, a nonhuman primate MECP2 loss-of-function mutant was recently created, resulting in viable female mutants (MECP2 loss-of-function in males was embryonic lethal) with endo/phenotypic similarity to human Rett syndrome patients[142]

The second group includes ***Fmr1 mutant mice*** which model Fragile X syndrome (FXS), the most commonly inherited genetic disease causing intellectual and developmental disabilities.[143] Approximately 25-50%[144] of FXS patients also display ASD symptoms, and FXS account for 5% of ASD cases.[145] While both sexes are affected by FXS, females usually show a milder phenotype due to a compensatory effect of female cells also harboring a “normal” X chromosome. Prevalence estimates for FXS in the total population are variable; however, the estimated of individuals with the full mutation (FM), which indicate the presence of an expansion to over 200 repeats, is approximately 1 in 7,000 males and 1 in 11,000 females.[146] The majority of FXS cases[147] are determined by the expansion of CGG·CCG repeat in the 5′-untranslated region

(UTR) of the FMR1 gene on the X chromosome, which induces the hypermethylation of the proximal CpG island, and the transcriptional silencing of the gene. This causes a decrease in the amount of the corresponding protein encoded by FMR1, FMRP, which is an RNA-binding protein involved in RNA metabolism[148] and localization[149]. The *Fmr1* mutant mouse was created in 1994 by means of the deletion of coding exon 5[150], which is different from the mechanism involved in human FXS. However, in *Fmr1* KO mice, the deletion has the same effect on the expression of the gene, i.e. the loss of FMRP expression. At the phenotypic level, male *Fmr1* KO mice show more or less severe ASD-associated behavioral deficits, depending on the genetic background of the animals.[151] Interestingly, in mice, pharmacological treatment with the selective mGlu5 inhibitor CTEP improved some of the deficits, providing significant information about the pathological mechanism involved with the disorder.[152]

In the same group as FXS are mouse models for PTEN Hamartoma Tumor Syndrome, which is characterized by mutations in the tumor-suppressor gene phosphatase and tensin homolog (PTEN). Mutations in PTEN are observed in such disorders as Cowden Syndrome, Bannayan-Riley-Ruvalcaba, and Proteus syndromes. Affected individuals display macrocephaly, hamartomas, and symptoms linked to ASD and ID.[153] WES methods revealed that mutations in PTEN are present in non-syndromic cases of ASD.[154,155]

***Pten* conditional knockout mice** are used to investigate the effects on behavior induced by Pten loss of expression. *Pten* mutant mice show the macrocephaly characteristic of human patients, as well as reduced sociability, social novelty preference, and reciprocal social interaction. Moreover, increased motor activity and anxiety-like behavior are observed in this model.[156] Interestingly, treatment with rapamycin (an mTOR inhibitor) rescued both macrocephaly and behavioral deficits in *Pten* mutant mice.[157] A relevant characteristic of this model is the fact that

neurons which do not express Pten show alterations in synaptic plasticity and neuronal firing, with impaired long-term potentiation (LTP) and long-term depression (LTD) in the hippocampus.[157,158]

Additional mouse models of mutations in proteins involved in post-transcriptional mechanisms are **Tsc1/Tsc2 mutant** mice which model Tuberous sclerosis complex (TSC), an autosomal dominant neurodevelopmental syndrome resulting from mutation in Tsc1 or Tsc2 genes. These genes encode for proteins hamartin (Tsc1) and tuberin (Tsc2), important inhibitors of the mTORC1 translational control pathway.[159] About 50% of TSC individuals are also diagnosed with ASD, and TSC genetic alterations account for about 1-4% of ASD cases.[145] Neither *Tsc1* nor *Tsc2* homozygous KO mice are viable; therefore, heterozygous mice and conditional knockout mice have been produced to model TSC. For *Tsc1*, the most used models target exon 6-8[160] or exon 5-7[161]. *Tsc1* heterozygous mice show increased anxiety-like behavior, impaired learning and memory, and reduced social interaction, which is rescued by rapamycin administration.[162,163] Notably, cKo mice with deletion of exon 17-18 only in the Purkinje cells of the cerebellum show most of the ASD-like phenotypes, all rescued by treatment with rapamycin, supporting the hypothesis of a peculiar role of Tsc1 in the cerebellum in regard to ASD-associated impairments.[164] For *Tsc2*, both heterozygous mice and conditional knockout mice have been generated. These mice show reduced sociability and social novelty preference, as well as impaired reciprocal social interaction.[162,165] They also display learning and memory impairments, which are reversed by treatment with rapamycin.[162,166] cKo mice with deletion of exon 2-4 exclusively in Purkinje cells of the cerebellum have more intense ASD-like deficits than the heterozygous models.[165]

In the final group, characterized by mutations in voltage-gated ion channels, are models with mutations in the CACNA1C gene (encoding the voltage-gated calcium channel α1 C subunit) and in the SCN1A gene (encoding the voltage-gated sodium channel α1 subunit), which are responsible for Timothy Syndrome and Dravet Syndrome, respectively. **Timothy Syndrome** is a rare disease determined by a common *de novo* gain-of-function mutation in exon 8 of CACNA1C gene. This gene encodes for $Ca_V1.2$., which is a L-type voltage-gated calcium channel. Affected individuals show cardiac defects, ID, and comorbid ASD in approximately 40% of cases.[167] *Cacna1c* mutant mice (Ts2-neo mice) carry the point mutation pGly406Arg, which is responsible for the human disease. These mice show ASD-associate behavior, including repetitive behavior and reduced locomotor activity.[168,169]

Dravet syndrome is a rare form epilepsy determined by autosomal dominant mutations in the SCN1A gene, which encodes the voltage-gated sodium channel $Na_V1.1$. Affected subjects show severe epilepsy, ID, and ASD.[170] *Scn1a* heterozygous mice are characterized by decreased sociability and social novelty preference, stereotyped movements, increased anxiety-like behaviors, and hyperactivity[171,172].

2.5.2 Mouse Models of Single Gene Mutations Associated with Non-syndromic ASD.

Mouse models of single gene mutations associated with non-syndromic ASD include models with mutations in the genes encoding for neurexins (NRXN), neuroligins (NLGN), and SHANK proteins. Each of these proteins participate in activity-dependent synaptogenesis, synapse maintenance, and synaptic function.[173,174] At synapses, presynaptic neurexins interact with their

counterparts on the postsynaptic membrane, including neuroligins, SHANK and members of the leucine-rich repeat transmembrane (LRRTM) protein family, to ensure synaptic formation and remodeling.[175]

The neurexin gene family in humans includes five members: NRXN1, NRXN2, NRXN3[176] and two NRXN4 genes, CNTNAP1 and CNTNAP2 (Contactin-associated protein 1 and 2).[177] The neuroligin gene family in humans includes five members: NLGN1, NLGN2, NLGN3, NLGN4 or NLGN4X, and NLGN4Y.[178] The SHANK gene family is comprised of three members: SHANK1, SHANK2, and SHANK3. The SHANK family members codify multiple mRNA splice variants, thus generating several protein isoforms, the SHANK/ProSAP proteins.[179] SHANK proteins interact with N-Methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-type (AMPA) glutamate receptors, within the post-synaptic density, acting as scaffolding proteins.[180]

***Cntnap2* mutant mice** are homozygous for mutations in *Cntnap2*. Heterozygous mutations in this gene have also been associated with ASD or ASD-related phenotypes.[181,182] *Cntnap2* homozygous mice (mutation in exon1), show hyperactivity, repetitive behavior, reduced sociability, and seizures, as well as a reduced number of cortical interneurons and oxytocinergic neurons in the hypothalamus. Intriguingly, treatment with the FDA-approved drug risperidone (discussed) above reverses the hyperactivity phenotype observed in *Cntnap2* KO mice yet fails to correct *Cntnap2* KO social deficits.[183] In contrast, exogenously provided (intranasal) or endogenously evoked (via chemo genetic strategies) oxytocin treatment corrects *Cntnap2* KO social deficits while leaving the hyperactivity phenotype untouched[184]. Taken together, these results are suggestive of a dissociable phenotypic presentation that could be due to divergent

underlying etiologies of the hyperactivity versus social phenotypes in the *Cntnap2* KO mice and, potentially, CNTNAP2 human patients.

***Nrxn1α* mutant mice** have mutation in exon1 of the Nrxn1gene and are deficient for Nrxn1α isoform. Gene mutations and copy-number variants in this gene have been linked to ASD and schizophrenia.[185-187] Nrxn1α mutant mice display anxiety-like behavior and increased locomotor activity, but no impairments in social interaction or social novelty preference.[188]

***Nlgn* mutant mice** include different models with mutations in each of the members of the neuroligin gene family. Some studies reported mutations in NLGN3 and 4 in ASD patients.[100,101] *Nlgn1 homozygous mice* (mutations in exon1 and 2) display normal sociability and social novelty, but alterations in pain sensitivity, and increased repetitive behavior.[189] *Nlgn2 mutant mice* (mutations in exon1) display normal social behavior, without increase in repetitive behavior. Instead, they show increased anxiety-like behavior and deficient motor coordination.[190] *Nlgn3* homozygous mice (mutations in exons 2 and 3) show reduced preference for social novelty, increased locomotor hyperactivity, but they normal levels of anxiety-like behavior.[191] *Nlgn4* null mutant mice display decreased social interaction and increased repetitive behavior, without alterations in anxiety-like behavior or motor coordination.[192]

***SHANK* mutant mice[193]** include models with mutations in each of the members of the SHANK (also known as ProSAP) gene family. Shank3 haploinsufficiency is associated with Phelan McDermid syndrome (PMS).[194] Mutations is SHANK3 have been associated with 1-2% of cases of ASD, and are associated with a NDDs called Phelan-McDermid syndrome[195]. Sporadic cases of ASD have been associated with mutations in all the SHANK genes.[196,197] *Shank1 mutant mice* (mutations in exons 14-15) have been reported to show very mild ASD-associated phenotypes

and also increased anxiety-like behaviors and hyperactivity.[198,199] *Shank2 mutant mice* include two models, one with mutations in exons 6-7[200] and the other one with mutation in exon 7[201]. The first model shows an ASD-associated phenotype as well as increased anxiety and hyperactivity, which are reversed by the treatment with NMDAR agonists and mGluR positive allosteric modulator. The second model replicated the ASD-related behavioral alterations observed in the first, as well as anxiety-like behavior and increased locomotor activity. SHANK3 gene is constituted by 22 exons and many protein isoforms arise from mechanisms as alternative splicing and different promoter activation.[202] *Shank3* isoform-specific knockout mice target different regions of exons, thus obtaining isoform-specific knockout[203-206] animals but not total knockout. These discrete models display some differences in the phenotypic effect of the specific mutations, likely due to the fact that different SHANK3 isoforms have different functions and expression patterns, both spatial and temporal, in the brain. However, all the *Shank3* models show some degree of consistency in the impairment of social behavior, as well as communication deficits, and repetitive behavior, thus proving that, despite its complexity, the investigation of the effects of SHANK3 mutations represent a promising avenue for understanding the molecular basis of ASD.[202]

2.5.3 Mouse Models of Human Copy Number Variations (CNVs).

CNVs at human chromosome 15q11-q13. This locus carries several variants associated with different diseases such as Prader-Willi Syndrome (PWS), Angelman Syndrome (AS), and some cases of sporadic ASD. In ASD, both maternal and paternal increased CNVs are observed with variable penetrance.[207] This region contains at least 15 genes; UBE3A is duplicated in the AS. The corresponding protein is the ubiquitin-protein ligase (UBE3A), which has an important role in

synaptic plasticity.[208] Indeed, an important target of Ube3A is Arc, which has a crucial role in AMPA receptor internalization.[209] Ube3a BAC transgenic mice carry one or two additional copies of UBE3A gene, and the severity of phenotypic alterations is proportional to the increase in the dosage.[210]

CNVs at human chromosome 16p11.2. This locus carries CNVs, both insertion and deletions, and was discovered by genome-wide approaches. It is thought to contribute to roughly 1% of non-syndromic ASD. In this case, the number of genes located in this region are >20, with unclear individual contribution to the ASD-associated characteristics. Mouse models carrying either a deletion or a duplication *(Df(7)16* and *Dp(7)16 mice)*[211] of a specific region in this locus show no ASD-like phenotypes.

2.6 Epigenetics of ASD.

Despite its multifactorial etiology, ASD aggregates in families, and, therefore, is considered a disorder with strong genetic root.[212] Some twin studies[213] reported that 60% of monozygotic (MZ) twins were concordant for autism versus no dizygotic (DZ) twins. However, a broad review[214] of several twin studies[95] performed in a period between 1977 and 2011 concluded that the concordance rate for ASD in MZ twin ranges from 36% to 96%. Subsequent studies[215,216] acknowledged that non-genetic factors play an important role in the etiology of the disease.

Recently, the hypothesis that epigenetic mechanisms might causally contribute to the development of ASD[217,218], therefore explaining the existence of disease-discordant MZ twin pairs, has gained ground in the scientific and medical communities, setting the stage for large-scale

epigenomic studies. Epigenetic modifications influence chromatin architecture and conformation, the accessibility of genes to transcriptional complexes, and gene expression. They include DNA methylation, histone modification and regulation, and transcriptional gene silencing by means of long non-coding RNAs (lncRNAs) and small non-coding RNAs (sncRNAs), such as siRNA and MiRNA.[219] The etiology of epigenetic alterations is complex and can be the effect of primary stochastic phenomenon or of either environmental factors or DNA mutations.[220] Precise spatial and temporal regulation of gene expression during discrete developmental stages is crucial for the correct establishment of excitatory and inhibitory synaptic connections, activity-dependent responses, and neuronal specification.

Chromatin remodeling enzymes, such as those belonging to the chromodomain helicase DNA-binding (Chd) family, have specific roles in the distinct stages of neurodevelopment, and mutations in these genes have been implicated in ASD and other NDDs.[221-223] Some studies reported a significant epigenetic variability in MZ twins[224,225]. Considerable differences in DNA methylation patterns have been found in MZ twins discordant for phenotypically complex disorders, such as schizophrenia and bipolar disorder.[226] In regard to ASD, the analysis[227] of lymphoblastoid cell lines derived from ASD-discordant MZ twin pairs' peripheral blood lymphocytes revealed many ASD-relevant loci with differential methylation profiles. In particular, two of these loci were found to be hypermethylated, and the corresponding transcripts resulted downregulated in post-mortem analysis of ASD brains. A genome-wide analysis of DNA methylation performed in ASD MZ pairs revealed that DNA methylation at specific CpG sites had a significant variability within ASD-discordant MZ twin pairs. This study also attempted to establish a quantitative association between epigenetic alterations in specific genes and the severity

of ASD phenotype.[228] Consistently, additional studies[229-231] looked at DNA methylation patterns in ASD subjects, and found relevant differences compared to non-affected individuals.

Furthermore, ASD individuals carry mutations in genes which encode for proteins involved in epigenetic modification. A *de novo* mutation in the HIST1H1E gene, which encodes for the linker histone H, has been reported in ASD patients[232]. This mutation disrupts chromatin organization and leads to downregulated protein expression. Intriguingly, in the same study, a review of a database of genes associated with ASD, (SFARI GENE)[129], led to the observation that almost 20% of these genes codify for proteins involved in epigenetic mechanisms and chromatin remodeling. Another gene linked to both ASD and ID[233,234] is SETD5, a member of the SET-domain family encoding for histone methyltransferase (HMT). SETD5 haploinsufficiency has been proved to be implicated development of ASD/ID[235,236], however the precise mechanisms by which the mutation causes the disease has not been fully elucidated, yet. A recent study suggested that SETD5 has a crucial role in the fine regulation of gene expression during early development, being implicated both in synaptic plasticity and cell fate determination.[235] Another body of research in the context of ASD epigenetics includes the investigation of microRNAs (miRNA) in ASD pathology. These short stretches of RNA are known to regulate mRNA translation and degradation. Several studies showed that, in animal models of ASD, impairments in miRNA synthesis are associated with the disease.[237,238] Other studies proposed that the expression profiles of miRNA in the serum is paired with those expressed in the tissues, and, therefore, these specific miRNA patterns can be used as biomarkers of disease.[239]

2.7 Convergence of Primary Insults: Two Primary Mechanisms Underlying ASD.

Each of the mouse models for ASD described in the previous section (models of single gene mutations associated with syndromic ASD, non-syndromic ASD, and CNVs) have been used to investigate ASD risk genes, and have uncovered a significant portion of ASD biology.[240] However, the pathophysiological mechanisms and molecular pathways involved in the disease and symptom manifestation are not yet fully understood. ASD-related genes have pleiotropic effects across various brain areas. Their expression is finely regulated, following specific patterns of expression, both in terms of space and time across development.

Modelling the contribution of a single gene in an animal system certainly provides useful insights with regard to some elements of the human disease, but it does not necessarily lead to full comprehension of the role of that specific gene and protein in the pathophysiology of the disease in genetically heterogeneous human patient populations. This is particularly true for non-syndromic autism, in which, despite the notion that a specific genetic mutation might significantly increase the risk of ASD, there is no clear association between a known mutation and the clinical manifestations of the disease, as instead happens in the case of syndromic ASD.

An alternative and, in some respects, complementary approach is represented by functional studies which identify and manipulate one or more key cellular and molecular pathways on which both common and rare variants associated with ASD converge. Such an approach can shed light on potential shared pathophysiological mechanisms, regardless of the initiating insult.[241,242] Taken together, human genetic studies of ASD reveal that genes involved with the disease belong to at least two main clusters: (1) the regulation of mRNA translation and protein synthesis[243] and (2) the regulation of synaptic structure and function.[244] The cellular pathways controlling these

paramount neuronal functions play a critical role in shaping healthy brain organization, from the modeling/re-modelling of neural circuits to regulating activity-dependent synaptic plasticity, and ultimately, behavior. Consequently, their disruption can result in severe dysfunction.[245]

2.7.1 Dysfunction of Synaptic Proteins.

The average age of ASD diagnosis in the U.S. is between 3 and 4 years, an age characterized by a strong experience-dependent refinement of neural circuits and synaptic connections.[246] Indeed, the formation of new synaptic connections between neurons initiates as a diffuse phenomenon which subsequently undergoes activity-dependent refinement in later stages of development. In this regard, two main groups of proteins play a major role in the establishment of neuronal circuits.

The first group includes scaffolding/anchoring proteins localized at the postsynaptic density, which is a complex network within the postsynaptic membrane where multiple proteins and enzymes interact to orchestrate the spatial and functional distributions of neurotransmitter receptors.[193,245] The second group includes cell-adhesion molecules (CAMs), which are involved in regulating the arrangement of both presynaptic and postsynaptic terminals by means of trans-compartmental signaling.[247 248] An increased ratio of excitatory to inhibitory (E/I) neuron activity is currently one of the most widely accepted conceptual frameworks used to investigate the cellular and molecular mechanisms underlying the changes occurring in the brain of ASD individuals. The E/I hypothesis suggests that the autistic brain carries an abnormal increase in excitatory inputs and/or an abnormal decrease of inhibitory inputs onto the neurons, and that this imbalance leads to deficits in cortical function, resulting in overexcitation which is manifested as epilepsy, a common feature associated with ASD.[249] The E/I theory implicates pathways involved in the

formation of inhibitory and excitatory synapses, mechanisms of synaptic plasticity, cell-to-cell signaling, and many others.[250] At the circuit level, E/I balance targets the interplay between local and global circuits[251]. Indeed, many studies suggest that ASD is characterized by brain network dysfunction.[252,253] Functional imaging studies suggest that ASD-related deficits in brain connectivity are sustained by both long-range underconnectivity and short-range overconnectivity phenomena in cortical areas.[249,254-256] However, the idea of the coexistence of local overconnectivity and global underconnectivity, which results in the inability to distinguish real signal from general noise, as a pathophysiological model for ASD is challenged by other studies showing the presence of a mix of under- and overconnectivity at the local level[257,258], as well as overconnectivity or mixed patterns at the global level.[259-261] The inconsistency of the studies focused on the mechanisms underlying the disrupted brain connectivity in ASD, might be the results of heterogeneity both of the methods/study designs and of the subjects included in the studies. It is also possible that the over- and underconnectivity phenomena might be differentially involved in relation to specific core ASD domains.[262] Specific synaptic E/I imbalances might occur in different cell types, brain areas and at different developmental stages, therefore, a more detailed and cell type-targeted investigation of synaptic events is needed to unravel the mechanisms involved in connectivity impairments.[263] Despite the lack of a consensus on the spatiotemporal distribution of abnormal connectivity patterns across the brain, the involvement of CAMs and scaffolding protein in synaptic dysfunction is widely accepted.[87,244]

Regarding the postsynaptic scaffolding/anchoring proteins, SHANK proteins are considered the master scaffold proteins. They are located in the postsynaptic compartment of excitatory synapses and play a crucial role in synaptic organization and stabilization.[264] As mentioned before, from the three SHANK genes originate several mRNA splice variants, which

lead to the generation of multiple protein isoforms. These isoforms play multiple roles, including glutamate receptor trafficking, activity-dependent signaling, and spine morphogenesis.[265] SHANK proteins bind to cytoskeleton component and other scaffolding elements, such as PSD-95-binding proteins (SAPAP), Homer family of scaffolding proteins, cortactin and F-actin, which mediate the interaction with neurotransmitter receptors. [180,266-268] Multiple genomic studies reported the association between ASD individuals and mutations in the three SHANK genes.[197,269-271] As described before, mouse models of SHANK mutations provided evidence of deficits in social behavior and anxiety-like phenotypes. Interestingly, also other neuropsychiatric and neurodevelopmental disorders have been associated with genetic alterations in these genes.[193] Indeed, these mutations impair the formation and correct functioning of glutamatergic synapses, a mechanism involved also in the etiology of diseases such as schizophrenia and intellectual disability.[272]

Similarly, many genomic studies suggest an important role of CAMs in ASD phenotype.[273,274] CAMs organize pre- and postsynaptic contacts, in order to establish and maintain adhesion between the two compartments. An important family of CAMs includes neurexin and neuroligin proteins. Neurexins are encoded by three genes (NRXN1,2,3) which produce larger α -neurexins and smaller β – neurexins, with multiple isoforms arising from alternative splicing.[275,276] The other class of CAMs is composed by neuroligins, which are the endogenous ligands of neurexins[277], together with neurexophilins (neuropeptide-like proteins), and dystroglycan. Neuroligins are encoded by four genes (NLGN1, 2, 3, 4) and have an important role in synaptic function.[178] Neurexins and neuroligins create a bridge across the synaptic cleft, each one triggering the creation of a hemisynapse. Mutations in the genes encoding for these proteins have been shown to impair synaptic function in animal models, by altering excitatory synaptic transmission and

neurotransmitter release.[278] Multiple mutations and deletions in many of the genes encoding for neuroligins[100] and neurexins[279] have been identified in ASD patient. Interestingly, mutations in DLGAP2[280], which encodes for SHANK protein molecular partner SAPAP2, and in SYNGAP1[281,282], which encodes for SynGAP1, a synaptic RasGTPase-activating protein (RasGAP) which interacts with NMDA receptors and PSD-95, have also been found in ASD patients. Taken together, this broad group of mutations converge onto synaptic function-related proteins, thus strongly supporting the hypothesis that ASD pathogenesis is, at least in part, attributable to synaptic dysfunction.

2.7.2 Dysregulation of Translational Control.

A growing body of evidence suggests that impaired mRNA translation regulation and subsequent dysregulated protein synthesis is another common pathogenic mechanism involved in ASD pathogenesis. Numerous studies employing protein synthesis inhibitors, such as anisomycin, show that new protein synthesis is required for most forms of synaptic plasticity[283]. Indeed, fine regulation of mRNA translation, which primarily occurs at the point of mRNA translation initiation, is crucial to facilitating adaptive responses to rapid, dynamic changes in stimuli received by the brain. Mechanisms of transcriptional control define gene expression patterns under the influence of a myriad of stimuli which reach the neuronal cell body. Nonetheless, local mechanisms at synapses orchestrate the spatiotemporal regulation of mRNA translation in relation to many inputs, including neurotransmitter release. In fact, synaptic plasticity is a localized event, occurring in a subset of synapses, hence the necessity to restrict changes in mRNA translation only where necessary and at a particular moment in time.

Short-term synaptic plasticity phenomena often rely on modification of pre-existing proteins, and therefore are considered protein-synthesis independent.[284] However, the majority of long-lasting synaptic plasticity events, including long-term potentiation (LTP) and long-term depression (LTD), require changes in mRNA translation.[285] Synaptic activity, by means of activation of NMDA receptors (NMDAR), metabotropic glutamate (mGluR) receptors, TrkB receptors, and other postsynaptic receptors, induces modifications in the translation of locally enriched pools of mRNA or of specific transcripts, such as those distinguished by specialized UTR architecture. In eukaryotes, mRNA translation includes three steps: initiation, elongation, and termination. Initiation is the most tightly controlled phase, and therefore, is considered as rate-limiting step of protein synthesis. Translational initiation requires three events: (1) assembly of the 43S ribosomal preinitiation complex, (2) binding of the transcript to the 43S complex, and (3) the final assembly of the 80S ribosomal complex to facilitate mRNA scanning for the AUG start codon. The association between the 43S complex and the mRNA occurs by two distinct mechanisms: (1) the traditional binding of the 5′-seven-methylguanosine cap (m^7G) of the mRNA ("cap-dependent translation") or (2) binding between the ribosome and the internal ribosome entry site (IRES), a sequence within the mRNA 5′ untranslated region (UTR).[286] Both mGluRs and NMDARs are involved in the regulation of mRNA translation via multiple signaling pathways. For instance, upon NMDAR activation, brain-derived neurotrophic factor (BDNF) is released and induces the activation of TrkB receptor pathway promoting long-term potentiation of synaptic connections, while mGluR activation leads to activation of a distinct translational program resulting in long-term depression.[287]

In neurons, translational control is mediated by two primary pathways. The first involves the phosphorylation/dephosphorylation of the alpha subunit of eukaryotic initiation factor 2

(eIF2α) at Ser5, which is regulated by three kinases–protein kinase R (PKR), PKR-like endoplasmic reticulum kinase (PERK), and general control nonderepressible 2 (GCN2)–and phosphatase complexes anchored by protein phosphatase 1 (PP1) coupled with the adaptor proteins growth arrest and DNA damage-inducible protein (GADD34) and constitutive reverter of eIF2α phosphorylation (CReP)[288]. Dephosphorylation of eIF2α promotes general translation by increasing the availability of the ternary complex, while phosphorylation of eIF2α (p-eIF2α) inhibits the general translation, due to restricted ternary complex availability. Intriguingly, however, p-eIF2α promotes the translation of a specific subset of transcripts with multiple upstream open reading frames (uORFs) in their 5′ UTRs, such as ATF4, a CREB repressor, thus reducing late LTP (L-LTP) and long-term memory (LTM) formation and facilitating mGluR-mediated internalization of AMPAR leading to synaptic LTD.[289,290] In GABAergic neurons, p-eIF2α downregulates the translation of interferon-γ (IFN-γ), thus enhancing GABAergic transmission and decreasing neuronal excitability.[291]

The second major pathway involved in translational control is orchestrated by the mechanistic target of rapamycin complex 1 (mTORC1), whose activation leads to increased mRNA translation through phosphorylation of its main downstream effectors: p70 S6 kinases (S6K1 and S6K2) and the eukaryotic initiation factor 4E (eIF4E)-binding proteins (4E-BPs). mTOR is known as a master regulator of cell metabolism. Acting through two distinct complexes (mTORC1 and mTORC2), mTOR is involved in numerous cell functions, including protein synthesis, autophagy, and mitochondrial function and actin dynamics and ribosomal synthesis, respectively, throughout the lifespan. In neurons, it specifically contributes to neurite growth, neuronal progenitor proliferation, and synaptic plasticity. [292] mTORC1 is the converging point of different signaling pathways involved in growth, amino acid sensing, and energy sensing; its

activity influences many cellular processes, such as protein synthesis, autophagy, mitochondrial metabolism and biogenesis, and lipid synthesis. In the context of protein synthesis, mTORC1 regulates the assembly of eIF4F complex by means of 4E-BPs phosphorylation, which is necessary for cap-dependent translation. As in the case of eIF2α, mTORC1 is also involved in the synaptic response to L-LTP[293] and mGluR-LTD-inducing[294] stimuli.

A third mechanism of control is present in the elongation step of mRNA translation.[295] This process has also been implicated in synaptic plasticity and is enacted by eukaryotic elongation factor (eEF2K-eEF2) system. In neuronal cells, eEF2 is phosphorylated by eEF2K which is activated by mGluR1/5, calcium, or other protein kinases. When phosphorylated, eEF2 decreases its binding to the ribosome and thus reduces elongation. However, this mechanism increases translation of specific mRNAs such as CAMKIIα, brain-derived neurotrophic factor (BDNF), and cytoskeletal proteins, which are critical for synaptic plasticity.[296]

Many syndromic ASD patients, such as those enduring TSC, FXS, and PTEN syndrome, harbor mutations in genes encoding for proteins that regulate translation, thus converging on impaired protein synthesis as the molecular mechanisms underlying the disorders.[243] In fact, FMRP, PTEN, and TSC1/2 are all negative regulators of protein synthesis; meaning, loss of function of these genes leads to run away, exaggerated protein synthesis that results in synaptic and behavioral dysfunction. FMRP is an RNA-binding protein with many roles in the posttranscriptional regulation of mRNA, such as mRNA stability, dendritic localization, and translational modulation. FMRP inhibits both initiation and elongation steps depending on the specific transcript and the kind of stimuli received by the neuron.[297,298] In mice models, lacking of FMRP increases mTORC1 signaling.[299] Notably, disrupted FMRP function leads to impaired synthesis of many synaptic proteins involved in synaptic plasticity and ASD, including SHANK1

and SAPAPs[300], SHANK3, SynGAP1 and some neuroligins and neurexins.[148] FMRP loss induces a strong enhancement of LTP, both in terms of amplitude and persistence.[301] Interestingly, FMRP regulates the translation of other mTORC1 inhibitors, including TSC2 and PTEN.[148] TSC1 and TSC2 form a heterodimeric complex that can downregulate protein synthesis by inhibiting mTORC1 activity. This complex is influenced by the activity of several kinases, such as Akt and ERK [302], and inhibits mTORC1 by means of the activation of the small GTPase Ras homolog enriched in the brain (Rheb).[303] In Tsc2+/ mice, LTP is enhanced[166], while LTD is attenuated.[304] PTEN activation is responsible for the inhibition of PI3K signaling, which in turn leads to the inactivation of Akt, and subsequently, of mTORC1. PTEN deficiency determines constitutively activation of the Akt-mTORC1 pathway.[305] Interestingly, mutations in genes involved in the regulation of mRNA translation have been linked to NLGN dysregulation, thus revealing a connection between NLGNs and mTOR.[306]

In summary, several lines of evidence suggest that, in syndromic ASD models, exaggerated protein synthesis leads to synaptic abnormalities which are ultimately responsible for the behavioral deficits associated with ASD. This is further supported by studies[307] showing that, in FXS model mice that lacking FMRP, treatment with 4EGI-1, which prevents the interaction between eIF4E and eIF4G, two critical mediators of translational initiation, is able to correct deficits in hippocampus-dependent memory and spine morphology. On the other hand, deficits in protein synthesis are associated with cognitive dysfunction and intellectual disability, phenotypes often co-occurring with ASD, suggesting that tightly controlled regulation of mRNA translation is necessary for proper synaptic function and normal brain development.[245,288,308] The investigation of the mechanism underlying syndromic ASD strongly supports the mechanistic link between

synaptic dysfunction and translation abnormalities; an interesting hypothesis is that a similar convergence might represent one of the main mechanisms underlying nonsyndromic ASD.[89]

2.8 Limitations of a Strictly Genetic/Epigenetic Etiology of ASD.

A vast number of studies in the field demonstrate the genetic heritability of ASD plays an important role in the onset of the disease. Autistic trait and ASD-related phenotypes are heritable, with a heterogeneous distribution in the population. Clinical manifestations are diversified, with variable severity, and often overlapping with other neurological disorders, with whom ASD also shares many genetic defects. However, genetic, and epigenetic factors, despite being ascertained determinants of syndromic ASD, often fail to explain the plethora of deficits which characterized the autistic brain, especially in the case of nonsyndromic ASD.

Emerging evidence suggests that ASD severity seems is also influenced by environmental factors, whose precise involvement in the disease is still under investigation. The concept of 'environment' encompasses multiple factors, with various effects on clinical outcome. In fact, some environmental factors are considered detrimental and causal in at least some the ASD phenotypes, while others may exert a protective function, decreasing the risk of the disease, while still others can act as modulators of the severity of symptoms and clinical manifestations.[309]

If the genetic universe of ASD is considered broad and complex, the ensemble of all the environmental elements potentially involved in ASD is even more vast and more difficult to understand and classify.[310] Environmental factors potentially involved in ASD susceptibility include a wide array of phenomena occurring during pregnancy, such as infections, exposure to

chemical, physical elements, and medications, perinatal and obstetric events, psychosocial determinants, and many others.

In recent years, maternal dietary exposures have emerged as key factors for child neurodevelopment, as shown by both animal and human studies. In particular, maternal obesity has been proposed as a very strong risk factor for the development of deficits in social behavior and represent the main focus of the experimental work presented in this thesis. Indeed, dissecting the causal role of each of these factors in the onset of ASD, and how they individually and/or in combination interact with genetic and epigenetic factors, is of primary importance to develop preventive and treatment strategies.

Part III – Nongenetic Factors Contributing to the Etiology of ASD.

3.1 Genetics Fail to Completely Account for ASD.

The underlying etiology of ASD has been an area of intense investigation for several decades. In the beginning, research efforts focused on identifying what was thought to be a single underlying cause. In the 1960's, a popular theory identified lack of parental warmth as a determinant of ASD, emphasizing the role of nurturing during early childhood.[311] To many, this idea implied that mothers were to blame for their children's autism and was in contrast with another theory on the psychogenesis in ASD, which focused on biological factors and brain development, instead.[312] In the following decades, epidemiologic, genetic, and cytogenetic studies[313], together with data coming collected using neuroimaging-based approaches[314], unraveled the connection between ASD and profound alterations in brain development, providing a new vision of autism which rejected the idea of psychogenic factors as the disease-driving mechanisms. This new era of ASD etiology investigation has essentially been driven by the concept that mutations in a specific gene, or in a group of genes, were the main cause underlying autistic phenotypes. A great number of studies on the genetics of ASD, reviewed in the previous section, highlighted the incredible complexity of genetic alterations associated with ASD, with more and more studies providing evidence for new genes involved in the disease, yet failing to trace ASD back to a main genetic determinant.

One of the reasons behind this inability to identify a genetic cause for ASD is due to the misconception that genes act independently as the sole influence on an individuals' morphology and physiology, and therefore the pathological states. On the contrary, it is a well-established concept in biology that the phenotype is the result of a constant interplay between genes, epigenetic regulation, and environmental factors. The interaction between the genes and the environment can

either modulate the negative impact of risk-increasing genetic variants, or intensify their effect, thus increasing the likelihood of developing a specific disease, or presenting more severe symptoms. According to the liability threshold model[315], a disease becomes manifest only when the liability threshold is reached. In this regard, the genetic architecture of an individual can be considered the baseline of susceptibility, and a toxic environmental burden can have an additive effect, increasing the chances of reaching the threshold and developing the disease. In other words, an environmental factor(s) can be the "tipping point" leading to disease manifestation in a genetically susceptible individual. Therefore, to understand the pathophysiology of ASD and to provide new therapeutical targets, it is imperative to broaden the horizons, dropping the gene-centered perspective and embracing a far more complex and holistic view of etiology of ASD.

Given the increasing global prevalence of ASD[32], it is critical to develop new theories and apply new approaches in investigation into determinants of ASD. While improved diagnostic tools, refined diagnostic criteria, increased awareness, and other technical factors may partially explain the increase in ASD prevalence, emerging evidence is shedding a light on the important contribution of environmental factors, especially those related to maternal conditions during pregnancy. Further research into such environmental factors and their long-term effects on brain development, function, and behavior is warranted to deepen our understanding of the non-genetic causes of ASD.[316]

3.2 Nongenetic Factors Contributing to Disease Etiology: The Concept of the Exposome.

The definition of '*environmental factors'* encompasses a myriad of exposures, including chemical substances or physical agents, nutritional states, psychological and social conditions, infectious diseases, intrauterine environment, among many others.[316] Environmental factors can either be initiating factors, meaning with a key role in the onset of the disease, or secondary factors, which can influence the progression of the disease and/or the severity of its clinical manifestations. However, all environmental factors share a translationally relevant feature: they can be modified or mitigated, and therefore, represent a great opportunity for both preventative and therapeutic interventions. The sum of all the environmental factors encountered by individuals throughout the course of life from conception onwards constitutes the so-called '*exposome'*, a term first introduced by Christopher Paul Wild in 2005[317] to point out the necessity of complementing the investigation of genetic factors in disease etiology with a similar approach for all of the nongenetic determinants.

However, in contrast to the genome, the exposome is very dynamic and has a higher degree of variability, making the development of experimental approaches aimed to its investigation considerably more difficult. Wild then classified three different groups of nongenetic factors: (1) internal, (2) specific external, and (3) general external.[318] The first group includes endogenous factors described as '*processes internal to the body such as metabolism, endogenous circulating hormones, body morphology, physical activity, gut microflora, inflammation, lipid peroxidation, oxidative stress and ageing*.' The second group encompasses factors which have been previously associated with risk factors for disease such as *'radiation, infectious agents, chemical*

*contaminants and environmental pollutants, diet, lifestyle factors (*e.g.*, tobacco, alcohol), occupation and medical interventions.'* The third group consist of broad socio-economic factors and individual psychological conditions described as *"social capital, education, financial status, psychological and mental stress, urban–rural environment and climate."* Together, these factors can have a profound influence on short- and long-term individual health outcomes.

3.2.1 Pregnancy Exposome and Fetal Programming.

Of particular importance is the *pregnancy exposome*[319], intended as the sum of environmental factors to which the developing fetus is exposed during gestation. Intrauterine life represents a critical period in human growth and development, and exposure to harmful environmental factors during this time window can dysregulate developmental processes, potentially leading to permanent alterations in the individual's structure, physiology, and metabolism as well as brain development, function, and behavior. The idea that the pregnancy exposome has the potential to seriously impact fetal development led to the hypothesis that chronic disorders manifesting in postnatal/adult life may represent the result of environmental insults during the intrauterine period. The so-called "Barker hypothesis"[320], also known as also called the "Fetal Programming Hypothesis" support the '*developmental origins of health and disease* (DOHaD),' postulating that adverse events during gestation/early post-natal life are responsible for fetal programming of the structure and function of cells, tissues, and organs, which then predispose the individual to a plethora of health conditions, including behavioral and cognitive dysfunction, cardiovascular diseases, diabetes and other metabolic disorders, cancer, and many others. The origin of the fetal programming hypothesis was based on the observation that the areas in England and Wales with highest rate of neonatal mortality were also the areas with the highest death rates from coronary

heart disease.[321] As the leading cause of neonatal death at that time was low birthweight due to fetal malnutrition, Barker hypothesized that the low birthweight infants who survived were at increased risk of developing cardiovascular disease as adults. These findings were then replicated in several subsequent studies[322-324], leading to the hypothesis that maternal malnourishment during gestation triggers adaptive responses in the fetus aimed to maximize survival chances in a nutrient-restricted environment. However, if these same individuals are then exposed to increased caloric intake in postnatal life, the fetal adaptations can become detrimental and increase the risk for metabolic and cardiovascular diseases.

In more recent times, the escalating global epidemic of overweight and obesity has become a great health concern in many countries and has prompted research to move toward the investigation of maternal overnutrition effects on offspring health. Intriguingly, it has been shown that children exposed to maternal obesity during fetal development are at increased risk of developing metabolic disorders in the postnatal life.[325,326] These findings support the hypothesis that abnormal maternal nutritional status, either under– or overnutrition, have the potential to induce long-term detrimental effects on child's health.[327]

3.2.2 Disruption of Fetal Programming: ASD Originates in the Womb.

Studies discussed in the section above highlight the connection between the pregnancy exposome, particularly in terms of maternal diet, fetal programming, and increased susceptibility to metabolic impairments and cardiovascular disorders.[328] Similarly, early life programming might be one of the causal mechanisms underlying the onset of NDDs, including ASD.[329-331] The concept of intrauterine life as a developmental critical period, in which an organism's phenotype is extremely

vulnerable to the effects of environmental factors, is particularly relevant in the case of brain development.[332,333] Brain development is an exquisitely orchestrated process involving several critical stages that necessarily occur at specific times, such as neural tube formation, cell proliferation and migration, myelination, etc.[334] Neurogenesis, for example, is a highly coordinated and complex process, occurring at a very high rate during fetal life. The formation of synapses, one of the processes dysregulated in ASD, takes place mainly during the third trimester, therefore harmful events occurring during this developmental stage might play a critical role in the establishment of synaptic dysfunction. However, multiple studies have shown that ASD does not arise from a single insult during a specific stage of development but is rather the result of multistage brain-wide impairments occurring in both prenatal and early postnatal life. Indeed, several stages of brain development are affected in ASD, spanning from cell proliferation to neuronal migration, neurite outgrowth, spine formation, and many others.[335-339]

ASD subject-derived iPSC studies[340], in which multipotent stem cells derived from ASD patients are programmed to differentiate into neurons, have identified specific alterations in the ability of the ASD-derived cells to form neural rosettes and in the rate of cell type assignment in the compared to controls. Furthermore, in post-mortem analysis of brain tissues[341] showed that ASD-affected children have a higher number of cortical neurons compared to control subjects, suggesting a critical role for neuronal proliferation occurring between the third and the fifth month of gestation.[342,343] Other evidence suggest a strong involvement of disrupted organization of cortical layers ("cortical dysplasia") in ASD pathology[344,345]. Prenatal corticogenesis occurs in the second and third trimester of pregnancy and involves the coordinated expression of multiple genes which have been found to be associated with idiopathic ASD by GWAS[346] and other genetic studies. Analysis of spatiotemporal expression patterns of gene associated with ASD allowed for

the identification of critical time windows of brain development potentially involved in ASD onset: one corresponding to all three trimesters, enriched with genes with regulatory function also involved in neurogenesis , and the other corresponding to the third trimester/early postnatal period, enriched with genes involved in synaptic development, neurite outgrowth, and the formation of cortical networks.[329,341] Taken together, these findings support the hypothesis that disruption of processes involved in *in-utero* development can play a critical role in the pathogenesis of ASD.

Although genetic susceptibility is involved in alteration of the aforementioned mechanisms of brain development, environmental factors might have a role in the disruption of highly controlled mechanisms, impacting signaling pathways, immune function, hormonal regulation, metabolic processes, and energy homeostasis, with different effects based on the specific environmental insult, the specific period of intrauterine life, the molecular pathway involved, etc.[347] For these reasons, a deeper investigation into the mechanisms by which the pregnancy exposome may influence various pathways in brain development is crucial to understand the etiology of NDDs.

3.3 Nongenetic Factors Known to Contribute to ASD.

Several nongenetic factors have been proposed to interfere with the developing human brain and, therefore, contribute to autistic phenotypes.[309,310] In 2003, a comprehensive study aimed to assess the contribution of environmental factors in ASD was launched[348]; the CHARGE (Childhood Autism Risks from Genetics and the Environment) study highlighted an increased risk for ASD associated with maternal occupational exposure to solvents, and suggested that other chemicals might have a similar effect on neurodevelopment.[349] However, the contribution of environmental

factors to ASD is not limited to industrial solvents. Indeed, at least three main groups of factors have been investigated in a very large number of studies: maternal toxin exposure, maternal infection during pregnancy, and maternal diet/metabolic status.

3.3.1 Maternal Toxin Exposure.

Chemicals resulting from industrial processes severely impact air quality and many of them have been shown to have neurotoxic effects. The detrimental impact of these substances on brain function are particularly relevant during the extremely vulnerable pre- and postnatal developmental stages.[350,351] Indeed, exposure to even small amounts of certain chemicals, relatively harmless in adult individuals, can have a great impact in terms of neurodevelopmental toxicity and brain damage.[352] The placenta is permeable to a large number of environmental toxins[353], therefore the fetus has little or no protection against these agents, which can easily cross the baby's blood-brain barrier.[354-356] Brain development continues throughout childhood, therefore the detrimental effects of exposure to toxic substances are not limited to the intrauterine period, but instead extend across many years.[332] Moreover, in children, detoxification processes are way less efficient compared to those occurring in adults, thus increasing the potential damage of toxin exposure.[357]

Lead was the first chemical to be investigated in relation to fetal neurotoxicity, with multiple studies reporting its harmful effects on brain development.[358-360]

Methylmercury was identified as a developmental neurotoxin following investigation of the cause underlying the high rate of blindness and mental retardation in children living in Minamata, Japan. It was then found that maternal consumption of fish from contaminated waters

containing the mercury compound was responsible for children's brain damage, while no effects were reported in exposed adults.[361] As a result of this and other findings, japanese and international food regulatory agencies issued directives to reduce mercury release in the environment.[362]

Arsenic causes neuropathy in adults when ingested through drinking water.[363] Arsenic-related toxicity in the developing brain was initially reported in contaminated powdered milk in Japan.[364] Follow-up studies[365,366] confirmed the findings of epidemiological investigation carried in Japan.

Pesticides, such as insecticides and fungicides, are widely used in agriculture field and their residues are often found in fruits, vegetables, and other food products.[367] Other sources of these compounds include pest control products used in private and public spaces. Indeed, a large number of pesticides are specifically designed to produce neurotoxic effects.[368,369] Among these, organophosphates represent the most widely used compounds. Multiple studies[370-373] have reported that prenatal exposure to these compounds elicits neurodevelopmental deficits, including cognitive dysfunction and attention deficits. Interestingly, a recent study[374] reported that ASD risk arising from prenatal exposure to pesticides is diminished when mothers supplement their diet with high dose of folic acid before pregnancy, suggesting a protective role for folic acid against harmful chemical exposure.

Solvents, such as ethanol, are responsible for acute poisoning in adults. Children born to mothers drinking sufficient amounts of alcohol during pregnancy display severe cognitive impairments and behavioral dysfunction.[375,376] Some studies reported an association also between low alcohol consumption and developmental neurotoxicity.[377] Industrial solvents, such as toluene, have also been linked to cognitive dysfunction in children born to mothers exposed to these chemicals.[378,379]

Industrial exposure to polychlorinated biphenyls has been associated with mild toxicity in adult individuals. However, children whose mothers were exposed to these chemicals, developed severe behavioral impairments, as well as hormonal and immune dysfunction.[380-382]

In addition to the aforementioned chemicals, whose neurodevelopmental toxicity has been widely established, other substances are hypothesized to have similar effects on exposed pregnancies. Indeed, prospective epidemiological birth cohort studies reported that prenatal exposure to substances such as manganese[383,384], fluoride[385,386], and perchlorate[387] is potentially associated with behavioral deficits and psychiatric disorders. Further studies are needed to confirm the neurodevelopmental toxicity of such chemicals and, therefore, to include them in the list of hazards to human neurobehavioral development.

In addition, maternal use of some medications during the gestational period have also been reported to increase the risk of ASD. Exposure to valproic acid, a drug used to treat epilepsy and ameliorate the symptoms of bipolar disorder, during pregnancy is associated with increased frequency of both physical and mental deficits.[388-390] Studies conducted in animal models show that rodents treated with valproic acid present impaired social behavior in a three-chambered social test[391] and locomotor and repetitive/stereotypic-like hyperactivity.[392] Other studies suggested that valproic acid may interfere with neuronal migration during intrauterine brain development.[393] Still other studies[394] suggest that another class of medications, the selective serotonin reuptake inhibitors (SSRIs), which are used to treat depression, might have an association with ASD. However, others[395] yielded confounding results, reporting no association between *in utero* exposure to SSRI and increased risk of ASD. Thus, the impact of SSRIs on fetal brain development and behavior in the context of ASD remain an active area of investigation.

3.3.2 Maternal Infection During Pregnancy.

Several epidemiological studies suggest that *in utero* exposure to viral infections, particularly those requiring maternal hospitalization, is associated with increased risk of developing ASD. The idea that viral infections during the pregnancy increase offspring's risk to develop ASD was first introduced by epidemiological studies reporting increased frequency of NDDs in individuals born to mothers who experienced a viral infection during pregnancy. In the 1970s, an American child psychiatrist, Stella Chess, diagnosed symptoms of ASD in a group of children with congenital rubella syndrome, resulting from the 1963–1964 rubella epidemic in New York. She reported a prevalence of 7.41%, which was approximately 200 times higher than that of the general population in the US.[396,397] Subsequent studies in the following years showed similar findings, not only in relation to rubella infection, but also in response to maternal infection during pregnancy with other viruses, such as cytomegalovirus[398,399] and influenza.[400,401]

An important limitation of epidemiological studies is linked to the fact that individual-level data are not available; therefore, measurements of exposure to pathogens are often imprecise. Birth cohort studies helped to overcome this issue. In birth cohort studies, all of the events occurring during gestation, including maternal infections are recorded and collected together with medical history and, after birth, children can be monitored to assess developmental milestones as well as the onset of behavioral and cognitive deficits. Such studies have shown that maternal infections during the gestational period increase the risk of developing ASD in the offspring, as a result of the maternal immune activation (MIA) and related inflammatory responses which damage the developing brain independently from the specific class of pathogen (*e.g.*, of viral versus bacterial origin)[402-405] Notably, multiple studies suggest an association between ASD and dysregulation of inflammatory response: increased activation of microglia and astroglia[406], upregulation of markers

of inflammation[407], and alterations in genes involved in the immune system[408] have been found in ASD patients. To test the hypothesis of causality between gestational MIA and ASD and to investigate the biological mechanisms by which MIA interferes with fetal neurodevelopment, a large number of animal models have been established.[409] MIA animal models can be divided in four categories: (1) models based on maternal exposure to live pathogens during pregnancy, which are aimed at investigating the specific effect of a particular class of infectious agents, such as influenza virus, (2) models based on the exposure to agents which stimulates the innate immune system, including lipopolysaccharide (LPS), polyinosinic-polycytidylic acid (poly(I:C)), a synthetic analog of double-stranded RNA (dsRNA), a molecular pattern associated with viral infections, and bacterial endotoxin, which are aimed to assess the causal role of cytokines imbalance in neurodevelopmental impairment, (3) models in which the stimulation of the immune system is achieved by means of specific cytokines with the aim to assess the effects of single immunological agent on neurodevelopment, and (4) models based on the administration of immunological factors linked with the pathogenesis of the disorder[410], as in the case of ASD-associated maternal autoantibodies.[411]

In a study employing the first model, pregnant mice who received a dose of a neurotropic influenza virus gave birth to offspring displaying a wide array of behavioral deficits and neuropathological abnormalities, such as dysregulated gene expression and brain atrophy.[401,412,413]

In the second group of models, stimulation with LPS or Poly(I:C) mimics the immunologic response to bacterial or viral infections, thus inducing the release of several proinflammatory cytokines by means of the binding to toll-like receptors (TLR), which recognize common structures shared by many pathogens. These models have some advantages, such as the possibility to tune the magnitude of the cytokine release, and the very rapid effect of the stimulation which

allows to target specific timepoints of brain development. The concept of specific time-windows for fetal neurodevelopment is relevant also in the context of maternal infections: for instance, the development of the dopaminergic (DA) system, a neuronal network strongly implicated in ASD[414], occurs in early gestational period, and therefore MIA in these stages might selectively impair brain circuits and functions associated with the DA network.[415] Another important feature of LPS/Poly(I:C)-stimulated models relies on the fact that these molecules do not reach the fetus, thus allowing for the selective assessment of the indirect effects of maternal cytokines on fetal development. Indeed, once released, maternal cytokines reach the placenta, the amniotic fluid and the fetus itself.[416] Cytokines are crucial modulators of several processes associate with neurodevelopment[417,418], however an imbalance in maternal pro- and anti-inflammatory cytokines can have detrimental effects on the fetal brain. At the phenotypic level, animal stimulated with Poly(I:C) or LPS display several behavioral impairments typically associated with ASD[419]: repetitive self-grooming and stereotypies, restricted interests/cognitive inflexibility, and decreased sociability.[64,420-424]

The third model allows for the investigation of the role of specific cytokines in the disrupted maternal-fetal immunological balance, for instance, pro-inflammatory cytokine IL-6 was found to have a critical role for mediating the behavioral alteration in MIA offspring.[425] Similarly, IL-17 administration into the fetal brain, induced MIA-related impairments, even in the absence of maternal immune response.[424]

Regarding the molecular mechanisms underlying cytokine-mediated impairments in MIA offspring, IL-6[425], IL-17a[424], IL-1β[426] have been indicated as major contributors to MIA phenotypes. However, the precise molecular and cellular pathological mechanisms remain mostly unknown. One hypothesis is based on the regulatory function of many cytokines on the expression

of major histocompatibility complex I (MHCI) molecules in neurons; MHCI has a negative effect on synapse formation and synaptic plasticity[427], therefore its upregulation might lead to synaptic dysfunction.[428] This mechanism might also involve other crucial mediators of synaptic function, thus suggesting an interplay between immunological pathway activity in the brain and synaptic processes.[429] A potential point of convergence between immune and brain function (or dysfunction, in the case of MIA offspring) is represented by the mammalian target of rapamycin (mTOR) complexes one and two (mTORC1, mTORC2), powerful regulators of mRNA translation. Indeed, mTORC1 and mTORC2 are involved in the differentiation of Th1 and T helper 17 (Th17) and Th2 cells, respectively.[430] mTOR complexes inhibition induces T cells to differentiate into Treg cells[431], therefore, an interesting hypothesis suggest that mTOR hyperactivity in gut-brain-immune axis, one of the pathological mechanisms involved in ASD, might lead to a decrease of the Treg cell-associated anti-inflammatory cytokines, including IL-10 and TGF-β.[432] Recent research further suggests a role for the gut-immune-brain axis (which we discuss in great detail below)[433] in MIA: treatment of MIA offspring with the human commensal *Bacteroides fragilis* improved impairments in communicative, stereotypic, anxiety-like and hyperactivity MIA phenotypes.[64] In addition, cytokines have been proposed as markers of immune dysfunction in ASD individuals[434]: indeed, human studies have identified a significant increase in the levels of pro-inflammatory cytokines, including IL-6[407], TNFα[435], IFN-γ[436], IL-17[437], in the brain and in biological fluids, including serum and cerebrospinal fluid, of ASD individuals compared to controls. In contrast, levels of anti-inflammatory, regulatory cytokines such as IL-23[438] and TGF-β[439] have been found to be downregulated in ASD individuals. Similarly, analyses of serum[440,441] and amniotic fluid[402,442] from mothers who gave birth to autistic children reveal an increase of proinflammatory cytokines and chemokines compared to control subjects.

The fourth MIA model has been used to explore the effects of maternal autoantibodies on fetal development.[443] IgG antibodies are physiologically transferred from the maternal immune system to the developing fetus in order to provide a temporary immunity to newborn[444], however, if the mother experiences phenomena of auto-immunity, auto-reactive antibodies may reach the fetus and affect its development.[445,446] Epidemiological studies support the association between ASD and maternal autoimmune disorders.[447-449] Multiple studies investigated the association between ASD and major specific autoantibodies[450]: increased levels of folate receptor α autoantibody (FRAA)[451], anti-myelin associated glycoprotein (MAG)[452] and anti-myelin basic protein (MBP)[453] antibodies, anti-ribosomal P protein antibody[454], anti-nuclear antibody (ANA).[455] Mice exposed to human plasma[456] or IgG[457,458] collected from mothers of ASD children display ASD-related behavioral phenotypes. The investigation of the molecular and cellular mechanisms implicated in prenatal exposure to autism-specific maternal autoantibodies suggest that these antibodies interfere with the proliferation of cortical neural precursor cells.[411]

In summary, MIA models provide a powerful tool to demonstrate the causal relationship between MIA and ASD, and to elucidate the underlying mechanisms. Some limitations[459] of these models concern methodological variability observed across different studies, such as the specific species/strain used, timing and dosage of the immune-stimulating agent, and so on. Despite these limitations, taken together MIA models provide a strong rationale supporting the hypothesis of a strong contribution of maternal infections to the etiology of ASD.[460] Further development of these models might include the investigation of the interactions between MIA and other ASD contributors[461], such as genetic alterations and other environmental factors, and the transgenerational transmission of MIA phenotypes and brain alterations to subsequent generations.[462]

3.3.3 Maternal Diet/Metabolic Status.

The maternal nutritional and metabolic states play crucial roles in fetal development, including brain development. Notably, the importance of maternal diet is not limited to the gestational period but also instead bookends it, extending from the pre- and periconception period, for processes such as gamete function and placental growth[463], to the postnatal period, particularly in breastmilk-fed infants. Indeed, throughout all of these critical periods, proper maternal intake of several micronutrients, such as vitamins and minerals, has been shown to be crucial for normal, healthy development - particularly neurodevelopment - of the offspring.[309,464] Among others, vitamin D, folate, and iron sufficiency have been extensively investigated in the context of ASD and other NDDs.

Vitamin D plays an important role in several biological processes[465], including calcium homeostasis, which is also critical for maintaining a healthy pregnancy and correct fetal growth and development.[466] Low levels of vitamin D in maternal serum[467] are correlated with increased pregnancy complications, such as miscarriage[468], preeclampsia[469] and gestational diabetes[470], and higher risk of developmental deficits, including ASD, in offspring.[471-473] Folate availability is another potential risk factor for ASD. Folate supplementation during pregnancy is known to prevent birth defects, notably neural tube defects (NTDs).[474] The mechanism underlying this protective effect of folate is thought to involve DNA methylation, a critical process for epigenetic modifications during development.[475] Regarding the potential connection between folate deficiency and ASD, epidemiological studies supports the hypothesis that maternal folate status during pregnancy may increase the risk of autism spectrum disorders.[476,477] Similarly, iron has a crucial role in brain function and fetal development.[478] Iron deficiency, a frequent condition in pregnant women[479], has also been associated with increased risk of developing ASD.[480,481]

Beyond micronutrient intake, a growing body of evidence support the critical role of maternal dietary patterns that govern macronutrient intake, such as high-fat diet (HFD), high-carbohydrate diet (HCD), and metabolic conditions in the etiology of ASD and other NDDs. As discussed above, the *Barker hypothesis* supported the developmental origins of health and disease (DOHaD), correlated harmful fetal environment, such as maternal undernutrition, with the incidence of various disorders in the adulthood. Despite the initial skepticism of many members of the scientific community[482,483], the DOHaD hypothesis was subsequently supported by epidemiological studies investigating the reasons for the increased rate of mental illnesses in populations affected by famine, such as the Dutch famine (1944-45), the Irish potato famine (1845–52), and the Chinese famine (1959–61). These studies showed that extreme maternal caloric restriction during pregnancy not only affected cardiovascular and metabolic function in subsequent generations, but also mental health[484]. For instance, several studies on the Dutch famine determined that, in offspring of mothers exposed to the famine, there were increased rates of severe mental disorders, such as major affective disorder[485], antisocial personality disorder[486], and schizophrenia spectrum disorder[487], a decrease in cognitive performances,[488] and an increased tendency to addiction[489]. Similar results have been reported for the Chinese famine, with increased rate of schizophrenia.[490,491] However, in contrast to Barker's hypothesis, which postulated a correlation between low birthweight and incidence of cardiovascular disorders in the adult age, these studies found that intrauterine food restriction increased the risk for disease, independently of weight at birth. Notably, further epidemiological data suggests specific correlations between the trimester in which mothers are exposed to dietary restrictions and the specific disorder induced in the offspring.[492,493] For example, while infants exposed to maternal malnourishment exclusively during late pregnancy were born small yet never experienced diseases such as obesity in adulthood,

exposure to maternal malnutrition during the early pregnancy was associated with an increase in preterm birth, infant mortality during the first days of life, as well as obesity and cardiovascular disease in adulthood in those who survived. Together, these findings highlight the relevance of timing in the programming of future disorders and suggest a critical role for exposure during early gestation.

Alterations in maternal environmental factors, including maternal nutrition, can significantly influence fetal development by triggering mechanisms that affect fetal programming. The placenta is the interface between the maternal and fetal blood circulation. By regulating the transfer of nutrients and oxygen from the mother to the fetus, the placenta consequently plays a central role in fetal programming. The placenta receives signals from both the mother and the fetus, balancing fetal needs with maternal supply. Perturbations in the maternal compartment are sensed by the placenta, which in turn modulates blood flow and nutrient supply, and adaptively modifies hormonal release.[463] Excessive deprivation (undernourishment) or abnormal increase (overnutrition) in maternal nutrient intake at conception and throughout pregnancy impact the ability of the placenta to properly allocate necessary resources for fetal growth.[494] Nutrient sensing in the placenta occurs by means of multiple mechanisms, such as the one involving the mTORC1 translational control pathway[495] in the syncytiotrophoblast. mTORC1 activity is regulated by several factors, such as glucose, ATP, amino acids, cytokines, and hormones. Indeed, maternal adipokines[496], IL-6[497], TNF-α[498], leptin[499], adiponectin[500], insulin, etc. influence the placental nutrient transport and nutrient delivery to the fetus. Maternal undernourishment causes a decrease placental amino acid transport, which in turn drives intrauterine growth restriction (IUGR).[501,502] Maternal obesity and diabetes drive excess of nutrient supply to the placenta which results in fetal overgrowth[503,504] and increased risk for the infants to develop obesity and metabolic dysfunction

in adulthood.[505,506] This evidence[507] shows a U-shaped correlation between unbalanced maternal diet, either malnutrition or obesity, and increased risk for disease in the offspring, with animal studies also supporting the impact of maternal diet on offspring health.[508-510]

Maternal nutrient intake and metabolic status also contribute to offspring mental health disorders. Indeed, multiple epidemiological and animal studies[511,512] support a role for maternal nutrition in the etiology of neuropsychiatric[513-515] and neurodevelopmental disorders[512] as well as cognitive impairment.[516,517] Maternal obesity and diabetes[518] have been associated with increased risk of NDDs, including ASD and ADHD[519,520], in the offspring. For ASD, human studies support a link with maternal obesity[521-525] and diabetes.[525,526] Notably, some studies report an increased risk for ASD when mothers are affected by both obesity and diabetes.[527,528] Epidemiological findings are complemented by animal studies which can provide important insights into molecular mechanisms underlying the maternal diet-induced risk for ASD and other NDDs.[529] Animal models[530] of diet-induced obesity are widely used to assess the impact of maternal obesity and metabolic conditions on neurodevelopment and mental disorders. In these models, maternal HFD induces impaired synaptic plasticity[531], social behavior[63,532], and learning and memory[533-535] in the offspring with increased oxidative stress, insulin resistance, impaired neurogenesis[536,537] and neuronal connectivity.[538] Yet, the precise mechanisms underlying the impact of maternal diet on offspring neurodevelopment remains to be determined.

In this regard, multiple mechanisms and pathways have been proposed to explain the connection between maternal diet and offspring health outcomes, such as inflammation, epigenetic modification, and, more recently, alterations in the gut microbiome, a hypothesis I explore in depth in this dissertation. Obesity is accompanied by chronic low-grade inflammation. In obese individuals, the adipose tissue experiences a variety of modifications in order to meet the increased

demand of fat storage. Moreover, the excess of fat is ectopically stored in liver and skeletal muscle, which results in insulin resistance (IR).[539] These processes are associated with increased release of pro-inflammatory cytokines, such as IL-6, TNFα, and IL-1β, and hormones, such as leptin and adiponectin, which contribute to the inflammatory state.[539] IR, diabetes[512,540], increased circulating leptin levels[496,541] and inflammation[542] characterized pregnancy in obese and/or diabetic women. As seen in the context of MIA models, maternal inflammation negatively affects neurodevelopment. Altered levels of pro-inflammatory cytokines and hormones are responsible for dysregulated placental function and therefore placental hormone, cytokine and growth factor production which are critical for fetal development.[543,544] Pro-inflammatory cytokines and hormones can also pass the placenta and reach the fetus, contributing to the impairment of neural pathways of crucial importance for behavior and cognition[511,545,546], including serotoninergic[547] and dopaminergic[548] circuits, and the hypothalamic-pituitary-adrenal (HPA) axis.[549]

Several studies provide evidence of a role of epigenetic effects of maternal obesity and diabetes on offspring development and long-term health outcomes. These effects might act as mediators of the association between unbalanced maternal nutrition and neurodevelopmental programming of offspring. Epigenetic alterations have been associated with other maternal nutritional conditions, such as maternal starvation[550] and maternal deficiency of vitamins and cofactors[551,552], and are known to be involved in ASD and other NDDs.[218,553] Modifications in the fetal epigenome, including DNA methylation, acetylation, histone modifications, long non-coding RNAs, have been associated with maternal obesity[554] and maternal diabetes. [555-557] For instance, MHFD offspring display alterations in histone binding and expression of the oxytocin receptor (OXT-R) in the hippocampus[558] which has an important role in social behavior[559], hypermethylation in the regulatory regions of hypothalamic POMC gene[560,561] which is involved

in regulation of food intake[562], alteration of histone modifications and expression in the hippocampal leptin receptor (Lepr)[563] which is involved in synaptogenesis[564] and neural circuitry maturation.[565] Interestingly, in perinatal MHFD mouse models, changes in the expression of several epigenetic regulators in the offspring developing brains were found in association with anxiety-like phenotype.[566] Furthermore, studies in animal models support the notion that maternal diet-induced epigenetic alterations in the offspring does not occur only at the fetus level, but also in the gametes.[567,568]

Another putative connection between maternal obesity/diabetes and offspring health outcomes, including brain disorders, that has only recently come under investigation is the maternal gut-fetal brain axis. The maternal gut microbiome has an important role in prenatal and early postnatal offspring development.[569] This regulation occurs via three routes: (1) *in utero* exposure to metabolites produced by the maternal gut microbiome, (2) postnatal exposure to maternal microbially-derived metabolites through breastmilk consumption, and (3) postnatal mother-to-infant vertical transmission of gut microbiota via vaginal delivery, breastfeeding, and/or skin-to-skin contact. Both genetic and environmental factors determine microbiome composition and metabolite production which establish multiple interactions with the host. These interactions occur in regard to many host processes, and ultimately impact on health and disease conditions. A growing body of evidence suggests an important microbial contribution to brain development, behavior, and more general health outcomes.[507,570,571]

Maternal diabetes, obesity, and metabolic syndrome have been shown to induce significant changes in the composition of the gut microbiome, which in turn alter the microbially-derived metabolite profile in both the mother and developing fetus. Such alterations are poised to significantly impact fetal development and contribute to fetal programming.[572] Consequently, the

impact of maternal high-fat diet intake on long-term health outcomes in offspring, including brain function and behavior, as well as the underlying mechanisms, are the focus of the research contained in this dissertation.

The gut-brain axis, the role gut microbiome in health and disease, and the mechanisms by which maternal gut microbiome modulates fetal brain development and behavior will be discussed in detail in the following chapter.

Part IV – The Gut Microbiome in ASD.

4.1 General Introduction of the Microbiome.

In recent decades, interest in the role of the gut microbiome in human physiology and pathology has grown exponentially, with a dramatic increase in the number of published scientific studies including the term, "microbiome"; indeed, instances of 'microbiome' or 'microbiota' in titles or abstracts have grown from 10 to 13,000 over the last 40 years. The microbiome, a term suggested by Nobel laureate Joshua Lederberg[573], comprises the genetic material within a microbiota - the collection of microorganisms in a specific niche, such as the human gut. The human microbiome accounts for trillions of microorganisms and includes bacteria, archaea, viruses, and eukaryotic microorganisms that live in and on the human body and that profoundly impact all the physiological and pathological processes occurring in the host organism. Distinct body sites and organs–*e.g.*, the skin, oral cavity, lung, and gastrointestinal system–have specific microbial inhabitants, but the clusters residing in the gastrointestinal compartments have, to date, attracted the greatest attention in biomedical research.

In recent years, there has been a significant increase in the amount of information gathered about the structure and function of these microbial communities, thanks to data provided by multiple large-scale studies aimed at characterizing the human microbiome (*i.e.*, the NIH-funded Human Microbiome Project (HMP)[574] and the European Metagenomics of the Human Intestinal Tract (MetaHIT)).[575] These consortiums produced an almost complete set of genetic sequences for most microbes living in the human gut, revealing the incredible quantity and variability of these communities. The analyses of the composition of the gut microbiome revealed that only a few phyla are present, with more than 160 species–*Bacteroidetes* and *Firmicutes* account for 90% of gut microbiota, and therefore, are the most represented phyla, with *Proteobacteria*, *Actinobacteria*,

Fusobacteria, *Spirochaetes*, *Verrucomicrobia* and *Lentisphaerae* also present. *Firmicutes* include about 200 genera, with *Clostridium* the most represented. *Bacteroidetes* consists of predominant genera such as *Bacteroides* and *Prevotella.*[576] Converging evidence support the important role played by the gut microbiome in a large number of diseases, ranging from cancer to autoimmune disorders and mental disorders, not only in influencing the pathological mechanisms underlying these diseases, but also modulating the effects of many drugs and treatments.[577] For these reasons, identifying what makes a 'good' gut microbiome, intended as the specific pattern or *core*[578] of microorganisms associated with a healthy state in the human body, both in terms of species and their relative abundance, is considered pivotal to understanding microbial contribution to health and the pathogenesis of several diseases.

The most commonly used approach to investigate the alterations in the composition of the gut microbiome in disease states is based on phylogenetic characterization of the microbiome in affected subjects in comparison with healthy subjects. However, the identification of the hypothesized 'core' microbiome has been complicated by the great variability in gut microbiome architecture across individuals and also within the same individuals across the lifespan.[579] Initial studies[580,581] which attempted to identify the compositional core of the gut microbiome, failed to species-level core groups common among healthy individuals. Subsequent studies provided moderate advances in regard to the detection of a core microbiome.[582,583] However, further efforts in the characterization of the composition and functions of the microbiome in regard to human physiology are needed to increase our knowledge of how the microbiome promotes health and contributes to disease.[584,585]

As for the molecular analysis of the gut microbiome, several culture-independent genomic techniques have either been developed or improved in recent years, allowing for precise and broad

investigations of the myriad of organisms living in the gut.[586] Methods based on 16S rRNA sequencing as a phylogenetic marker are efficient and cost-effective approaches for microbiome analysis and have been extensively used in this field of research. However, they carry at least two main drawbacks: (1) 16S rRNA sequencing can only identify bacteria, and does not identify viruses or fungi, which also reside in the human gut, and (2) its resolution is limited to taxonomic classification at the genus level, thus providing only limited information. On the other hand, shotgun metagenomics-based methods allow for classification at the species- to strain-level, and, most importantly, account for all microorganisms (including archaea, fungi, and viruses) present in a specific sample. The drawbacks of this approach, however, are high cost and the need for more sophisticated bioinformatic tools required for this technique. Both of the approaches described above are limited to the analysis of genomic material and do not include other features of the microbial life, such as transcriptome, proteome, and metabolome, which are crucial to the comprehension of the complex microbial physiology and its dynamic interaction with the host.[587,588] Indeed, the direct assessment of transcripts or proteins in a specific microbial environment, by means of metatranscriptomics or metaproteomics, respectively, represent an essential tool in the investigation of human microbiomes. The information acquired in metatranscriptomics studies[589,590] can be combined with metagenomic dataset to provide more accurate evaluation of gene expression/repression in specific conditions or timepoints.

Metaproteomics[591] employs high-resolution mass spectrometry (MS) to characterize microbiome protein compositions. Additionally, MS-based approaches provide quantitative measurements of proteins produced by all the organisms residing in the microbiome, including host proteins. Another important aspect of microbiome research is the assessment of metabolic products present in the gut. Metabolomics approaches[592] based on nuclear magnetic resonance

(NMR) spectroscopy or mass spectrometry (MS) allow for the analysis of fecal or microbial intracellular metabolites. Several metabolites present in the gut are produced and secreted by host-microbiome co-metabolism, and often serve as signaling molecules for communications between the two compartments.[593]

Data from 'omics' studies[594] suggest several hypotheses which need to be confirmed in order to establish a causation between microbiome composition and human health. In this regard, experimental modeling, such as humanized gnotobiotic animal models[595] carrying specific set of microbial communities, allows for the investigation of the effects of multiple variables, including diet[596], environmental exposure[597], and genetic background, on microbe's transcriptomes, proteomes, and metabolomes, and, eventually, on health outcomes. These models can also provide a powerful platform for the assessment of therapeutic efficacy of drugs aimed to treat diseases associated with alterations in gut microbiome composition. All of the aforementioned experimental approaches help to shed light on the complex relationship between the microbiome and the host and on the clinical significance of changes in microbiome composition and structure, which will be discussed in the following paragraphs.

4.2 Host-Microbe Interactions in Health and Disease.

The interplay between the gut microbiota and the host is regulated by an elaborate network of metabolic, immune, and neuroendocrine interactions. This crosstalk is profoundly influenced by microbially-derived metabolites which have broad effects on different organs and regulate multiple cellular and molecular processes. During co-evolution, the gut microbiome established a symbiotic relationship with the host and is deeply involved in maintaining host homeostasis. The composition

of the gut microbiome is far from static, but is rather dynamic, undergoing constant reshaping under the influence of many different host factors, including diet, exposure to drugs, toxins, pathogens and pharmacological treatments, the immune system, physical and psychological conditions, and the microbiota itself. Normal variations and adjustments within the gut microbial community can evolve into a detrimental state–so-called "**dysbiosis**"[598]–in which the delicate balance existing between the bacterial population is disrupted by powerful stressors mediating a cascade of events that ultimately lead to a sharp decrease of microbial diversity and promote exponential growth of specific bacterial species or taxa. Dysbiosis modifies the pool of the metabolites produced and released by the microorganisms, and can, therefore, have significant repercussions on host physiology and health. Increasing knowledge about the relationship between the microbiome and the host also suggests that disease onset, development, and outcome might be modified by modulating the composition of the microbiome, mostly by means of precise dietary intervention or probiotic administration aimed at either increasing or decreasing the abundance of specific microorganisms which have been shown to be involved in pathological conditions.

In the following section, I described the role of the gut microbiota in regulating host metabolic function, immune function, and brain function and behavior in detail.

4.2.1 Metabolism.

Important micronutrients, including vitamins[599,600], are produced by the gut microbiota. For example, Vitamin K[601] is synthesized by specific groups of bacteria and contributes to reducing the risk of cardiovascular diseases.[602] Vitamins B5 and B12[603], which are exclusively produced by the gut microbiota, are involved in a multitude of biochemical processes, and their deficiency is

associated with multiple disorders.[604] Another important role the gut microbiota involves the metabolism of bile acids produced in the liver and released in the intestine to facilitate digestion and metabolism. A residual portion of these components are converted into secondary bile acids by means of enzymes expressed by the microbiota. Bile acids influence several hepatic processes[605], including glucose metabolism, and can activate GPCRs in multiple organs, thus regulating gene expression and signaling pathways.[606] Moreover, bile acids also regulate the composition[605] of the gut microbiome itself and help to prevent growth of infectious pathogens. Digestion is among the best-known functions of the gut microbiome, and it represents one of the mutually beneficial relationships between the host and the microbial population. The gut microbiota participate in the metabolism of dietary amino acids, carbohydrates, xenobiotics, and other micronutrients, thus supporting host metabolic energy yield.[607,608] Experiments involving germ-free mice[609] validate these findings, thus confirming the central role of gut microbiota on host metabolic processes. Microorganisms living in the gut, in particular commensal bacteria, are particularly involved in the fermentation of dietary fibers and unabsorbed starch, which leads to the production of **short-chain fatty acids** (SCFAs), fatty acids with fewer than six carbon atoms (such as butyrate, propionate, acetate and pentanoate). SCFAs represent an additional source of energy for the host, providing ~10% of host daily caloric requirements[610], and, contributing to ATP production in colonocytes.[611] Beyond this energy-providing function, SCFAs promote intestinal barrier integrity, counteract intestinal inflammation[612], and are thought to regulate gastrointestinal motility[613], adipogenesis[614,615], and central modulation of food intake.[616,617] For instance, positive effects of SCFAs on glucose homeostasis and satiety are mediated by promotion of glucagon-like peptide-1 (GLP-1) synthesis in intestinal cells by butyrate, and regulation of gluconeogenesis occurring in the gut by propionate[618]. Butyrate is also involved in increased

energy consumption via multiple mechanisms.[619] For these reasons, SCFAs are considered beneficial metabolites with an important role in the prevention of metabolic disorders and obesity.[620] Multiple studies provide evidence about the cellular and molecular mechanisms by which SCFAs exert these functions, including regulation of histone deacetylase[621], in the context of gene expression, and signaling through GPCRs.[622]

Given that the gut microbiota plays an important role in human energy homeostasis and metabolism, several studies investigated its contribution to pathological metabolic conditions, such as obesity-associated metabolic syndrome (MS) and type 2 diabetes (T2D). In the case of **obesity**, animal studies showed that microbiota transfer from obese mice to germ-free mice increased body weight and fat mass.[620,623] Similarly, antibiotic administration reduced obesity and metabolic disorders in obese animals.[624] Furthermore, **fecal microbiota transplants** (FMT) from human twins discordant for obesity resulted in reproduction of the donor phenotype in the recipient mice: mice that received an FMT from the lean twin remained lean, whereas, mice that received an FMT from the obese twin became obese.[625] Multiple studies reporting metagenomic characterization of the gut microbiome composition in different mouse models of obesity identified an increase in the ratio of the two most prevalent bacterial phyla, the *Firmicutes/Bacteroidetes* ratio, which is associated with high levels of fatty acid synthesis substrates. Human studies confirmed the role of gut microbiome in obesity, consistently showing decreased diversity in gut microbial communities[626,627], a condition associated with obesity and low-grade inflammation. However, the identification of a precise microbial signature linked to obesity remains under investigation, due to both methodological variability of the different studies and biological variability of the hosts.[628]

Identification of the molecular mechanisms underlying the association between alterations in the gut microbiome and increased risk of developing obesity is an area of active investigation.

Many studies focus on the production of a number of detrimental metabolites produced as a result of proteolytic fermentation occurring in the distal colon, as well as SCFAs produced in the proximal colon from carbohydrate fermentation.[629] Evidence suggests that SCFA synthesis and release into circulation is altered in obese individuals compared to controls. Indeed, in obese subjects a higher concentration of SCFAs is observed in the feces, resulting from impaired colonic fermentation patterns.[630] Intriguingly, recent studies in humans provided evidence that circulating, but not fecal, SCFAs promote insulin sensitivity, lipolysis, and GLP-1 production.[631] In contrast, a marked increase in circulating branched-chain amino acids (BCAAs)–valine, leucine, and isoleucine–has been associated with obesity, insulin resistance, and diabetes in humans.[632,633] The gut microbiome of obese individuals is characterized by an increase in bacteria with high BCAAs biosynthetic potential, such as *Bacteroides* (*B.*) *vulgatus* and *Prevotella* (*P.*) *copri.*[633] As mentioned above, gut microbiota also participates in bile acid metabolism in the gut, leading to the production of GPCR agonists and antagonists which are involved in glucose and lipid metabolism regulation. Interestingly, obesity seems to be associated with an enhanced bile acid synthesis and impaired transport[634]; however, further investigation is needed to elucidate the role of microbiome in obesity-related bile acid metabolism.

Metabolic Syndrome (MS) is a cluster of co-occurring conditions which are associated with increased risk of developing insulin resistance, type 2 diabetes, stroke, and cardiovascular disorders. These conditions include hypertension, dysglycemia, dyslipidemia, and abnormal fat accumulation. Intriguingly, the gut microbiome has been implicated in the development each of these conditions. In animal models of MS, administration of polysaccharides, such as pectin and exopolysaccharides, by acting on gut microbiome composition, improves several metabolic parameters, including reduction of lipid and glucose levels in the blood and reducing total body

weight and adipose tissue.[635,636] Human studies investigating the positive effect of polysaccharide administration on MS are limited[637], and further investigation is warranted.[638,639] Regarding the mechanisms underlying the contribution of the gut microbiome to MS pathology, the first evidence of the central role of the gut microbiome in metabolic syndrome came from studies showing that prolonged excess fat consumption increased intestinal barrier permeability, a condition known as 'leaky gut syndrome,' caused by dysfunctional tight junctions in the mucosal barrier.[640,641] This phenomenon leads to increased translocation of bacterial products and components, in particular pro-inflammatory lipopolysaccharides (LPS), and bacteria themselves into the circulation, thus inducing detrimental effects in distant tissues and organs.[642] Low levels of LPS are detectable in healthy individuals, however they tend to increase significantly following meals with high-fat content.[643] Obese subjects show high levels of LPS both after a meal and during fasting, and chronic levels of elevated LPS has been associate to insulin resistance.[644] Intestinal barrier defects are associated with low-grade inflammation, resulting from the increase in LPS released in the bloodstream, a condition known as metabolic endotoxemia. Indeed, circulating LPS binds to LPS-Binding Protein (LBP) leading to the formation of LPS–LBP–CD14 complex which activates toll-like receptor 4 (TLR4) expressed on the surface of innate immune cells. The result of TLR4 signaling is the nuclear factor-κB (NF-κB)[645] which induced the production and release of pro-inflammatory cytokines, including IL-6 and TNFα. Interestingly, elevated levels of these cytokines are found in individuals affected by MS[646], and lifestyle interventions acting on the gut microbiome show an improvement in metabolic endotoxemia in MS subjects.[647]

Type 2 diabetes mellitus (T2DM) is a form of diabetes characterized by high blood sugar, insulin resistance, and relative lack of insulin. Prolonged hyperglycemia leads to a wide range of damage to multiple organs.[648] Many studies suggest a role for gut microbiome dysbiosis in the

pathogenesis of T2DM. Indeed, the diabetic gut microbiome is characterized by an increase in the proportion of opportunistic pathogens and endotoxins-producing gram-negative bacteria and a decrease in of *Bacteroidetes/Firmicutes* ratio and SCFAs producing bacteria.[649,650] Furthermore, alterations in levels of mucin-degrading bacteria, such as *Akkermansia (A.) muciniphila*, which play a crucial role in gut barrier integrity maintenance, have been associated with diabetes in animal and human[651,652] studies. Animal[653,654] studies have revealed a positive effect of *A. muciniphila* supplementation in ameliorating several metabolic parameters. Other studies[655] report that metformin, the FDA-and EMA-approved first-line medication for the treatment of T2DM, ameliorate glucose homeostasis in obese mice by increasing *Akkermansia* spp. population.

The impact of gut microbiome alterations in T2DM is further confirmed by studies focused on the effects of prebiotic and probiotic administration on metabolic health outcomes. Prebiotics are non-digestible nutrients that are degraded by gut microbiota. They induce the growth of beneficial bacteria, such as *Bifidobacteria* and *Lactobacilli*, that positively impact host health. Indeed, both animal[656] and human[657] studies report that prebiotic administration lowers blood glucose levels, increases GLP-1 release, and modulates insulin levels. Probiotics, "*live microorganisms which when administered in adequate amounts confer a health benefit on the host"*[658], exert similar beneficial effects on glucose metabolism by modifying the gut microbiome composition.[656] Indeed, several clinical trials performed in recent years showed that administration of probiotics, especially *Lactobacillus* species, can be an effective therapy for T2DM.[657]

4.2.2 Immunity.

The interplay between the microorganisms residing in the gut and host immune system function is involved in a multitude of processes in both health and disease. On one hand, the gut microbiome profoundly influences the development of both the adaptive and innate immune systems, inducing broad systemic outcomes involving sites distal to the gut; on the other, the immune system finely tunes the symbiotic host-microbe relationship to avoid microorganism overgrowth while ensuring tolerance to non-detrimental stimuli.[659,660] Studies in animal models and humans have revealed that the gut microbiome plays a major role in host immune system development. Indeed, microbial colonization of the host's mucosa during early life profoundly shapes development and maturation of the immune system.[661] This "window of opportunity," coinciding with the first years of life, is particularly vulnerable to any alteration in the host-microbe relationship, with potentially long-lasting detrimental effects on host immunity and overall health. Microbial colonization of the infant gut begins upon parturition and is primarily dominated by colonization from the maternal microbiome. This colonization is influenced by a variety of factors[662], including delivery mode[663] (vaginal or c-section), breastfeeding[664], and early skin-to-skin mother-infant contact. Mother-to-newborn transfer of skin and vaginal microbiota seems to be the first act in the establishment of microbial populations in the gut[665], however, these strains are quickly replaced by maternal gut strains, which persist more stably over time.[666] While the existence of *in utero* colonization is a contentious area of debate in the field, existing evidence for microbial colonization in the womb has not been reproduced; however, the presence of maternal microbiome-derived products in the fetal circulation suggests a functional influence of the maternal gut microbiome on both general and immune system-related fetal development.[667]

Germ-free (GF) mice have been extensively used to explore the mechanisms underlying the relationship between the host immune system and the microbiome. Negative effects of the absence of microbiota on immune system function have been documented in studies[668,669] dating back to the 1960s reporting significant alterations in the architecture and function of lymph nodes and spleen of GF mice. Subsequent studies showed that IL-17+CD4+ T (Th17) lymphocytes, which have a crucial role in the modulation of immune responses[670], are absent from the small intestine of GF mice but can be restored following gut colonization with specific commensal microorganisms.[671,672]

Maternal immune activation (MIA) during pregnancy, achieved through the inoculation of pregnant mice with the viral infection-mimicking synthetic molecule poly(I:C), is associated with a spike in IL-17a levels in maternal circulation, which is responsible for behavioral deficits in the offspring. Intriguingly, this increase in IL-17a is a consequence of Th17 cell expansion in the gut and depends on the presence segmented filamentous bacteria (SFB). Indeed, animals lacking this commensal microorganism are protected from the abnormal release of IL-17a and do not display the associated phenotypical aberrations.[65,424] A substantial reduction in different antibody classes, including IgA[673] and IgE[674], is also observed in GF mice, with consequences for mucosal immunity and allergy susceptibility. Evidence provided by the aforementioned studies support the critical role of the microbiota in the development of a fully functioning host immune system and suggest that dysbiosis might have broad implications for host health and disease states.[675] However, the cellular and molecular mechanisms underlying the multiple interactions between the immune system and the microbiota remain under investigation and future studies to further elucidate the relationship are required.

Beyond its role in shaping the host's immune system during the developmental period, the gut microbiome is also strongly involved in maintaining homeostasis throughout life, in regard to both innate and adaptive immunity. In the gut[676], the immune system plays three important functions: (1) tolerance towards the vast group of microorganisms which characterized the normal, healthy gut microbiome, (2) surveillance towards potential pathogenic agents, and (3) prevention of commensal overgrowth escape into the body from the intestinal lumen. Intestinal mucosa represents an important barrier standing between the host and microbiota to facilitate the compartmentalization of resident microbes.[677] Additionally, enteric dendritic cells (DCs) are instructed to tolerate microbial antigens present in the gut.[678] Regarding the innate component of immune system, antimicrobial peptides (AMPs), also called host defense peptides (HDPs), are produced by secretory cells located in the small intestine (Paneth cells) and circulating phagocytic cells. These molecules are synthetized and released in response to both gram-positive and gram-negative bacteria, which play an important role in the protecting the host from pathogen invasion through the activation of TLR-MyD88 signaling in AMP-producing cells.[679] Therefore, AMPs represent one of the non-specific first line of defense against pathogenic microbes by regulating the composition of the gut microbiota and the function of intestinal barrier.[680]

Toll-like receptors (TLRs) are a group of pattern recognition receptors (PRRs) which recognize microbial antigens produced both by pathogens and commensal microbiota. They are involved in host protection from deleterious microorganisms and control over potential overgrowth of commensal ones, while promoting host-microbial symbiosis.[681] Among others, TLR5 is particularly important in the regulation of gut microbiome composition, and its deficiency is associated with the onset of several metabolic dysfunctions, such as hyperglycemia, hyperlipidemia, insulin resistance, and obesity.[682] Another class of PPRs which is strongly

involved in the interplay between the gut microbiome and the immune system is represented by NOD-like receptors (NLRs). NOD2 is a peptidoglycan-activated cytosolic bacterial sensor which has an important role in preventing inflammatory processes in the small intestine by reducing the expansion of *B. vulgatus*.[683]

The expression of antimicrobial peptides is only one of the multiple mechanisms by which gut microbiota activates the innate immune system[684]. Indeed, recent evidence support a role for gut microbiota metabolites in activating the inflammasome, a multiprotein intracellular complex that detects pathogenic microorganisms and sterile stressors, and that activates the highly pro-inflammatory cytokines interleukin-1β (IL-1β) and IL-18.[685] Interleukin expression, in particular IL-22, IL-17, and IL-10, is another mechanisms used by the innate immune system to maintain tissue homeostasis. Indeed, multiple studies[686-688] provide evidence for the significant contribution of the gut microbiome to enhancing this protective response against pathogens. In addition to regulating the innate immune system, emerging evidence suggests that the gut microbiota also influence adaptive immune function. For instance, CD4+ regulatory T cells, in particular Th17 cells[689], appear to be regulated by SCFAs and their differentiation is impaired in GF mice.[690] Similarly, SCFAs are required for CD8+ cytotoxic T cell transition in memory cells.[691] Intriguingly, *Lactobacillus* (*L.*) *reuteri* plays a crucial role in reprogramming intraepithelial CD4+ T cells in immunoregulatory cells. Indeed, *L. reuteri* produces indole derivatives of tryptophan which bind to the aryl-hydrocarbon receptor in CD4+ T cells.[692] Microbiota-produced secondary bile acids have been found to be implicated in RORγ+ regulatory T cell function.[693] Furthermore, multiple studies showed that B cells, another important component of adaptive immunity, are strongly involved in gut microbiota shaping by means of multiple processes associated with B cells-produced secretory IgA antibodies.[694,695]

Several environmental factors modify gut microbiome composition. Diet and antibiotics have been extensively shown to induce significant perturbations in gut microbial ecology, changes which are reflected in the complex interactions between the microbiota and the host immune system. Indeed, diet- or antibiotic-driven gut microbial dysbiosis impairs host immune function. Recent investigations show that human subjects treated with broad-spectrum antibiotics before and after flu vaccine administration not only dramatically decreased both bacterial load and species diversity, but also impaired the production of neutralizing antibodies and increased inflammatory signatures.[696] Antibiotics are also responsible for decreases in microbiota-produced SCFAs, which in turn causes abnormal activation of macrophages in the gut, leading to enhanced proinflammatory T helper 1 (T_H1) responses in the colon.[697] Regarding diet-induced dysbiosis of the gut microbiome, high-fat diet is known to promote the overgrowth of bacteria which exert detrimental effects on the immune system, exacerbating inflammatory responses associate with intestinal disorders, autoimmune diseases in the CNS[698], and cancer.[699]

More broadly, a growing body of evidence supports the notion that alterations in the interplay between host immune system and the gut microbiome contribute to the onset of many immunity-related disorders affecting various organs and systems. Metabolic and cardiac diseases, such as diabetes, metabolic syndrome, atherosclerosis, and obesity, are characterized chronic low-grade inflammation which is thought to be triggered and/or sustained by detrimental gut microbiome-produced metabolites reaching other organs as a result of structural and functional alterations in gut barrier permeability.[700] However, the precise cellular and molecular mechanisms by which bacterial metabolites participate in the regulation of immunometabolism are still under investigation and represent a promising new avenue for prevention and treatment of metabolic disorders. Gut dysbiosis has been also implicated in systemic autoimmune disorders such as

rheumatoid arthritis, whose pathogenesis has not been fully elucidated yet. Recent research focuses on microbially-derived metabolites, in particular SCFAs, as contributors to immune dysregulation in rheumatoid arthritis.[701] Immune dysregulation in the CNS is also thought to be linked to alterations in metabolites produced by microorganisms living in the gut. The primary immune cells in the CNS, microglial cells, display unique properties which differ from other macrophagic cells in other parts of the body, and alterations in microglia function are associated with multiple pathological neurological conditions.[702] Interestingly, studies in GF mice suggest a critical role for the gut microbiome in microglia maturation. Indeed, GF mice display severe defects in microglia function, partially restored by microbial colonization.[703]

Collectively, the aforementioned studies provide important insights in the complex crosstalk between the microbiome and the immune system. However, further mechanistic studies are required to fully understand the molecular and cellular processes involved in the interplay between the gut microbiome and host physiology, which might then lead to development microbiome-based therapies to treat immune dysfunction associated with the broad range of disorders. More detailed information about the role of immune system in the bidirectional communication between gut bacteria and the brain communication between bacteria living in the gut and the brain, and in microbiota-mediated brain function alterations, will be provided in the following paragraphs.

4.2.3 Brain Function and Behavior.

In the last decade, many studies at the intersection of neuroscience and microbiology have begun to unravel the crucial contribution of resident microbial communities to the development and

homeostasis of neurological structures and functions. Multiple mechanisms are involved in the interplay between the gut and the brain, the so-called gut-brain axis, including immunological, metabolic, neuronal, and chemical signaling, with the gut microbiome playing a crucial role in communication. The knowledge provided by these new studies contributed to establish a new approach in the investigation on brain disorders. Indeed, it is now well accepted that brain processes are strongly influenced by microbial communities in the gut and their products both in health and in disease state. The modulatory effect of gut microbiome on the gut-brain axis have been explored primarily using GF animals[704], fecal microbiota transplantation (FMT), and antibiotic-driven changes in the gut microbiome composition.[705] The modulatory effects of the gut microbiota on brain function and behavior[706] have been described by a large body of preclinical literature investigating the pathological mechanisms underlying several brain disorders, including depression[707] and anxiety[708], behavioral dysfunction[709], learning and memory impairment[710], and others.[711] The amount of available information about human brain-microbiota interactions are more limited due to the greater complexity of human brain and microbiota, the influence of several both genetic and nongenetic variables, and technical limitations regarding study design and data collection and interpretation. Nonetheless, in the last few years, a growing interest in human microbiome research has been observed.[712] Indeed, deciphering the mechanisms by which the brain and gut interact is imperative to understand the physiopathology of many neuropsychiatric conditions, including neurodevelopmental, mood, and neurodegenerative disorders, and to improve existing therapeutic interventions by implementing microbiota-based treatments.[713]

The communication channels along the gut-brain axis will be described in the next paragraphs, with a particular focus on how perturbations in these pathways impact brain function and behavior.

4.3 The Gut-Brain Axis.

The famous statement "*all disease begins in the gut*" has been attributed to the Greek physician Hippocrates, who is commonly considered the father of modern medicine, over 2,000 years ago. While the attribution of this sentence to Hippocrates has been questioned, the general idea behind this claim has caught the attention of generations of scientists, resulting in an exponential increase in the research focused on the microbiome-gut-brain axis (MGBA), particularly in the past 10 years.[714] The concept of MGBA refers to the complex network of connections which support the bidirectional conversations occurring between the brain and the bacteria living in the gut. This communication has a crucial role in correct functioning of both gastrointestinal and central nervous system, and occurs via neurotransmitters, neuropeptides, or microbial-derived products. The MGBA includes the following components: the CNS, the autonomic nervous system (ANS), the enteric nervous system (ENS), the hypothalamic-pituitary-adrenal axis (HPA), the blood-brain barrier, neurotransmitters, hormone and neuropeptides, and the elements forming the gut microenvironment, including the gut microbiota and the epithelial barrier with the ensemble of their bioactive products, the immune and the entero-endocrine systems. Regarding the route of communications between the brain and the gut, it is possible to identify neuroanatomic pathways, neuroendocrine-HPA axis pathways, and immunological pathways (**Fig. 2**).

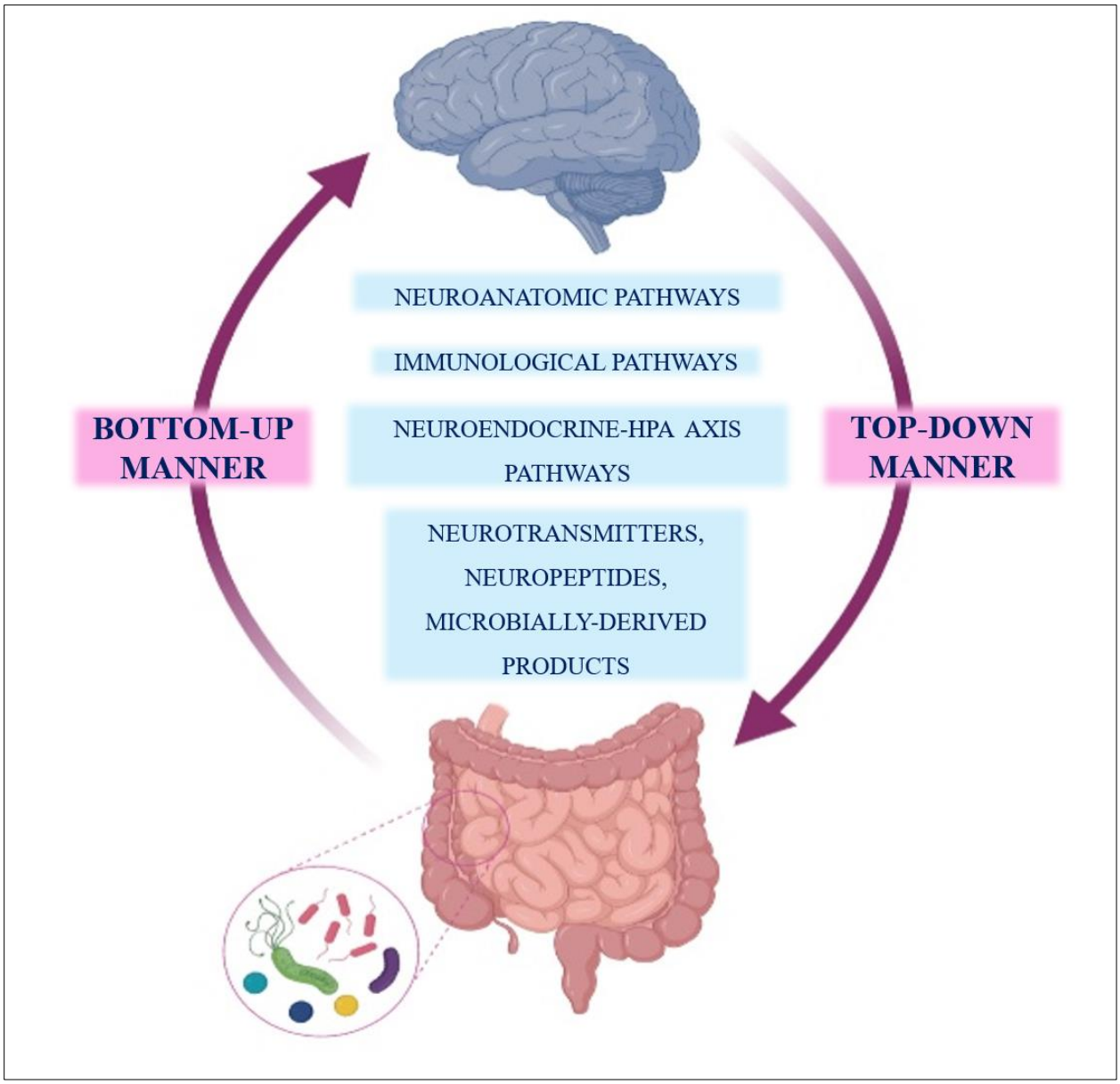
NEUROANATOMIC PATHWAYS
IMMUNOLOGICAL PATHWAYS
BOTTOM-UP MANNER
NEUROENDOCRINE-HPA AXIS PATHWAYS
TOP-DOWN MANNER
NEUROTRANSMITTERS, NEUROPEPTIDES, MICROBIALLY-DERIVED PRODUCTS

Figure 2. The bidirectional communication in the MGBA. The brain and gut with its microbiota regulate each other via multiple routes of communications: (1) neuroanatomic pathways, (2) immunological pathways, (3) neuroendocrine-HPA axis pathways. Top-down and bottom-up communications are mediated by neurotransmitters, neuropeptides, and microbial-derived products. (Figure adapted from Zhao, L., Xiong, Q., Stary, C.M. et al., 2018[715].)

4.3.1 Neuroendocrine-Hypothalamic-Pituitary-Adrenal Axis Pathway.

The neuroendocrine system regulates many processes in the human body and has a critical role in organ development and function. It includes the hypothalamus, the pituitary gland, and the target organs. Six primary neuroendocrine pathways have been identified: the hypothalamic–pituitary–thyroid axis (HPT), hypothalamic–neurohypophyseal axis (HN), the hypothalamic-pituitary-liver axis, the hypothalamic–pituitary–gonadal axis (HPG), the hypothalamic-pituitary-prolactin axis, and hypothalamic–pituitary–adrenal axis (HPA).

Together with others, the HPA is thought to be strongly connected with the gut microbiome in the context of the gut brain axis. This neuroendocrine structure is constituted by the hypothalamus, the pituitary gland, and the adrenal glands.[716] The HPA axis is involved in the response to psychological and physical stressors: the hypothalamus receives stimuli to produce and release corticotrophin-releasing hormone (CRH) which induces the secretion of adrenocorticotropic hormone (ACTH) from the pituitary gland; ACTH stimulates the adrenal cortex to release of glucocorticoids, mineralocorticoids, and catecholamines, which then regulate many other pathways in order to ensure appropriate response to the stressor. The release of hormones from the adrenal cortex also triggers a negative feedback mechanism which occurs via binding of the same hormones to receptors expressed in specific brain regions, thus eventually leading to the inhibition of the whole process.[717] A growing body of evidence documents bidirectional communication between the neuroendocrine-HPA axis system and the gut microbiota. The precise mechanisms by which the microorganisms living in the gut influences HPA axis and, more broadly, the neuroendocrine system are not completely fully understood; however, multiple lines of investigation suggest that several microbiota-derived products, such as SCFAs, gastrointestinal hormones, neurotransmitters and their precursors, might exerts direct

modulatory effects on the neuroendocrine system. Furthermore, gut microbiota also act on the immune system, in particular on inflammatory pathways, which in turn can influence the HPA axis. Indeed, the interplay between the HPA axis and the gut microbiome is interconnected with other systems in the gut-brain axis, including the ANS and ENS, the blood-brain-barrier, and the immune system.[718]

Investigation of disorders associated with alterations in both the neuroendocrine system and the gut microbiome, such as irritable bowel syndrome (IBS) and depression, have identified increased HPA axis activity in affected individuals. Intriguingly, the HPA axis is thought to be able to alter both the composition of the gut microbiome and the permeability of intestinal barrier, leading to dysbiosis and bacterial translocation which might support chronic low-grade inflammation states associated with both IBS[719,720] and depression[721,722]. The activity of the HPA axis is strongly influenced by negative early life events which may cause increased responsiveness of the axis to stressful inputs in post-natal life.[723] Dysregulation of the HPA axis can also drive functional alterations in the activity of the innate immune system: hyperactivity of axis induces compensatory downregulation of glucocorticoid signaling in the immune cells, thus decreasing the effectiveness of the anti-inflammatory action of cortisol.[724] On the other hand, proinflammatory mediators can upregulate the activity of HPA axis pathways, thus reinforcing the reciprocal influence between the HPA axis and the immune system.[725] Interestingly, the gut microbiota appears to play a role in this immune-neuroendocrine interplay. Stress-induced neuroendocrine mediators can alter the permeability of the intestinal barrier and promote the diffusion of microbial metabolites which can contribute to inflammatory processes.[726] It has also been suggested that the gut microbiome can trigger the activation of the HPA axis and the immune system by means of bacteria-derived metabolites, such as peptidoglycan[727] and LPS, thereby affecting brain function

and behavior. This effect might be especially relevant when occurring during neonatal life, leading to permanent alterations in the stress response and inflammatory mechanisms.[728] Additional evidence for the reciprocal influence between the gut microbiome and the neuroendocrine-HPA axis is provided by studies in GF mice which show altered expression of CRF and GR in the brain and elevated levels of corticosterone in the blood[704], while colonization in specific time windows with *B. infantis* or *E. coli* can, respectively, decrease or augment HPA axis activation.[704] The underlying mechanisms is thought to be mediated by immune-related factors which therefore might connect the alterations in gut compositions with dysregulation of the stress response. GF mice also display neuroendocrine alterations which are often associated with impairments in synaptic plasticity and behavioral alterations.[729] While dysbiosis can trigger abnormal activation of HPA axis and immune system, other studies support a positive influence of pre- and probiotics in modulation of both stress and immune response.[717,718] For instance, administration of the probiotic *Bifidobacterium pseudocatenulatum* CECT 7765 to rats during a specific time window in postnatal development attenuated maternal separation (MS) stress-induced neuroendocrine alterations.[730] Another study employing the same strain found that the treatment reduced levels of pro-inflammatory, stress-induced cytokines in the gut and in the serum, thus providing an additional link between the microbiota, the stress response, and the immune system.[731] Intriguingly, *B. pseudocatenulatum* administration to HFD-fed mice reduced neuroendocrine dysfunction in the brain and in the gut.[732] In yet another study, a combination of different probiotics tested in HFD-fed rats induced a decrease of depression-like behavior and HPA axis activation, shown as a downregulation of CRF receptor expression in the hippocampus, and changes in cytokine profile.[733] Studies conducted in human subjects showed that probiotic administration may attenuate the stress response, measured as urinary free cortisol levels.[734,735] In addition to

probiotics, prebiotics have also been shown to improve the functionality of the neuroendocrine-HPA axis in the context of the microbiota–gut–brain axis. Indeed, administration of fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) induced a decrease in (1) corticosterone plasma levels. (2) expression of CRF receptors in the hippocampus, and (3) depression- and anxiety-like behavior.[736]

4.3.2 Neuroanatomic Pathways.

Two main neuroanatomic pathways, both at the level of the spinal cord, connect the gut and the brain: one involves the ANS and the ENS, and the other one ASN and the vagus nerve (VN). The ENS, a large division of the peripheral nervous system (PNS), is an important neuronal network positioned at the intersection between the host and the microorganisms living in the gut. Anatomically, the ENS is organized in two groups of ganglia: the myenteric (Auerbach's) and submucosal (Meissner's) plexuses, localized between the inner and outer layers of the muscularis externa, and in the submucosa. Functionally, the ENS controls peristalsis and the secretion of enzymes and gastrointestinal hormones, such as gastrin and secretin. In the context of the gut-brain axis, sensory neurons located in the myenteric plexus are in contact with the microorganisms residing in the gut lumen and form synapses with the enteric motor neurons and vagal terminals. Interestingly, the electrophysiological properties of these neurons are influenced by changes in the gut microbiome. Indeed, sensory neurons of GF mice display altered excitatory function, which can be restored by microbial colonization.[737] Furthermore, administration of *L. reuteri* in rats increases neuronal excitability in the ENS.[738] ENS maturation occurs during the postnatal stage, at the same time the microbiota colonizes the gut; therefore, changes in the gut microbiome are

reflected in ENS function. This process is mediated by the activity of pattern recognition receptors (PRRs), such as TLRs, on ENS neurons which recognize microbial component, including LPS.

Studies in GF mice showed that ENS defects driven by the absence of gut microbiota might involve serotonin (5-HT): microbial colonization of GF mice correlates with the upregulation of the expression of 5-HT and its receptors in enteric neurons. Additionally, direct treatment with 5-HT4R agonists restores normal gut innervation in GF mice.[739] The crucial role played by the gut microbiota in the development and functioning of the ENS is further supported by studies investigating the effects of antibiotic-mediated depletion of gut bacteria. These investigations[740] revealed that antibiotic treatment affected multiple features of the ENS, including its morpho-functional structure and neurochemistry; glial cells were significantly decreased, while the amount of substance P (a neuropeptide acting as a neurotransmitter and as a neuromodulator, commonly co-expressed with ACh in enteric motor neurons) a–containing neurons and the expression of TLR2 were enhanced. Additionally, antibiotic-treated mice showed increased amount of fecal taurocholic acid and a decrement of cholic acid. Alterations in the fecal bile acid profile have been also found in the BTBR T+ (Itpr3tf/J) mouse strain, one of the most commonly used mouse models of ASD.[741] Regarding the effect of specific bacterial strains, it has been observed that different microorganisms[742] exert different effects on ENS neuronal activity that are the result of distinct mechanisms. These same studies showed that an intact intestinal epithelial structure is required to properly transmit luminal microbial inputs to the ENS, thus providing mechanistic insights into microbiota-ENS interaction. Emerging evidence suggest that the ENS can also influence gut microbiota, thus implying that alterations in the ENS might drive dysbiotic states in microbial community living in the intestine. However, further studies are required to elucidate the detrimental effects of ENS dysfunction on the gut microbiome.[743]

The final primary communication channel along the MGBA is a neuroanatomic pathway anchored by the vagus nerve (VN). The VN is the tenth cranial nerve and is one of the main structures of the parasympathetic nervous system (PSNS), which, together with the sympathetic nervous system (SNS), forms the ANS. The ANS regulates many important functions in humans, such as digestion, heart rate, endocrine regulation, and immune response.[744] The ANS gathers information from the gut which are then conveyed to the CNS in a bottom-up manner, and, in turn, the CNS produces responses sent to the gut via the ANS in a top-down manner. The VN is the most direct connection between the brain and the gut, providing both bottom-up and top-down communications. Encompassing 80% of afferent and 20% of efferent fibers, whose cell bodies are located in the nodose ganglia, the VN collects signals from various inner organs, including the gut, and sends them to solitary nucleus (SN), an important hub for microbiota-gut-brain signaling, and from there to other brain stem nuclei and forebrain structures, thus establishing an important connection between the gut and the brain.[745] Anatomically, the vagal fibers reaching the gut are located: (1) in the smooth muscles lining the gut, (2) within the mucosal layer without crossing the epithelium, and (3) in proximity of enteroendocrine cells (EECs), therefore, vagal terminals do not come in direct contact with the microorganisms residing in the lumen. However, even in the absence of a direct interaction with the microbiota, vagal terminals, thanks to the expression of receptors for several microbial metabolites, are able to sense changes occurring in luminal microbial populations. Similarly, EECs carry receptors for LPS[746] and SCFAs[747], and release serotonin and gut hormones which directly reach the VN and, subsequently, the brain. Both human[748] and animal studies revealed the crucial importance of bidirectional vagal connection for normal brain function and behavior. Work conducted in rodent models showed that either partial or total vagotomy induced alterations in brain functions associated with various neuropsychiatric

disorders, such as anxiety-like and fear-related behavior[749], learning[750], locomotor activity[751] and sensorimotor gating.[752] These data are further support by other studies in which the stimulation of the vagus nerve produces a wide range of effects on brain processes associated with anxiety and depression[753], stress[754], and the reward system, which is implicated in many mental disorders.[755] Intriguingly, a growing body of evidence suggest that gut microorganisms can modify brain function via vagal fibers. For instance, *Campylobacter jejuni* administration in mice induces c-Fos activation, a marker of intense neuronal activity, in the VN[756], and can increase anxiety-related behavior.[757] Similarly, *Lactobacillus rhamnosus* supplementation increased the firing rate of vagal afferents[758] and reduced anxiety- and depression-related behavior, an effect abolished in vagotomized mice.[759]

4.3.2.1 The VN in Microbiota-mediated Behavioral Alterations in ASD Models.

Epidemiological evidence supports the hypothesis that maternal obesity during pregnancy is one of the main risk factors for the onset of neurodevelopmental disorders, such as ASD, in the offspring.[521,760,761] Given the growing prevalence of maternal obesity, which now affects two in five pregnancies in the United States and one in five globally[762-764], recent studies investigated the relationship between maternal high-fat diet (MHFD)-induced obesity and offspring neurobehavioral deficits in mice.[63,765] Indeed, understanding the cellular and molecular mechanisms underlying maternal obesity-driven deficits in offspring brain function and behavior, is crucial to identify new therapies to prevent or moderate adverse mental health outcomes in affected offspring. These investigations found that, consistent with previous studies[623], mothers fed a HFD had a shift in gut microbiome composition, with a loss of diversity and significant differences in community structure, when compared to mothers fed a regular diet (RD).

Interestingly, the same alterations were found in MHFD offspring despite the fact that they were fed a RD after weaning. Additionally, MHFD descendants showed social deficits, which were prevented by co-housing MHFD with MRD mice. Whole genome shotgun sequencing identified a significant reduction in abundance of the commensal *L. reuteri* in MHFD group, whereas its reintroduction selectively reverted behavioral deficits in MHFD offspring, further supporting the strong connection between the gut microbiome and behavior. *L. reuteri* is known to promote oxytocin production, a hormone associated with social behavior[766] and involved in ASD pathology.[767,768] Oxytocin is produced in the paraventricular nuclei (PVN) of the hypothalamus. MHFD offspring showed less oxytocin immunoreactive neurons compared to MRD offspring, which were restored by *L. reuteri* treatment.

The investigation of the molecular and cellular mechanisms underlying the impact of maternal obesity, and subsequent dysbiosis of the gut microbiome, on offspring brain function and behavior unraveled a central role for vagal terminals in the etiology of maternal environmental factor-associated social behavioral deficits. Indeed, it was found that social interaction-induced LTP in dopaminergic neurons in the VTA, a critical process for normal behavior that is mediated by oxytocin release in the VTA, was impaired in MHFD offspring. *L. reuteri* treatment restored synaptic plasticity in the VTA. Additionally, the communication between the gut and brain through which *L. reuteri* promotes social behavior requires VN integrity. Indeed, *L. reuteri* failed to restore normal social behavior in vagotomized mice. These findings suggest that targeted microbial treatments might represent a new non-invasive therapeutic strategy for childhood disorders[769], including ASD. Additionally, selective administration of bacteria, such as *L. reuteri*, to restore oxytocin levels in the brain could also be more effective than intranasal oxytocin administration

itself[50], which requires high doses of hormone and could potentially lead to oxytocin receptor desensitization over time.

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4.3.3 Systemic and Mucosal Immune Regulation (Immunological Pathways).

The gut microbiota is strongly involved in priming the immune system to recognize potentially dangerous pathogens, as noted above. The vast array of bacterial products allows the host immune system to identify the multitude of bacteria living in the gut, and to sense alterations in the homeostatic balance of the microorganisms. Additionally, the interplay between the gut microbiota and the host immune cells leads to the production of cytokines, neurotransmitters, neuropeptides, and other mediators which can influence the activity of brain by acting on vagal and spinal afferent fibers. In this regard, one of the key mechanisms of interaction between the microbiota, the immune system and the brain involves microglial cells (**Fig. 3**). Microglia are considered innate sentinel immune cells which regulates inflammatory processes in the brain by the release of pro- and anti-inflammatory cytokines and chemokines. Importantly, the function of microglia in the CNS is not limited to immune surveillance, but also include the regulation of several neuronal events, including CNS maturation and synaptic plasticity. As noted in previous sections, gut microbiome composition is crucial to microglia maturation and differentiation.[703,770] The maternal microbiome in particular has an important role in defining microglial properties during prenatal life, and the absence of gut microbiota, as in the case of GF mice, differentially impacts microglial function in male and female animals, with males being more affected.[771]Microglia is also able to mobilize monocytes from the periphery to enter the brain. This process appears to be mediated by systemic TNF-α signaling, which leads to microglial activation and subsequent recruitment of activated monocytes.[772,773] Interestingly, the trafficking of monocytes from the spleen might be

modulated by microbiota produced SCFAs, which bind to free fatty acid receptor type 2 (FFAR2) expressed on peripheral lymphocytes.[774]

Adaptive immunity is also shaped by the gut microbiome, with implications for brain function and behavior. In the context of stress-induced disruptions in brain function, it has been shown that, in mice, shifts in the composition of the gut microbiota, in particular increased abundance in *Clostridium*, determine alterations in the immunoregulatory responses which exert ultimately negative effects on the brain.[775] Another study in B and T cell-deficient *Rag1*$^{-/-}$ mice showed that these mice displayed impairments in memory and anxiety, manifested by reduced c-Fos expression in the hippocampus and hyperactivation of the HPA axis, and dysbiosis of the gut microbiome. Remarkably, these alterations were reversed by pretreatment with probiotics (specifically a combination of *L. rhamnosus* and *L. helveticus*), thus showing that adaptive immune cells play an important role in both intestinal and brain homeostasis, and that probiotics can bypass the immune-related deficit in the MGBA.[776]

In the context of neurodevelopmental disorders, several studies conducted in maternal immune activation (MIA) mouse models highlight that certain gut maternal bacteria, namely segmented filamentous bacteria (SFB), promote T helper 17 (Th17) cell differentiation which is associated with multiple inflammatory conditions and behavioral and cortical abnormalities in the offspring.[65] Interestingly, treatment of MIA offspring with specific commensal bacteria to induce changes in the gut microbiome composition or with microbially-derived metabolites have been shown to ameliorate both gastrointestinal and behavioral dysfunction.[64]

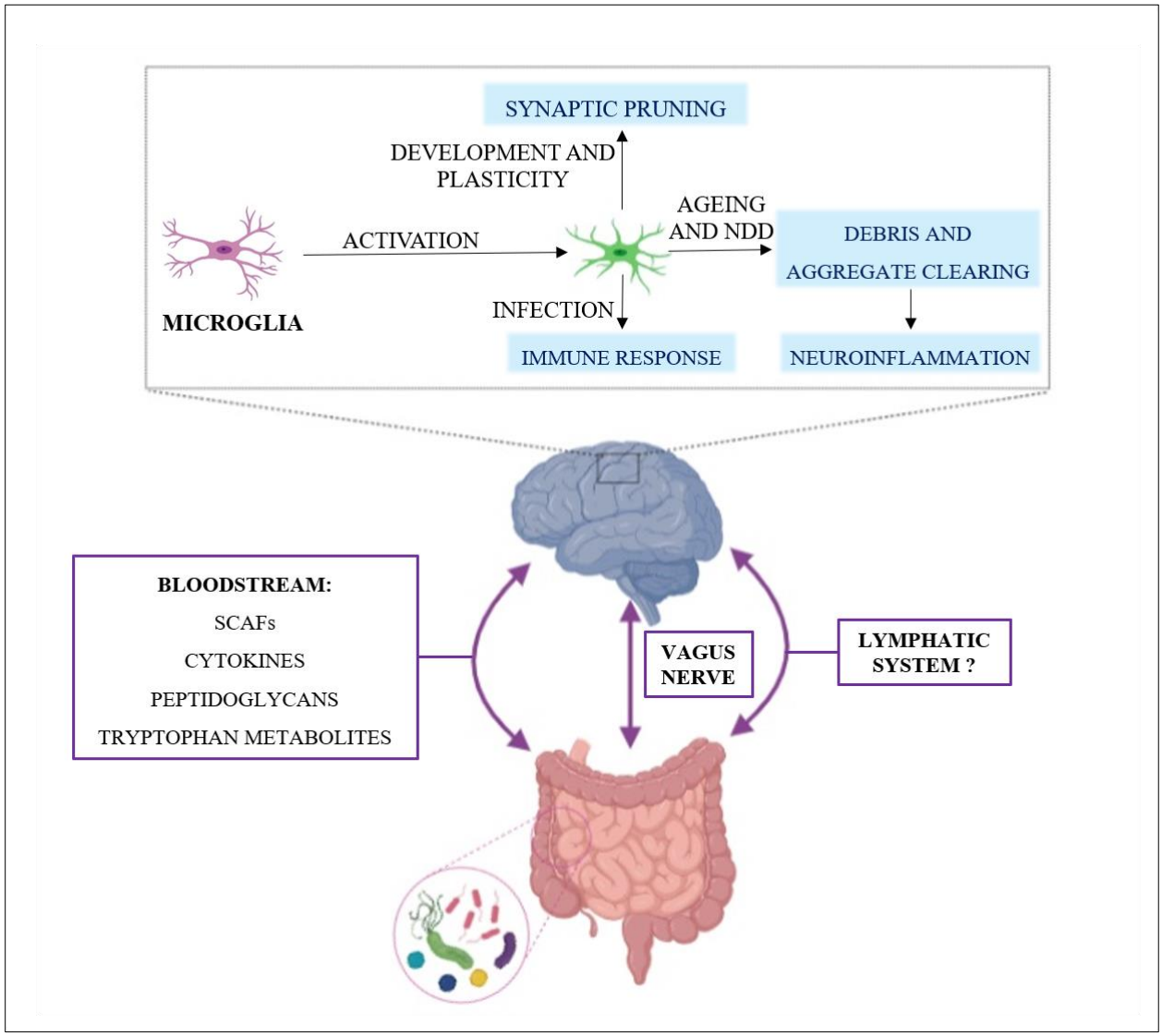
SYNAPTIC PRUNING
DEVELOPMENT AND PLASTICITY
AGEING AND NDD
DEBRIS AND AGGREGATE CLEARING
ACTIVATION
MICROGLIA
INFECTION
IMMUNE RESPONSE
NEUROINFLAMMATION
BLOODSTREAM:
SCAFs
CYTOKINES
PEPTIDOGLYCANS
TRYPTOPHAN METABOLITES
VAGUS NERVE
LYMPHATIC SYSTEM ?

Figure 3. Mechanisms for microbiota regulation of neuroimmune signaling. Activated microglia modulates a variety of processes within the brain, including neuroinflammation, response to infection, synaptic plasticity and remodeling, and debris and aggregate clearance. These multiple roles of microglia in the brain make them crucial targets to rescue pathological events in NDDs and ageing. Microglia activation and function can be mediated by several factors produced in the gut as a result of the interactions between the host and the gut microbiome, such as cytokines, tryptophan metabolites, bacterial-derived cell wall components (peptidoglycans) and bacterial-derived metabolites (SCFAs). Signals from the gut can reach the brain through the bloodstream, the vagus nerve and potentially the newly discovered meningeal lymphatic system. The discovery that microglia activation is regulated by the gut microbiota suggests the possibility for microbiota-based therapies for many brain disorders. *(adapted from Cryan, J., Dinan, T., 2015[770])*

4.3.4 Microbially-Derived Neuroactive Metabolites.

Neurotransmitters, neuropeptides, and microbial-derived products play an important role in the regulation of the MGBA. Different neuropeptides, including substance P, calcitonin gene-related peptide, neuropeptide Y (NPY), somatostatin, and others, contribute to the regulation of the mutual relationship between the microbiota and the host. Neuropeptides can modulate the activity of gut microbiota and, conversely, microbial control of amino acids availability and gut hormone release regulates the synthesis of neuroactive peptides.[777] Similarly, neurotransmitters exert pleiotropic effects on the gut microbiome, and, interestingly, the gut microbiota itself represents a source of neurotransmitters. Indeed, dopamine, norepinephrine, GABA, serotonin, and histamine have all been proved to derive from microorganisms living in the gut and to influence brain function.[778] Among microbially-derived metabolites[779], branched chain amino acids (BCAAs) and short chain fatty acids (SCFAs) have recently gained a great deal of attention because of their ability to influence various processes in the host, including those involved in the regulation of brain function and behavior. The role of BCAAs and SCFAs in the context of animal and human metabolism has been discussed in previous paragraphs of this thesis. Here, their functions in regard to the gut-brain-axis will reviewed in detail.

4.3.4.1 Short Chain Fatty Acids (SCFAs).

Altered levels of fecal SFCA have been associated in several brain disorders, including ASD.[780,781] SCAFs produced in the gut exert a number of local effects, as the promotion of intestinal barrier integrity and the protection from intestinal inflammation.[612,782] However, SCFAs can cross the intestinal distribl barrier and can be transported through the blood stream and be imported inside

the cells, where they are metabolized as an energy source via the Krebs cycle. Additionally, SCAFs can also play multiple signaling roles in the context of the gut-brain-axis, summarized in (**Fig.4**).[783] Indeed, SCFAs affects brain physiology through multiple indirect mechanisms and it also possible that these metabolites can cross the blood–brain barrier, thank to elevated expression of monocarboxylate transporters MCTs on endothelial cells[784] , even though additional research is needed to clarify the direct effects of SCFAs on brain physiology.

SCFAs, especially butyrate, can enhance the inhibitory effect of histone deacetylase, which have been implicated in the pathogenesis of several neuropsychiatric disorders.[785] Acute doses of butyrate have been proven to ameliorate cognitive performance[786,787] and ASD-like social dysfunction[788] in animal models.

SCFAs bind to several G protein-coupled receptors (GPCR), with GPR43 and GPR41, later renamed free fatty acid receptor 2 (FFAR2) and FFAR3, respectively, being the most studied in regard to the gut-brain-axis.[789] FFAR3 is expressed in the PNS and the BBB. Of particular relevance for the gut-brain axis signaling, propionate-induced FFAR3 activation on vagal fibers led to an increase in the activity of the dorsal vagal complex, parabrachial nuclei, and hypothalamus.[618] These data suggest that SCFAs can directly influence brain activity through the vagus nerve, however more investigations are required to unravel the effects of SCFAs on brain function and behavior. SCFAs are also able to stimulate enteroendocrine signaling. Indeed, FFARs receptors are expressed on colonic enteroendocrine L cells, and their activation leads to the production of GLP-1 and PYY in the circulation.[790,791] Each of these hormones directly regulates appetite and food intake in the CNS.[792,793] Additionally, in mice, GLP1 improved learning and memory[794], and increased neuroplasticity and reduced microglia activation in the hippocampus.[795]

SCFAs have been shown to modulated intestinal mucosal immunity[796] and are thought to also affect the peripheral immune system which then might be linked to brain function modulation. SCFA oral administration promoted peripheral regulatory T cell differentiation in mice[797] and FFAR agonists have been shown to modulate human monocyte inflammatory pathway, by decreasing the release of pro-inflammatory cytokines.[798] Another study[799] suggested that microbially derived SCFAs can influence brain function, specifically hippocampal neurogenesis, by acting on monocytes, which then could represent crucial mediators of signaling from the periphery to the brain. SCFAs have been also implicated in various neuropsychiatric disorders, including ASD; however, the role of SCFAs in ASD is controversial. ASD subjects have been reported to carry either increased or decreased SCFAs in fecal samples.[780,800] Higher levels of SCFAs have been found in a valproic acid mouse model of ASD[801], while the beneficial effect of butyrate on social dysfunction has been reported in BTBR mouse model of ASD.[788]

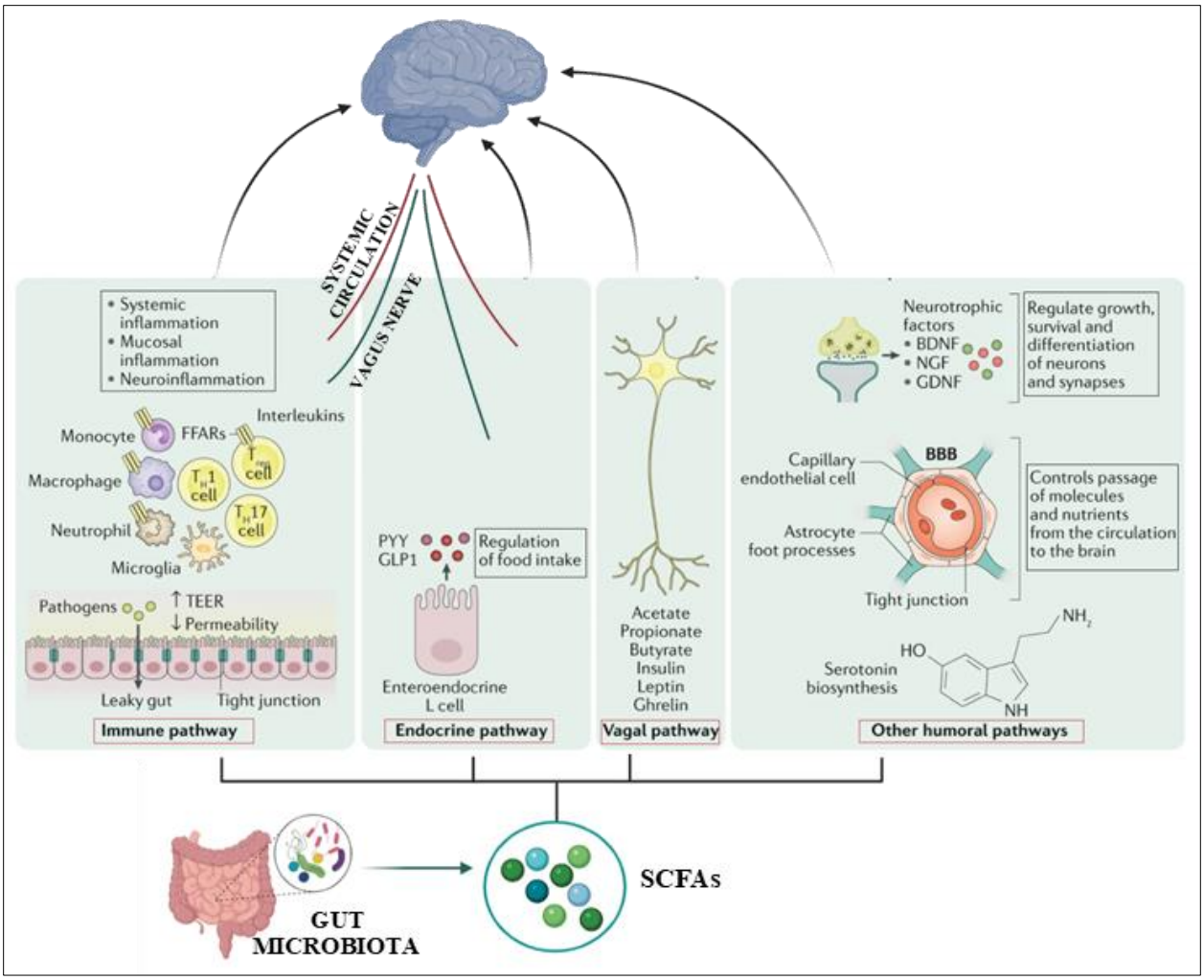
SYSTEMIC CIRCULATION
VAGUS NERVE
• Systemic inflammation
• Mucosal inflammation
• Neuroinflammation
Monocyte
FFARs
Interleukins
Macrophage
Neutrophil
Microglia
Pathogens
↑ TEER
↓ Permeability
Leaky gut
Tight junction
Immune pathway
PYY
GLP1
Regulation of food intake
Enteroendocrine L cell
Endocrine pathway
Acetate
Propionate
Butyrate
Insulin
Leptin
Ghrelin
Vagal pathway
Neurotrophic factors
• BDNF
• NGF
• GDNF
Regulate growth, survival and differentiation of neurons and synapses
Capillary endothelial cell
BBB
Astrocyte foot processes
Tight junction
Controls passage of molecules and nutrients from the circulation to the brain
Serotonin biosynthesis
Other humoral pathways
GUT MICROBIOTA
SCFAs

Figure 4. Gut–brain pathways through which SCFAs modulate brain function. Gut microbiota-produced SCFAs are thought to influence brain function through immune, endocrine, vagal and other humoral pathways. SCFAs locally interact epithelial cells and immune cells in the gut by means of the activation of free fatty acid receptors (FFARs) or by inhibiting histone deacetylases thus regulating intestinal mucosal immunity and barrier function. By modulating interleukin secretion, SCFAs participate in the regulation of systemic inflammation. SCFAs also influence microglia function and neuroinflammation. In the context of endocrine pathways, SCFAs influence the secretion of gut hormones such as glucagon-like peptide 1 (GLP1) and peptide YY (PYY) from the enteroendocrine L cells. By activating receptors on the vagal nerve, SCFAs can signal to the brain. BBB integrity is promoted by SCFAs via monocarboxylate transporters located on endothelial cells. In the brain, SCFAs regulate the production of neurotrophic factors. (*Figure adapted from Dalile et al., 2019*[783])

4.3.4.2 Branched Chain Amino Acids (BCAAs).

Branched chain amino acids (BCAAs)–valine, leucine, and isoleucine–are essential amino acids which must be introduced with the diet or produced by the microbiota. They are key nitrogen donors which play an important role in intercellular and interorgan nitrogen transfer. A growing body of evidence suggests that BCAAs play essential roles in the regulation of several biochemical mechanisms besides simple nutrition.[802] Unlike other AAs, BCAAs are not metabolized in the liver[803], but instead their oxidation [transamination via the branched-chain aminotransferases (BCAT) isozyme] initially occurs in peripheral tissues, especially in skeletal muscle (SK), where they can modulate several physiological processes, such as protein synthesis and degradation, glucose homeostasis, hormonal regulation and nutrient-sensing signaling pathways, including, phosphoinositide 3-kinase-protein kinase B (PI3K-AKT), mammalian target of rapamycin (mTOR) pathways. Transamination is followed by irreversible oxidative decarboxylation of the α-keto acid products, which is catalyzed by the branched-chain α-keto acid dehydrogenase enzyme complex (BCKDC) in the liver.

BCAAs can stimulate initiation of mRNA translation and can reduce protein degradation.[804] The mechanism by which BCAAs stimulate protein synthesis is thought to involve mTOR signaling pathway.[805] Among the three BCAAs, Leucine[806] seems to be the most effective in increasing mRNA translation through the phosphorylation and subsequent activation of the main effectors of mTOR pathway, elF4E-BP1 and p70 S6 kinase. In the context of glucose homeostasis, BCAAs stimulate glycogen synthesis[807] and glucose uptake by SK and liver by insulin-independent mechanism which involves PKC and PI3-kinase pathways rather than mTOR pathway.[808] The CNS is very sensitive to AA and nitrogen levels because either deprivation or accumulation of AAs can be toxic and disrupt protein synthesis and neurotransmitter production

in the brain. Plasma BCAAs are transported into the brain and other regions of the CNS by means of a by a transporter, located at the blood–brain barrier (BBB) on CNS capillary endothelial cells, shared by many large neutral amino acids, such as aromatic amino acids (ArAAs) TRP, TYR, and PHE.[809] BCAAs are nitrogen donors in Glu and Gln brain metabolism. The Glu/Gln cycle involves both astrocytes and neurons, regulates neuronal Glu amount, and prevents excess Glu in the synaptic cleft. During neurotransmission, astrocytes remove the excess of Glu and convert it in the non-neuroactive Gln, which is then released in the extracellular space where is internalized in presynaptic neurons.[810] The nitrogen transfer cycle in the brain is illustrated in (**Fig. 5**).

While BCAAs are required for protein and neurotransmitter synthesis as well as energy production in the brain, exaggerated intake of BCAAs can cause neurotoxicity. Maple syrup urine disease (MSUD)[811], for example, is an inherited disorder of BCAA catabolism, manifested as abnormal increase of BCAA concentration in blood, cerebrospinal fluid, and urine, and characterized by cognitive dysfunction.[812] The detrimental effects of elevated BCAA levels on neuronal cells is also suggested by studies[813,814] reporting hyperexcitability of cortical neurons by BCAAs which leads to excitotoxicity. High BCAA levels might also responsible for the impairment of microglia, the main macrophage population in the brain. Alterations in microglia function can contribute to neurotoxicity. Indeed, microglia can either increase or attenuate inflammatory response, by acquiring pro- inflammatory (M1) or anti-inflammatory (M2) phenotypes. Some studies[815] found that high levels of BCAAs can promote the acquisition of a phenotype which is intermediate between M1 and M2 and might lead to a low-grade inflammatory state, while decreasing the response to local damage, thus increasing the susceptibility to neurodegeneration. BCAAs have been implicated in numerous physiological and pathological processes in the body, such as immune pathways. Immune cells can internalize BCAAs and

oxidize them[816]. BCAA deficiency causes impairments in the innate immunity, especially in the intestinal mucosa, and reduces resistance to pathogens.[817] The growing interest in metabolic regulation of immune response prompted the investigation of the role of BCAAs, especially leucine, in the activation and function of T cells.[818] Some studies showed that leucine is critical for mTORC1-mediated regulation of T cell activation and differentiation. One of the main enzymes responsible for BCAA metabolism is the cytosolic branched-chain aminotransferase (BCATc), which plays an important role in the negative feedback regulation of mTORC1 pathway. The loss of this enzyme determines an increase in availability of leucine, which in turn determines T cell hyperactivation.[819] BCAA dysregulation has been implicated in metabolic and neuropsychiatric disorders, including, insulin resistance (IR), T2DM, and mental disorders, such as ASD. High levels of BCAAs have been associated with increased rick for metabolic abnormalities and development of IR[820] and have been suggested to be an indicator of pre-diabetic states.[821] Indeed, one of the effects of elevated BCAAs is the hyperactivation of mTOR/p70S6K pathway and phosphorylation of IRS-1 on multiple serine sites, which is thought to contribute to IR and cardiovascular disease.[822] Regarding T2DM, human studies[823,824] suggest that high intake of BCAAs might be associated with elevated risk of diabetes.

BCAA dysregulation has been proposed to contribute to the pathogenesis of maternal environmental factor-associated and syndromic autism. Indeed, studies[811] found that the risk for ASD was increased in children, boys in particular, whose mothers presented metabolic conditions and elevated BCAA levels during pregnancy. Additionally, coding variants in the large amino acid transporter (LAT) gene, which is responsible for the transportation of tryptophan and BCAA across the blood-brain barrier, have been found in ASD patients.[825,826] As mentioned above, BCAAs cannot be synthetized and must be supplemented by the diet. Another important source of

BCAAs is represented by the microbiota. Indeed, select microorganisms living in the gut carry all enzymes necessary for BCAA synthesis, and BCAAs are essential for the growth and survival of many bacteria species. Indeed the components of the BCAA synthesis pathway have been proposed as targets for the development of new antibacterial agents, due the fact that this pathway in not present in mammals which may reduce toxicity.[827]

Since the microbiota can both synthetize and metabolize BCAAs, it can be considered as a master regulator of BCAA homeostasis in the host.[828] Species like *P. copri* and *B. vulgatus* have a great biosynthetic potential for BCAAs, and their abundance positively correlates with insulin resistance[633]. A similar association with IR was seen in the case of reduced capacity for bacterial BCAA uptake driven by a decreased proportion of many species, including *Butyrivibrio crossotus* and *Eubacterium siraeum*.[633] Other studies showed that, in mice, BCAA supplementation modified gut microbiome composition and improved host metabolism. Indeed, mice fed with BCAAs displayed decreased Bacteroidetes and increased Firmicutes, and a reduction in LPS-binding protein which was correlated with lower inflammation.[829] Since the potential beneficial effects of BCAAs remain under debate, further investigations are required to better elucidate the role of BCAAs in the pathogenesis of metabolic and nonmetabolic disorders.

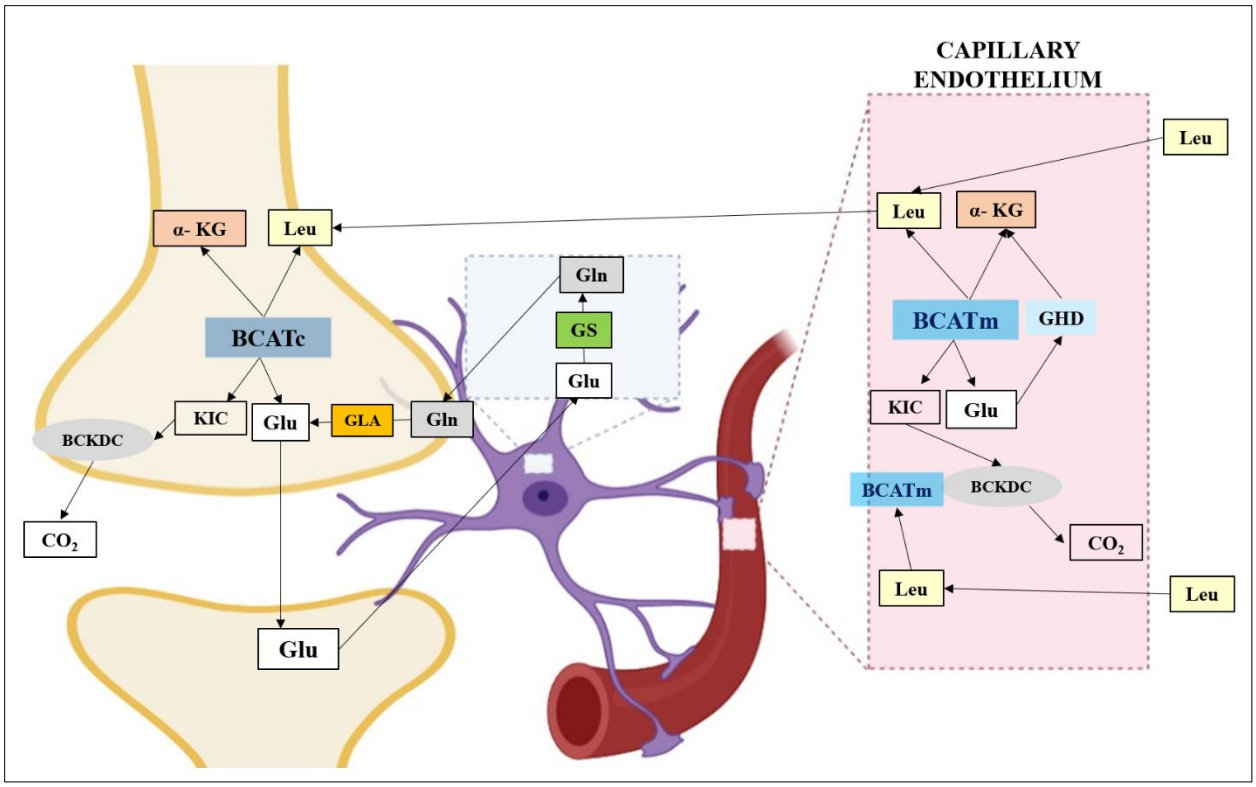
CAPILLARY
ENDOTHELIUM
Leu
α- KG
Leu
Gln
GS
Glu
BCATc
KIC
Glu
GLA
Gln
BCKDC
CO2
Glu
Leu
α- KG
BCATm
GHD
KIC
Glu
BCATm
BCKDC
CO2
Leu
Leu

Figure 5. Schematic of human brain nitrogen transfer. Leucine (Leu) enters the capillary endothelium of BBB for transamination or delivery to neurons. Inside the endothelium, BCAAs are oxidated and transaminated by mitochondrial branched-chain aminotransferase (BCATm) and branched-chain α-keto acid dehydrogenase enzyme complex (BCKDC). In the neuron, Leu is transaminated by cytosolic branched-chain aminotransferase (BCATc) to produce glutamate (Glu) and α-ketoisocaproate (KIC). Glu is released in synaptic cleft. Remaining Glu is transported in the astrocyte and converted in glutamine (Gln) by glutamine synthase (GS) and transported back into the neuron, where is converted in Glu by glutaminase (GLA). α-ketoglutarate (α-KG), glutamate dehydrogenase (GDH). (Figure adapted from Sperringer *et al*., 2019[810].)

4.4 Behavior and The Microbiota-gut-brain Axis.

The MGBA is implicated in the regulation of several physiological and pathological processes occurring in the body, including those influencing brain function and behavior. Indeed, the MGBA influences brain processes such as food intake, social behavior, cognition, fear, and stress-related behavior. Disruptions in the MGBA are connected to several diseases, such as ASD and other NDDs, major depressive disorder, schizophrenia, anxiety, bipolar disorder, addiction, Alzheimer disease (AD) and other neurodegenerative disorders and many others. (**Fig.6**) [830] Social behavior and cognition, the two most pertinent of these processes with regard to this thesis, will be discussed in greater details in the following sections.

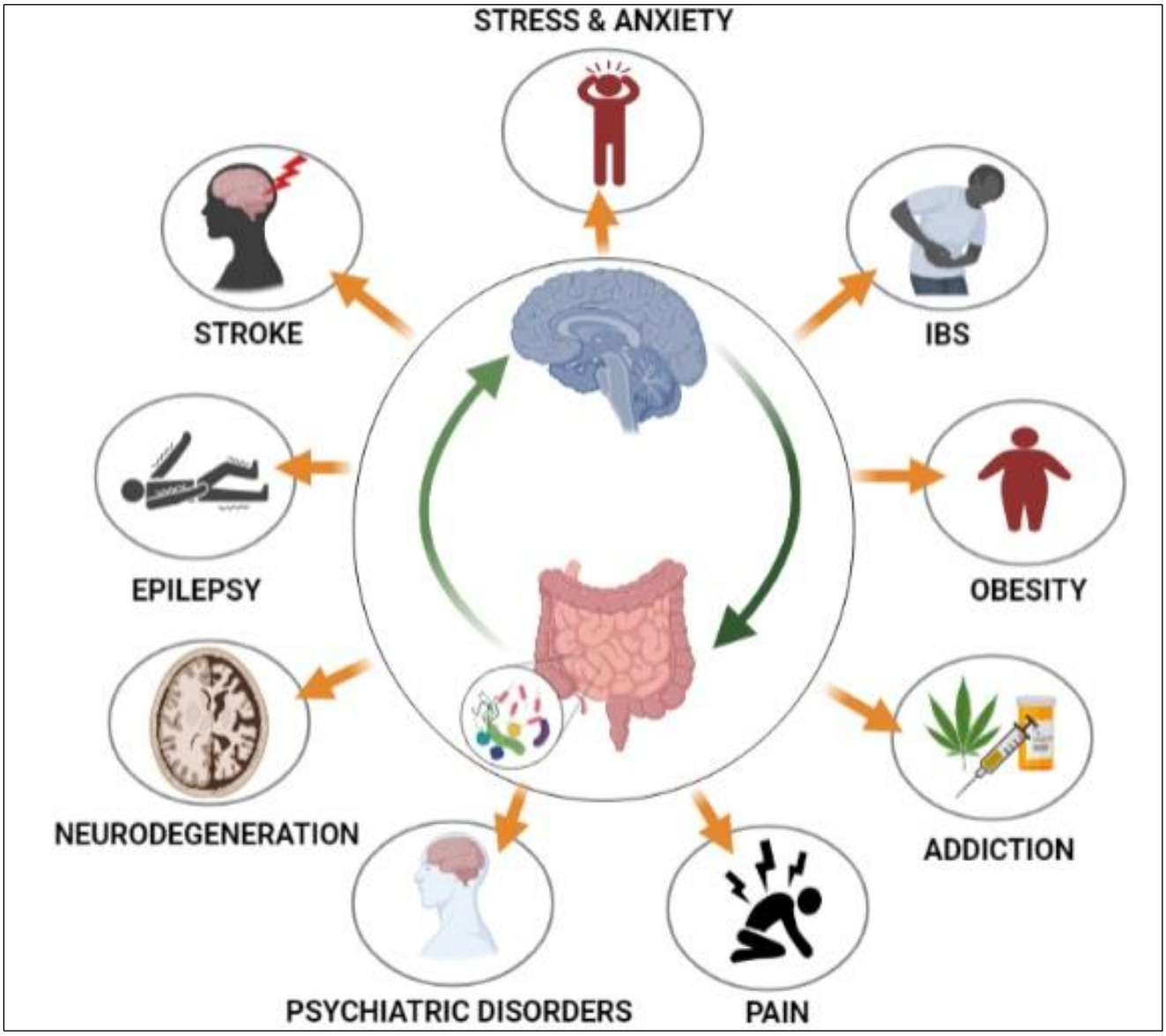
STRESS & ANXIETY
STROKE
IBS
EPILEPSY
OBESITY
NEURODEGENERATION
ADDICTION
PSYCHIATRIC DISORDERS
PAIN

Figure 6. An outline showing the array of diseases the MGBA is involved in. The growing number of studies related to the MGBA began to unravel the important role that this axis has in many physiological and pathological processes occurring in the host, in particular those related to brain function and behavior. Examples of disorders linked to alterations in the MGBA include psychiatric disorders, neurodegeneration, pain, stress and anxiety, irritable bowel syndrome (IBS), stroke, epilepsy, addiction, and obesity. **(***adapted from Cryan, J. F. & Dinan, T. G., 2019*[830])

4.4.1 Social behavior.

Many of the most fundamental of human experiences, such as romance, require one to engage in reciprocal social interactions with new individuals; yet, this ability is impaired in people affected by disorders of social dysfunction, including ASD, schizophrenia, and social anxiety. To develop effective treatments for disorders of social dysfunction, it is critical to first understand the underlying mechanisms. Heretofore, most research into social behavior has focused exclusively on the brain; however, our recent[63,765,831] and current work suggests that gut microbiota make critical contributions to host social behavior.

The rewarding nature of social novelty is crucial in inducing exploratory behavior, thus promoting learning and normal cognitive development. As noted above, MHFD-induced dysbiosis of the gut microbiome is responsible for social deficits in the offspring, as a result of the disruption of oxytocin signaling in the mesolimbic dopaminergic reward systems. These findings are corroborated by investigations in GF mice and antibiotic treated animals. Indeed, GF mice show reduced interest in novel conspecifics, compared to colonized mice, and are unable to distinguish between novel conspecifics over familiar ones, thus displaying altered social behavior.[63,765,832,833] Similar results have been obtained in antibiotic-treated mice in which the reduction in the gut microbiome diversity is linked to social dysfunction.[834,835] Commensal microbiota-produced bacterial peptidoglycan (PGN) can translocate from the gut to the brain and influence social behavior. Indeed, KO mice for PGN-recognition protein 2 (*Pglyrp2*), which is highly expressed in the mouse developing brain, displayed increased sociability and preference for social novelty in the three-chamber social test. These mice also carried altered expression levels of gene with a crucial role in synapse formation, such as BDNF and c-Met, in the striatum.[727] Interestingly, while other studies highlighted marked alterations in the transcriptome in the amygdala of GF rodents[836],

Pglyrp2 KO mice did not exhibit significant gene expression changes in this brain region. Sickness behavior in mice with induced liver inflammation was reduced by the administration of probiotic mixture VSL#3 (consisting of *Streptococcus, Bifidobacterium,* and *Lactobacillus),* which decreased circulating levels of proinflammatory cytokines, such as TNFα, without altering the degree of liver inflammation.[772] These findings suggest that one of the possible mechanisms by which the gut microbiota regulates social behavior is represented by the modulation of immune function in the brain.

4.4.2 Cognition.

Studies in GF mice revealed that the absence of gut microbiota severely impair host cognition, including altered working memory.[710,837,838] Similar results have been obtained with antibiotic treatment.[834,839] In both GF and antibiotic-treated mice, behavioral deficits are accompanied by dysregulation of synaptic plasticity modulators, such as BDNF.[705] Data from experiments conducted in other animal models, such as diet-induced obesity (DIO) and transgenic AD[840-842] models, further confirmed the association between alterations in the gut microbiome and cognitive deficits. Regarding DIO models, a large body of evidence reported that HFD negatively impacts the gut microbiota and leads to cognitive impairments. Fecal microbiota transplant (FMT) from HFD-fed mice to regular diet mice induced cognitive deficits and altered the composition of the gut microbiome.[843] Other studies showed an increased Firmicutes/Bacteroidetes (F/B) ratio after HFD treatment, which was associated with cognitive impairments.[844,845] The mechanisms underlying gut dysbiosis-determined cognitive alterations, however, remain poorly understood. Disruption in inflammatory pathways[846,847] has been proposed to explain diet-induced detrimental effects on cognition, as well as alterations in biochemical pathways involving SCFAs produced by

the microbiota.[848] The connection between dysbiosis of the gut microbiome and cognitive deficits is further supported by studies exploring the positive effects of pre- and probiotic administration on restoring cognitive performance. For instance, *Lactobacillus* strains have been shown to ameliorate spatial memory impairments in multiple mice models.[849-851] Similarly, treatments with *Bifidobacterium*[852], a combination of *Bifidobacterium* and *Lactobacillus*[853], or VSL#3 mixture[799], restored memory impairments in multiple mouse models.

While the number of human studies in the context of gut microbiome effects on cognition is limited, some investigations provide promising results. For example, a recent study showed that gut microbiome composition was associated with cognitive development in a cohort of 1-year old infants[854]. Three different groups were identified based on the microbiota composition: elevated levels of *Faecalibacterium*, *Ruminococcaceae*, and *Bacteroides*, with the latter showing the highest level of performance assessed by the Mullen scales of early learning. Probiotic treatments have been shown beneficial effects on cognition in human subjects. For instance, a probiotic mixture containing *B. longum* and various *Lactobacillus* strains ameliorated both cognitive performance and metabolic status in a randomized, double-blind and controlled trial on AD patients.[855]

4.4.3 The Maternal Gut Microbiome Modulates Offspring Brain Development and Behavior.

A growing number of studies in the field suggest that the maternal gut microbiome is a primary determinant of fetal and post-natal development, especially in the context of brain function and behavior.[856] The importance of maternal gut microbiome in pre- and early postnatal offspring development was introduced in Part III of this chapter, with three possible routes for the interaction between the microorganisms living in the maternal gut and the fetus, being: (1) *in utero* exposure to metabolites produced by the maternal gut microbiome, (2) postnatal exposure to maternal microbially-derived metabolites through breastmilk consumption, and (3) postnatal mother-to-infant vertical transmission of gut microbiota via vaginal delivery, breastfeeding, and/or skin-to-skin contact. Mother to- infant vertical transmission of the gut microbiome is a critical event for the achievement of developmental milestones in the neonate.[857] Multiple factors influence the extent to which maternal microbiota colonize the newborn, including the mode of delivery and length of gestation.[666,858] Investigation of mother-to-infant strain transmission revealed that newborn gut is initially colonized by strains localized in the maternal vaginal (for cesarean-delivered babies) and skin microbiota; however, these strains are eventually replaced by maternal gut strains, which are more stable in the offspring gut.[666]

Nonetheless, the influence of maternal gut microbiota on offspring development is not limited to the postnatal period, but instead begins during the intrauterine life. Indeed, nutrients and other substances in maternal circulation can reach the fetus by crossing the placental barrier and influence fetal development. Pregnancy is characterized by several adaptations that the mother's body undergoes to accommodate the growing fetus. Maternal metabolism must be adjusted to fetal requirement and shifts from an anabolic condition which characterized the first two trimesters of

pregnancy, to a catabolic one during the third trimester in which nutrient transfer to the fetus is boosted. The latter is achieved through the increase in maternal insulin resistance, a condition which determines a rise in glucose and free fat availability.[859]

The initial phases of pregnancy coincide with crucial steps in fetal brain formation, including synaptogenesis and neural migration, which occur at specific time points in discrete brain regions.[860] These highly regulated developmental trajectories are particularly sensitive to nutrient availability and are strongly affected by adverse events occurring at maternal-fetal interface.[861] Since the gut microbiome has a crucial role in the regulation of host metabolism and immune function, it has the potential to broadly impact on the physiology of gestation. The gut microbiome is a critical interface for nutrition: host diet determines nutrient availability to the microbiota, and therefore, continually shapes microbiota composition, which is then mirrored on microbiota global metabolome. This collection of all microbially-derived metabolites has pleiotropic effects on host function both in physiological and pathological status. This influence is extended to the fetus during the gestational period; therefore, the composition and the community structure of the microorganisms are ultimately critical to proper fetal growth and development.[862] Intriguingly, the maternal gut microbiome undergoes significant modification across the duration of the pregnancy. Recent studies describe quantitative and qualitative microbial changes which occur in this period and which seem to run in parallel with the gestational metabolic changes described above. For instance, during final phase of gestation, an increase in Actinobacteria and Proteobacteria is observed, together with a sustained inflammatory state.[862]

Despite the fact that additional investigation is required to unravel the precise microbiota-host interplay occurring during gestation and the underlying molecular and cellular mechanisms, recent studies[863] in rodents revealed that, during pregnancy, the maternal gut microbiota is master

regulator of circulating metabolites not only in the mother but also in the fetus, including the fetal brain. In the context of brain development, these metabolites are crucial for axonal outgrowth in the thalamus and the establishment of thalamocortical connections. Intriguingly, some of these metabolites, such as trimethylamine N-oxide (TMAO)[864,865] have been associated with other brain disorders and neuronal function, while other, such as imidazole propionate (IP)[866], are involved in the activation of mTOR which is suggested to promote axonal growth capacity.[867] Maternal conditions leading to dysbiotic states of the gut microbiome alter the maternal microbially-derived metabolite profile which is then reflected on the developing fetus. Such perturbations in the microbial ecology of the maternal gut can contribute to fetal programming[569], and therefore, be a concurring factor for the onset of various disorders in the offspring. Indeed, the first 1000 days of life, the period from conception to 2 years of age, are particularly important for the development of metabolic pathways, immune system, neural circuits, and many others.[868]

Adverse environmental events, including maternal over- or undernutrition, can interfere with proper development. Indeed, environmental disturbances occurring before and during pregnancy can affect maternal gut microbiome, and therefore, impact on the fetus. For instance, adverse events can prompt abnormal inflammatory and stress response which are thought to involve the gut microbiome, as in the case of adverse childhood experiences (ACEs) which induce dysregulations in the glucocorticoid-immune response in humans.[869] The effects of maternal infection during pregnancy in animal models have been reported in previous paragraphs. In regard to brain function and behavior, MIA models showed deficient social and communicative behavior, as well as increased levels of repetitive behaviors[421,870]. SCFA availability has a crucial role on metabolism and immunity in adult experimental animal models and has been extensively explored. Indeed, studies show that SCFA production by the maternal gut microbiome fluctuates during

pregnancy and it is crucial for regulatory T cell development in the offspring.[667] As reviewed in previous paragraphs, maternal diet plays a crucial role for the onset of behavioral dysfunction.[63,765] Taken together, these studies suggest the pivotal role of maternal gut microbiome in maternal infection-derived fetal programming and fetal development. Yet, the underlying mechanism by which alterations in maternal gut microorganisms impact offspring physiology remains unresolved, as well as their implications on other cognitive and metabolic dysfunctions in the offspring.

Part V - Aims of the Study.

Maternal obesity predisposes offspring to a wide array of adverse long-term health outcomes. Given the increasing prevalence of obesity and overweight in women of childbearing age, further investigation into the mechanisms by which maternal obesity impacts offspring health is needed. Our lab and others have reported that, in mice, HFD consumption by mothers induces significant changes in the maternal and offspring gut microbiome, most notably characterized by a decrease in overall microbial diversity. We found that MHFD-induced dysbiosis in the offspring gut microbiome is causally related to social dysfunction, a hallmark of both autism and schizophrenia, and underlying synaptic plasticity deficits in the hypothalamic oxytocinergic-mesolimbic dopaminergic social reward circuit, which is evolutionarily conserved between mice and humans. Importantly, precision reconstitution with *L. reuteri*, the species most reduced by MHFD consumption, can rescue autism-like social deficits in MHFD offspring. However, whether MHFD-induced dysbiosis in offspring gut microbiome is also casually related to other maternal obesity-related offspring pathological phenotypes, such as cognitive and metabolic dysfunction, is unknown.

The first part of our study was aimed at testing the novel hypothesis that vertical transmission of maternal obese-type gut microbiota serves as the primary driver of cognitive and metabolic dysfunction in offspring and identify the underlying mechanisms. We then investigated whether prenatal modulation of the maternal gut microbiome could prevent behavioral and metabolic dysfunction in offspring (**Chapter 2**).

In the second part of our study, we investigated the impact of MHFD-induced dysbiosis on brain development and behavior across multiple generations. While detrimental effects of MHFD on the F_1 generation have been extensively reported, the ramifications of maternal obesity, particularly in the context of diet-induced dysbiosis of the gut microbiome, on subsequent

generations remain almost completely unexplored. Previous studies suggest that, in mice, diet-induced loss of taxa within the gut microbiome is magnified over generations, thus supporting the idea that diet can produce long-lasting and heritable 'scars' on the gut microbiome. In light of these findings, we tested the hypothesis that high-fat diet-induced dysbiosis of the gut microbiome persists across generations, propagating alterations in brain function and behavior in progeny, even in the absence of direct exposure to MHFD (**Chapter 3**).

Understanding the cellular and molecular mechanisms underlying maternal obesity-associated long-term health risks in offspring is imperative to develop effective preventative treatments for children affected by maternal obesity. The investigation of MHFD-induced changes in the gut microbiome and their effects on offspring health presented in this thesis provides has the potential to guide a better identification of at-risk individuals and to aid in the development of both preventive and treatment strategies.

Chapter II : Vertically Transmitted Opportunistic Pathogens Impair Cognitive and Metabolic Dysfunction in Maternal High-Fat Diet Offspring.

Vertically Transmitted Opportunistic Pathogens Impair Cognitive and Metabolic Dysfunction in Maternal High-Fat Diet Offspring.

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1. Introduction.

Obesity prevalence is escalating worldwide.[871] In the United States (US)[872], the recent rise in obesity rates has been disproportionately among women[872]–between 2005 and 2015, obesity incidence increased by 5% in women but only 2% in men[764,872]–particularly those of childbearing age[873]. The growing proportion of women of childbearing age[874] of obese/overweight metabolic status is of particular concern given that maternal obesity predisposes offspring to adverse long-term health outcomes[875], including metabolic dysfunction[876] and neurodevelopmental disorders.[877] Yet, due to our limited understanding of the underlying pathophysiology, there is no consensus on strategies for mitigating long-term health risks to children affected by maternal obesity *in utero.*[878-880] Obesity is a complex multifactorial disorder, with both genetic and nongenetic factors contributing to its onset.[881] However, obesity is considered to be primarily induced by overconsumption of food. Indeed, a recent study[882] using the phenotype-discordant monozygotic twin (MZ) pair method confirmed overeating as the main cause of a higher body mass index (BMI). Western pattern diet (WPD)[883], which is characterized by a high intake of red meat, sugar, fat, salt, and refined grains, and lower complex carbohydrates consumption, together with poor lifestyle choices, is considered a major *culprit* in the *obesity* epidemic[884]. Given that diet-derived nutrient availability influences the composition of the gut microbiome[885,886], whose role in regulating host physiology has been extensively supported by multiple studies,[635,887,888] it is important to understand the relationship between the two. Data from our lab and others shows that WPD significantly alters the gut microbiome, inducing persistent imbalance of the gut's microbial community ("dysbiosis"), in rodents, non-human and human primates. Emerging evidence suggests that the resulting dysbiosis is involved in the pathogenesis of multiple disorders of immune[889], metabolic[890], and brain function.[891]

We[63,765] and others[892,893] have shown that high-fat diet (HFD) consumption in a mouse model of diet-induced obesity evokes similar changes in gut microbiome composition as those observed in an obese-type human gut microbiome. The maternal gut microbiota regulates both prenatal and early postnatal offspring development.[894] During pregnancy, the maternal gut microbiota provide metabolites essential for fetal growth, immune cell maturation, and neural circuit formation.[569,895] At parturition, maternal vaginal and skin microbes pioneer colonizing the infant gut, but only transiently, and are then replaced by maternal gut strains that persist throughout development.[666] Such mother-to-offspring "vertical transmission" of the gut microbiome is critical to developmental milestones in the neonate, including innate immunity and metabolism[896] as well as higher brain function to support complex behaviors, as evidenced by abnormal social behavior in germ-free mice.[833]

Given that the offspring gut microbiome is acquired from the mother and is influenced by her diet and body composition during gestation[897,898], we recently investigated whether maternal high-fat diet (MHFD)-induced dysbiosis of the gut microbiome could compromise offspring brain development and behavior by altering offspring gut microbiome composition.[63] Indeed, our data showed that MHFD induces persistent, functional changes in the offspring gut microbiome that are causally related to offspring social dysfunction and related deficits in synaptic plasticity in the ventral tegmental area (VTA), a dopaminergic reward circuit locus in the brain. Intriguingly, precision reconstitution with *L. reuteri*, the species most reduced by MHFD consumption, rescued autism-like social deficits in MHFD offspring. Yet, the underlying mechanism by which MHFD microbes impact host physiology remains unresolved and whether MHFD-induced changes in the offspring gut microbiome are causally related to cognitive and metabolic dysfunction in the offspring is unknown.

Here, we tested the novel hypothesis that that MHFD-induced dysbiosis of the offspring gut microbiome is the primary driver of cognitive and metabolic disorders in affected offspring. Our metagenomic whole genome shotgun sequencing results led us to focus our study on the contribution of the opportunistic pathogen *B. vulgatus*, the species whose abundance was most increased in the MHFD offspring gut microbiome, to the cognitive and metabolic phenotypes in MHFD offspring, given that increased *B. vulgatus* abundance is associated with high-fat diet consumption, increased fat mass, and insulin resistance in humans.[633,899-902] We specifically investigated its effect on the host serum metabolome, identifying two pathways by which *B. vulgatus* can influence both host cognitive and metabolic function. Finally, we aimed to determine whether targeting opportunistic pathogenic bacteria by means of maternal pharmacological interventions, *i.e.*, the FDA-approved antidiabetic drug metformin, which has been shown to induce functional changes in the host gut microbiome that are causally related to its ability to treat metabolic dysfunction in humans and mice[903,904], could improve cognitive and metabolic function in MHFD offspring. This study provides key insight into the effects of dysbiosis caused by maternal diet-induced obesity on long-term health outcomes in offspring. Moreover, it has the potential to drive a paradigm shift in the practice of antenatal care for women of overweight and obese status and to significantly improve health outcomes in children exposed to maternal obesity *in utero*.

2. Experimental Procedures.

Mice and Maternal Diet. C57BL/6N mice were obtained from Taconic Laboratories (B6) and were kept on a 12-hour light/dark cycle and had access to food and water *ad libitum*. Six-week-old females were placed on either a regular diet (RD) consisting of 13.4% kcal from fat, 30% kcal from protein, and 57% kcal from carbohydrates (Lab Diets, #5001) or HFD consisting of 60% kcal from fat, 20% kcal from protein, and 20% kcal from carbohydrates (Research Diets, #D12492). Maternal weight was measured weekly. After 4 weeks on diet, females were paired with C57BL/6N adult males to produce subject offspring. Resulting offspring were weaned at 3 weeks of age and all placed on RD, regardless of maternal diet (RD or HFD). All behavioral tests were performed on offspring starting at 7 weeks of age. Animal care and experimental procedures were approved by The University of Texas Medical Branch's Institutional Animal Care and Use Committee in accordance with all guidelines set forth by the U.S. National Institutes of Health.

Body composition analysis. Whole body composition was performed using the EchoMRI™ whole body composition analyzer (EF-037) provided by the UTMB Rodent *In vivo* Assessment (RIVA) Core to accurately measure total, lean, and fat mass, as well as free and total water.

16s rRNA Gene Sequencing Stool samples were aseptically collected in sterile 2mL Eppendorf tubes, immediately placed on dry ice, and stored at -80°C until further processing. Bacterial DNA was extracted and sequenced by the Alkek Center for Metagenomics and Microbiome Research using adapted from protocols developed for the NIH-Human Microbiome Project[574], as described previously[63]. Briefly, bacterial genomic DNA was extracted using MagAttract PowerSoil DNA Kit (Qiagen) followed by PCR amplification of the 16S rDNA V4

region. The primers used for amplification include MiSeq adapters and single-end barcodes allowing for pooling and direct sequencing of PCR products. Sequencing was performed on the Illumina MiSeq platform using the 2 x 250 bp paired-end protocol yielding overlapping paired-end reads. The 16S rRNA gene read pairs were demultiplexed and merged using USEARCH v7.0.1090[905], allowing zero mismatches and a minimum overlap of 50 bases. Merged reads were trimmed at first base with Q5. A quality filter was applied to the resulting merged reads and reads containing above 0.05 expected errors were discarded. 16S rRNA gene sequences were clustered into Operational Taxonomic Units (OTUs) at a similarity cutoff value of 97% using the UPARSE algorithm.[906] OTUs were mapped to an optimized version of the SILVA Database[907] containing only the 16S v4 region to determine taxonomies. Abundances were recovered by mapping the demultiplexed reads to the UPARSE OTUs. A custom script constructed a rarefied OTU table from the output files generated in the previous two steps for downstream analyses of alpha-diversity, beta-diversity, and phylogenetic trends using the *Agile Toolkit for Incisive Microbial Analyses* (ATIMA) platform (https://atima1.jplab.net).

Behavioral Assays. Behavioral assays were performed as previously described[63] and are briefly described below. Animals were acclimated for 30–60 minutes prior to all behavioral experiments. Apparatuses were spot cleaned with 70% EtOH after each animal and thoroughly cleaned at the end of the day. AnyMaze automated software version 4.99 (Stoelting) was used.

Reciprocal social interaction. Mice were placed in a neutral, 25 cm^3 Plexiglass arena with either a familiar or stranger age- and sex-matched conspecific matched for maternal diet. The time a pair of mice engaged in social interaction (close following, touching, nose-to-nose sniffing, nose-to-anus sniffing, and/or crawling over/under each other) over ten minutes was recorded by a blinded human observer and analyzed via AnyMaze.

Crawley's three-chamber test for sociability and preference for social novelty. Crawley's three-chamber test for sociability and preference for social novelty was performed as described.[908] Animals were habituated for 10 minutes in a 60 X 40 X 23 cm arena divided into three inter-connected chambers. Sociability was measured during a second 10-minute interval in which the test subject could interact either with an empty wire cup (empty) or a wire cup containing an age- and sex-matched stranger conspecific (mouse 1). Interactions were automatically scored by AnyMaze software and by an independent observer. Empty cup placement in the left or right chamber during the sociability period was counterbalanced between trials. Preference for social novelty was assayed by introducing a second stranger mouse (mouse 2) into the previously empty wire cup. Interactions were again recorded by AnyMaze software and an independent observer.

Contextual fear conditioning. Mice were first habituated to the contextual fear conditioning chamber (Coulburn, Actimetrics) for twenty minutes. 24h later, during the training phase of the protocol, mice were placed in the and allowed to explore for 3 minutes. An auditory stimulus (80dB white noise) was then presented for 30 seconds as the auditory conditioned stimulus (CS). A co-terminating foot shock (0.5 mA, 2 seconds duration), applied via the floor grid (the unconditioned stimulus; US), was delivered during the last 2 seconds of the CS. A second presentation of the paired CS and the US was delivered at the 4 minutes mark and the animal was left in the cage for another 30s. At both 24h and 48h post-conditioning, animals were re-introduced to the chamber for 5 min in the absence of US to evaluate the contextual fear memory. Acquisition and analysis of freezing behavior, our readout of mouse memory, was automatically performed in FreezeFrame/View software (Actimetrics).

Open field test. Animal test subjects were gently lowered into an open arena (40 X 40 X 20 cm) and allowed to freely explore for 10 minutes. AnyMaze automatically measured distance

traveled, speed, and position in the arena, as well as time spent in the center of the arena (defined as the interior 20 X 20 cm).

Glucose tolerance and insulin sensitivity tests. For intraperitoneal glucose tolerance tests (IPGTT), mice were fasted for 6 hours and then baseline blood glucose measurements were acquired with an ACCU-CHEK® blood glucose monitoring system, using approximately 5 µl of blood obtained *via* tail vein puncture. Intraperitoneal injections of 20% glucose solution at a dose of 2 g glucose/kg body weight were then administered to each mouse in rapid succession.[909] Blood glucose was measured again 15, 30, 60, and 120 minutes after glucose injection and then the mice were re-fed. For intraperitoneal insulin tolerance tests (IPITT), mice were fasted for 4.5 hours and then baseline blood glucose measurements were acquired from each mouse. Intraperitoneal injections of 0.25 IU of human recombinant insulin (MP Biomedicals, #193900) solution at a dose of 0.75 IU insulin/kg body weight were then administered to each mouse in rapid succession.[910] Blood glucose was measured again at 15, 30, 60, and 120 minutes after insulin injection and then the mice were re-fed. Curves displaying blood glucose concentration over time were generated, and statistical analysis of difference between groups and area under the curve (AUC) were calculated using GraphPad Prism 8 software (La Jolla, CA).

Whole Genome Shotgun Sequencing (WGS). WGS was performed as previously described.[63] Briefly, individual libraries constructed from each sample were loaded onto the HiSeq platform (Illumina) and sequenced using the 2 × 100 base pair (bp) pair-end read protocol. Illumina paired-end libraries were constructed from total genomic DNA isolated from each sample. The DNA was sheared into approximately 400–600 bp fragments followed by ligation of Illumina adaptors containing molecular barcodes for downstream de-multiplexing. These products were then amplified through ligation-mediated PCR (LM-PCR) using KAPA HiFi DNA Polymerase

(Kapa Biosystems, Wilmington, MA, USA). Following bead purification with Agencourt AMPure XP (Beckman Coulter, Brea, CA, USA), quantification and size distribution of the LM-PCR product was determined using the LabChip GX electrophoresis system (PerkinElmer, Akron, OH, USA). Libraries were pooled in equimolar amounts at six samples per pool and prepared for sequencing with TruSeq PE Cluster Generation Kit (Illumina). Each library pool was loaded onto one lane of a HiSeq 2000 flow cell spiked with 1% PhiX control library. Sequencing files were de-multiplexed with CASAVA version 1.8.3 (Illumina). Quality filtering, trimming and de-multiplexing was carried out by a custom pipeline containing Trim Galore and Cutadapt (Martin, 2011) for adaptor and quality trimming, and PRINSEQ (Schmieder and Edwards, 2011) for low complexity filtering and sequence deduplication. In addition, Bowtie2 v2.2.1 was used to map reads to MetaPhlAn markers for the classification of bacterial species (Segata et al., 2012).

***B. vulgatus* growth and administration to mice.** *B. vulgatus* ATCC 8482 was obtained from the American Type Culture Collection (ATCC) and was cultured in supplemented BHI consisting of Bacto Brain Heart Infusion broth (BD, #237500) + 2% yeast extract (Fisher Bioreagents, #BP1422-500) + 0.2% L-Cysteine (Sigma, #C7352-100G), at 37˚C, in an Anaerobe Systems AS-580 anaerobic chamber with a 5% H_2, 5% CO_2, and 90% N_2 anaerobic gas mixture. For growth analysis in the presence of varying concentrations of metformin, metformin hydrochloride (TCI, #M2009) was added to supplemented BHI to final concentrations of 0 mM (vehicle), 10 mM, or 20 mM and then filter sterilized. Growth curves were generated by culturing *B. vulgatus* in 10 ml of supplemented BHI for 24 hours, withdrawing 100 µl of culture at specified intervals to measure OD_{600nm} using a BioTek Epoch plate reader with untreated, clear plastic 96 well plates. Treatment of mice with *B. vulgatus* was performed by lacing Mott's® No Sugar Added Applesauce with concentrated *B. vulgatus*. To do this, 15 ml of *B. vulgatus* per animal was

grown for 24 hours in supplemented BHI, then concentrated and suspended in 100 µl of PBS. The resulting *B. vulgatus* suspension was then rapidly mixed in 1 ml of applesauce and left on food pellets in the bottom of each mouse cage. Vehicle controls were given applesauce with 100 µl of PBS per 1 ml applesauce in the same manner. *B. vulgatus*-laced applesauce or vehicle control applesauce was administered at 5:00 PM, 3 days per week. To determine the viability of *B. vulgatus* in sugar-free applesauce, *B. vulgatus* was cultured and suspended in applesauce as described[64]. The applesauce suspension was then serially diluted in PBS and plated on BHI agar plates and incubated anaerobically at 37˚C for 48 hours, after which time colonies were counted to calculate colony forming units per milliliter (CFU/ml).

***B. vulgatus*-specific 16S rDNA quantification by qPCR.** Mouse fecal pellets were obtained in a sterile environment and immediately frozen at -80˚C before further use. Bacterial DNA was isolated from fecal pellets with the QIAamp PowerFecal DNA Kit (QIAGEN, #12830-50) using a bead homogenizer to lyse bacteria. Relative abundance of *B. vulgatus* was quantified by qPCR using PowerUp SYBR Green Master Mix (Applied Biosystems, #A25742) on an Applied Biosystems StepOnePlus Real-Time PCR system. Abundance of *B. vulgatus* specific 16S rDNA relative to universal bacterial 16S rDNA was calculated using the comparative Ct method (2-ΔΔCt method). Primer sequences used for qPCR analysis were *B. vulgatus* 16S Forward: 5'-CGATTGGTCTGGCACGTATG-3'; *B. vulgatus* 16s Reverse: 5'-ACTTCATTGTCACGCA CATTCAT-3'; Universal bacterial 16S Forward: 5'-ACTCCTACGGGAGGCAGCAGT-3'; Universal bacterial 16S Reverse: 5'-ATTACCGCGGCTGCTGGC-3'.

Metformin treatment. For administration of metformin to mice, human-equivalent dose of 200 mg/kg/day metformin hydrochloride[904] was added to mouse water bottles to a final volume of 300 ml. Metformin-laced water was replenished 3 times per week.

KEGG pathway analysis. A map of the BCAA biosynthesis pathway was adapted from the Kyoto Encyclopedia of Genes and Genomes (keg.jp). Each gene encoding a protein involved in BCAA biosynthesis, identified by KEGG, was searched in the NCBI Gene (https://www.ncbi.nlm.nih.gov/gene/) for presence in both *B. vulgatus* and *L. reuteri.*

BCAA analysis. To quantify BCAA concentration in mouse serum, mouse blood was isolated by cardiac puncture under deep anesthesia once behavioral and body composition tests were complete. Blood serum was isolated by centrifugation in SST Microtainer tubes (BD, #365967) and snap-frozen over dry ice. The samples were stored at -80C until used. Total BCAA concentration was quantified using a colorimetric Branched Chain Amino Acid Assay Kit (Abcam, #ab83374), and colorimetric change was measured at λ_{max} = 450 nm on an Epoch plate reader (BioTek).

Western blotting. Once behavioral and body composition tests were complete, region-specific brain tissue isolates (hippocampus, VTA, somatosensory cortex, and hypothalamus) were rapidly collected, snap-frozen over liquid nitrogen, and stored at -80 °C until further processing. Samples were homogenized in buffer containing [200 mM HEPES, 50 mM NaCl, 10% Glycerol, 1% Triton X-100, 1 mM EDTA, 50 mM NaF, 2 mM Na_3VO_4, 25 mM β-glycerophosphate, and Pierce protease inhibitor cocktail (Thermo Scientific)]. Sample protein content was quantified with Pierce rapid gold BCA protein assay kit (Thermo Scientific) and a total of 60 µg of protein/sample was resolved on SDS–PAGE (12%) and transferred onto 0.45 µm nitrocellulose membranes

(Biorad). Membranes were blocked in 5% BSA in 1x TBS + 0.2% Tween (Biorad) for 1 h, followed by incubation with primary antibodies diluted in 5% BSA in 1x TBS + 0.2% Tween overnight at 4 °C. Membranes were then washed with 1x TBS-T five times and incubated with fluorescent secondary antibodies for 1h at RT, and washed with 1x TBS-T three times. Bands were detected with Odyssey® Fc imaging System. *Antibodies.* Primary antibodies for Western blotting were rabbit anti-β-Actin Antibody #4967, mouse anti-S6 Ribosomal Protein #2317, rabbit anti Phospho-S6 Ribosomal Protein (Ser240/244) #2215, mouse anti-eIF2α #2103, rabbit anti-Phospho-eIF2α (Ser51) #9721 (all from Cell Signaling and Technology Laboratories, Danver, MA). Secondary antibodies were IRDye® 800CW Goat anti-Rabbit and IRDye® 680RD Goat anti-Mouse IgG (Licor).

Immunofluorescence. Mice were deeply anesthetized by inhalation of isoflurane and perfused transcardially with 10 mL 0.9% phosphate-buffered saline followed by 30 mL 4% paraformaldehyde in 0.1M phosphate buffer (PFA). Brains were post-fixed in 4% PFA at 4°C overnight, then cryoprotected in 30% sucrose 0.1M PB. 30 μm-thick coronal slices containing the hippocampal formation were obtained from frozen tissue using a sliding blade microtome and then transferred to ice cold PBS. Slices were blocked with 5% normal goat serum, 0.3% Triton X-100 0.1M PB (PBTgs) for 1 hr rocking at RT and then incubated in primary antibodies (mouse anti-S6 Ribosomal Protein Cell signaling #2317 1:200; rabbit anti Phospho-S6 Ribosomal Protein (Ser240/244) Cell signaling #2215 1:200) diluted in PBTgs rocking at 4°C for 24 hr. Slices were then washed three times with 0.3% Triton X-100 0.1M PB. Primary antibodies were visualized using secondary goat anti-rabbit Alexa Fluor 488 (ThermoFisher Scientific, #A-11034) and goat anti-mouse Alexa Fluor 594 (ThermoFisher Scientific, #A-11032) antibodies (1:1,000 dilution). Slices were incubated in secondary antibodies rocking in the dark for 1h at room temperature.

Five-minute final washes with each of PBTgs, 0.1M PB, and 0.05M PB preceded mounting onto 2% gelatin (Sigma-Aldrich, #G9391)-coated coverslips. Nuclei were visualized using Vectashield H-1200 with DAPI (Vector Labs, #H-1200). Fluorescent imaging and data acquisition was performed on an Axio Imager.Z2 microscope (Carl Zeiss Imaging) mounted with an Axiocam 506 6MP mono digital camera (Carl Zeiss Imaging) and Apotome.2 structured illumination slider (Carl Zeiss Imaging). Images were captured using ZEN acquisition software (Carl Zeiss Imaging). All images within a given dataset were acquired at identical exposure times, within a given channel, to allow comparison of signal intensity. In some images, contrast and brightness were linearly adjusted using Photoshop (Adobe). Image processing was applied uniformly across all images within a given dataset.

Statistical analysis. Power analyses to establish group size were performed in GPower 3.1. Data were analyzed and visualized using GraphPad Prism version 8.4.3 for Mac OS X, GraphPad Software, San Diego, California USA, www.graphpad.com. Data are presented as mean ± SEM. P-values are presented in the figure legends; n values are provided in the figures. $p < 0.05$ was considered significant. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Outliers were identified using Grubbs' test and excluded from analysis for that assay. For behavioral experiments, statistics were based on the two-sided unpaired Student's *t* tests, one- or two-way ANOVA with Bonferroni post hoc analysis to correct for multiple comparisons, unless otherwise indicated.

3. Results.

High-fat diet-feeding alters the composition of the gut microbiome while exerting minimal effects on total body weight at four weeks on diet.

Previous studies[63,911], performed by our group and others, investigating the effects of HFD consumption on maternal and offspring gut microbiome composition were based on maternal diet feeding schedule in which mating of dams occurred after eight weeks of HFD regimen, a time point at which the animals displayed a significant weight gain compared to regular diet (RD)-fed mice. However, we reasoned that if the behavioral and metabolic phenotypes in MHFD offspring were causally related to MHFD-induced dysbiosis, then they should manifest in MHFD offspring dependent on the timing of maternal gut microbiome dysbiosis, not on the timing of maternal obesity onset. Furthermore, the eight-week-long feeding schedule does not allow for the dissociation of the effects of diet-induced changes in the gut microbiome from maternal weight gain. To account for this, we reduced the maternal diet feeding schedule to only four weeks HFD exposure prior to mating to produce MHFD offspring yet minimize the effects of maternal adiposity. All offspring were placed on regular diet after weaning (**Fig. 1a**). At the time of mating, dam total body mass was not significantly different between the HFD- and the RD-fed groups (**Fig. 1b**). Similarly, data body mass analysis revealed no significant differences in lean mass between HFD- and RD-fed mice after four weeks on diet (**Fig. 1c**). However, compared to RD-fed dams, HFD-fed females showed a significant increase in fat mass within one week on diet that is sustained at four weeks on diet (**Fig.1d**).

To assess temporal alterations in the gut microbiome in HFD-fed female mice, we performed longitudinal metagenomic 16S ribosomal RNA (rRNA) gene amplicon sequencing of fecal microbiota to determine the microbial composition and community structure of the fecal microbiome of RD-fed *vs*. HFD-fed. Principal coordinates analysis of unweighted UniFrac distances from the averaged, rarefied 16S rRNA gene sequencing dataset, a method to assess the community structure based on operational taxonomic unit (OTU) presence/absence, found that a single week of HFD consumption is sufficient to substantially shift maternal gut ecology. Indeed, as early as 1 and 4 weeks on diet (**Fig. 1e, i**), fecal microbiota from RD-fed mice cluster separately from fecal microbiota of HFD-fed mice, illustrating a significant divergence between the community structures. To further assess the HFD-driven changes in the gut microbiome, we evaluated two α-diversity indices, including the number of observed OTUs and Shannon index. Both measures demonstrate significantly decreased α-diversity at 1 week on diet (**Fig. 1f-g**), which is maintained at 4 weeks (**Fig. 1j-k**). Additionally, we assessed the ratio of phyla Firmicutes to Bacteroidetes (F:B ratio). Importantly, increased F:B abundance has been reported to be associated with dysbiosis of the gut microbiome in both obesity[912-914] and autism[60,915], even though other studies did not observe any modifications of the F:B ratio or even reported decreased F:B ratio in ASD[916,917] or obese individuals[918,919], suggesting that compositional changes at family, genus, or species level, might be more relevant than this parameter.[920] This comparison of changes in major phyla revealed that, while at 1 week on diet there are no significant differences in the F:B ratio between RD- and HFD-fed (**Fig. 1h**), the F:B ratio is increased in the HFD-fed group compared to RD-fed mice at 4 weeks on diet the precise time at which we began mating the dams (**Fig. 1l**). Taken together, these data suggest that four weeks of HFD results in pathologically-associated alterations in the composition of the gut microbiome, including decreased α-diversity and

increased F:B ratio. The lack of significant changes in body weight at these time points also minimizes the effect of increased adiposity in this model, compared to other commonly used models which extend HFD-feeding by additional weeks.

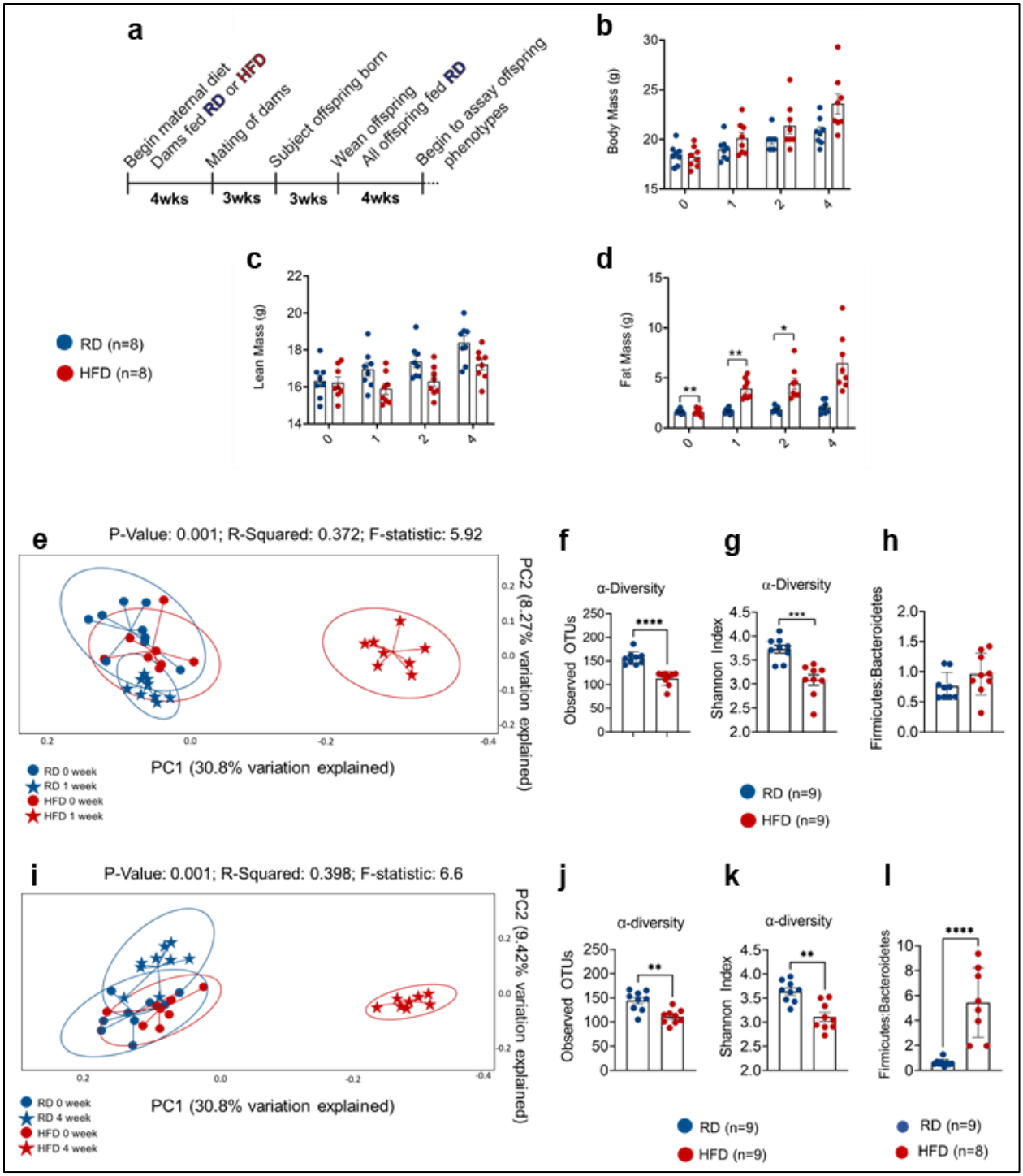
a
Begin maternal diet
Dams fed RD or HFD
Mating of dams
Subject offspring born
Wean offspring
All offspring fed RD
Begin to assay offspring phenotypes
4wks
3wks
3wks
4wks
b
Body Mass (g)
c
Lean Mass (g)
d
Fat Mass (g)
RD (n=8)
HFD (n=8)
e
P-Value: 0.001; R-Squared: 0.372; F-statistic: 5.92
PC1 (30.8% variation explained)
PC2 (8.27% variation explained)
RD 0 week
RD 1 week
HFD 0 week
HFD 1 week
f
α-Diversity
Observed OTUs
g
α-Diversity
Shannon Index
RD (n=9)
HFD (n=9)
h
Firmicutes:Bacteroidetes
i
P-Value: 0.001; R-Squared: 0.398; F-statistic: 6.6
PC1 (30.8% variation explained)
PC2 (9.42% variation explained)
RD 0 week
RD 4 week
HFD 0 week
HFD 4 week
j
α-diversity
Observed OTUs
k
α-diversity
Shannon Index
RD (n=9)
HFD (n=9)
l
Firmicutes:Bacteroidetes
RD (n=9)
HFD (n=8)

Figure 1. Four weeks HFD-feeding increases fat mass and shifts gut microbiome composition in female mice. (**a**) Schematic of the maternal diet regimen and breeding. Female breeders are started on diet at six weeks of age. (**b**) RD- and HFD-fed mice total body mass is not significantly different at 4 weeks on diet (*week 1, p=0.9859, t=0.4557; week 2, p=0.4545, t=1.574; week 3, p=0.3951, t=1.717; week 4, p=0.1194, t=2.504*) (**c**) No significant differences in lean mass were observed between RD- and HFD-fed mice at four weeks on diet (*week 1, p=0.9994, t=0.2018; week 2, p=0.1510, t=2.272; week 3, p=0.1082, t=2.451; week 4, p=0.1104, t=2.451*) (**d**) HFD-fed mice showed a significant increase in fat mass within one week on diet that was sustained at four weeks on diet (*week 1, p=0.9994, t=0.2018; week 2, p=0.1510, t=2.272; week 3, p=0.1082, t=2.451; week 4, p=0.1104, t=2.451*) (**e**) Principal coordinates analysis (PCoA) of unweighted UniFrac distances from the averaged rarefied 16S rRNA gene amplicon sequencing dataset showed that fecal microbiome samples from RD-fed mice clustered separately from those of HFD-fed mice after one week on diet. (*$p < 0.001$, $R_2 = 0.372$, F-statistic=5.92*) (**f**) A decrease in α-diversity, measured as total number of OTUs, is observed the HFD-fed females compared to the RD-fed counterpart after one week on diet (*Mann-Whitney U = 0, P = <0.0001*) and (**g**) α-diversity, represented in terms of Shannon index, is also decreased in HFD-fed dams (*Mann-Whitney U = 2, p= 0.0002*). (**h**) No significant phylum-level (Firmicutes:Bacteroidetes ratio) were observed changes after one weeks on diet (*Mann-Whitney U = 22, p= 0.1135*). (**i**) PCoA of unweighted UniFrac distances from the averaged rarefied 16S rRNA gene sequencing dataset showed that showed that fecal microbiome samples from RD-fed mice clustered separately from those of HFD-fed mice after four weeks on diet. (*$p < 0.001$, R2 = 0.398, F-statistic=5.92*) (**j**) Decrease α-diversity (total number of OTUs) was again seen in HFD mice and was maintained at four weeks on diet (*Mann-Whitney U = 7, P = 0.0019*). (**k**) α-diversity (Shannon index) is also

decreased in HFD females at four weeks on diet (*Mann-Whitney U = 7, P = 0.0019*). (**l**) Phylum-level changes characterized by a significant increase in the Firmicutes:Bacteroidetes ratio are evident after four weeks on diet (*Mann-Whitney U = 0, p<0.0001*).

Four weeks MHFD intake induces dysbiosis of gut microbiota, social deficits in offspring.

Recently, we demonstrated that eight weeks HFD-feeding prior to conception induces autism-like social dysfunction in MHFD offspring, a finding corroborated by epidemiological evidence showing that maternal obesity is associated with autism spectrum disorder (ASD)[761,921] and other neurodevelopmental disorders in humans.[521] Here, to further validate our novel murine diet-induced maternal obesity model, we performed: *(1)* longitudinal metagenomic 16S rRNA gene amplicon sequencing to assess the microbiota composition and community structure in the feces of MHFD fed and maternal regular diet (MRD) offspring, and *(2)* a battery of behavioral tests aimed to assess social behaviors in the offspring of RD- and HFD-fed dams. Principal coordinates analysis of unweighted UniFrac distances from the averaged, rarefied 16S rRNA gene sequencing dataset revealed that MRD offspring fecal microbiota was significantly different from MHFD offspring fecal microbiota (**Fig. 2a**), consistent with what we previously reported in the eight-week-fed, HFD model and with what we in the four-week HFD-fed dams (**Fig. 1i,e**). The MHFD offspring gut microbiome exhibits decreased α-diversity, measured as number of observed OTUs (**Fig. 2b**) and increased evenness (and therefore, decreased diversity), as measured by the Shannon diversity index (**Fig. 2c**), compared to the MRD offspring group.

We next investigated the effects of maternal HFD on MHFD offspring social behavior. We evaluated reciprocal social interactions by recording the amount of time a pair of mice, unfamiliar with each other, spent interacting in a neutral arena (**Fig. 2d**). Pairs of MHFD offspring spent less time interacting with each other than MRD pairs (**Fig. 2e**). To further assess MHFD offspring social behavior we performed Crawley's three-chamber (3C) social test (**Fig. 2f -h**) to evaluate (1)

sociability by comparing the time mice spent interacting with an empty cup versus one containing a mouse and (2) preference for social novelty by measuring the time mice spent interacting with a familiar versus a stranger mouse. Unlike MRD offspring, MHFD offspring demonstrated decreased interest in social interaction and our previous findings (**Fig. 2j**) and loss of preference for social novelty (**Fig. 2k**), each consistent with the results of the reciprocal social interaction test. We did not observe any differences in locomotor activity, in terms of distance travelled, during the habituation phase of the 3C test (**Fig. 2i**), between the two groups. Taken together, these data indicate that four-weeks maternal HFD intake is sufficient to induce a shift in the gut microbiota of MHFD offspring and to invoke the pathology underlying social deficits in MHFD offspring.

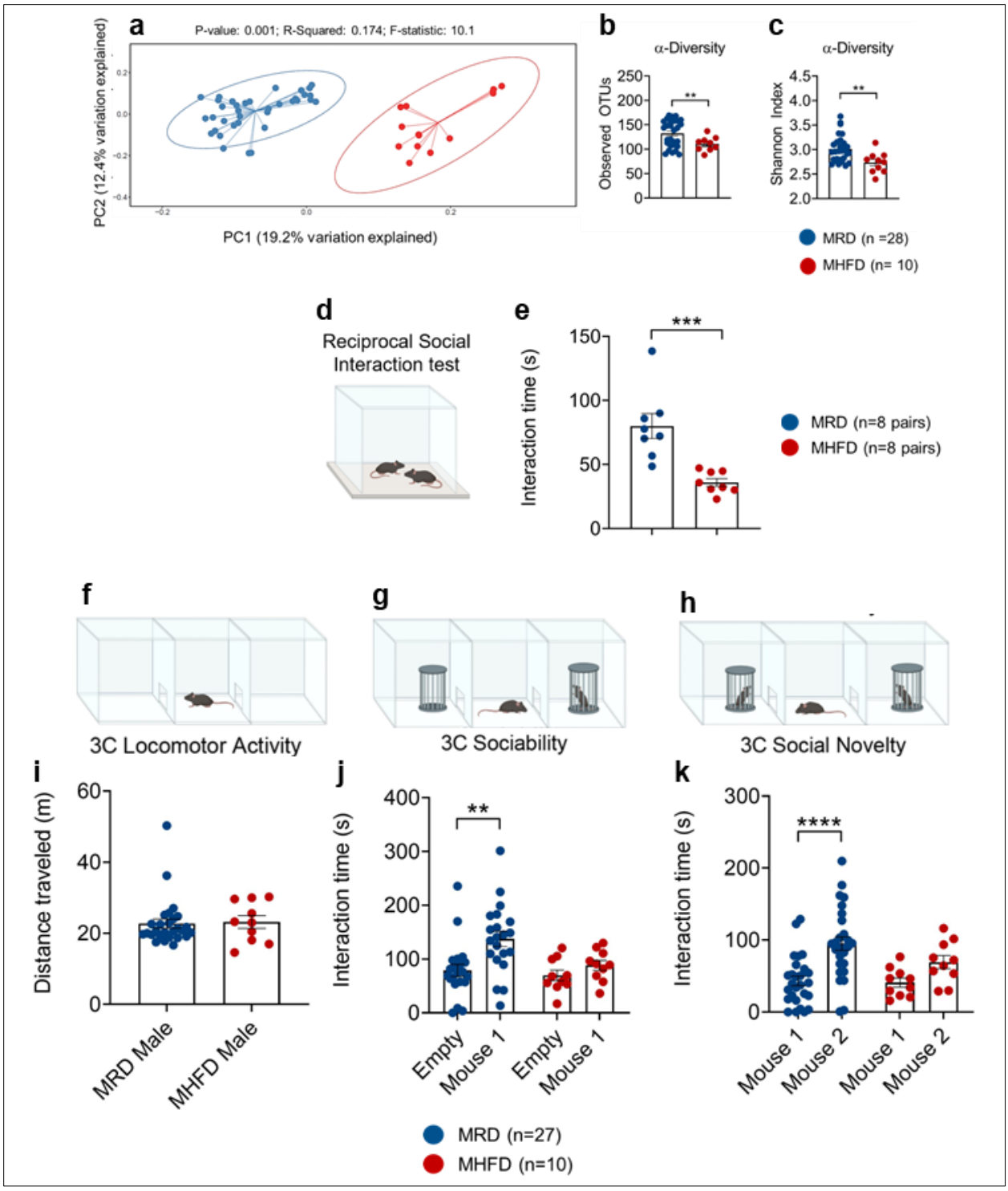
a
P-value: 0.001; R-Squared: 0.174; F-statistic: 10.1
PC2 (12.4% variation explained)
PC1 (19.2% variation explained)
b
α-Diversity
Observed OTUs
c
α-Diversity
Shannon Index
MRD (n =28)
MHFD (n= 10)
d
Reciprocal Social Interaction test
e
Interaction time (s)
MRD (n=8 pairs)
MHFD (n=8 pairs)
f
3C Locomotor Activity
g
3C Sociability
h
3C Social Novelty
i
Distance traveled (m)
MRD Male
MHFD Male
j
Interaction time (s)
Empty
Mouse 1
k
Interaction time (s)
Mouse 1
Mouse 2
MRD (n=27)
MHFD (n=10)

Figure 2. Four weeks MHFD intake induces dysbiosis of gut microbiota, social deficits in offspring. (**a**) Principal coordinates analysis (PCoA) of unweighted UniFrac distances from the averaged rarefied 16S rRNA gene dataset revealed that MRD offspring fecal microbiota cluster separately from MHFD offspring fecal microbiota. (*$p < 0.001$, $R_2 = 0.174$, F-statistic=10.1*). (**b**) A decrease in α-diversity, measured as total number of OTUs, is observed in the MHFD group compared to the MRD mice (*Mann-Whitney U = 31, P <0.0001*) and (**c**) α-diversity, measured as Shannon index, is also decreased in MHFD mice (*Mann-Whitney U = 58, P = 0.0055*) (**d**) schematic of the reciprocal social interaction test (**e**) MHFD offspring showed reduced interaction time compared to MRD offspring controls in the reciprocal social interaction test. (*Mann-Whitney U = 0, p=0.0002*). (**f-h**) schematic of the three-chamber test. (**f,i**) no changes in distance travelled between MHFD- and MRD offspring controls (*Mann-Whitney U = 116, p=0.5330*). (**g,j**) MHFD mice show impaired sociability compared to the MRD mice (*MHFD, p=0.6783, t =0.7879, MRD, p= 0.0052, t = 3.122; maternal diet effect F1,70 = 5.277, p =0.0246)* (**h,k**) social novelty preference is decreased on the MHFD offspring compared to the MRD offspring (*MHFD, p=0.1944, t = 1.655, MRD, p < 0.0001, t = 4.493; maternal diet effect F1,70 = 15.59, p=0.002*). (Data in **a**–**j** reflect MRD and MHFD male cohorts)

MHFD-induced shift in the gut microbiome results in cognitive deficits, but not anxiety-like behavior in offspring.

Given that maternal obesity is associated with cognitive deficits in human offspring[877,922,923], we sought to determine whether we could model maternal HFD-associated cognitive deficits in offspring of HFD-fed female mice. To this end, we tested both MRD and MHFD offspring in the hippocampus-dependent contextual fear conditioning (CFC) paradigm, which is a well-established tool for assessing memory performance, by applying a foot-shock after a tone is played and then assessing freezing behavior after the same tone is played in the same context 24 hours later.[924] Our data show that MHFD offspring underperformed controls in the CFC paradigm (**Figs. 3a-b**), indicated by a significant decrease in the amount of time spent freezing during the test stage, demonstrating that MHFD offspring develop hippocampal-dependent memory impairments. To further explore the effect of MHFD on offspring behavior, we performed the open field test to assess hyperactivity and anxiety-like behavior (**Fig. 3c**). Consistent with data from the habituation phase of the 3C test (**Fig. 2i**), we did not observe any change in the distance travelled (**Fig. 3d**) nor in the time spent in the center which is associated with anxiety-like behavior in mice (**Fig. 3e**).

To determine the causal relationship between MHFD offspring gut microbiome dysbiosis and cognitive function, we assessed whether co-housing MHFD with MRD offspring rescued MHFD hippocampus-dependent memory. The co-housing protocol allows for coprophagic behavior of rodents, which results in consistent transfer of gut microbiota between co-housed mice, via the fecal-oral route.[625] In our experiments, we co-housed one MHFD offspring mouse with three MRD offspring mice at the time of weaning (3 weeks of age), while control cages contained either four MHFD offspring or MRD offspring (**Fig. 3f**). We found that co-housing with MRD

offspring rescued hippocampal dependent-memory in MHFD offspring in the CFC paradigm (**Fig. 3h-i**). To assess whether co-housing with MRD offspring could also correct the alterations in the MHFD offspring gut microbiome, we examined the composition of MHFD offspring co-housed with MRD controls and observed an increase in α-diversity, measured as number of OTUs present in the fecal samples, thus suggesting that co-housing was sufficient to rescue the microbial phylogenetic profile of MHFD offspring (**Fig. 3g**). Taken together, these findings suggest that MHFD-drive dysbiosis of the gut microbiome is causally related to offspring cognitive impairment.

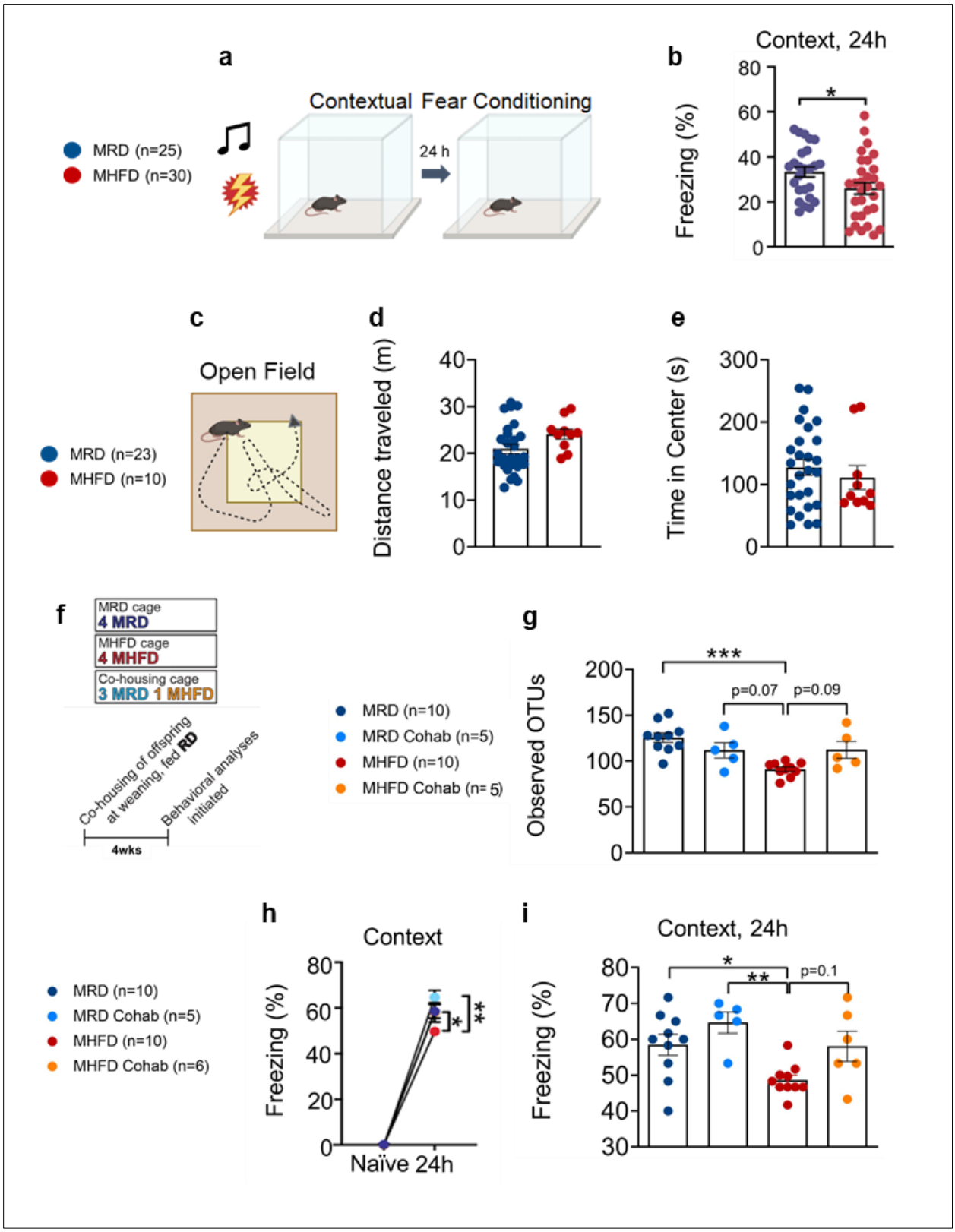
a
Contextual Fear Conditioning
MRD (n=25)
MHFD (n=30)
24 h
b
Context, 24h
Freezing (%)
c
Open Field
MRD (n=23)
MHFD (n=10)
d
Distance traveled (m)
e
Time in Center (s)
f
MRD cage
4 MRD
MHFD cage
4 MHFD
Co-housing cage
3 MRD 1 MHFD
Co-housing of offspring at weaning, fed RD
Behavioral analyses initiated
4wks
MRD (n=10)
MRD Cohab (n=5)
MHFD (n=10)
MHFD Cohab (n= 5)
g
Observed OTUs
p=0.07
p=0.09
h
Context
Freezing (%)
Naïve 24h
MRD (n=10)
MRD Cohab (n=5)
MHFD (n=10)
MHFD Cohab (n=6)
i
Context, 24h
Freezing (%)
p=0.1

Figure 3. Four weeks MHFD intake induces cognitive deficits in MHFD offspring, which can be rescued by co-housing MHFD with MRD offspring. (**a**) Schematic of contextual fear conditioning (CFC) paradigm. (**b**) MHFD offspring long-term memory performance was decreased relative to MRD offspring (ordinary one-way ANOVA, *Mann-Whitney U=422, p=0.0079*) (**c**) Schematic of the open field test. (**d**) No significant changes in distance travelled (*Mann-Whitney U=80, p=0.0616*) or time spent exploring the center of the open field arena (**e**) (*Mann-Whitney U=115, p=0.5110*) was found between the two groups. (**f**) Schematic of the co-housing experiment: MHFD and MRD offspring were weaned into one of three cage arrangements for four weeks prior to perform behavioral tests. (**g**) Co-housing with MRD offspring increased α-diversity (total number of OTUs) of the MHFD offspring gut microbiome (*ordinary one-way ANOVA, F=8.80, p=0.0003*). (**h-i**) MHFD offspring performance in the contextual fear conditioning test was rescued by co-housing with MRD offspring (*ordinary one-way ANOVA, F=5.49, p=0.0045*). (Data in **a-i** reflect MRD and MHFD male cohorts).

MHFD induces metabolic dysfunction in offspring.

Given that maternal obesity predisposes offspring to metabolic syndrome, we aimed to assess whether MHFD affected offspring metabolic function. We began by measuring total body weight of both MRD and MHFD offspring at eight and 20 weeks of age. We observed a small but significant decrease in MHFD offspring weight at eight weeks of age compared to MRD group, regardless of sex (**Fig. 4a-b**), consistent with the literature.[925] Interestingly, at 20 weeks of age MHFD offspring weight was comparable to MRD offspring weight in both males and females (**Fig. 4c-d**).

Next, we investigated glucose homeostasis in the two groups. To this end, we performed the intra-peritoneal glucose tolerance test (IPGTT) (**Fig. 4e**) which is used to assess the rate of glucose clearance from the blood. Impaired ability to efficiently clear glucose from circulation is a hallmark of diabetes and is associated with increased adiposity.[926] Although MRD and MHFD offspring glucose tolerance is comparable at eight weeks of age regardless of sex (**Fig. 4f,g**), at 20 weeks of age both male and female MHFD offspring exhibit delayed clearance of glucose from the blood after receiving a glucose bolus, compared to MRD offspring controls (**Fig. 4h,i**).

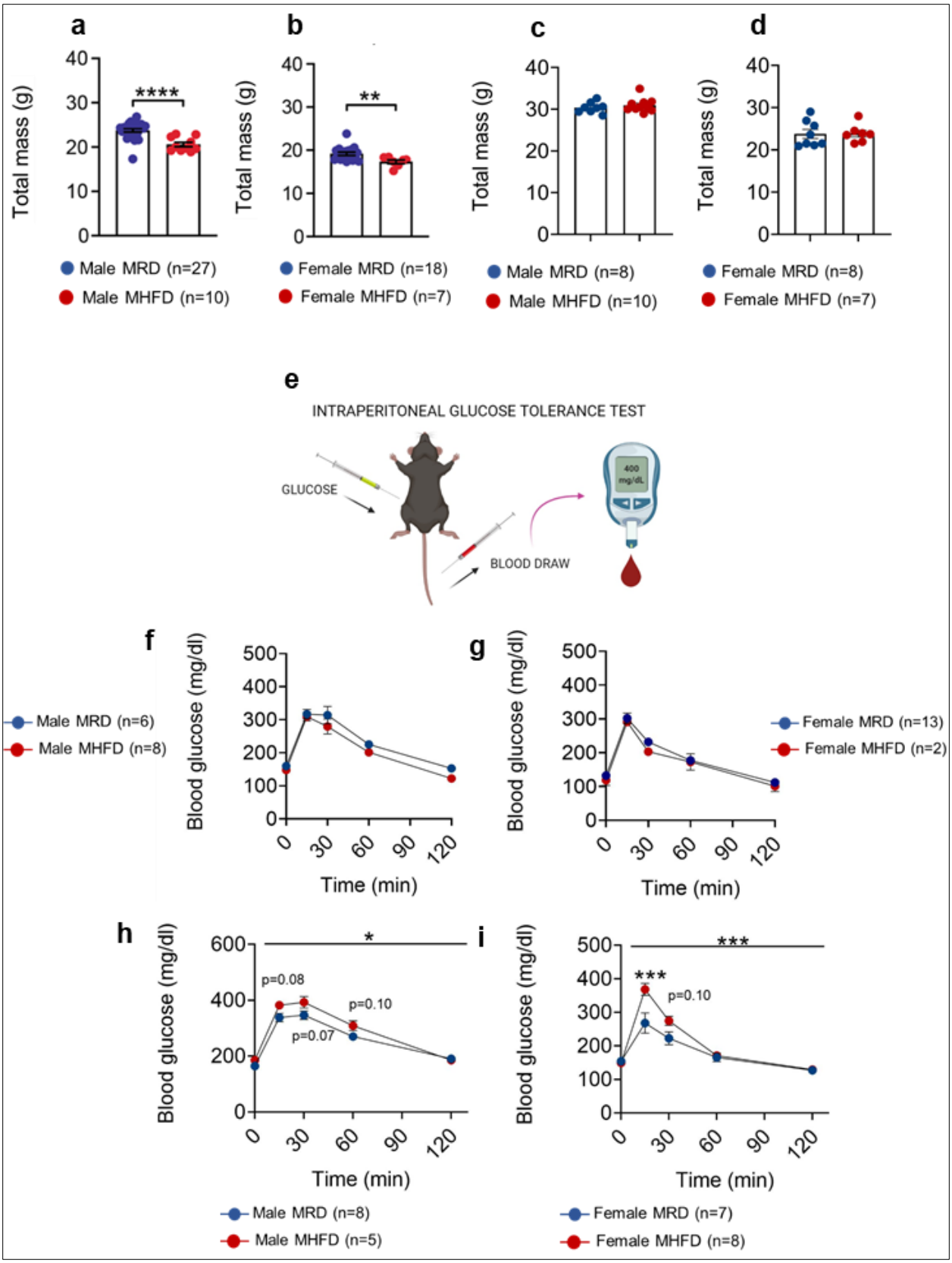
a
b
c
d
Total mass (g)
****
**
Male MRD (n=27)
Male MHFD (n=10)
Female MRD (n=18)
Female MHFD (n=7)
Male MRD (n=8)
Male MHFD (n=10)
Female MRD (n=8)
Female MHFD (n=7)
e
INTRAPERITONEAL GLUCOSE TOLERANCE TEST
GLUCOSE
BLOOD DRAW
400 mg/dL
f
g
Blood glucose (mg/dl)
Time (min)
Male MRD (n=6)
Male MHFD (n=8)
Female MRD (n=13)
Female MHFD (n=2)
h
i
*
***
p=0.08
p=0.10
p=0.07
Male MRD (n=8)
Male MHFD (n=5)
Female MRD (n=7)
Female MHFD (n=8)

Figure 4. MHFD offspring weight and glucose tolerance. Male (**a**) and female (**b**) MHFD offspring weight was significantly lower than MRD offspring weight at eight weeks of age (*females, p=0.0069, Mann-Whitney U=20; males, p<0.0001, Mann-Whitney U=25.50*). (**c,d**) For both sexes, MHFD offspring weight is comparable to MRD offspring weight at 20 weeks of age (*females, p=0.6117, Mann-Whitney U=23; males, p=0.6179, Mann-Whitney U=34*). (**e**) Schematic of the intra-peritoneal glucose tolerance test (IPGTT) measuring fasted blood glucose levels over a two-hour period following an intraperitoneally injected glucose challenge. (**f,g**) MRD and MHFD offspring glucose tolerance is comparable at eight weeks of age for both males (**f**) and females (**g**). (**h,i**) Compared to MRD offspring, MHFD offspring glucose tolerance is impaired at 20 weeks of age for both males (**h**) (*15mins, p=0.08, t =2.538, 30mins, p= 0.07, t = 2.260; maternal diet effect F1,8 = 5.445, p =0.0479)* and females (**i**). (*15mins, p=0.0003, t =4.638; maternal diet effect F 4,23 = 8.3990, p =0.0002)*

HFD alters the abundance of specific bacteria in both the maternal and offspring gut.

To identify changes in the gut microbiome of MRD *vs*. MHFD offspring at the level of single species, recently[63] we performed metagenomic whole genome shotgun (WGS) sequencing of fecal samples from MRD and MHFD offspring. Our results showed that, despite an overall decrease in microbial diversity, the abundances of particular taxa are significantly increased in the gut of MHFD offspring, such as the opportunistic pathogen *B. vulgatus* (**Fig. 5a**). Remarkably, *B. vulgatus* abundance was increased by >1,700-fold in MHFD vs. MRD offspring, representing 12% of the content of the MHFD offspring fecal microbiota, instead of <1% of MRD offspring fecal microbiota. Here, we performed 16S ribosomal RNA gene amplicon sequencing of maternal and offspring fecal samples which provided insights about the effect of HFD regimen on the abundance of specific bacteria at the genus level. Within one week on HFD, we observed an increase in genus *Bacteroides*, to which *B. vulgatus* belongs (**Fig. 5b**), in the maternal gut microbiome, which was maintained at four weeks on HFD (**Fig. 5c**). Remarkably, we observed a similar increase in the MHFD offspring (**Fig.5d**). These data show that MHFD induces an increase in *Bacteroides* which is then transmitted to, or at least recapitulated in, the offspring, thus propagating diet-induced alterations into the subsequent generation.

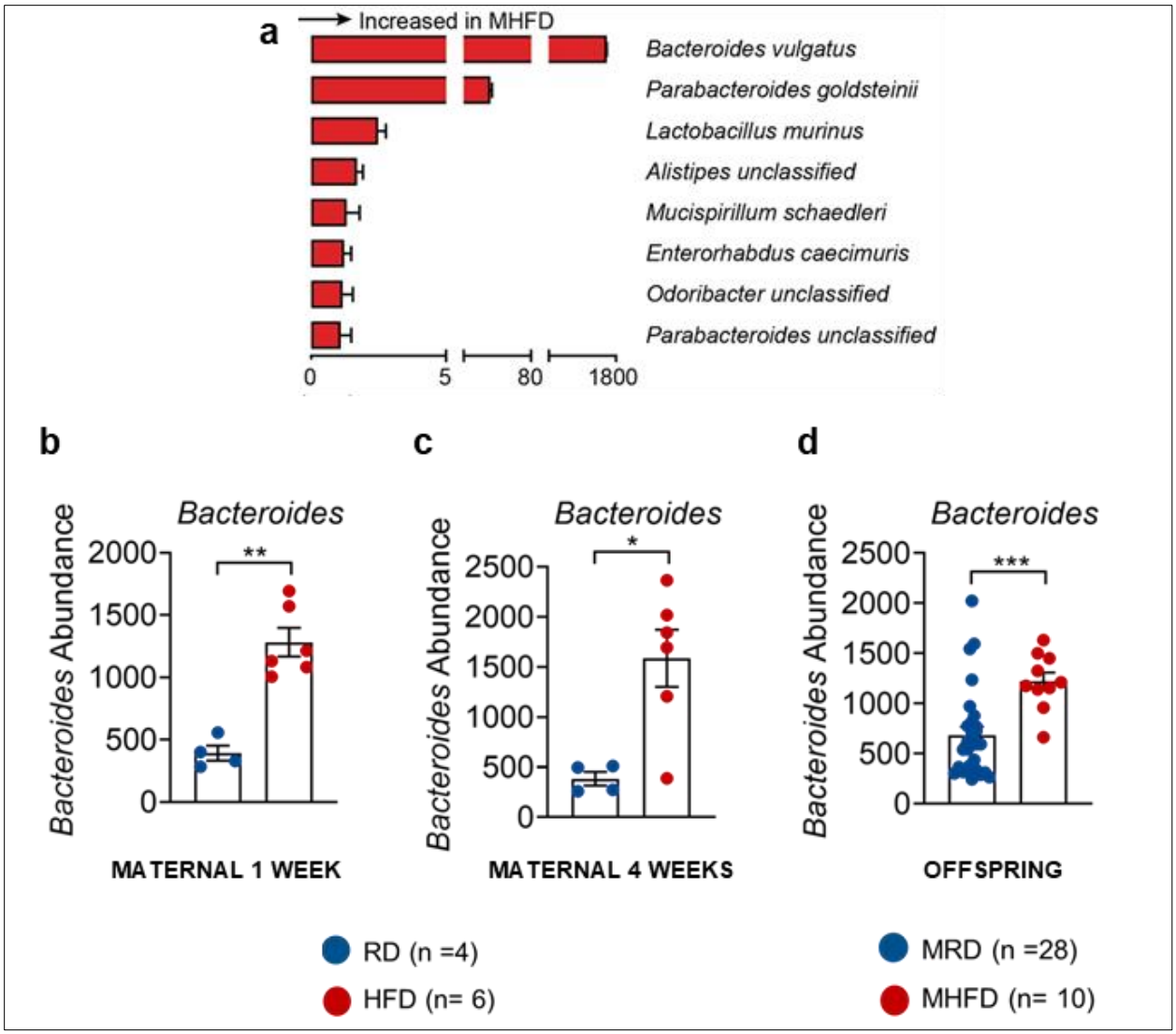
a
Increased in MHFD
Bacteroides vulgatus
Parabacteroides goldsteinii
Lactobacillus murinus
Alistipes unclassified
Mucispirillum schaedleri
Enterorhabdus caecimuris
Odoribacter unclassified
Parabacteroides unclassified
0
5
80
1800
b
Bacteroides
Bacteroides Abundance
2000
1500
1000
500
0
**
MATERNAL 1 WEEK
c
Bacteroides
Bacteroides Abundance
2500
2000
1500
1000
500
0
*
MATERNAL 4 WEEKS
d
Bacteroides
Bacteroides Abundance
2500
2000
1500
1000
500
0
***
OFFSPRING
RD (n =4)
HFD (n= 6)
MRD (n =28)
MHFD (n= 10)

Figure 5. MHFD increases the abundance of genus *Bacteroides* in both maternal and offspring gut microbiome. (**a**) Metagenomic shotgun sequencing of fecal samples from both MHFD and MRD offspring (from 8-week on diet dams) identified species whose relative abundance was increased in the MHFD offspring microbiota Among these, *B. vulgatus* was the most dramatically increased in the MHFD microbiota population, compared to the MRD microbiota. (two-sample Student's *t*-test; P=0.0069, *t*=2.95). (**b**) 16S ribosomal RNA gene amplicon sequencing of maternal fecal samples revealed an increased abundance of *Bacteroides* in HFD- vs. RD-fed dams at 1 week on diet (Mann-Whitney $U = 0$, $P = 0.0095$). (**c**) The increase in *Bacteroides* is maintained in HFD-fed dams at 4 weeks on diet (*Mann-Whitney U =2, p=0.0381*). (**d**) MHFD offspring fecal samples contained a higher proportion of *Bacteroides* compared to MRD offspring (*Mann-Whitney U = 42, p=0.0007*). (Data in **a,d** reflect MRD and MHFD male cohorts)

Increased *B. vulgatus* is responsible for cognitive and metabolic deficits in MHFD offspring.

Given that *B. vulgatus* abundance is increased in the MHFD offspring microbiome and *B. vulgatus* is a known opportunistic pathogen associated with autism[60], childhood obesity[60,901], and insulin resistance[633] in humans, we decided to focus our study on this species to determine whether specific microbial taxa altered in the MHFD offspring gut microbiome were casually related to the cognitive and metabolic phenotypes observed in the MHFD descendants.

To this end, we cultured *B. vulgatus* (an obligate anaerobe) in anaerobic conditions (**Fig. 6b**) and established its viability in sugar-free applesauce, the vehicle by which administered it (or the control treatment, growth media) to mice **(Fig. 6c**). At 72h post-inoculation, we successfully recovered live *B. vulgatus* from fecal matter of treated mice, demonstrating that *B. vulgatus* successfully colonizes its host (**Fig. 6d**). To perform our experiment, we introduced live *B. vulgatus* into the diet of conventionally colonized MRD offspring at weaning (**Fig. 6a**) then performed a longitudinal study of offspring behavior and metabolic function. Remarkably, we determined that colonization with *B. vulgatus* alone is sufficient to induce MHFD offspring-like metabolic and cognitive behavioral phenotypes. Indeed, long-term *B. vulgatus* treatment impairs glucose tolerance, as determined by IPGTT and glucose area under the curve (AUC), which is an index of whole glucose excursion after glucose loading (**Fig. 6e,f**), but does not induce insulin resistance, as determined by intraperitoneal insulin tolerance test (IPITT) (**Fig. 6g**), in 20-week-old hosts. These data suggest *B. vulgatus* treatment decreases glucose tolerance, without impairing insulin function. To assess the effect of *B. vulgatus* on cognitive function, we performed the open field test in treated mice *vs*. media-treated controls and found that *B. vulgatus* treatment did not

alter host locomotor activity (**Fig. 6i**) or anxiety-like behavior (**Fig. 6j**). However, *B. vulgatus*-treated host memory performance was decreased relative to media-treated mice at 24h (**Fig. 6l**) and significantly impaired at 48h (**Fig. 6m–n**) post-training in the CFC paradigm. Taken together, these data show that the selective increase in *B. vulgatus* in the gut microbiota of MHFD offspring accounts, at least in part, for their cognitive and metabolic dysfunction.

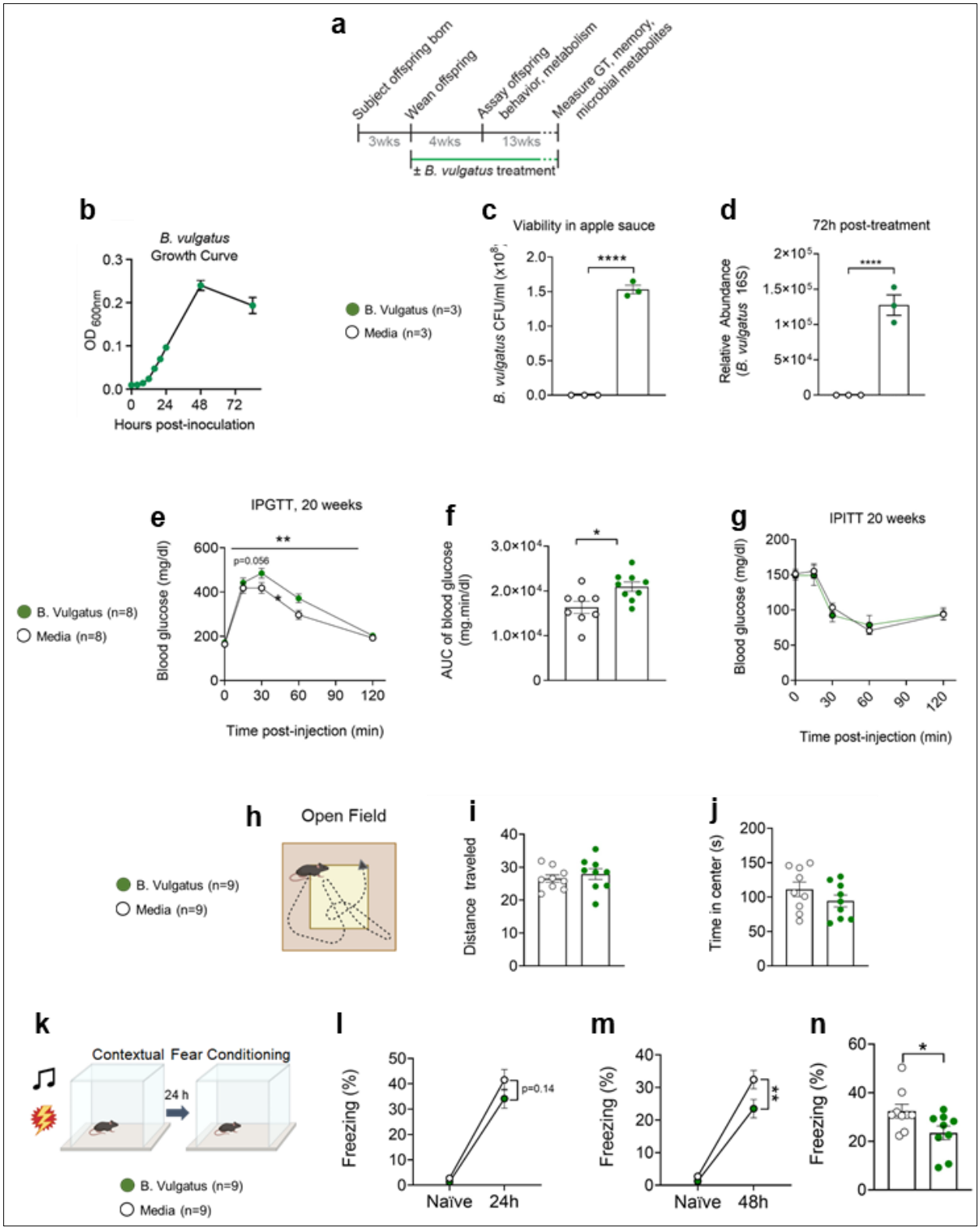
a
Subject offspring born
Wean offspring
Assay offspring behavior, metabolism
Measure GT, memory, microbial metabolites
3wks
4wks
13wks
± B. vulgatus treatment
b
B. vulgatus Growth Curve
OD 600nm
Hours post-inoculation
B. Vulgatus (n=3)
Media (n=3)
c
Viability in apple sauce
B. vulgatus CFU/ml (x10^8)
****
d
72h post-treatment
Relative Abundance (B. vulgatus 16S)
****
e
IPGTT, 20 weeks
Blood glucose (mg/dl)
Time post-injection (min)
B. Vulgatus (n=8)
Media (n=8)
**
p=0.056
f
AUC of blood glucose (mg.min/dl)
*
g
IPITT 20 weeks
Blood glucose (mg/dl)
Time post-injection (min)
h
Open Field
B. Vulgatus (n=9)
Media (n=9)
i
Distance traveled
j
Time in center (s)
k
Contextual Fear Conditioning
24 h
B. Vulgatus (n=9)
Media (n=9)
l
Freezing (%)
p=0.14
Naïve
24h
m
Freezing (%)
**
Naïve
48h
n
Freezing (%)
*

Figure 6. Colonization with the opportunistic pathogen *B. vulgatus* impairs glucose metabolism and cognitive behavior in MRD offspring. (**a**) Schematic of *B. vulgatus* treatment. (**b**) Growth curve of *B. vulgatus* in anaerobic culture. (**c**) *B. vulgatus* suspended in applesauce and exposed to atmospheric O_2 for 10 minutes was isolated and re-grown in an anaerobic environment, indicating survival between the anaerobic chamber and the host gastrointestinal system (*$p<0.0001$, $t=24.50$*). (**d**) Live *B. vulgatus* was detected in host feces 72h-post-inoculation by qPCR targeting the 16S rRNA gene (*$p<0.0001$*). (**e-f**) Long-term *B. vulgatus* treatment impaired glucose tolerance, measured by IPGTT, in 20-week-old-hosts represented as change in (**e**) blood glucose over time (30 min, *$p=0.056$, $t=2.590$; 60 min, $p=0.0253$, $t=2.884$; treatment factor, $p=0.0021$, $T(1,75)$*=10.17) and (**f**) area under the curve (AUC) (*$p=0.0148$, $t=2.754$*). (**g**) Insulin tolerance was normal in *B. vulgatus*-treated 20-week-old hosts (*$p=0.3108$*). (**h**) Schematic of the open field test. (**i,j**) No significant changes in distance travelled or time spent exploring the center of the open field arena between *B. vulgatus* vs. media treated groups. (*i, $p < 0.5034$, $t = 0.6846$; j, $p < 0.2337$, $t = 1.238$*). (**k**) Schematic of the contextual fear conditioning paradigm. (**l-n**) *B. vulgatus*-treated host memory performance was decreased relative to media-treated mice at 24h (*$p=0.14$, $t=1.856$*) **(l)** and significantly impaired at 48h (**m,n**) post-training in the contextual fear conditioning paradigm (*m, $p= 0.096$, $t=3.032$; n, $p=0.0395$,* t=2.242). (Data in **d-n** reflect MRD and MHFD male cohorts.)

Maternal metformin treatment throughout pregnancy and lactation alters the community structure of the maternal and offspring gut microbiota.

If the increase in *B. vulgatus* abundance is causally related to cognitive and metabolic dysfunction in MHFD offspring, then we reasoned that targeted reduction of maternal *B. vulgatus* load during pregnancy would rescue offspring phenotypes. To this end, we employed the FDA-approved antidiabetic drug metformin, which has been reported to induce functional changes in the host gut microbiome, including negative selection of *Bacteroides* species, that are causally related to its ability to treat metabolic dysfunction.[903,904] We first established the ability of metformin to inhibit growth of *B. vulgatus in vitro* (**Fig. 7a**), consistent with previous reports[904], then treated the dams with metformin at a human-equivalent dose (200mg/kg/day in drinking water, *ad libitum*)[904], from the start of the diet regimen (RD or HFD), through pregnancy and lactation, until offspring weaning (**Fig. 7b**). To determine whether maternal metformin treatment could rescue MHFD offspring health outcomes by altering the composition of the maternal and/or offspring gut microbiota, we performed 16S rRNA gene amplicon sequencing on fecal samples collected from dams and offspring. Consistent with our previous findings[63], unweighted UniFrac analysis of the 16S dataset showed significant effects of maternal diet, either RD or HFD, on both the maternal (**Fig. 7c-d**) and offspring (**Fig. 7c-e**) fecal microbial community structure. Furthermore, we observed an effect of metformin on the fecal microbiota of both RD- and HFD-fed dams (**Fig. 7c-d**) and their offspring (**Fig. 7c-e**), albeit to a lesser extent than maternal diet.

To evaluate how diet and metformin treatment act on maternal gut microbiome across time, *i.e.*, weeks on diet, we performed a longitudinal analysis of 16S rRNA gene amplicon sequencing data and found that maternal α-diversity of HFD-fed dams showed a progressive decrease of

microbial diversity over the four weeks on diet, which is exacerbated by metformin treatment (**Fig. 8a**). We also observed genus-level changes driven by maternal diet and metformin among dams. Indeed, maternal diet resulted in a decrease in the abundances of genera *Parabacteroides* and *Lactobacillus* (**Fig. 8c,d**), while increasing the abundance of genus *Bacteroides* (**Fig. 8b**). Metformin treatment paradoxically increased the abundance of *Parabacteroides* in HFD dams (**Fig. 8c**) but did not significantly alter either *Bacteroides* **(Fig. 8b)** or *Lactobacillus* **(Fig. 8d)** abundance in metformin *vs.* vehicle treated HFD mice.

MHFD induced a decrease in α-diversity in the offspring , regardless of maternal metformin treatment. (**Fig. 9a**). Additionally, neither diet nor metformin affected genus *Lactobacillus* abundance (**Fig. 9d**). Interestingly, however, while MHFD increased the abundance of *Parabacteroides*, maternal metformin treatment restored the *Parabacteroides* abundance in the MHFD + metformin group (**Fig. 9c**). Finally, consistent with our working hypothesis, maternal metformin significantly decreased the amount of *Bacteroides* in the gut of MHFD offspring; although, it had the opposite effect in MRD offspring (**Fig. 9b**). Together, our data suggest that maternal diet and metformin treatment induce long-lasting changes in both the maternal and offspring gut microbiota composition.

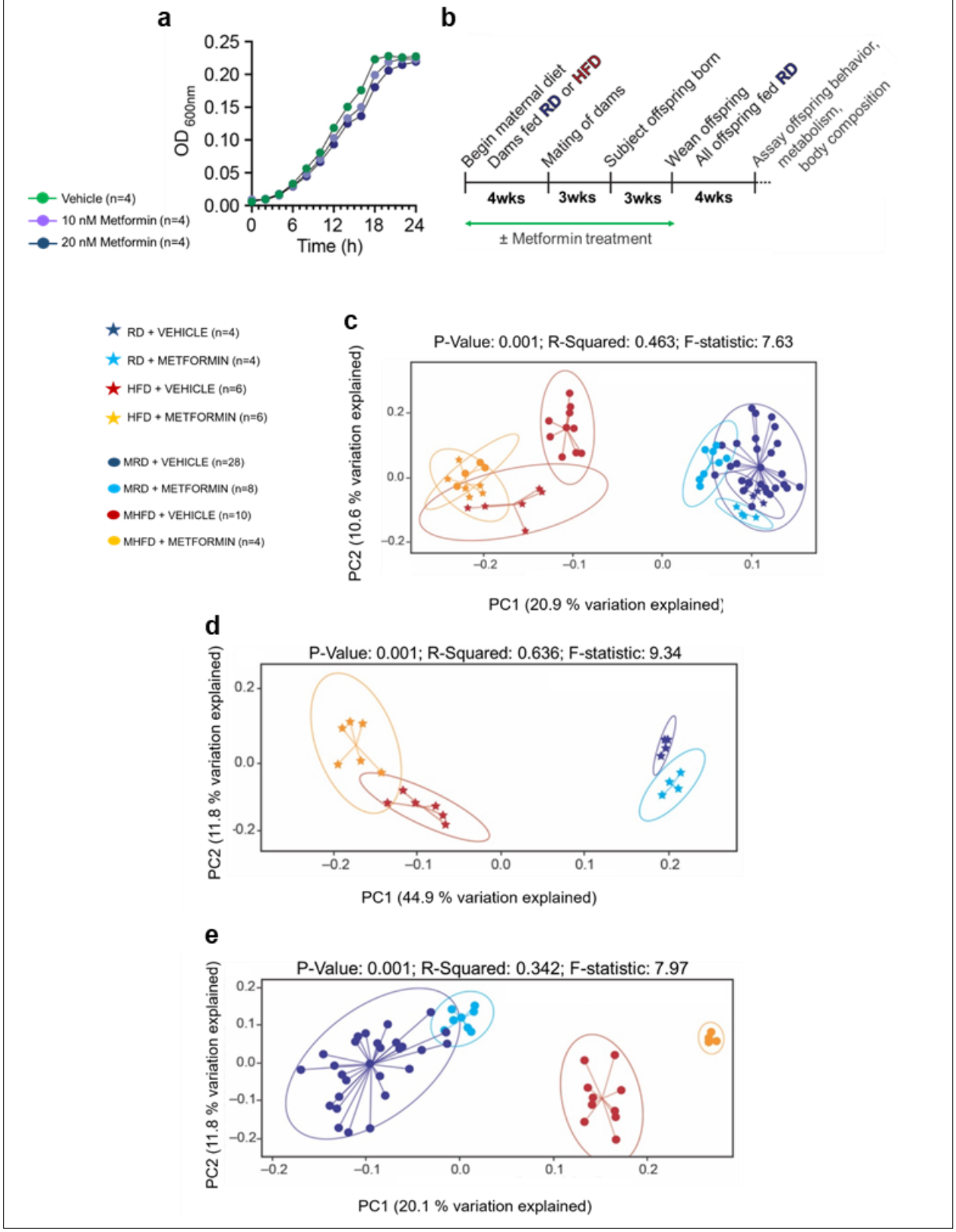
a
OD 600nm
0.25
0.20
0.15
0.10
0.05
0.00
0
6
12
18
24
Time (h)
Vehicle (n=4)
10 nM Metformin (n=4)
20 nM Metformin (n=4)
b
Begin maternal diet
Dams fed RD or HFD
Mating of dams
Subject offspring born
Wean offspring
All offspring fed RD
Assay offspring behavior, metabolism, body composition
4wks
3wks
3wks
4wks
± Metformin treatment
RD + VEHICLE (n=4)
RD + METFORMIN (n=4)
HFD + VEHICLE (n=6)
HFD + METFORMIN (n=6)
MRD + VEHICLE (n=28)
MRD + METFORMIN (n=8)
MHFD + VEHICLE (n=10)
MHFD + METFORMIN (n=4)
c
P-Value: 0.001; R-Squared: 0.463; F-statistic: 7.63
PC2 (10.6 % variation explained)
PC1 (20.9 % variation explained)
d
P-Value: 0.001; R-Squared: 0.636; F-statistic: 9.34
PC2 (11.8 % variation explained)
PC1 (44.9 % variation explained)
e
P-Value: 0.001; R-Squared: 0.342; F-statistic: 7.97
PC2 (11.8 % variation explained)
PC1 (20.1 % variation explained)

Figure 7. Maternal metformin treatment inhibits *B. vulgatus* growth *in vitro* and modifies the composition of the maternal and offspring gut microbiota. (**a**) Growth curve of *B. vulgatus* cultured in the absence of metformin (total area=2.872) or in the presence of 10 mM (total area=2.635) or 20 mM metformin (total area=2.474) in MRS media, anaerobically, for 24 hours. (**b**) Schematic of maternal metformin treatment and sample collection timeline. (**c–e**) Principal coordinates analysis of unweighted UniFrac distances from the 16S rRNA gene amplicon sequencing dataset showed significant effects of maternal diet and treatment on the maternal (**c,d**) and offspring (**c, e**) gut microbiota community structure. (*b, P-Value: 0.001; R-Squared: 0.463; F-statistic: 7.63; c, P-Value: 0.001; R-Squared: 0.636; F-statistic: 9.34; d, P-Value: 0.001; R-Squared: 0.342; F-statistic: 7.97*). (Data in **e** reflect MRD and MHFD male and female combined cohorts).

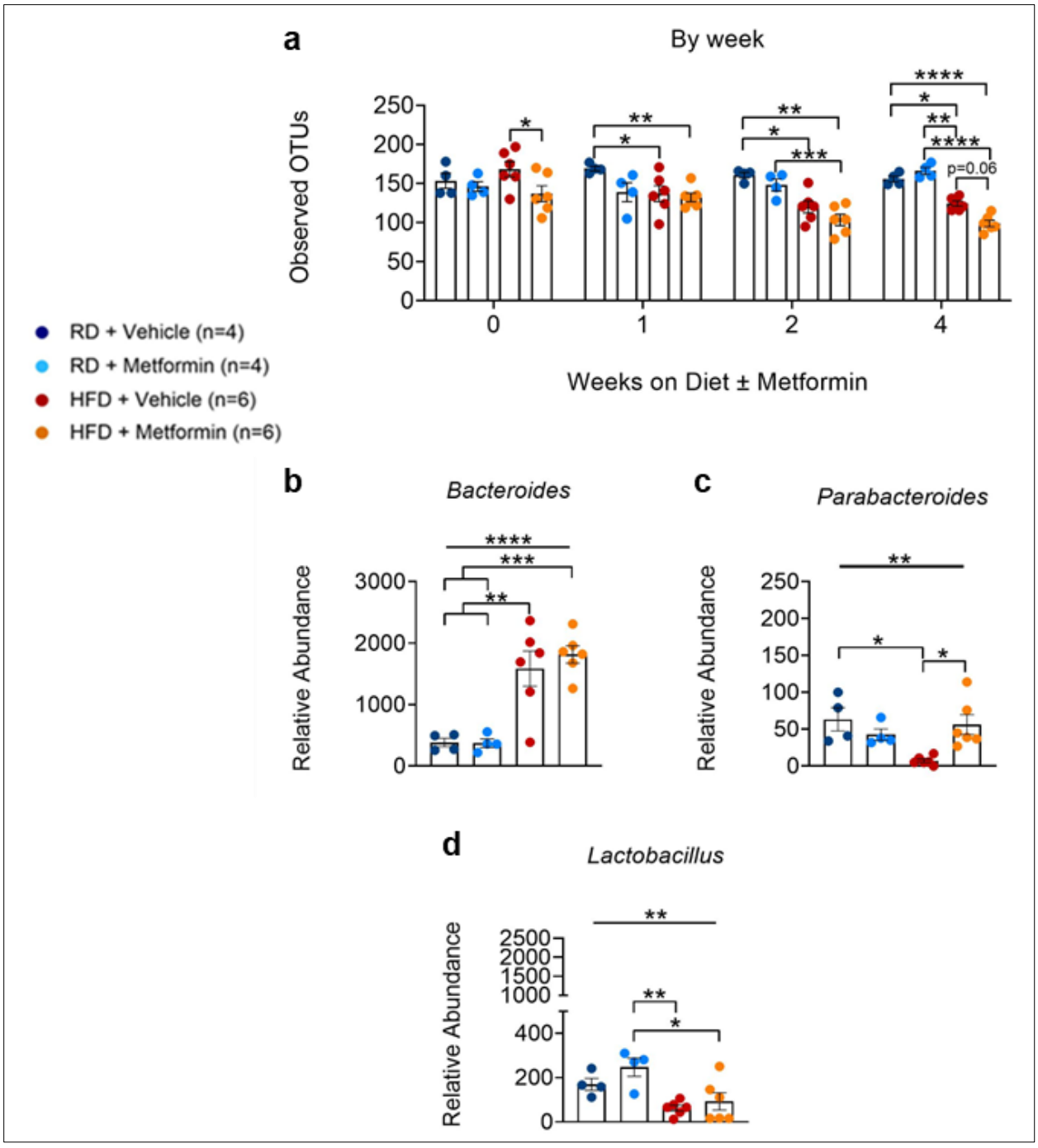
a
By week
Observed OTUs
250
200
150
100
50
0
*
**
*
**
*
***
*
**
****
****
p=0.06
0
1
2
4
Weeks on Diet ± Metformin
RD + Vehicle (n=4)
RD + Metformin (n=4)
HFD + Vehicle (n=6)
HFD + Metformin (n=6)
b
Bacteroides
Relative Abundance
3000
2000
1000
0
****
***
**
c
Parabacteroides
Relative Abundance
250
200
150
100
50
0
**
*
*
d
Lactobacillus
Relative Abundance
2500
2000
1500
1000
400
200
0
**
**
*

Figure 8. Maternal metformin treatment modifies the abundance of select genera in the maternal gut microbiota. (**a**) Longitudinal investigation of maternal gut microbiome composition showed that HFD decreased α-diversity (number of observed OTUs) of the maternal fecal microbiota, and metformin treatment increased this loss of diversity, data shown by week. (0 weeks, HFD + Veh. vs. HFD + Met. p=0.0135; 1 week, RD + Veh. vs. HFD + Veh. p=0.0266, RD + Veh. vs. HFD + Met. p=0.0083; week 2, RD + Veh. vs. HFD + Veh. p=0.0031, RD + Veh. vs. HFD + Met. p<0.0001, RD + Met. vs. HFD + Veh. p=0.0009; week 4, RD + Veh. vs. HFD + Veh. p=0.0336, RD + Veh. vs. HFD + Met. p<0.0001, RD + Met. vs. HFD + Veh. p=0.0022, RD + Met. vs. HFD + Met. p<0.0001) (**b-d**) Phylogenetic analysis revealed genus-level changes for *Bacteroides* (**b**) (ordinary one-way ANOVA F=14.47 p<0.0001). *Parabacteroides* (**c**) (ordinary one-way ANOVA F=5.913 p=0.0065) and *Lactobacillus* (**d**) (ordinary one-way ANOVA F=6.490 p=0.0044) in fecal microbiota, driven by maternal diet and metformin treatment. (Data in **a-d** reflect MRD and MHFD male and female combined cohorts)

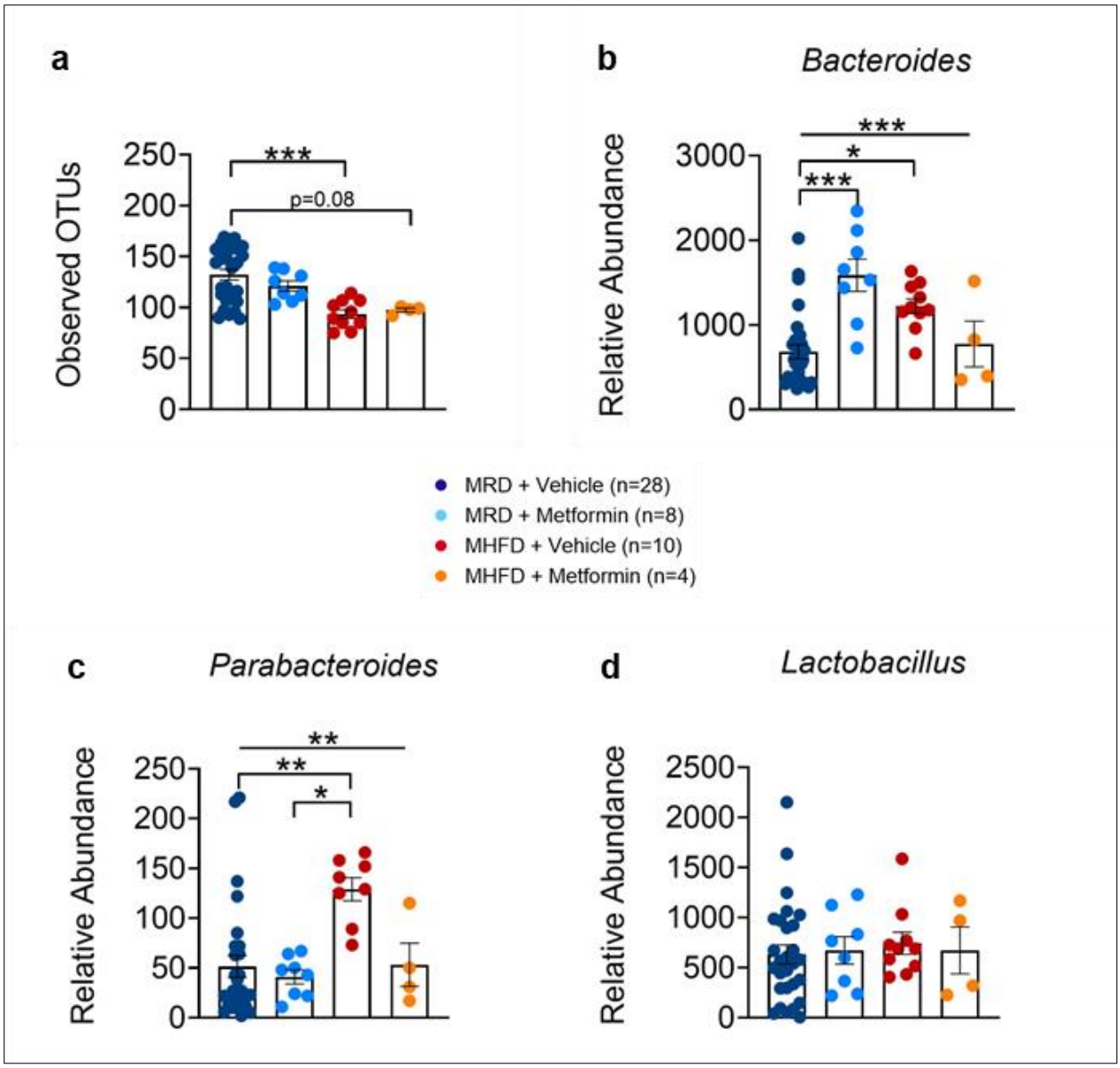
a
Observed OTUs
250
200
150
100
50
0
***
p=0.08
b
Bacteroides
Relative Abundance
3000
2000
1000
0
***
*
***
MRD + Vehicle (n=28)
MRD + Metformin (n=8)
MHFD + Vehicle (n=10)
MHFD + Metformin (n=4)
c
Parabacteroides
Relative Abundance
250
200
150
100
50
0
**
**
*
d
Lactobacillus
Relative Abundance
2500
2000
1500
1000
500
0

Figure 9. Maternal metformin treatment modifies the abundance of select genera in the offspring gut microbiota. (**a**) Regardless of metformin treatment, MHFD induced a decrease in α-diversity (number of observed OTUs) in offspring (ordinary one-way ANOVA F=8.665 p=0.0001) (**b-e**) Phylogenetic analysis revealed genus-level changes for *Bacteroides* (**b**) (MRD + Veh. vs. MRD + Met. p=0.0008; MRD + Veh. vs. MHFD + Veh. p=0.0180) and *Parabacteroides* (**c**) (MRD + Veh. vs. MHFD + Veh. p=0.0012; MRD + Met. vs. MHFD + Veh. p=0.0338)in fetal microbiota, driven by maternal diet and metformin treatment, while no changes were observed in *Lactobacillus* abundance (**d**). (Data in **a-d** reflect MRD and MHFD male and female combined cohorts)

Maternal metformin treatment throughout pregnancy and lactation rescue offspring metabolic and cognitive deficits.

Given that (*1*) metformin inhibits *B. vulgatus* growth (**Fig. 7a**)[904], (*2*) metformin has been shown to rescue cognitive and social deficits in a mouse model of Fragile X Syndrome (FXS)[927,928], and (*3*) our data showing the ability of metformin to alter maternal and offspring gut microbiome (**Figs. 7**,**8,9**), we aimed to determine whether maternal metformin treatment could rescue MHFD offspring metabolic and cognitive function. MRI-based body composition analysis revealed that low total (**Fig. 10a**) and lean (**Fig. 10b**) body mass at 8 weeks of age in MHFD offspring was corrected by maternal metformin treatment. Furthermore, maternal metformin treatment increased fat mass in MHFD, but not MRD, offspring (**Fig. 10c**), while offspring hydration ratio [(total water – free water)/lean mass] was unaffected by maternal diet or metformin treatment (**Fig. 10d**). Taken together, these results support the idea that maternal metformin treatment is effective in preventing some MHFD-associated changes in adolescent offspring body composition but does significantly increase fat mass exclusively in the offspring of HFD-fed females.

To determine if maternal metformin treatment likewise rescued MHFD offspring cognitive performance, we performed the CFC paradigm (**Fig. 10h**) and found that MHFD offspring contextual fear memory at 24h post-training was on par with MRD controls in the maternal metformin, but not vehicle, group (**Fig. 10i-j**). Additionally, maternal metformin treatment rescued mild hyperactivity in the open field task (**Fig. 10e**) in this cohort of MHFD offspring (**Fig. 10f**), while no change in anxiety-like behavior was detected in MRD or MHFD offspring ± maternal metformin (**Fig. 10g**). We also assessed whether metformin ameliorated MHFD offspring social deficits and found that maternal metformin treatment improved MHFD offspring sociability (**Fig.**

10o) and preference for social novelty performance (**Fig. 10p**) in the 3C task, while it had no effect on offspring locomotor activity in the habituation phase (**Fig. 10n**). Taken together, these results show a beneficial effect of maternal metformin treatment on both social and cognitive deficits in MHFD offspring. Furthermore, they provide evidence that targeting of the maternal gut microbiome is a potential therapy for preventing the onset of behavioral symptoms, and potentially the underlying molecular-to-circuit-level causes, in children exposed to MHFD-induced obesity *in utero*.

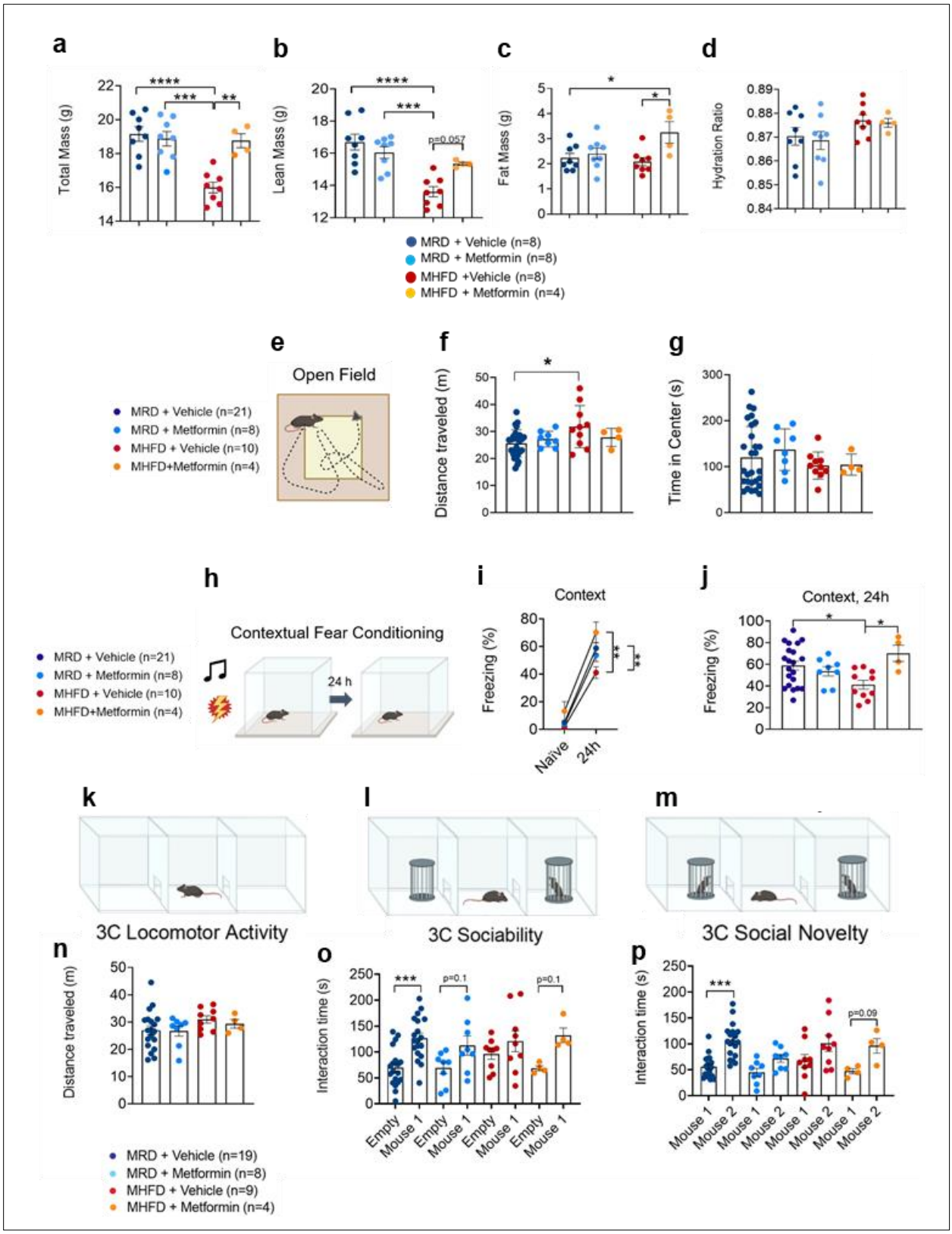
a
b
c
d
Total Mass (g)
Lean Mass (g)
Fat Mass (g)
Hydration Ratio
p=0.057
MRD + Vehicle (n=8)
MRD + Metformin (n=8)
MHFD +Vehicle (n=8)
MHFD + Metformin (n=4)
e
Open Field
MRD + Vehicle (n=21)
MRD + Metformin (n=8)
MHFD + Vehicle (n=10)
MHFD+Metformin (n=4)
f
Distance traveled (m)
g
Time in Center (s)
h
Contextual Fear Conditioning
24 h
i
Context
Freezing (%)
Naive
24h
j
Context, 24h
k
3C Locomotor Activity
l
3C Sociability
m
3C Social Novelty
n
Distance traveled (m)
MRD + Vehicle (n=19)
MRD + Metformin (n=8)
MHFD + Vehicle (n=9)
MHFD + Metformin (n=4)
o
Interaction time (s)
Empty
Mouse 1
p=0.1
p
p=0.09
Mouse 2

Figure 10. maternal metformin treatment ameliorates body mass composition and behavioral deficits in the MHFD offspring. (**a-d**) MRI-based body composition analysis revealed that the loss of total (**a**) (*MRD + Veh. vs. MHFD + Veh., p=<0.0001; MHFD + Veh. vs. MRD + Met., p=0.0001; MHFD + Veh. vs. MHFD + Met., p=0.0019;*) and lean (**b**) (*MRD + Veh. vs. MHFD + Veh., p=<0.0001; MHFD + Veh. vs. MRD + Met., p=0.0006; MHFD + Veh. vs. MHFD + Met., p=0.0574;*) body mass induced by MHFD in the offspring is rescued by maternal metformin treatment, while fat mass is increased in the MHFD + maternal metformin group **(c)** (*MRD + Veh. vs. MHFD + Met., p= 0.0432; MHFD + Veh. vs. MHFD + Met., p=0.0169;*). Offspring hydration ratio [(total water – free water)/lean mass] is unaffected by maternal diet or metformin treatment (**d**). (**e**) schematic of the open field test. (**f**) Mild hyperactivity in the open field test in this cohort of MHFD offspring was rescued by maternal metformin treatment. (MRD + Veh. vs MHFD + Veh., p=0.0155; MRD + Veh. vs. MHFD + Met., p=0.9977) (**g**) No change in anxiety-like behavior was detected in MRD or MHFD offspring ± maternal metformin. (**h**) schematic of the contextual fear conditioning paradigm. (**i**,**j**) Maternal metformin treatment rescued MHFD offspring cognitive performance at 24h in the CFC paradigm. (**j**, *MHFD + Met. vs MHFD + Veh., p=0.0140, Mann- Whitney U=3, MRD + Veh. vs MHFD + Veh., p=0.0116, Mann- Whitney U=116*) (**k-m**) schematic of the three-chamber test. Maternal metformin treatment had no effect on offspring locomotor activity in the habituation phase of the 3C test (**k,n**), improved MHFD offspring sociability (**l,o**) (*MRD + Veh. p= 0.0002, t = 4.332; MRD + Met. p=0.1, t=2.120; MHFD + Met. p=0.1, t=1.300; maternal diet effect F1,72 = 19.94, p =0.0001)* and preference for social novelty performance in the 3C test (**m,p**) (*MRD + Veh. p= 0.0002, t = 4.332; MHFD + Met. p=0.09, t=2.304; maternal diet effect F1,72 = 26.23, p<0.0001)*. (Data **a**–**p** reflect observations combined male and female MRD and MHFD cohort)

MHFD offspring show BCAA dysregulation which is rescued by maternal metformin treatment.

To identify the mechanism by which maternal high-fat diet (MHFD)-associated opportunistic pathogenic bacteria, such as *B. vulgatus*, impair host cognitive and metabolic function, we focused on the offspring serum metabolome, specifically on branched-chain amino acids (BCAA; valine, leucine, and isoleucine). BCAA dysregulation is associated with metabolic disorders[929,930], syndromic[825,826] and maternal environmental factor-associated autism[921], and maple syrup urine disorder[931] (a disease characterized by cognitive dysfunction). Furthermore, other studies[633] suggest that the increased concentration of circulating BCAAs observed insulin-resistant individuals is correlated with the concurrent increase in the abundance of *B. vulgatus* in their gut microbiome. Indeed, based on the Kyoto Encyclopedia of Genes and Genomes (KEGG; https://www.genome.jp/kegg/) pathway analysis[932], *B. vulgatus* possesses all genes required for complete biosynthesis of BCAAs from pyruvate, but not L-threonine. Conversely, *L. reuteri*, the species most reduced in the MHFD offspring group[63], possesses only one gene encoding an enzyme involved in BCAA biosynthesis, indicating that it is insufficient in BCAA biosynthesis (**Table 1**, **Fig. 11**). We performed a colorimetric assay to quantify the amount of BCAA in the serum of 9-week-old MRD *vs*. MHFD mice which revealed a 28% increase in circulating BCAA in MHFD offspring serum compared to MRD offspring, which is restored by maternal metformin treatment (**Fig. 12a**). Importantly, we detected this significant increase in circulating BCAA concentration *prior* to the onset of glucose intolerance, suggesting that the increased BCAA precedes metabolic dysfunction in MHFD offspring and may therefore be causally related to its onset, as is implicated in human studies. Given that *B. vulgatus,* which is significantly increased

in MHFD offspring (**Fig. 5a**), is genetically capable of producing BCAAs from pyruvate (**Fig. 11**) and that the MRD and MHFD offspring are on an identical diet from weaning, we reasoned that increased serum BCAA concentration in MHFD offspring is directly due to the increase in *B. vulgatus* in the intestinal microbiome.

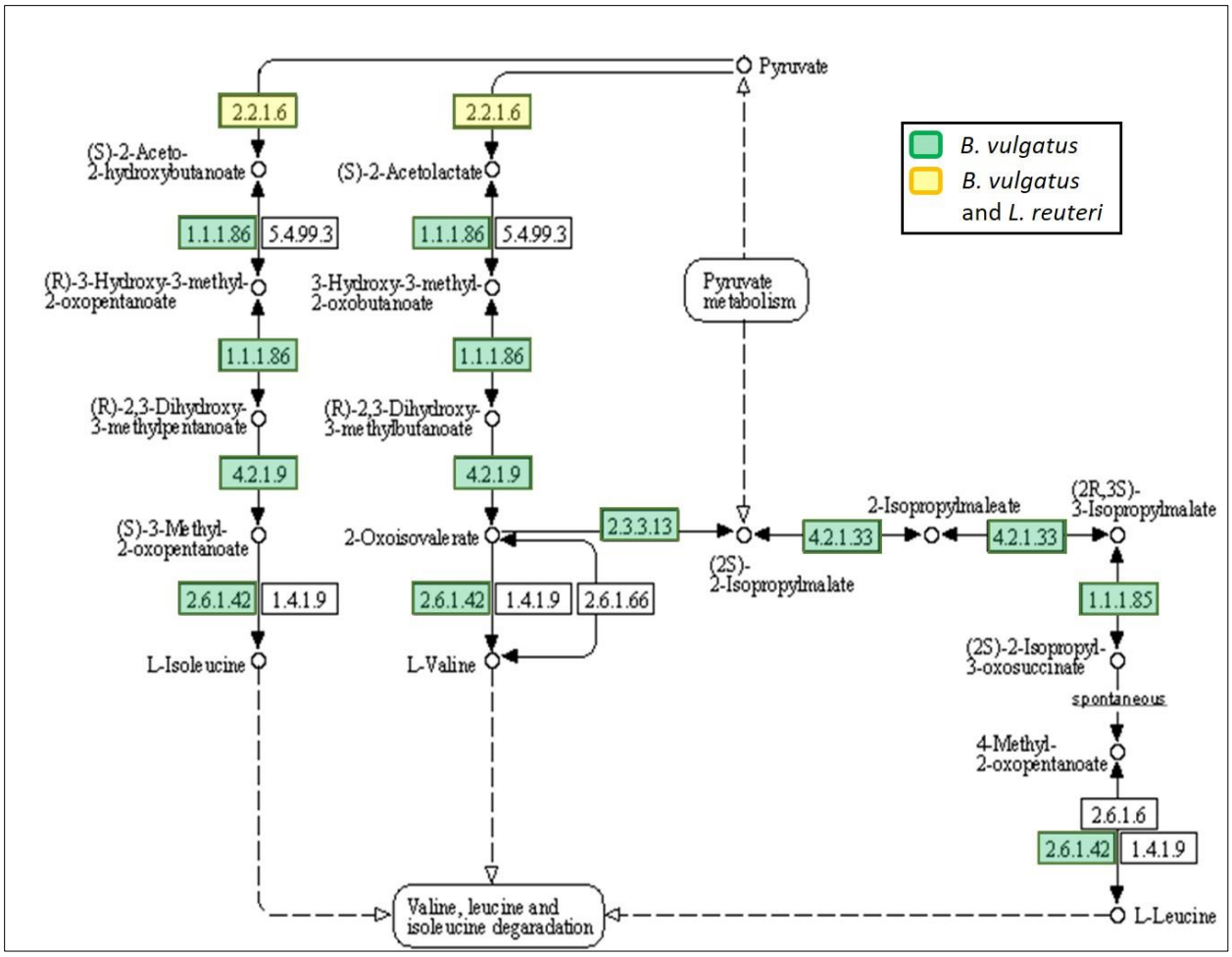
Pyruvate
2.2.1.6
2.2.1.6
(S)-2-Aceto-2-hydroxybutanoate
(S)-2-Acetolactate
1.1.1.86
5.4.99.3
1.1.1.86
5.4.99.3
(R)-3-Hydroxy-3-methyl-2-oxopentanoate
3-Hydroxy-3-methyl-2-oxobutanoate
Pyruvate metabolism
1.1.1.86
1.1.1.86
(R)-2,3-Dihydroxy-3-methylpentanoate
(R)-2,3-Dihydroxy-3-methylbutanoate
4.2.1.9
4.2.1.9
(S)-3-Methyl-2-oxopentanoate
2-Oxoisovalerate
2.3.3.13
2-Isopropylmaleate
4.2.1.33
4.2.1.33
(2R,3S)-3-Isopropylmalate
(2S)-2-Isopropylmalate
2.6.1.42
1.4.1.9
2.6.1.42
1.4.1.9
2.6.1.66
1.1.1.85
L-Isoleucine
L-Valine
(2S)-2-Isopropyl-3-oxosuccinate
spontaneous
4-Methyl-2-oxopentanoate
2.6.1.6
2.6.1.42
1.4.1.9
Valine, leucine and isoleucine degaradation
L-Leucine
B. vulgatus
B. vulgatus and L. reuteri

Figure 11. BCAA biosynthesis pathway capability in *B. vulgatus* and *L. reuteri*. A diagram of the BCAA biosynthesis was adapted from the Kyoto Encyclopedia of Genes and Genomes (KEGG; https://www.genome.jp/kegg/) to display biosynthesis of BCAAs from pyruvate. Enzymes that are encoded by genes possessed by *B. vulgatus* are highlighted in yellow or green. Enzymes encoded by genes possessed by both *B. vulgatus* and *L. reuteri* are highlighted in yellow. The presence of acetolactate synthase in *L. reuteri*, an organism that possesses no other genes encoding BCAA biosynthesis proteins is explained by the enzymes utility in maintaining cellular pH by way of eliminating intracellular pyruvate.[933]

Substrate	Enzyme (EC Number)	*B. vulgatus*	*L. reuteri*
Pyruvate	Acetolactate synthase (2.2.1.6)	+	+
	Keto acid isomeroreductase (1.1.1.86)	+	-
	Dihydroxyacid dehydratase (4.2.1.9)	+	-
	Isopropylmalate synthase (2.3.3.13)	+	-
	Isopropylmalate isomerase (4.2.1.33)	+	-
	Isopropylmalate dehydrogenase (1.1.1.85)	+	-
	Branched-chain aminotransferase (2.6.1.42)	+	-
Threonine	L-threonine dehydratase/deaminase (4.3.1.27)	-	-

Table 1. BCAA biosynthesis pathway enzymes expressed by *B. vulgatus* and *L. reuteri.* Enzymes involved in the biosynthesis of BCAAs from pyruvate or L-threonine initial substrates are shown. *B. vulgatus* and *L. reuteri* are indicated to have (+) or not have (-) the encoding gene. Information collected from the KEGG database (https://www.genome.jp/kegg/).

mTORC1 translational control pathway activity is upregulated in the hippocampus of MHFD offspring.

Given that (*1*) dysregulation of amino acid homeostasis can cause cognitive[432,825,826] and metabolic dysfunction[632,633,934,935] (*2*) BCAAs are essential amino acids provided exclusively by diet and microbiota[936], (*3*) MRD and MHFD offspring are fed an identical diet post-weaning, (*4*) *B. vulgatus* has biosynthetic potential for BCAAs[633], and that (*5*) *B. vulgatus* abundance (**Fig. 5a**,**d**) and circulating BCAA concentration (**Fig. 12a**) are both increased in MHFD offspring, we hypothesized that the mechanism by which MHFD-induced dysbiosis of the gut microbiome causes cognitive and metabolic dysfunction in MHFD offspring is by generating excess BCAA availability which thus dysregulates host protein synthesis and, thereby, impairs synaptic plasticity and insulin signaling, respectively .[288] To begin to test this hypothesis, we performed Western blotting and immunostaining to assess the activity of the mTORC1 and eIF2α translational control pathways in MRD *vs*. MHFD offspring hippocampal tissue harvested at approximately 12 weeks of age. Consistent with our hypothesis, we observed elevated mTORC1 (**Fig.12b-d,h**)., but not eIF2α (**Fig.12e-g,i**), pathway activity in MHFD offspring hippocampi.

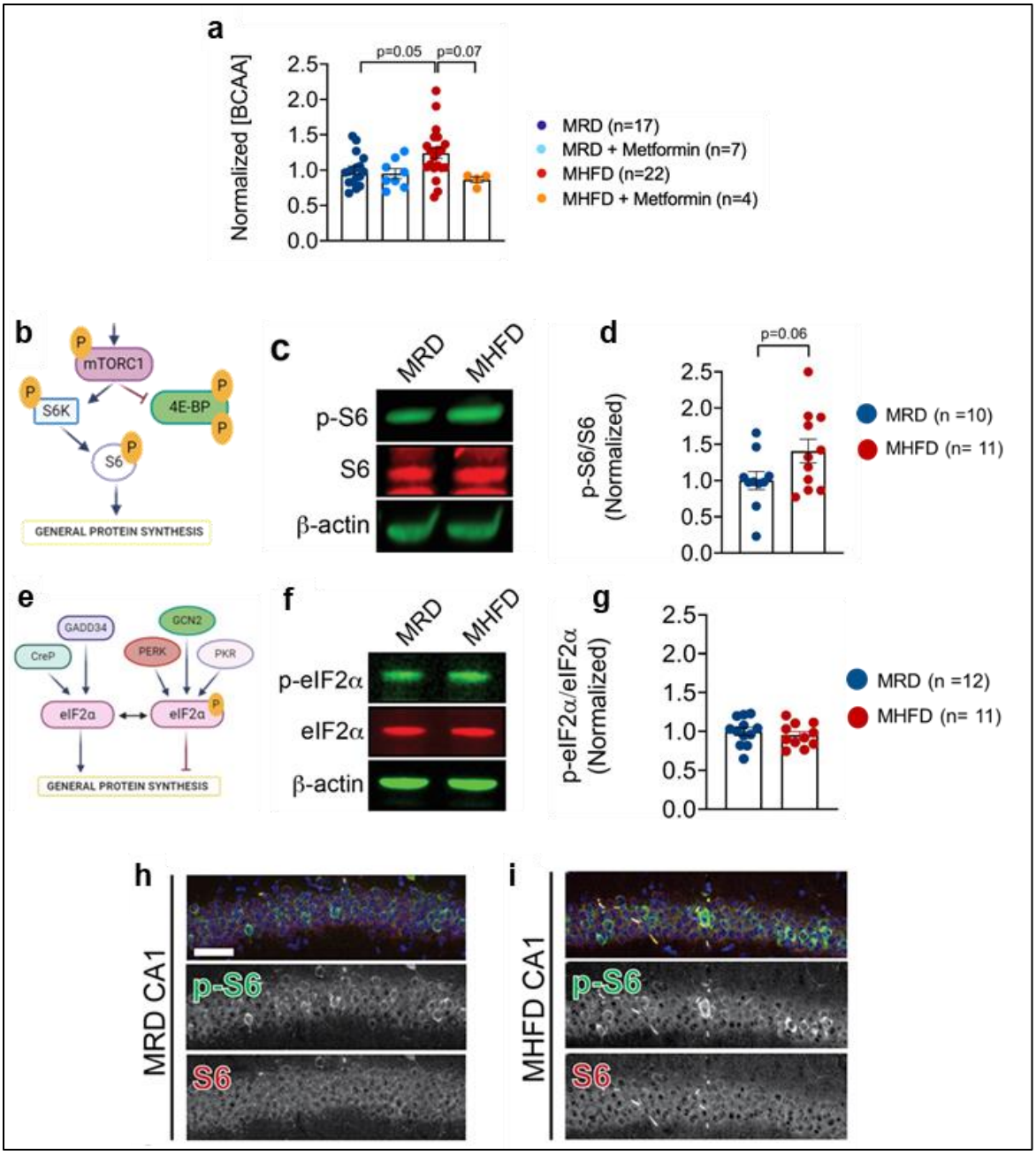
a
Normalized [BCAA]
p=0.05
p=0.07
2.5
2.0
1.5
1.0
0.5
0.0
MRD (n=17)
MRD + Metformin (n=7)
MHFD (n=22)
MHFD + Metformin (n=4)
b
mTORC1
S6K
4E-BP
S6
GENERAL PROTEIN SYNTHESIS
c
MRD
MHFD
p-S6
S6
β-actin
d
p=0.06
p-S6/S6 (Normalized)
MRD (n =10)
MHFD (n= 11)
e
GADD34
CreP
GCN2
PERK
PKR
eIF2α
GENERAL PROTEIN SYNTHESIS
f
MRD
MHFD
p-eIF2α
eIF2α
β-actin
g
p-eIF2α/eIF2α (Normalized)
MRD (n =12)
MHFD (n= 11)
h
MRD CA1
p-S6
S6
i
MHFD CA1
p-S6
S6

Figure 12. Circulating BCAAs are elevated in MHFD offspring, restored by maternal metformin treatment; mTORC1 translational control pathway activity is elevated in the hippocampus of MHFD offspring. (**a**) Maternal metformin restored MHFD offspring circulating BCAA concentration (*MRD vs. MHFD, p=0.05; MHFD vs. MHFD +metformin, p=0.07*). (**b,e**) schematics of the mTORC1 (**b**) and eIF2α (**e**) translational control pathways. (**c-g**) Phospho-specific Western blots of hippocampal lysates showing an increase in the activity (phospho vs. total ratio) of mTORC1 **(c,d)** (p=0.06, t=1.947) but not eIF2α (**f,g**) (p=0.04928, t=0.6980) pathway. (**h**,**i**) Immunostaining for phospho- and total-S6 in the hippocampus of MRD (**h**) *vs.* MHFD (**i**) offspring. Scale bar, 50μm. (Data in **a** reflect MRD and MHFD male and female combined cohorts; data in **b–i** reflect MRD and MHFD male cohorts.)

4. Discussion.

Maternal obesity is associated with a higher risk for many pregnancy complications, including preeclampsia, gestational diabetes, and delivery of large-for-gestational-age (LGA) infants.[937] In addition to detrimental effects on the mother *per se*, a growing body of evidence, including human epidemiological data, suggests that maternal obesity is an important risk factor for multiple adverse long-term health outcomes in progeny[762,875], including cognitive and metabolic dysfunction; yet, the underlying mechanisms remained poorly understood. Our investigation of the mechanisms by which maternal obesity contributes to the onset of chronic disorders in the offspring reveals a crucial role for the gut microbiome in mediating the effects of maternal diet on offspring health.[938] Indeed, maternal obesity during pregnancy determines substantial changes in the composition and community structure of maternal gut microbial ecology (**Fig. 1e-l**)[939,940], which is considered a powerful regulator of human physiology and pathology. These changes are reflected on the profile of microbially-derived metabolites, key factors in host-microbiota crosstalk which can influence prenatal and early postnatal offspring development.[941] Indeed, given that the maternal gut microbiome is vertically transmitted to the offspring, changes in the maternal gut microbiome composition and structure can be inherited by the offspring, thus propagating the detrimental effects of dysbiotic gut microbial communities. Therefore, therapeutic modulation of the maternal gut microbiome in obesity represents an unorthodox opportunity for intervention to prevent the onset of several chronic disorders, including mental health disorders, in the offspring. Understanding the mechanisms by which dysbiosis of the gut microbiome negatively impact on offspring health is imperative to develop such interventions.

In recent work[63] we showed that eight weeks of MHFD intake induces dysbiosis of the gut microbiome in both mothers and offspring in mice, and that these persistent, functional changes in the composition and community structure of the gut microbiome are ultimately responsible for offspring social behavior deficits. Our finding are consistent with emerging epidemiological data showing that mothers affected by obesity and/or gestational diabetes were more likely to have children with ASD and other NDDs.[521,942] In this study, we modified the maternal diet feeding schedule, mating the dams after four weeks of HFD regimen, a time point at which HFD maternal total body mass is not significantly increased relative to RD controls, allowing us to isolate the effects of diet-induced changes in the gut microbiome from maternal weight gain (**Fig. 1a**–**d**). We found that four weeks HFD-feeding is sufficient to induce marked changes in the maternal gut microbiome, including a general loss of diversity and phylum-level changes characterized by a significant increase in the F:B ratio . These alterations are reflected in the gut microbiome of MHFD offspring (**Fig. 2a**–**c**) and are associated with social dysfunction (**Fig. 2d**–**k**), suggesting that long-lasting, transmissible changes in the gut microbiome occur after a relatively short period on HFD, even in the absence of significant alterations in total body mass. Furthermore, similar to what we previously observed after eight weeks of maternal HFD-feeding[63], these changes negatively impact on offspring social behavior.

Our further investigation of the effects of MHFD on offspring health outcomes, revealed that, in our mouse model, MHFD not only impacts on offspring social function, but is also causally implicated in the onset of cognitive deficits and metabolic alterations in the offspring (**Figs. 3, 4**). Indeed, we showed that MHFD offspring underperformed controls in the CFC paradigm (**Fig. 3a**, **b**), identifying hippocampal-dependent memory impairments, consistent with previous reports[943]. Conversely, based on the results obtained in the open field test and of habituation phase of the 3C

(Fig. 2i), we did not observe changes in locomotor activity or anxiety-behavior (**Fig. 3d,e**). As discussed in the introduction chapter of this thesis, increased anxiety, which is considered as one of the several ASD-related symptoms, is reported in a variable proportion of ASD patients, ranging between 1.47% and 54% across studies. Similarly, mouse models of ASD, either genetic or nongenetic, do not always report increased anxiety as one of the observed behavioral phenotypes.[944] In the context of MHFD models of ASD, it has been shown[945] that the effect of perinatal HFD on offspring anxiety-like phenotype varies based on the age of the offspring mice, with a decrease in anxiety-like behavior in adolescence, and an increase in adulthood, suggesting that other factors might play an important role in the onset of anxiety-like behavior.

Additionally, consistent with literature[925], we reported a small but significant decrease in MHFD offspring weight at 8 weeks of age, regardless of sex (**Fig. 4a**,**b**). While we found that juvenile MHFD offspring low body weight is normalized in adulthood (**Fig. 4c**,**d**), despite indistinguishable weight profiles, adult MHFD offspring show impaired glucose metabolism at 20 weeks of age for both males and females (**Fig. 4h,i**). These findings demonstrate that MHFD induces metabolic dysfunction in offspring, consistent with human studies which report maternal obesity as a predisposing factor for offspring child- and adulthood obesity and type 2 diabetes.[876,946,947] The casual relationship between MHFD-mediated dysbiosis of the maternal and/or offspring gut microbiome and offspring cognitive function was demonstrated by our co-housing experiment in which one MHFD mouse was co-housed with three MRD mice (**Fig. 3f**). Similar to our previous finding that the co-housing protocol corrected social deficits in MHFD offspring[63], here we showed that co-housing of MHFD with MRD offspring initiated post-weaning rescued hippocampal dependent memory in the CFC paradigm and microbial phylogenetic profile in MHFD offspring (**Fig. 3g**–**i**). Together, these data indicate that MHFD-induced changes in the

gut microbiome are responsible, at least in part, for impaired hippocampus-dependent memory in MHFD offspring and that restoration of the MHFD gut microbiome is sufficient to rescue memory performance in MHFD offspring.

Recently, we demonstrated that precision reconstitution with the species most reduced by MHFD consumption, *L. reuteri*, was sufficient to rescue ASD-like social dysfunction and related deficits in synaptic plasticity in the VTA.[63,765] However, the impact of species whose abundance is increased by MHFD consumption on host phenotypes remains completely unexplored. Our metagenomic WGS sequencing of MRD and MHFD offspring fecal samples showed that, despite an overall decrease in microbial diversity, the abundances of particular taxa are significantly increased in the gut of MHFD offspring, such as the opportunistic pathogen *B. vulgatus* (**Fig. 5**). In light of this data, we determined whether colonization of conventional mice with the opportunistic pathogen *B. vulgatus* was sufficient to induce cognitive and metabolic deficits in the recipients. Remarkably, we found that colonization with *B. vulgatus* alone at the time of weaning is sufficient to induce MHFD offspring-like metabolic and cognitive behavioral phenotypes in conventional mice (**Fig. 6 e–n**).

Next, we aimed to determine whether targeting opportunistic pathogenic bacteria could improve cognitive and metabolic function in MHFD offspring. In particular, we focused on maternal pharmacological intervention with the FDA-approved antidiabetic drug metformin. Our results showed that maternal metformin treatment induced significant changes in the maternal (**Fig. 7,8**) and offspring gut microbiota composition (**Figs. 7,9**) and rescued MHFD offspring alterations in body composition, long-term memory performance, and social behavior (**Fig. 10**).

Of note, metformin treatment seems to reduce the increase in the abundance of the genus Parabacteroides observed in MHFD offspring compared to MRD mice. Intriguingly, based on the

WGS sequencing data (**Fig. 5a**) *P. goldsteinii*, is the second most elevated species in the MHFD offspring. Conversely, HFD dams treated with metformin showed a significant increase in the abundance of Parabacteroides genus, consistent with previous literature.[948] Interestingly, a recent study[949] showed the dominance of Parabacteroides genus in offspring born to HFD dams at the age of 1 and 6 months, and established a correlation with impaired exploratory behavior and memory in adulthood. Additionally, an increase in Parabacteroides has been reported also in children born to obese mothers.[950] *Bacteroides distasonis, Bacteroides goldsteinii* and *Bacteroides merdae* have been reclassified as *P. distasonis*, *goldsteini*i, and *merdae*, thus introducing a new genus in order to accommodate the phylogenetic differences with the Bacteroides genus.[951] *P. goldsteinii and P. distasonis* have been reported to alleviates obesity and metabolic dysfunction[952] Our data are in line with the existing literature, showing the differential effect of maternal HFD and metformin treatment on Parabacteroides abundance in dams and offspring, however, further research is required to elucidate the role of Parabacteroides genus and its species on offspring metabolism and brain function.

Finally, we investigated the mechanisms by which the MHFD-associated opportunistic pathogen *B. vulgatus* impairs host cognitive and metabolic function. Host-microbe interactions mediated by microbially-derived, low molecular weight (LMW) bioactive molecules[593,953] are known to regulate host metabolic[633] and brain function.[64] Changes in the composition of the gut microbiome, as in the case HFD-induced increase of opportunist pathogens, can determine the increase of specific opportunistic pathogen-derived LMW bioactive molecules in host serum metabolome, which might be ultimately involved in host cognitive and metabolic dysfunction. Intriguingly, a previous study[633] analyzing the serum metabolome of individuals affected by insulin-resistance showed that BCAA concentration is elevated in IR-subjects and that this

increase is driven by species in the gut microbiome with enriched biosynthetic potential for BCAAs, specifically *P. copri* and *B. vulgatus.* Given that (*1*) *B. vulgatus* is increased in the gut of MHFD offspring (**Fig. 5**), (*2*) *B. vulgatus* treatment is sufficient to induce MHFD offspring-like cognitive and metabolic dysfunction (**Fig. 6**), and (*3*) is genetically capable of producing BCAAs from pyruvate (**Fig. 11**, **Table 1**), and (*4*) elevated circulating maternal BCAA in obese women is associated with increased risk of ASD in offspring[921], we hypothesized that increased serum BCAA concentration in mice which have been exposed to MHFD is directly due to the increase in *B. vulgatus* in the intestinal microbiome and that the increase in circulating BCAAs might be responsible for the phenotypes observed in MHFD mice. Our hypothesis is supported by a significant increase in the concentration of circulating BCAAs that we observed in MHFD offspring serum–a change on par with the 20% increase in BCAA concentration observed in the breastmilk of obese women[954]–which is normalized by maternal metformin treatment (**Fig. 12a**). Future experiments aimed to test our hypothesis include direct feeding of mice with *B. vulgatus,* followed by the assessment of serum BCAA by mass spectroscopy.

Importantly, AAs, including BCAAs, are known to modulate the activity of translational control pathways, including the mTORC1 pathway, via multiple mechanisms based on the specific types of AAs.[955,956] Intriguingly, hyperactivation of mTORC1 pathway in neurons has been suggested to play a role in the pathogenesis of several brain disorders, including ASD[432] and cognitive disorders.[957,958] Indeed, enhanced mTOR activation leads to an increase in the translation of proteins involved in synaptic formation and maintenance, which may lead to impaired synaptic activity and neural network connectivity.[959,960] In this regard, our assessment of hippocampal activity of mTORC1 pathway revealed the presence of mTORC1 pathway hyperactivity in MHFD offspring compared to controls (**Fig. 12b–i**) which we postulate is driven by the increase in

mTORC1 modulators, namely *B. vulgatus*-derived BCAAs. Additionally, in the context of metabolic disorders, dysregulated BCAA levels have been associated with various disorders[961], altered glucose metabolism[962], insulin resistance[963] , T2D[964,965] a cardiovascular disorder. Indeed, BCAAs have been reported to play a crucial role in regulating metabolic processes occurring in different organs, such as liver, skeletal muscle and adipose tissue, with different effects depending on catabolic or anabolic status.[966] Interestingly, many of the metabolic pathways regulated by BCAAs involves the activity mTORC1, which is a master regulator of cell growth and metabolism.[966] For instance, it has been proposed[930,967] that persistent nutrient signaling might induce IR by means of BCAA-induced hyperactivation of mTORC1 and its downstream effector S6K1 in skeletal muscles. Hyperactivation of mTORC1 and its downstream effector S6K1 might then lead to phosphorylation and degradation of insulin receptor substrate 1 and 2 (IRS1 and IRS2) thus impairing normal insulin signaling effects, including glucose uptake and glycogen accumulation.[968,969]

Based on the results obtained by the present study, we propose a model (**Fig. 13**) in which MHFD-driven increase in the abundance of opportunistic pathogens in the maternal gut microbiome, specifically *B. vulgatus*, is responsible for the alteration of maternal serum metabolome (*e.g.*, a significant increase in BCAA availability) which can cause dysregulation of protein synthesis in the brain of the developing fetus, thus leading to synaptic deficits underlying cognitive and social behavior impairments. Similarly, increased BCAA levels might lead to persistent activation of mTORC1 signaling pathway in skeletal muscles, thus interfering with glucose homeostasis (**Fig. 13a**). The detrimental effect of the dysbiosis in the maternal gut microbiome on offspring development are not limited to the prenatal period, but due to the mother-to-infant vertical transmission of the gut microbiome, opportunistic pathogens can be transmitted

from the maternal to the offspring gut microbiome, thus interfering also with the early post-natal development of offspring (**Fig. 13b**). Thus, targeting the MHFD-derived increase in the abundance of *B. vulgatus* with maternal metformin treatment (**Fig. 14a, b**) in women of obese status during pregnancy might restore normal BCAAs availability and therefore prevent mTORC1 dysregulation both in the brain and in the skeletal muscles, and ultimately decrease the risk for offspring metabolic and behavioral dysfunction in children exposed to MHFD *in utero*.

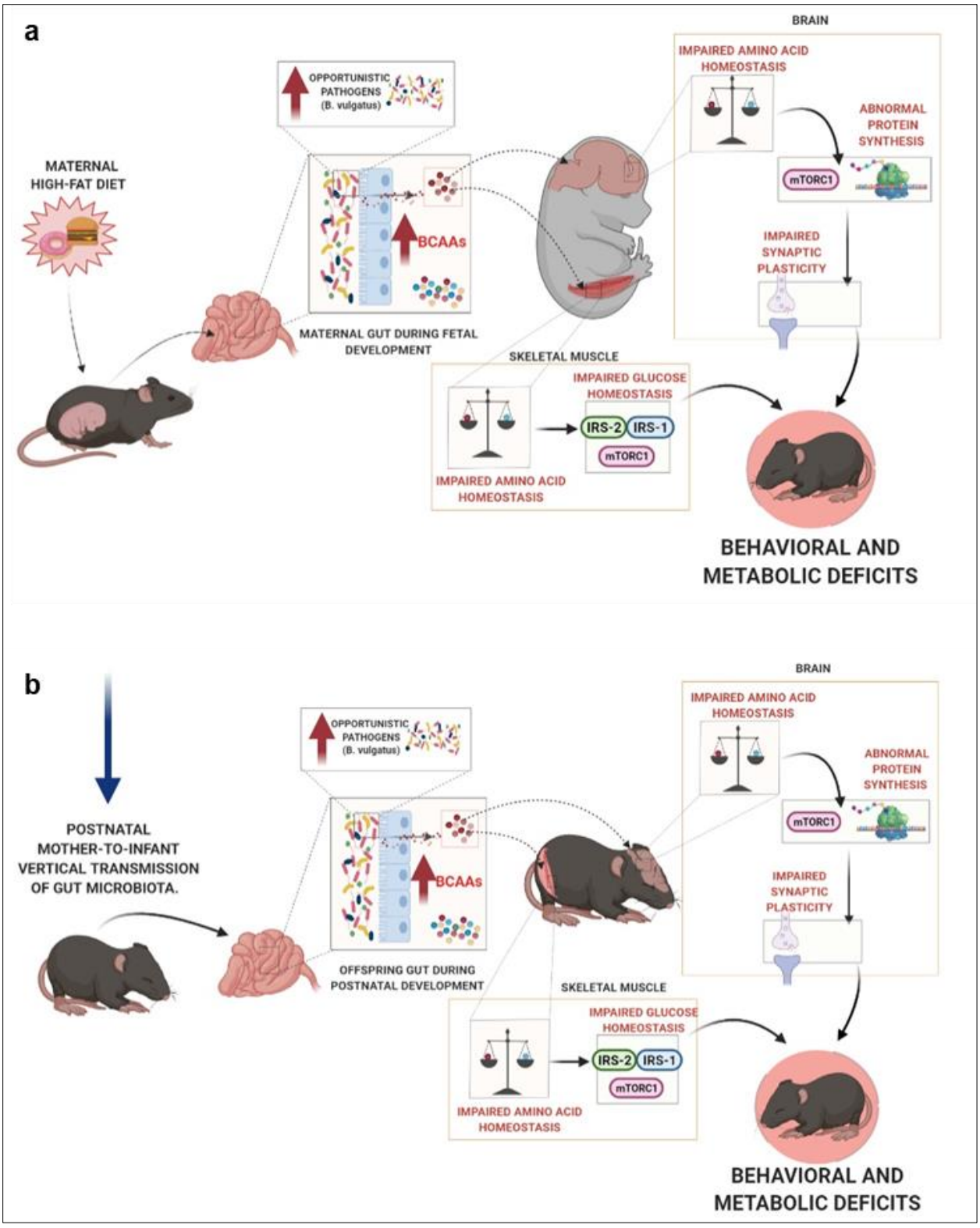
a
BRAIN
IMPAIRED AMINO ACID HOMEOSTASIS
ABNORMAL PROTEIN SYNTHESIS
mTORC1
IMPAIRED SYNAPTIC PLASTICITY
OPPORTUNISTIC PATHOGENS (B. vulgatus)
MATERNAL HIGH-FAT DIET
BCAAs
MATERNAL GUT DURING FETAL DEVELOPMENT
SKELETAL MUSCLE
IMPAIRED GLUCOSE HOMEOSTASIS
IRS-2
IRS-1
mTORC1
IMPAIRED AMINO ACID HOMEOSTASIS
BEHAVIORAL AND METABOLIC DEFICITS
b
POSTNATAL MOTHER-TO-INFANT VERTICAL TRANSMISSION OF GUT MICROBIOTA.
OFFSPRING GUT DURING POSTNATAL DEVELOPMENT

Figure 13. MHFD-driven increase in the abundance of the opportunistic pathogen *B. vulgatus* in the maternal and offspring gut microbiome determines behavioral and metabolic impairments in the offspring by dysregulating amino acid homeostasis. (**a**) During fetal development, the HFD-driven increase in the abundance of B. vulgatus in the maternal gut microbiome increases the microbiota biosynthetic potential for BCAAs. Increased amount in circulating BCAAs determines impairments amino acids homeostasis in *(1)* the brain, where the activity of the master regulator of protein synthesis, mTORC1, abnormally increases, leading to alterations in protein synthesis, including proteins involved in synaptic plasticity, and ultimately to dysfunction in neuronal circuits associated with social behavior and cognition, and *(2)* in skeletal muscles where hyperactivated mTORC1 impairs insulin signaling, leading to metabolic dysregulation. (**b**) Since the offspring gut microbiome is acquired from the mother after birth, opportunistic pathogens, such as *B. vulgatus,* are transmitted from the mother to the offspring, therefore perpetuating BCAA-induced alterations in amino acids homeostasis and translational control pathways in the post-natal developmental period.

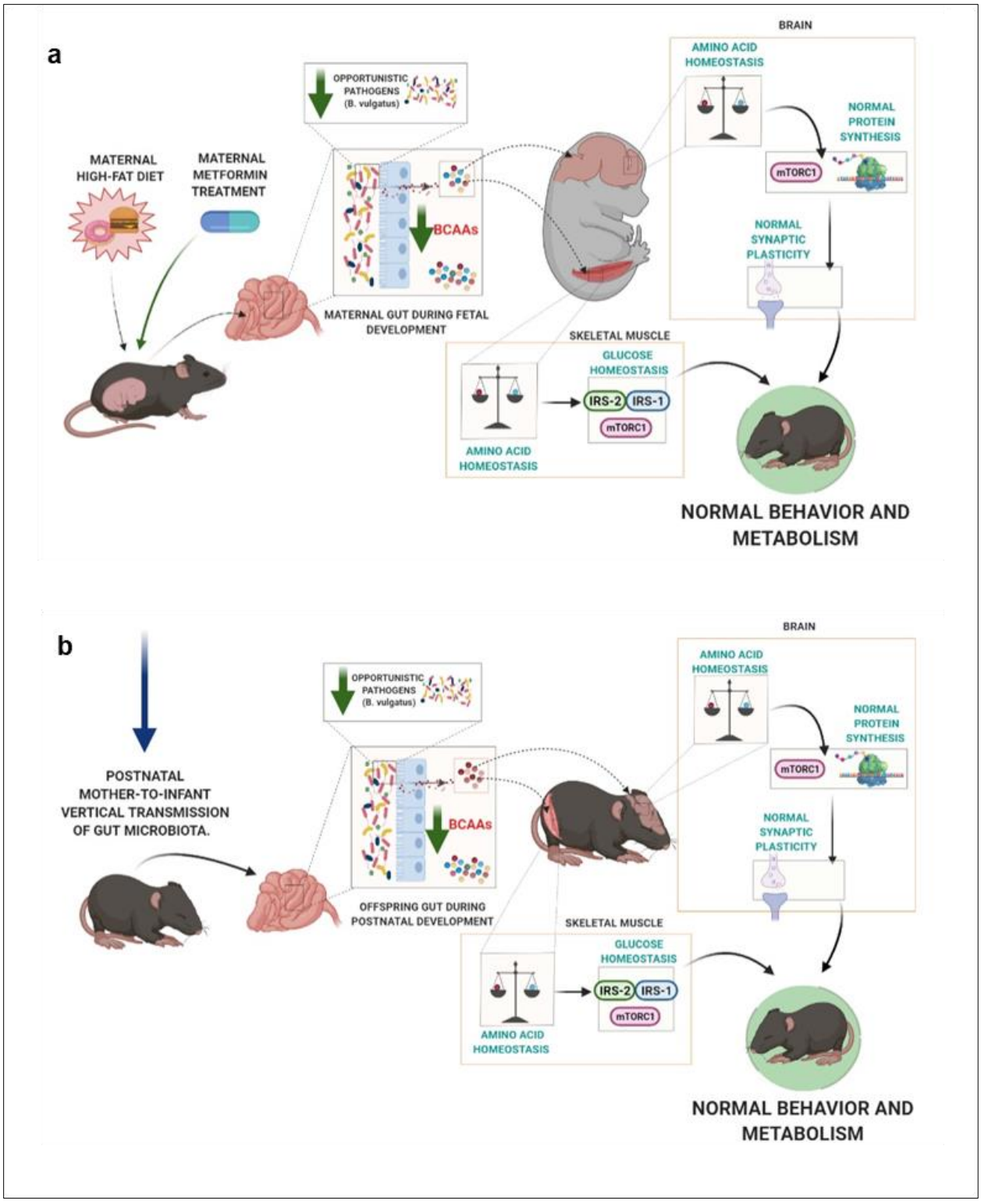
a
MATERNAL HIGH-FAT DIET
MATERNAL METFORMIN TREATMENT
OPPORTUNISTIC PATHOGENS (B. vulgatus)
BCAAs
MATERNAL GUT DURING FETAL DEVELOPMENT
BRAIN
AMINO ACID HOMEOSTASIS
NORMAL PROTEIN SYNTHESIS
mTORC1
NORMAL SYNAPTIC PLASTICITY
SKELETAL MUSCLE
GLUCOSE HOMEOSTASIS
IRS-2
IRS-1
mTORC1
AMINO ACID HOMEOSTASIS
NORMAL BEHAVIOR AND METABOLISM
b
POSTNATAL MOTHER-TO-INFANT VERTICAL TRANSMISSION OF GUT MICROBIOTA.
OPPORTUNISTIC PATHOGENS (B. vulgatus)
BCAAs
OFFSPRING GUT DURING POSTNATAL DEVELOPMENT
BRAIN
AMINO ACID HOMEOSTASIS
NORMAL PROTEIN SYNTHESIS
mTORC1
NORMAL SYNAPTIC PLASTICITY
SKELETAL MUSCLE
GLUCOSE HOMEOSTASIS
IRS-2
IRS-1
mTORC1
AMINO ACID HOMEOSTASIS
NORMAL BEHAVIOR AND METABOLISM

Figure 14. Maternal metformin treatment rescues MHFD-associated offspring phenotypes by inhibiting MHFD-induced increase of *B. vulgatus* in the maternal gut microbiome and the associated increase in circulating BCAAs. (**a**) Maternal metformin treatment during pregnancy and lactation inhibits HFD-driven increase in the abundance of *B. vulgatus* in the maternal gut microbiome, thus preventing the BCAA-induced disruption of amino acid homeostasis and dysregulation in protein synthesis in the brain and in skeletal muscle. (**b**) maternal metformin treatment prevents the transmission of opportunistic pathogens to the offspring, thus contributing to offspring normal brain development and metabolism.

Chapter III : Diet-induced Dysbiosis of the Gut Microbiome in the Maternal Lineage Drives Multigenerational Impacts on Metabolism and Behavior.

Diet-induced Dysbiosis of the Gut Microbiome in the Maternal Lineage Drives Multigenerational Impacts on Metabolism and Behavior.

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1. Introduction.

Neurodevelopmental disorders (NDDs), such as autism spectrum disorders (ASD), include a large group of disorders characterized by deficits ranging from specific limitations of learning and memory, language and speech, behavior, or motor skills, to more general impairment of social abilities and intelligence.[970] NDDs are associated with multiple negative psychological and social outcomes for the subjects affected and their family and with high economic costs relative to the complex medical and nonmedical needs of the increasing number of children with these disabilities. NDDs affect >3% of children worldwide and their prevalence is increasing, therefore they represent significant global public health concern.[71] Recent studies[87,309] have shed light on the multifactorial etiology of neurodevelopmental disorders (NDDs), such as autism spectrum disorders (ASD), which are determined by the interaction of both genetic and nongenetic factors. This complex etiology is reflected in the clinical heterogeneity and phenotypic variability in patients with NDDs, making identification of causal mechanisms contributing to their onset difficult. Yet, unraveling the underlying causes of NDDs is essential to the development of appropriate preventive/therapeutic strategies. Among nongenetic factors contributing to NDDs, several studies identified maternal obesity as a strong risk factor for the development of such disorders.[875,877] However, the precise pathophysiological mechanisms underlying maternal obesity-associated long-term health risks in offspring have not been fully elucidated. The idea that maternal obesity may influence and impair offspring neurodevelopment is supported by epidemiological studies reporting that mothers with obesity and/or diabetes mellitus were more likely to have a child affected by ASD.[521,921,942,971,972] This epidemiological evidence, together with the increasing prevalence of obesity and overweight in women of childbearing age – it is estimated that two out of every five pregnancies in the US are carried by obese/overweight mothers[874,973]–

linked to dietary patterns[974,975] provide strong rationale for further investigation into the mechanisms by which maternal obesity impacts offspring health. A better understanding of these mechanisms will aid in the identification of preventative therapeutic measures to reduce the prevalence of neurodevelopmental and disorders in children born to women of obese status. Diet-induced obesity is associated with significant changes in the gut microbiome, most notably characterized by a decrease in overall microbial diversity.[886,913,976,977] Furthermore, a growing body of evidence suggests that maternal high-fat diet (MHFD) is able to induce alterations in gut microbial ecology in offspring.[63,939,940] Indeed, several studies reported the association between maternal obesity and alteration in the composition of gut microbiome in offspring in both human[950] and non-human primates.[940]

Importantly, several studies proposed gut dysbiosis as a major contributor to brain disorders, including cognitive dysfunction[839,849,978], neurodegeneration[979,980], and cerebrovascular diseases.[981,982] Despite the anatomical distance between the gut and the brain, the existence of a complex communication network between the gut and the central nervous system (CNS) – the so-called "gut-brain axis" – has been widely demonstrated. In this context, the gut microbiome regulates the bidirectional communication between the gastrointestinal (GI) tract and the CNS through multiple immunologic, endocrine, and metabolic mechanisms.[830,891] The gut microbiome establishes a symbiotic relationship with the host early on in life which evolves throughout the individuals' lifespan.[983,984] Perturbations in the composition of the gut microbiome may affect brain function[985], and, when occurring during early in development, may lead to adverse neurodevelopmental and mental health outcomes. Indeed, multiple studies[60,986-988] showed the presence of dysbiosis of the gut microbiota in individuals affected by NDDs.

Considering the strong impact that MHFD may have on offspring gut microbial ecology and the link between gut dysbiosis and NDDs, MHFD may represent a great risk factor for the development of NDDs. Recently[63], working in a mouse model for diet-induced obesity, we demonstrated that MHFD intake throughout gestation and lactation produces alterations in the offspring gut microbiota, in particular, a marked decrease in the commensal species *L. reuteri*. Interestingly, *L. reuteri* has been shown to regulate the level of oxytocin level[989], a hormone that plays a crucial role in modulating social behavior through its impact on the mesolimbic dopaminergic reward system, in both the hypothalamic nucleus of the brain and in the periphery.[63,990] Our investigation into hypothalamic oxytocin levels and the function of the mesolimbic dopaminergic reward system in MHFD offspring revealed a considerable reduction in oxytocin immunoreactivity in the paraventricular nucleus (PVN) of the hypothalamus and an impairment at the level of social interaction-induced long-term potentiation (LTP) in dopaminergic (DA) neurons of the ventrolateral ventral tegmental area (VTA). Interestingly, different treatments, including the modulation of the microbial community structure by co-housing MHFD with MRD offspring or precision reconstitution with *L. reuteri*, restored social behavior in MHFD offspring. Taken together, these results suggest a strong connection between MHFD, gut microbial ecology, and social dysfunction, and support the idea that a specific combination of probiotics might represent a potential cost-effective and non-invasive treatment for NDDs including ASD.

While detrimental effects of MHFD on the first generation of offspring have been reported by our lab and others, few studies have focused on the implications of maternal obesity on subsequent generations, which remains almost completely unexplored. Previous studies primarily focused on transgenerational effects of maternal nutritional status mediated by epigenetic modifications, particularly DNA methylation. This phenomenon emerged in the context of studies

investigating the mechanisms behind the increased rate of mental illnesses in population affected by famine, such as the Dutch famine (1944–45)[487,489], the Irish potato famine (1845–52)[991], and the Chinese famine (1959–61)[490,491]. Children with prenatal exposure to famine during gestation had an increased risk to develop obesity, cardiovascular diseases, and psychiatric disorders (e.g., schizophrenia and depression). In some cases, this effect lasted across at least 2 generations.[992] It was proposed that extreme maternal conditions, such as exposure to famine, induced modifications in fetal epigenetic programming and that these changes persist across generations.

Determining the transgenerational impacts of maternal conditions in human population presents a variety of epidemiological challenges, including the long generation times, as well as the heterogeneity in experience that exists within populations. The design and feasibility of such studies is incredibly complex. For these reasons, animal studies represent an indispensable tool in elucidating such long-term effects of maternal nutritional state on offspring health across multiple generations. Oh et al. investigated the cross-generation effect of neonatal macrosomia in rats. The main risk factor for neonatal macrosomia, a condition in which infants are unusually large for their gestational age, is poorly controlled gestational diabetes or preexisting diabetes mellitus in the mothers. Oh et al. found that the macrosomic female offspring of diabetic mothers develop glucose intolerance during pregnancy and, as a result, give birth to macrosomic pups, which exhibit a similar impairment in glucose metabolism through adulthood and propagate the abnormal phenotype of the parents. Interestingly, in the third generation, both males and females showed a similar abnormal weight gain during postnatal life. A recent study[993] showed that mice consuming a diet with a low content of microbiota accessible carbohydrates (MAC) displayed a loss of taxa within the gut microbiota over generations. Loss of diversity was only partially restored by the re-introduction of MACs. The studies support the idea that, understandably, maternal metabolic status

has a strong influence on offspring health in both humans and animals that can persist across multiple generations.

In the present study, we test the hypothesis that MHFD-induced dysbiosis of the gut microbiome and related social dysfunction persists across generations, even in the absence of direct maternal exposure to HFD. Here we report that MHFD not only affects the first generation, but also causes disease-relevant pathological effects in the second generation. Our findings have great clinical significance in that they suggest a need to consider multi-generational maternal lineage metabolic dysfunction and obesity as strong risk factors for the development of NDDs in humans, independent of direct maternal diet, lifestyle, and medical conditions, thus helping to identify new at-risk individuals, with potential implications on both preventative and therapeutic strategies.

2. Experimental Procedures.

Mice and Maternal Diet. C57BL/6N mice were obtained from Taconic Laboratories (B6) and were kept on a 12-hour light/dark cycle and had access to food and water *ad libitum*. Six-week-old females were placed on either a regular diet (RD) consisting of 13.4% kcal from fat, 30% kcal from protein, and 57% kcal from carbohydrates (Lab Diets, #5001) or HFD consisting of 60% kcal from fat, 20% kcal from protein, and 20% kcal from carbohydrates (Research Diets, #D12492). Maternal weight was measured weekly. After 4 weeks on diet, females were paired with C57BL/6N adult males to produce subject offspring. Resulting offspring were weaned at 3 weeks of age and all placed on RD, regardless of maternal diet (RD or HFD). F_1 MRD and MHFD offspring were monogamously mated to generate the F_2 cohorts. All F_2 offspring were weaned at 3 weeks of age and placed on RD, regardless of maternal diet All behavioral tests were performed on offspring starting at 7 weeks of age. Animal care and experimental procedures were approved by The University of Texas Medical Branch's Institutional Animal Care and Use Committee in accordance with all guidelines set forth by the U.S. National Institutes of Health.

Body composition analysis. Whole body composition was performed using the EchoMRI™ whole body composition analyzer (EF-037) provided by the UTMB Rodent *In vivo* Assessment (RIVA) Core to accurately measure total, lean, and fat mass, as well as free and total water.

16s rRNA Gene Amplicon Sequencing Stool samples were aseptically collected in sterile 2mL Eppendorf tubes, immediately placed on dry ice, and stored at -80°C until further processing. Bacterial DNA was extracted and sequenced by the Alkek Center for Metagenomics and Microbiome Research using adapted from protocols developed for the NIH-Human Microbiome

Project[574], as described previously[63]. Briefly, bacterial genomic DNA was extracted using MagAttract PowerSoil DNA Kit (Qiagen) followed by PCR amplification of the 16S rDNA V4 region. The primers used for amplification include MiSeq adapters and single-end barcodes allowing for pooling and direct sequencing of PCR products. Sequencing was performed on the Illumina MiSeq platform using the 2 x 250 bp paired-end protocol yielding overlapping paired-end reads. The 16S rRNA gene read pairs were demultiplexed and merged using USEARCH v7.0.1090[905], allowing zero mismatches and a minimum overlap of 50 bases. Merged reads were trimmed at first base with Q5. A quality filter was applied to the resulting merged reads and reads containing above 0.05 expected errors were discarded. 16S rRNA gene sequences were clustered into Operational Taxonomic Units (OTUs) at a similarity cutoff value of 97% using the UPARSE algorithm.[906] OTUs were mapped to an optimized version of the SILVA Database[907] containing only the 16S v4 region to determine taxonomies. Abundances were recovered by mapping the demultiplexed reads to the UPARSE OTUs. A custom script constructed a rarefied OTU table from the output files generated in the previous two steps for downstream analyses of alpha-diversity, beta-diversity, and phylogenetic trends using the *Agile Toolkit for Incisive Microbial Analyses* (ATIMA) platform (https://atima1.jplab.net).

Behavioral Assays. Behavioral assays were performed as previously described[63] with modifications briefly described below. Animals were acclimated to the behavioral suit for 30–60 minutes prior to all behavioral experiments. Apparatuses were spot cleaned with 70% EtOH after each animal and thoroughly cleaned at the end of the day. AnyMaze automated software version 6.33 was used for data acquisition and processing.

Reciprocal social interaction. Mice were placed in a neutral, 25 cm^3 Plexiglass arena with either a familiar or stranger age- and sex-matched conspecific matched for maternal diet. The time

a pair of mice engaged in social interaction (close following, touching, nose-to-nose sniffing, nose-to-anus sniffing, and/or crawling over/under each other) over ten minutes was recorded by a blinded human observer and analyzed via AnyMaze (Stoelting).

Crawley's three-chamber test for sociability and preference for social novelty. Crawley's three-chamber test for sociability and preference for social novelty was performed as described.[908] Animals were habituated for 10 minutes in a 60 X 40 X 23 cm arena divided into three inter-connected chambers. Sociability was measured during a second 10-minute interval in which the test subject could interact either with an empty wire cup (empty) or a wire cup containing an age- and sex-matched stranger conspecific (mouse 1). Interactions were automatically scored by AnyMaze software and by an independent observer. Empty cup placement in the left or right chamber during the sociability period was counterbalanced between trials. Preference for social novelty was assayed by introducing a second stranger mouse (mouse 2) into the previously empty wire cup. Interactions were again recorded by AnyMaze software and an independent observer.

Open field test. Animal test subjects were gently lowered into an open arena (40 X 40 X 20 cm) and allowed to freely explore for 10 minutes. AnyMaze automatically measured distance traveled, speed, and position in the arena, as well as time spent in the center of the arena (defined as the interior 20 X 20 cm).

BCAA analysis. To quantify BCAA concentration in mouse serum, mouse blood was isolated by cardiac puncture under deep anesthesia once behavioral and body composition tests were complete. Serum was isolated by centrifugation in SST Microtainer tubes (BD, #365967) and snap-frozen over dry ice. The samples were stored at -80C until used. Total BCAA concentration was quantified using a colorimetric Branched Chain Amino Acid Assay Kit (Abcam,

#ab83374), and colorimetric change was measured at λ_{max} = 450 nm on an Epoch plate reader (BioTek).

Immunoassays. To quantify IL-17A concentration in mouse serum, mouse blood was isolated by cardiac puncture under deep anesthesia once behavioral and body composition tests were complete. Blood serum was isolated by centrifugation in SST Microtainer tubes (BD, #365967) and snap-frozen over dry ice. The samples were stored at -80C until used. Serum was thawed on ice, diluted 1:2 in 1X assay diluent A, and measured in duplicate for levels of IL-17A (BioLegend, Catalog # 431304) according to the manufacturer protocols.

Immunofluorescence. Mice were deeply anesthetized by inhalation of isoflurane and perfused transcardially with 10 mL 0.9% phosphate-buffered saline followed by 30 mL 4% paraformaldehyde in 0.1M phosphate buffer (PFA). Brains were post-fixed in 4% PFA at 4°C overnight, then cryoprotected in 30% sucrose 0.1M PB. 30 μm-thick coronal slices containing the hypothalamic paraventricular nucleus (PVN) were obtained from frozen tissue using a sliding blade microtome and then transferred to ice cold PBS. Slices were blocked with 5% normal goat serum, 0.3% Triton X-100 0.1M PB (PBTgs) for 1 hr rocking at RT and then incubated in primary antibodies (rabbit anti-Oxytocin Immunostar #20068 1:1000; mouse anti-NeuN Millipore #MAB377 1:400) diluted in PBTgs rocking at 4°C for 24 hr. Slices were then washed three times with 0.3% Triton X-100 0.1M PB. Primary antibodies were visualized using secondary goat anti-rabbit Alexa Fluor 488 (ThermoFisher Scientific, #A-11034) and goat anti-mouse Alexa Fluor 594 (ThermoFisher Scientific, #A-11032) antibodies (1:1,000 dilution). Slices were incubated in secondary antibodies rocking in the dark for 1h at RT. Five-minute final washes with each of PBTgs, 0.1M PB, and 0.05M PB preceded mounting onto 2% gelatin (Sigma-Aldrich, #G9391)-coated coverslips. Nuclei were visualized using Vectashield H-1200 with DAPI (Vector Labs, #H-

1200). Fluorescent imaging and data acquisition was performed on an Axio Imager.Z2 microscope (Carl Zeiss Imaging) mounted with an Axiocam 506 6MP mono digital camera (Carl Zeiss Imaging) and Apotome.2 structured illumination slider (Carl Zeiss Imaging). Images were captured using ZEN acquisition software (Carl Zeiss Imaging). All images within a given dataset were acquired at identical exposure times, within a given channel, to allow comparison of signal intensity. In some images, contrast and brightness were linearly adjusted using Photoshop (Adobe). Image processing was applied uniformly across all images within a given dataset.

Statistics. Power analyses to establish group size were performed in GPower 3.1. Data were analyzed and visualized using GraphPad Prism version 8.4.3 for Mac OS X (GraphPad Software, San Diego, California USA). Data are presented as mean ± SEM unless otherwise indicated. P-values are presented in the figure legends, and N values are provided in the figures. $p < 0.05$ was considered significant, where $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$. Outliers were identified using Grubbs' test and excluded from analysis for that assay. Data without repeated measures (Open Field test, BCAA, and ELISA), were visualized as column data but analyzed using a two-tailed nested t-test to account for litter effects. No significant differences between litters were determined. For Crawley's Three-Chamber assay, a mixed-effects model with repeated measures was applied with the Sidak correction for multiple comparisons. Random (litter) effects with a variance of zero were removed from the model and data were fit to a simpler model (cf. graphpad.com). In cases where data were not normally distributed and no outliers were identified (cf. graphpad.com), the data were transformed and re-analyzed. For the Reciprocal Social assay, an unpaired, two-tailed t-test was performed unless otherwise indicated. In cases where data were not normally distributed, the non-parametric Mann-Whitney test was used.

3. Results.

Deficits in social behavior persist through the second generation of MHFD.

In Chapter 2 of this thesis and a previous study[63] we reported the detrimental effects of either eight or four weeks of MHFD on first generation (F_1) offspring social behavior, cognitive function, and metabolism, which are mediated by diet-induced dysbiosis in the maternal gut microbiome. Since *(1)* previous studies suggest that diet-induced alterations in gut microbial ecology are transferred to subsequent generation, therefore producing long-lasting and heritable 'scars' on the gut microbiome, and (2) the ramifications of maternal obesity, particularly in the context of diet-induced dysbiosis of the gut microbiome, on subsequent generations remain almost completely unexplored, we sought to determine whether the MHFD-associated social dysfunction observed in the F_1 generation persists in second generation (F_2) offspring , even in the absence of direct exposure to MHFD. Moreover, given that males are disproportionately diagnosed with NDDs, including ASD[994], and that our knowledge of the role of biological sex in ASD risk is very limited, we intentionally conducted all of the experiments here reported were conducted comparing males and females F_2 descendants of regular- versus HFD-fed F_0 dams in order to assess potential sexually dimorphic outcomes. In the present study, regular diet control (MRD) and MHFD F_1 offspring were used to produce, respectively, MRD- descendant and MHFD-descendant F_2 offspring (**Fig. 1a**). F_2 offspring were weaned and caged according to maternal lineage, and both groups were placed on regular diet (RD) at weaning (**Fig. 1b**). To determine if the MHFD-descendant F_2 generation displayed dysfunction of social behavior, we conducted a battery of behavioral tests designed to assess social and anxiety-like behavior in male and female F_2

descendants of regular- *versus* high-fat diet F_0 dams. First, we performed Crawley's three-chamber (3C) test to evaluate (1) sociability by comparing the time mice spent interacting with an empty cup versus one containing a mouse (**Fig. 2b, Fig. 3b**), and (2) preference for social novelty by measuring the time mice spent interacting with a familiar versus a stranger mouse (**Fig. 2c, Fig. 3c**). We did not observe any differences in locomotor activity, in terms of distance travelled, during the habituation phase of the 3C test (**Figs. 2a, 3a**), for either sex or diet (**Figs. 2d, 3d**).

MHFD F_2 males showed a significant impairment in sociability (**Fig. 2e**) and loss of preference for social novelty compared to the MRD group (**Fig. 2f**). These findings were further supported by the time mice spent in the two chambers automatically measured by the computer (**Fig. 2g,h**). Interestingly, when we examined female F_2 offspring sociability we observed a strong preference in MRD females and a weaker, yet still statistically significant preference in MHFD females (**Fig. 3e**). Neither MRD nor MHFD female F_2 offspring showed a statistically significant preference for interaction with a novel over a familiar mouse (**Fig. 3f**), though the time MHFD F_2 female offspring spent in the social (mouse 1) *versus* empty chamber did not rise to the level of significance, while that of the F_2 MRD female offspring did (**Fig. 3g,h**), suggesting a weak social dysfunction phenotype.

Next, we performed the reciprocal social interaction test and recorded the amount of time a pair of unfamiliar mice spent interacting in a neutral arena (**Fig. 4a**). Pairs of MHFD male offspring spent significantly less time interacting with each other than MRD pairs (**Fig. 4b**), while no statistically significant differences in interaction time were observed between MRD and MHFD female F_2 offspring – consistent with the 3C data (**Fig. 4c**). Additionally, similar to what we previously observed for F_1 offspring, the results of the open field test (**Fig. 4d**) showed no differences between F_2 MRD or MHFD in hyperactivity, as measured by distance traveled, (**Fig.**

4e) or anxiety-like behavior, measured as time spent in the center, (**Fig. 4f**), regardless of sex. Taken together, these data indicate that MHFD-induced social deficits are transmitted to the F_2 generation, despite having no direct exposure to MHFD.

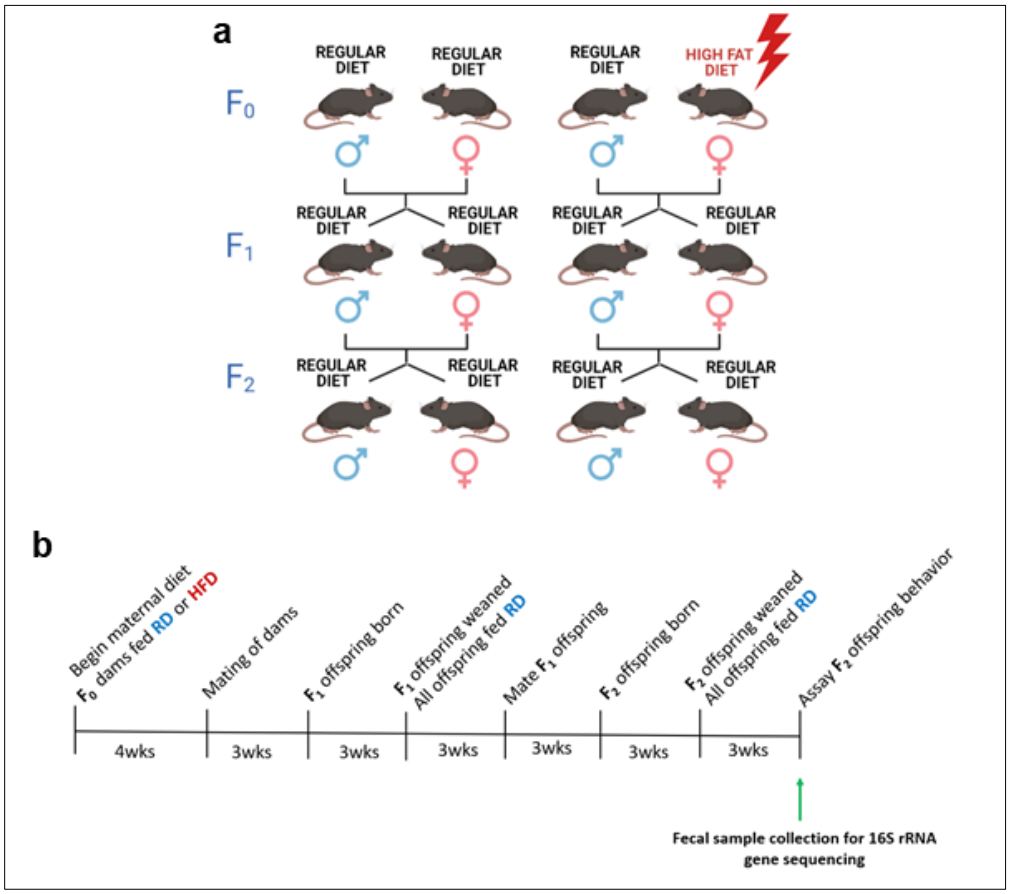
a
REGULAR DIET
REGULAR DIET
REGULAR DIET
HIGH FAT DIET
F0
F1
F2
b
Begin maternal diet F0 dams fed RD or HFD
Mating of dams
F1 offspring born
F1 offspring weaned All offspring fed RD
Mate F1 offspring
F2 offspring born
F2 offspring weaned All offspring fed RD
Assay F2 offspring behavior
4wks
3wks
3wks
3wks
3wks
3wks
3wks
Fecal sample collection for 16S rRNA gene sequencing

Figure 1. Breeding schematic. (**a**) Control (MRD) and MHFD F_1 offspring were used to produce, respectively, MRD- descendant and MHFD-descendant F_2 generations. (**b**) F_2 offspring were weaned and caged according to maternal lineage at three weeks of age, and both groups were then placed on regular diet. Stool samples were collected aseptically during handling three weeks after weaning and three days prior to performing behavioral tests.

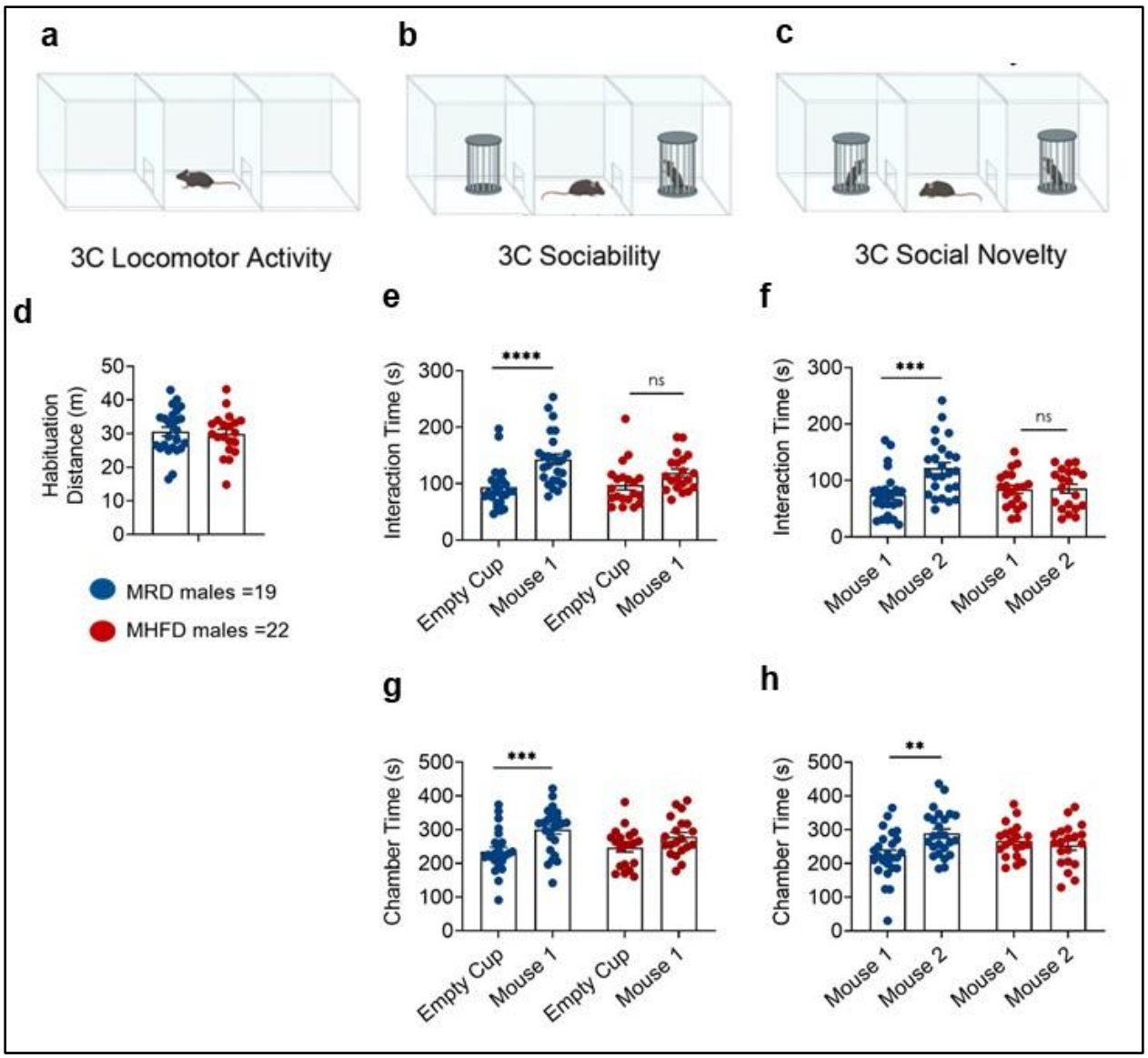
a
b
c
3C Locomotor Activity
3C Sociability
3C Social Novelty
d
e
f
Habituation Distance (m)
50
40
30
20
10
0
MRD males =19
MHFD males =22
Interaction Time (s)
300
200
100
0
****
ns
Empty Cup
Mouse 1
***
Mouse 2
g
h
Chamber Time (s)
500
400
**

Figure 2. Male MHFD-descendant F_2 generation display reduced sociability and loss of preference for social novelty. (**a, b, c**) Schematic depicting the three stages of Crawley's three-chamber test–habituation (**a**), sociability (**b**), and preference for social novelty (**c**). (**d**) No changes in distance travelled between MHFD- and MRD male offspring (*p= 0.7186, t=0.3626*). (**e**) MHFD male mice show impaired sociability compared to the MRD mice (*MRD, p=0.0001, t=4.517; MHFD, p=0.1651, t=1.735*). (**f**) Social novelty preference is decreased on the MHFD male offspring compared to the MRD offspring (*MRD, p=0.0003, t=4.208; MHFD, p=0.9916, t=0.1159*). (**g**) Time mice spent in the chamber with the empty cup vs. the chamber with the cup containing mouse 1 (*MRD, p=0.0007, t=3.740; MHFD, p=0.1932, t=1.654*). (**h**) Time mice spent in the chamber with the empty containing mouse 1 vs. the chamber with the cup containing mouse 2 (*MRD, p=0.0013, t=3.534; MHFD, p=0.7804, t=0.6284*).

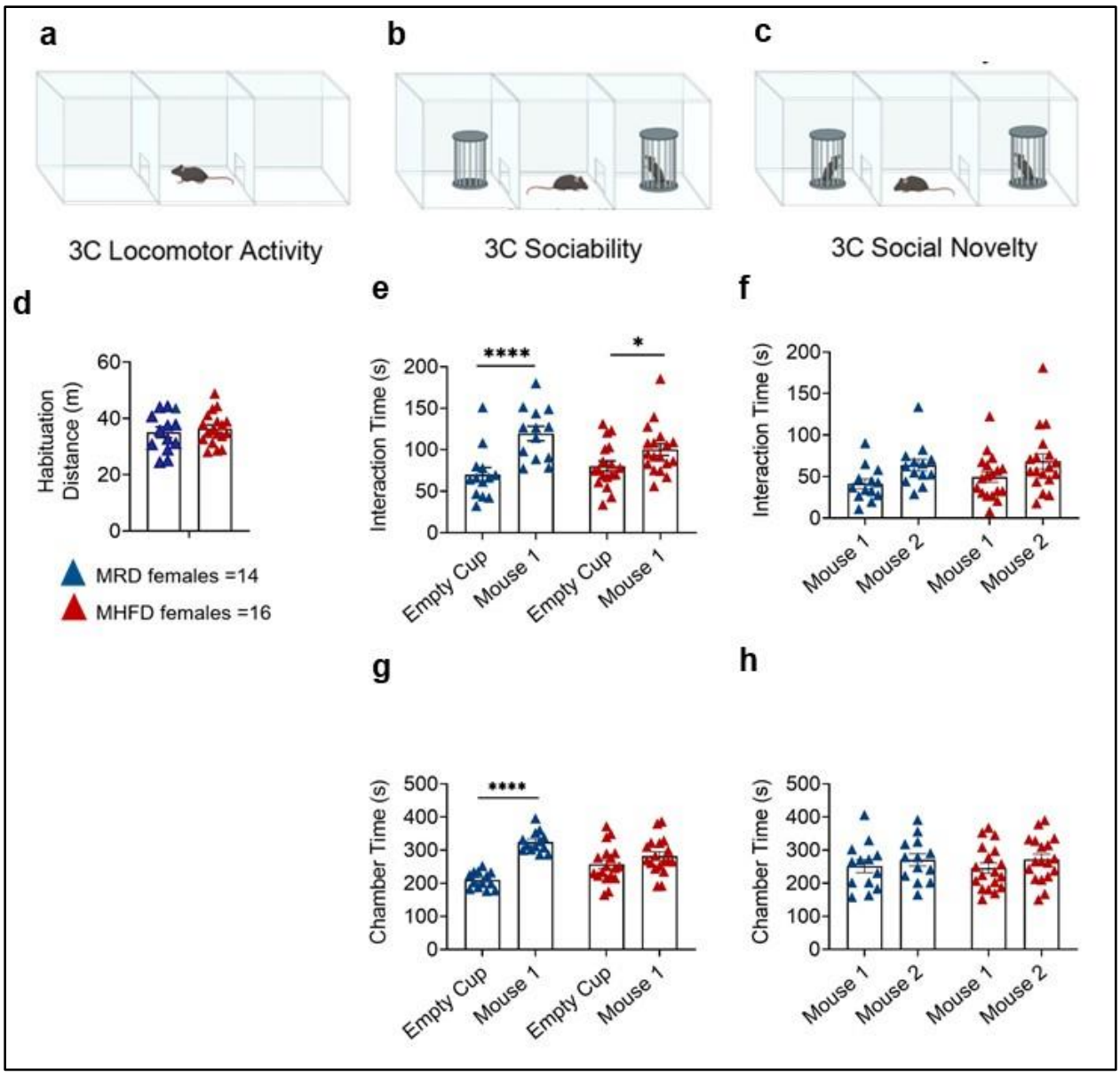
a
b
c
3C Locomotor Activity
3C Sociability
3C Social Novelty
d
Habituation Distance (m)
60
40
20
0
MRD females =14
MHFD females =16
e
Interaction Time (s)
200
150
100
50
0
****
*
Empty Cup
Mouse 1
Empty Cup
Mouse 1
f
Interaction Time (s)
200
150
100
50
0
Mouse 1
Mouse 2
Mouse 1
Mouse 2
g
Chamber Time (s)
500
400
300
200
100
0
****
Empty Cup
Mouse 1
Empty Cup
Mouse 1
h
Chamber Time (s)
500
400
300
200
100
0
Mouse 1
Mouse 2
Mouse 1
Mouse 2

Figure 3. Female MHFD-descendant F_2 generation display reduced sociability but no changes in preference for social novelty. (**a, b, c**) Schematic depicting the three stages of Crawley's three-chamber test–habituation (**a**), sociability (**b**), and preference for social novelty (**c**). (**d**) No changes in distance travelled between MHFD- and MRD female offspring (*p= 0.8379, t=0.2113*). (**e**) Sociability is decreased, yet still statistically significant, in MHFD female offspring compared to MRD (*MRD, p<0.0001, t=5.346; MHFD, p=0.0341, t=2.528*). (**f**) Neither MRD nor MHFD female offspring showed a statistically significant preference for social novelty (*MRD, p=0.1139, t=1.928; MHFD, p=0.1160, t=1.920*). (**g**) Time mice spent in the chamber with the empty cup vs. the chamber with the cup containing mouse 1 (*MRD, p<0.0001, t=6.333; MHFD, p=0.2037, t=1.634*). (**h**) Time mice spent in the chamber with the empty containing mouse 1 vs. the chamber with the cup containing mouse 2 (*MRD, p=0.7183, t=0.7285; MHFD, p=0.4252, t=1.182*).

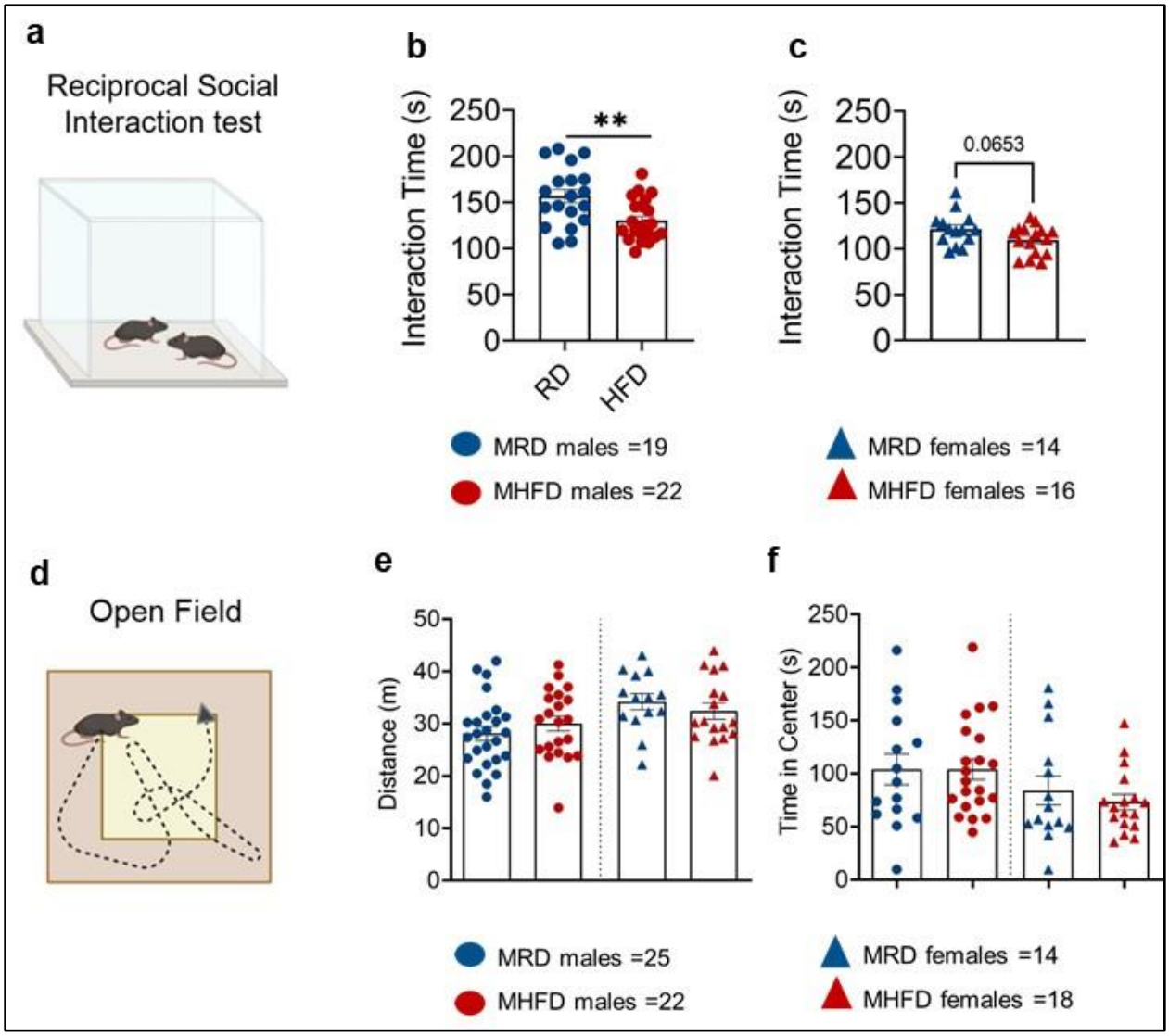
a
Reciprocal Social Interaction test
b
Interaction Time (s)
250
200
150
100
50
0
**
RD
HFD
MRD males =19
MHFD males =22
c
Interaction Time (s)
250
200
150
100
50
0
0.0653
MRD females =14
MHFD females =16
d
Open Field
e
Distance (m)
50
40
30
20
10
0
MRD males =25
MHFD males =22
f
Time in Center (s)
250
200
150
100
50
0
MRD females =14
MHFD females =18

Figure 4. MHFD-descendant F_2 generation display reduced social interaction but not anxiety-like behavior. (**a**) Schematic of the reciprocal social interaction test. (**b**) Male MHFD offspring showed reduced interaction time compared to MRD offspring controls in the reciprocal social interaction test (*p= 0.0049, t=3.024*) **(c)** No statistically significant difference in the interaction time between MHFD and MRD female mice (*p=0.0653, t=1.918*) (**d**) Schematic of the open field test. (**e,f**) No significant changes in distance travelled (*males, p = 0.3934, t=0.8882; females p=0.4807, t=0.5470*) (**e**) or time spent exploring the center of the open field arena (*males, p= 0.7805, t=0.2856; females p=0.5273, t=0.6608*) (**f**) was found between MRD and MHFD F_2 cohorts, regardless of sex.

F_0 parent HFD consumption alters F_2 offspring body mass composition.

After 4 weeks on HFD, F_0 female total and lean mass was not significantly different between the HFD- and the RD-fed groups (**Chapter 2, Fig. 1b,c**). However, compared to RD-fed dams, HFD-fed females show a significant increase in fat mass (**Chapter 2, Fig.1d**). In the F_1 generation, we observed a decrease in MHFD offspring weight at 8 weeks of age compared to MRD group, regardless of sex (**Chapter 2, Fig. 4a-b**), while at 20 weeks of age MHFD offspring weight was comparable to MRD offspring weight in both males and females (**Chapter 2, Fig. 4c-d**).

Here, we performed BMA on the F_2 offspring at the end of the battery of behavioral tests to explore body mass composition of these mouse cohorts. Data obtained showed that both F_2 male and female offspring showed no differences in total (**Fig. 5a,d**) or lean mass (**Fig. 5b,e**) regardless of sex. However, we did observe a significant increase in fat mass in both males (**Fig. 5c**) and females (**Fig. 5f**) F_2 MHFD offspring, thus showing that the effects of MHFD regimen on total weight and body mass composition are extended to generations beyond F_1.

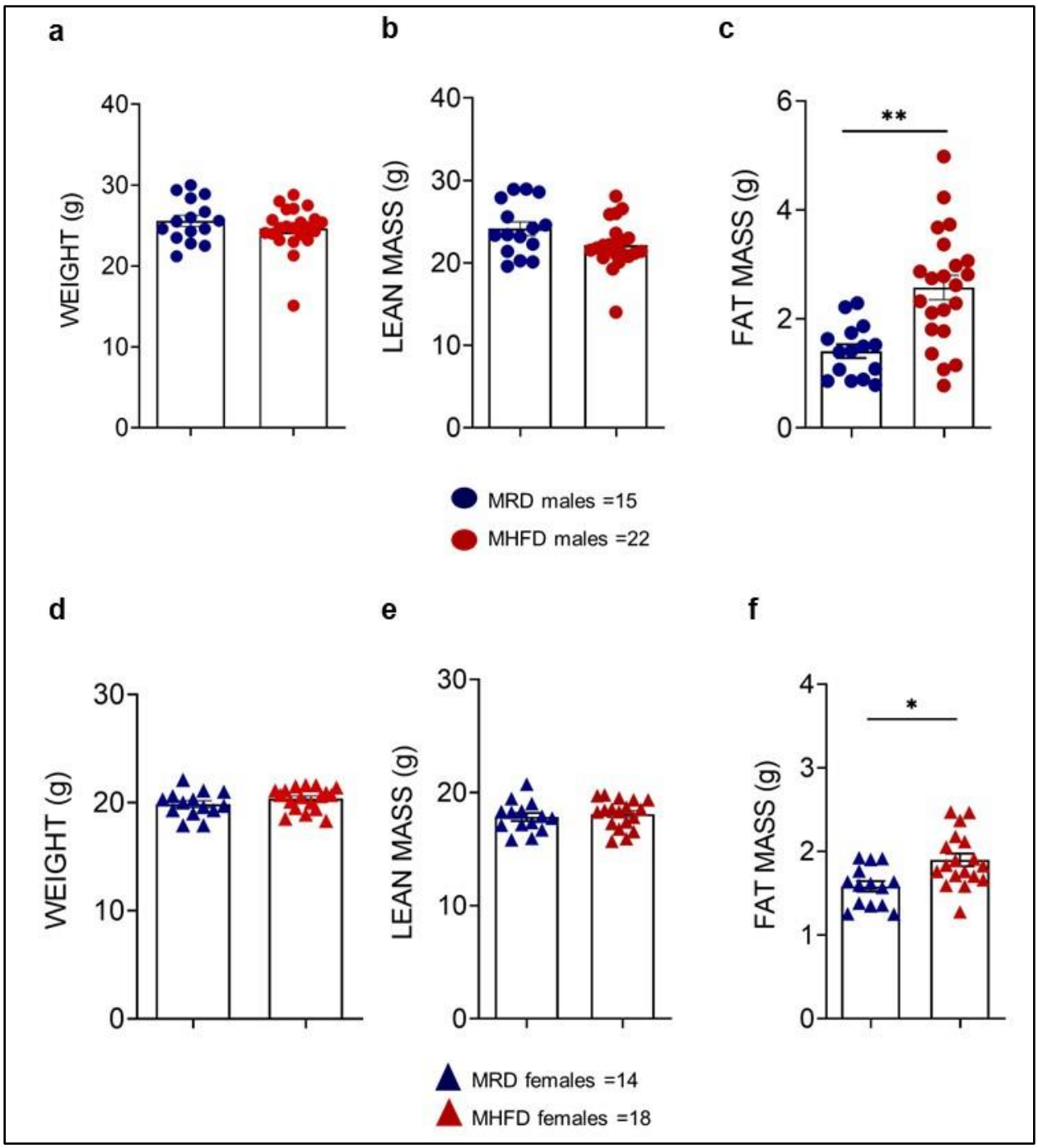
a
b
c
WEIGHT (g)
LEAN MASS (g)
FAT MASS (g)
**
MRD males =15
MHFD males =22
d
e
f
*
MRD females =14
MHFD females =18

Figure 5. MHFD-induced alterations in body mass composition are visible in the F_2 generation. (**a-c**) F_2 male offspring total (**a**) and lean mass (**b**) are unaffected by F_0 MHFD, while fat mass (**c**) is increased in the MHFD group (*a, p=0.5055, t=0.6971; b, p=0.3014 t=1.105; c, p=0.0151, t=3.082*). Similarly, no differences in F2 female offspring total (**d**) and lean mass (**e**), however, MHFD group fat mass (**f**) is increased compared to the MRD group (*d, p=0.4487, t=0.6371; e, p=0.7721 t=0.2996; f, p=0.0699, t=2.091*).

MHFD-induced dysbiosis of the gut microbiome observed in the F_1 generation persists in the F_2 generation.

As social deficits persist in MHFD F_2 offspring, especially among males, we hypothesized that the dysbiosis of the gut microbiome that we have previously observed in HFD-fed dams and F_1 offspring, could be reflected also in the second generation. To interrogate microbial gut composition and community structure of both our F_1 and F_2 cohorts, fecal samples were collected during mouse handling, prior to the start of behavioral tests, and sent to our collaborators at the Alkek Center for Metagenomics and Microbiome Research (CMMR) to extract bacterial DNA and perform 16S rRNA gene sequencing.

The assessment of the community structure of gut microbiota by means of principal coordinate analysis (PCoA) based on weighted UniFrac distances, a β-diversity measure which considers the phylogenetic relationships between samples and the abundance of operational taxonomic units (OTUs), revealed no significant differences across diets/generations (**Fig.6a**). However, PCoA based on unweighted analysis of UniFrac distances, which is not affected by OTU abundance, and consider only their presence/absence, revealed statistically significant clusters. Indeed, fecal microbial communities of F_1 MHFD females clustered together, separately from both F_1 and F_2 MRD cohorts, as well as F_2 MHFD offspring. While F_2 MHFD offspring communities clustered together, however, we observed a shift of this community towards the F_2 MRD cohort, demonstrated by partially overlapping 95% confidence intervals between the two groups. Of note, as expected, F_1 and F_2 MRD community show low degree of variation, appearing more similar to each other than MHFD groups (**Fig. 6b**).

Next, we used different metrics to summarize the "within-sample" diversity (alpha-diversity). Observed OUT count, Shannon index, and Inverse Simpson index were used to this end. The number of observed OTUs, which describes the richness of each microbial community, showed a significant decrease in F1 MHFD subjects compared to the F1 MRD group (**Fig. 6c**), although no differences were found for Shannon, an estimator for both species' richness and evenness, but with weight on the richness, and Inverse Simpson indexes, which instead considers species evenness more than species richness in its measurement. (**Fig. 6d,e**). The comparisons between F2 MHFD *versus* MRD males or females, showed no statistically significant differences were observed in observed OTUS, Shannon, and Inverse Simpson index (**Fig7a-f**).

Since the differences in observed OTUs found between diets in the F_1 generations were not reproduced in the F_2 groups, we therefore examined generational differences in alpha diversity within each respective diet. While, as expected, no generational differences were observed in MRD mice (**Fig. 7j-l**), observed OTUs in both F_2 MHFD males and females, were significantly increased compared to those found in the MHFD F_1 mice (**Fig. 7g**). Conversely, no differences were observed for Shannon and Inverse Simpson indexes for either MHFD or MRD generations (**Fig. 7h,i**).

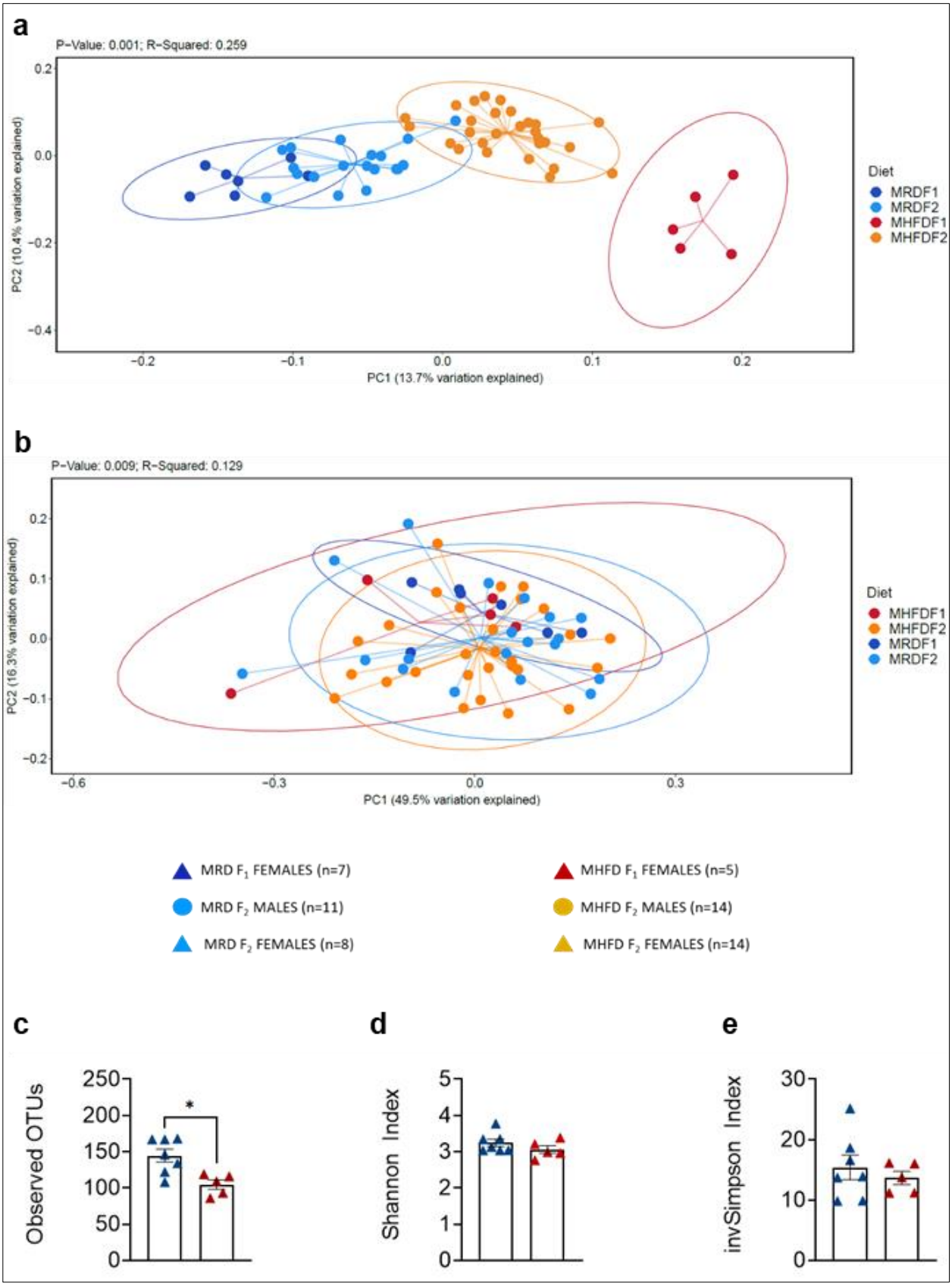
a
P−Value: 0.001; R−Squared: 0.259
PC2 (10.4% variation explained)
PC1 (13.7% variation explained)
Diet
MRDF1
MRDF2
MHFDF1
MHFDF2
b
P−Value: 0.009; R−Squared: 0.129
PC2 (16.3% variation explained)
PC1 (49.5% variation explained)
Diet
MHFDF1
MHFDF2
MRDF1
MRDF2
MRD F1 FEMALES (n=7)
MRD F2 MALES (n=11)
MRD F2 FEMALES (n=8)
MHFD F1 FEMALES (n=5)
MHFD F2 MALES (n=14)
MHFD F2 FEMALES (n=14)
c
Observed OTUs
d
Shannon Index
e
invSimpson Index

Figure 6. MHFD F_2 offspring microbiota clusters separately from MHFD F_1 and MRD F_2 gut microbiota. (**a**) Principal coordinates analysis (PCoA) of unweighted UniFrac distances from the averaged rarefied 16S rRNA gene dataset revealed that MHFD F_2 offspring fecal microbiota cluster separately from MHFD F_1 offspring microbiota, while it partially overlaps with the MRD F_2 group (*$p = 0.001$, $R^2 = 0.259$, F-statistic=6.4*). (**b**) PCoA of weighted UniFrac distances from the averaged rarefied 16S rRNA gene dataset showed overlap between the four diet/generation cohorts (*$p = 0.006$, $R^2 = 0.129$, F-statistic=2.73*). (**c**) A decrease in α-diversity, measured as total number of OTUs, is observed in the MHFD F1 group compared to the F1 MRD dams (*Mann-Whitney U = 3, $p = 0.0164$*) , while no differences are observed in Shannon (**d**) (*Mann-Whitney U = 10, $p = 0.2677$*), and inverse Simpson (**e**) (*Mann-Whitney U = 15, P = 0.7551*).

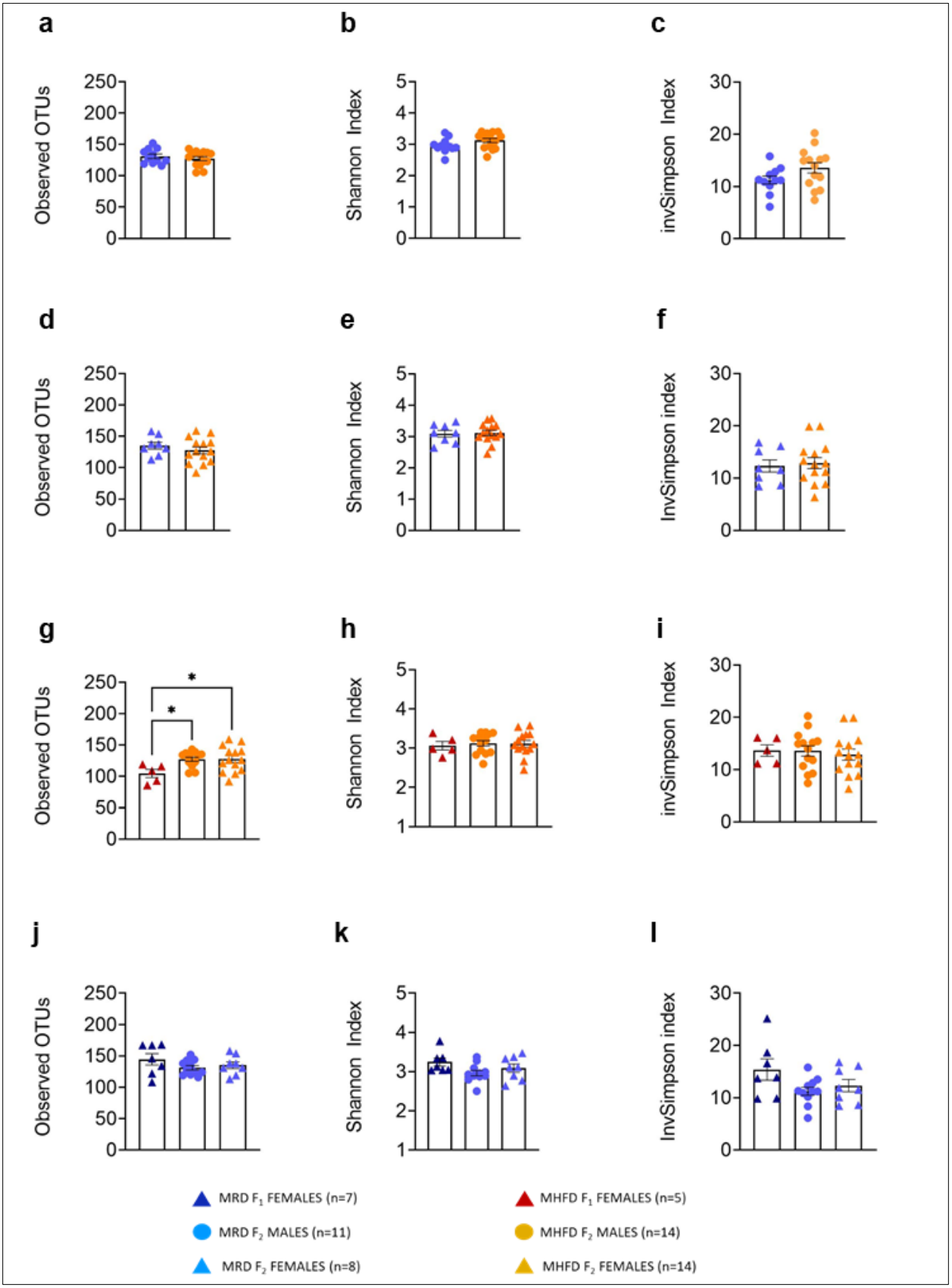
a
b
c
d
e
f
g
h
i
j
k
l
Observed OTUs
Shannon Index
invSimpson Index
InvSimpson index
MRD F1 FEMALES (n=7)
MRD F2 MALES (n=11)
MRD F2 FEMALES (n=8)
MHFD F1 FEMALES (n=5)
MHFD F2 MALES (n=14)
MHFD F2 FEMALES (n=14)

Figure 7. MHFD F_2 offspring shows a partial recovery in α-diversity

No differences in observed OTUs (**a, d**), Shannon index (**b,e**) and inverse Simpson (**c,f**) are observed for either F_2 males or females between diets (*Mann-Whitney U = 65, p = 0.5263; Mann-Whitney U = 45, p = 0.4920; Mann-Whitney U = 45, p = 0.1656; Mann-Whitney U = 55, p = 0.9734; Mann-Whitney U = 49, p = 0.1341; Mann-Whitney U = 53, p = 0.8676).* While no differences observed OTUs (**j**), Shannon (**k**) and inverse Simpson (**l**) indexes are observed between MRD F_1 and MRD F_2 males and females (*ordinary one-way ANOVA F=1.583, p=0.0856; F=2.480, p=0.1059; F= 1.365, p= 0.2753*), both male and female MHFD F_2 show an increase in the number of observed OTUs (**g**) compared to the MHFD F_1 dams (*ordinary one-way ANOVA F=4.058, p=0.0276).* No differences across MHFD generations are observed in Shannon (**h**) and inverse Simpson (**i**) indexes. (*ordinary one-way ANOVA F=0.08900, p=0.9151; F=0.1594, p=0.8533)*

Circulating BCAA concentration is comparable in F_2 MHFD and MRD offspring.

Our assessment of behavior and body mass composition in the F_2 generation revelead that social dysfunction and metabolic alterations observed in the F_1 offspring persist across generation. The next step in our study was to identify the molecular and cellular mechanisms underlying the behavioral and metabolic phenotypes observed in the F_2 offspring. In this light, we begin to investigate the maternal and offspring serum metabolome. Indeed, changes in gut microbiota composition and structure are reflected in circulating metabolites in the host, and alterations in the metabolome impact host physiology.[995] In our previous study, we reported that MHFD induced a concurrent decrease in *L. reuteri* and increase in *B. vulgatus* abundance in the intestinal microbiome of the F_1 offspring. In Chapter 2, we hypothesized that *B. vulgatus*-induced dysregulation in concentration of circulating BCAAs, a group of essential AAs which have been implicated in ASD[825,826] and metabolic disorders[929,930], might be one molecular mechanism underlying the metabolic and behavioral impairment observed in the F_1 MHFD offspring. Indeed, we identified a 28% in the concentration of BCAAs in the serum of the F_1 MHFD mice (**Chapter 2, Fig. 12a**). Our assessment of BCAA concentration in the serum of the F_2 offspring, however, showed no significant differences between MRD- and MHFD-descendent F_2 offspring (**Fig. 8a, b**). While this data suggests that other microbially-derived serum metabolites might be implicated in the etiology of F_2 offspring behavioral and metabolic alterations, notably we have yet to assess cognitive phenotypes in the F_2 generation and it is possible that the elevated BCAA levels in the F_1 generation are solely linked to the cognitive, but not the social, deficits, in the MHFD offspring.

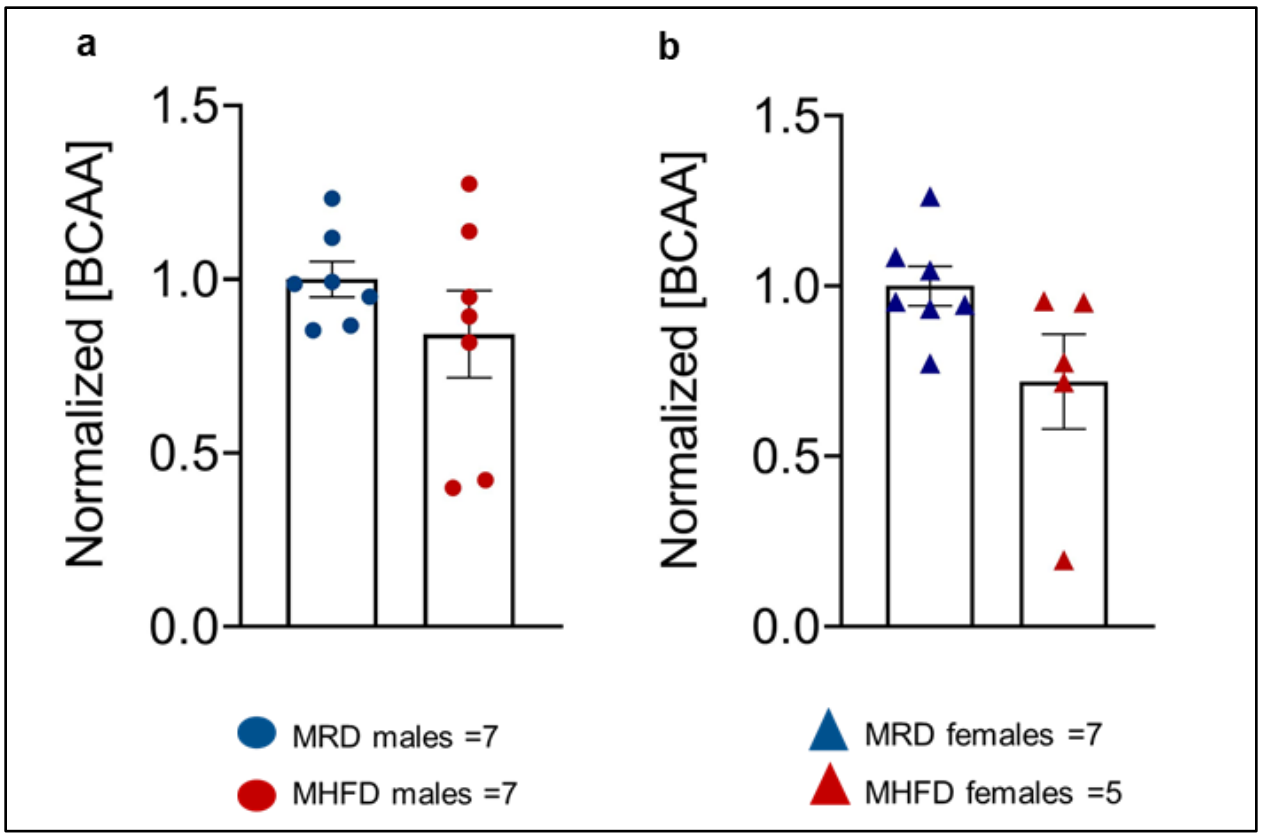
a
b
Normalized [BCAA]
1.5
1.0
0.5
0.0
MRD males =7
MHFD males =7
MRD females =7
MHFD females =5

Figure 8. The concentration of circulating BCAA in the F_2 offspring generation is unaffected by F_0 maternal diet. (**a**) Normalized BCAA concentration in the serum of MHFD and MRD F_2 male offspring showed no differences between the two groups (*p=0.2653, t=1*). (**b**) Similar results were obtained the analysis of BCAA concentration in the serum of MHFD and MRD F_2 female offspring (*p=0.0635, t=2.087*).

The concentration of circulating IL-17a is increased in HFD-fed dams but comparable in the F_2 MHFD and MRD offspring.

Bidirectional communication between gut microbiota and the brain occurs through multiple channels (**Chapter 1, Fig. 2**), including via modulation of host immune function. Given that gut microbiota influences the development and homeostasis of the immune system, we investigated whether alterations in host immune function, specifically dysregulation of cytokine profiles and immune cell differentiation, might be involved in the behavioral and metabolic impairments observed in our F_2 cohorts. In this light, we assessed the cytokine concentration in the serum of the F_2 offspring, focusing our attention on IL-17a–a cytokine whose levels are increased in the serum of autistic children[437,996] and other environmental models of ASD.

Previous studies investigating the effects of maternal immune activation (MIA) during pregnancy on offspring neurodevelopment in mice showed that T-helper 17 (T_H17) cells and the effector cytokine interleukin-17a (IL-17a) were involved in the MIA-induced ASD-like behavioral abnormalities in offspring.[424] Based on the influence of different cytokines, naïve CD4 T-cells can differentiate to various T helper cells (Th), including T_H17 cells and regulatory T (Treg) cells, whose functions are completely different. Indeed, while Th17 cells are involved in inflammatory and autoimmune phenomena, Treg cells induce anti-inflammatory responses.[997] Given that the microbiome plays a crucial role in the differentiation of several intestinal immune cells, changes in the composition of the gut microbiome, including those driven by MHFD, could influence the Th17/Treg balance and thus inflammation.[998]

Given that MIA offspring also show dysbiosis of the gut microbiome, and that treatment with the commensal species *Bacteroides fragilis* ameliorates both inflammation and ASD-like

phenotypes[64], we hypothesized that a similar mechanism might be involved in the context of our MHFD model of diet-induced dysbiosis in the maternal gut microbiome. Specifically, we hypothesized that MHFD-induced dysbiosis could trigger an abnormal immune response and cytokine imbalance which ultimately contributes to behavioral alterations in the offspring. First, we aimed to investigate whether a HFD regimen could drive an increase in IL-17a. Our data showed that IL-17a is significantly elevated by one week on HFD compared to RD (**Fig. 9a**), concurrently with the observed shift in HFD-driven gut (fecal) microbiome composition (**Chapter 2, Fig. 1e-h**) and at gestational day 14.5 in HFD-fed compared to RD-fed dams (**Fig. 9b**), the precise developmental stage at which MIA-induced increase in IL-17a results in offspring social dysfunction.[424] Next, we collected the serum of F_2 offspring after completing the battery of behavioral tests to investigated the levels of IL-17a. However, we found no significant differences between MHFD and MRD mice, regardless of sex (**Fig. 9c, d**), suggesting that alternative cytokines might be involved in the onset of F_2 offspring behavioral and metabolic alterations.

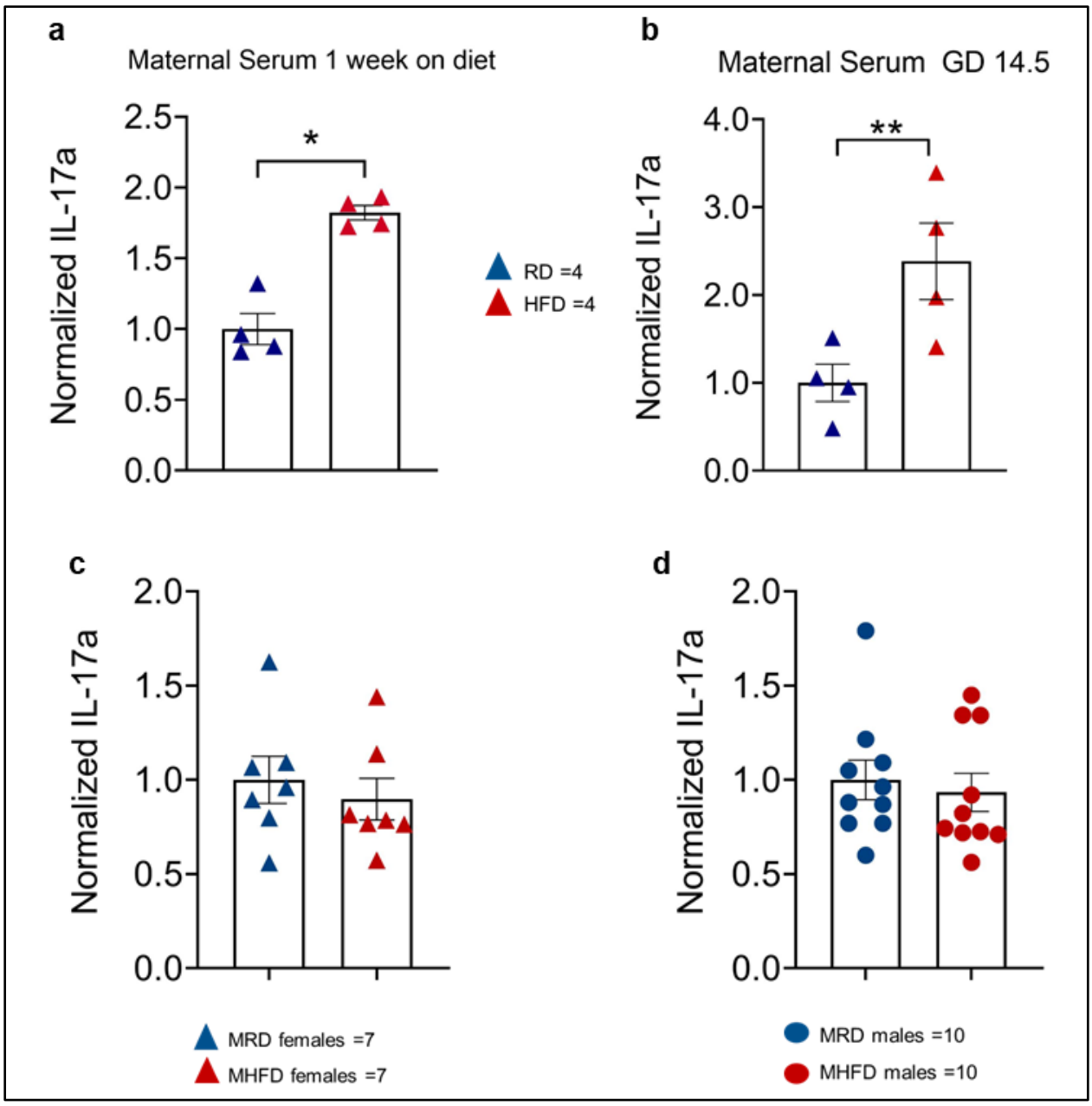
a
Maternal Serum 1 week on diet
Normalized IL-17a
2.5
2.0
1.5
1.0
0.5
0.0
*
RD =4
HFD =4
b
Maternal Serum GD 14.5
Normalized IL-17a
4.0
3.0
2.0
1.0
0.0
**
c
Normalized IL-17a
2.0
1.5
1.0
0.5
0.0
MRD females =7
MHFD females =7
d
Normalized IL-17a
2.0
1.5
1.0
0.5
0.0
MRD males =10
MHFD males =10

Figure 9. The concentration of circulating proinflammatory cytokines is increased in HFD-fed dams, while no differences are observed in F_2 generation mice. (**a,b**) Normalized IL-17a concentration in the serum of HFD-fed females is increased after 1 week on diet (**a**) (*p=0.0286*, Mann-Whitney U=0) and at GD14.5 (**b**) (*p=0.0288, t=2.860*) compared to RD-fed group. (**c,d**) Normalized IL-17a concentration in the serum of MRD and MHFD F2 male offspring (**c**) (*p=0.4359, Mann-Whitney U=30*) and female (**d**) (*p=0.4557, Mann-Whitney U=18*) offspring is not significantly different.

Oxytocin levels are decreased in the paraventricular nucleus of the hypothalamus of F_2 maternal HFD lineage offspring.

Since *L. reuteri* has been shown to promote oxytocin (OT) levels by a vagus nerve-mediated pathway[999], in our recent work, we hypothesized that the decrease in *L. reuteri* that we observed in the gut microbiome of the F_1 MHFD offspring was casually related to their social impairment. Interestingly, we found that reconstitution with *L. reuteri* rescues the MHFD-induced reduction in OT levels in the paraventricular nucleus of the hypothalamus (PVN), where OT is primarily synthetized, of F_1 MHFD offspring.[63]

Furthermore, in subsequent work, we found that genetic deletion of oxytocin receptor expression in dopaminergic neurons attenuated the effect of *L. reuteri* on social dysfunction in the *Shank3B*$^{-/-}$ genetic mouse model of ASD.[765] Therefore, we measured oxytocin levels in the PVN of the F_2 offspring and found a mild, yet significant decrease in the MHFD lineage, but not control (**Fig. 10a**), F_2 offspring (**Fig. 10b**). These results suggest that the deficits in the oxytocinergic-dopaminergic social reward circuit identified in the F_1 MHFD offspring persist in F_2, despite lack of direct exposure to HFD.

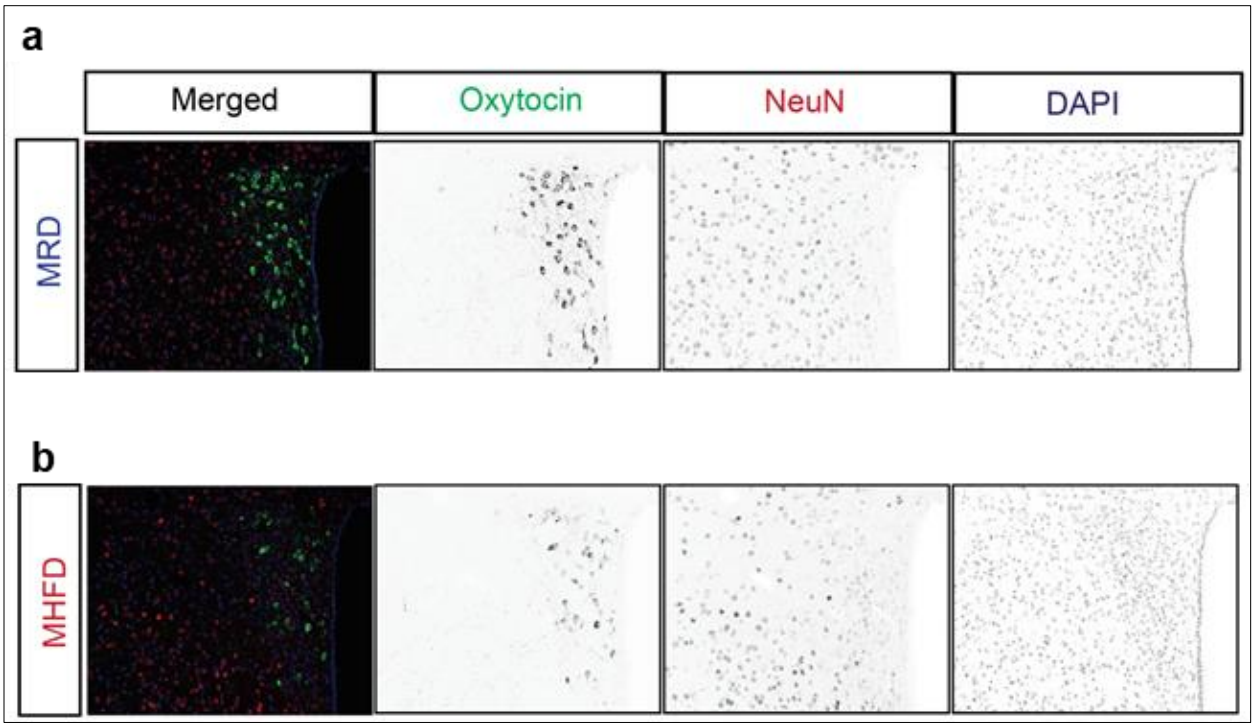
a
Merged
Oxytocin
NeuN
DAPI
MRD
b
MHFD

Figure 10. Oxytocin levels are decreased in the PVN of MHFD F_2 offspring. (**a,b**) Oxytocin immunoreactivity was reduced in the PVN of MHFD (**b**) versus MRD F_2 offspring (**a**). In the PVN of MRD (**a**) and MHFD (**b**) offspring, NeuN cell number immunoreactivity similar. (Data in **a,b** reflect MHFD and MRD male cohorts).

4. Discussion.

NDDs, such as ASD, represent a significant global public health concern and unraveling the underlying causes of NDDs is essential to the development of appropriate preventive/therapeutic strategies. Many studies, including human epidemiological studies, point at maternal obesity, often accompanied by diabetes, as one of the primary nongenetic risk factors underlying the development of NDDs in progeny.[525] Given the increasing prevalence of obesity and overweight status in women of childbearing age, further investigation into the mechanisms by which maternal obesity impacts offspring health is needed.

We have previously shown that, in mice, HFD consumption induced significant changes in maternal gut microbial ecology, including a significant decrease in diversity. Interestingly, similar alterations were found in adolescent MHFD offspring, despite their being fed a regular diet (RD) from the time of weaning onward. Furthermore, the MHFD F_1 generation displayed impaired social behavior, rescued by the selective re-introduction of *L. reuteri*, the species we found to be most significantly reduced in the MHFD offspring microbiota.[63] In **Chapter 2**, we provided new evidence supporting the hypothesis that the detrimental effects of MHFD on offspring health are not limited to impairments in social behavior, but also include cognitive deficits and metabolic alterations, consistent with comorbidities in patient populations[1000]. Since maternal diet-induced alterations in gut microbiome composition are transferred to the first generation, we reasoned that a similar mechanism, directly related to microbial dysbiosis, might impact F_2 offspring and induce behavioral and metabolic deficits in the absence of direct exposure to MHFD. Our hypothesis is supported by previous work showing the heritability of the gut microbiome across multiple generations in a mouse model consuming a diet with limited availability of microbiota accessible

carbohydrates (MACs). When compared to high-MAC diet group, the MAC-deprived mice displayed a loss of taxa in the gut microbiome. Intriguingly, this effect was magnified over generations.[993]

Here, we investigated (1) the impact of MHFD on F_2 social behavior and body mass composition, and (2) the potential mechanisms underlying these behavioral and metabolic phenotypes. Regarding the former, we showed that male offspring in the MHFD-descendant F_2 generation display reduced sociability and loss of preference for social novelty (**Figs. 2**,**3**). Indeed, while MHFD male F_2 offspring showed a clear loss of preference in sociability stages (**Fig. 2e,g**), we observed a strong preference in MRD females and a weaker, yet still statistically significant preference in MHFD females (**Fig. 3e,g**), thus suggesting that MHFD-induced impairments in sociability are relatively less pronounced in females than males. We speculate that this points to the idea of "female resiliency," which is supported by human studies showing that females affected by ASD tend to show fewer restricted and repetitive behaviors and better social communication skills than males with ASD.[33,1001] Also of note, while MHFD male F_2 offspring showed a clear loss of preference in the social novelty stage (**Fig. 2f,h**), neither MRD or MHFD female F_2 offspring showed a statistically significant preference for interaction with a novel over a familiar mouse (**Fig. 3f,h**). We hypothesize that this reflects a "social redundancy" in which females are "engineered" to be social, so as to ensure survival of offspring, therefore making a preference more difficult to emerge. A similar conclusion can be drawn from the results of the reciprocal social interaction test, in which the time spent interacting was lower for pairs of MHFD male offspring than MRD pairs (**Fig. 4b**), while pairs of MRD and MHFD female F_2 offspring showed no statistically significant difference in interaction time (**Fig. 4c**). As we previously showed in the F_1 offspring, the analysis of open field test data showed that neither males or females showed clear

signs of hyperactivity or anxiety-like behavior (**Fig. 4e**,**f**), thus reinforcing the consistency of our experimental model.

Additionally, both male and female mice of the MHFD F_2 offspring generation displayed increased fat mass (**Fig. 5c,f**), as measured by BMA performed at the end of the battery of behavioral tests, while total (**Fig. 5a,d**) and lean mass (**Fig. 5b,e**) were comparable between the MHFD and MRD descendants. This finding is of particular interest, since the F_2 mice were all fed a regular diet and, most importantly, unlike the F_1 generation offspring, they were never exposed directly to a maternal high-fat regimen. The increase in fat mass that we observed in F_2 MHFD descendants will spur future investigation of the molecular and cellular mechanisms underlying the potential changes in the metabolic status which leads to alterations in the body mass composition.

As mentioned above in this chapter, we postulated that the behavioral and metabolic impairments in the F_2 generation are the result of the multigenerational transfer of a dysbiotic gut microbiome from F_0 HFD-exposed mothers. To assess whether the F_2 offspring also harbor a dysbiotic gut microbiome, we collected fecal samples from our F_2 generation dam and offspring cohorts to perform 16S rRNA gene sequencing, the most used method for identification, classification, and quantitation of microbes within complex biological mixtures.

The analysis of F_2 MHFD gut microbiota composition at 6–8 weeks of age revealed a partial recovery in microbial diversity compared to their mothers for both males and females, demonstrated by the absence of differences in observed OTU count between MRD and MHFD F_2 cohorts. This might suggest that the decrease in α-diversity induced by parental HFD regimen would be eventually recovered under the effect of a regular diet over the course of two generation. However, observed OTU count is a qualitative metric, which does not account for species

abundance or other features of a microbial community. The assessment of the similarity between the communities, performed by unweighted analyses of UniFrac distances suggest that MHFD F_2 gut microbiota is indeed shifted towards MRD F_2 group, but yet distinct from both the MRD F_2 and MHFD F_1 microbiota. It is therefore possible to hypothesize that the third generation of MHFD descendants would be completely overlapping with MRD offspring.

This presumed amelioration of the MHFD F_2 gut microbiota composition and structure appear not sufficient to prevent the onset of behavioral dysfunction, as indicated by the behavioral tests that we performed. To fully explore the mechanisms underlying this apparent mismatch between gut microbiota diversity and behavioral phenotypes, a more integrated approach, which account for multiple features of the gut microbiota, including qualitative taxa differences and functional role (metabolic pathways), is needed. Importantly, it must be noted that microbial composition here described was determined by 16S rRNA gene sequencing from fecal samples, which does not include mucosa-associated microorganisms, technically far more complex to isolate, but equally informative about the structure of the gut microbiota.[1002] Additionally, offspring brain development is influenced by both the maternal and offspring gut microbiome, during pre- and early post-natal life, respectively. Therefore, while the offspring post-natal gut microbiome might have at least partially recovered from the dysbiotic status induced by antenatal diet at the time of our assessment (6 weeks of age) , the maternal one might still be influenced by these alterations, thus providing a suboptimal fetal environment. To elucidate the evolution of the pregnant gut microbiome, its relationship with the diet, and its influence on offspring neurodevelopment, we suggest performing a longitudinal metagenomic sequencing and functional metagenomic profiling of the maternal gut microbiome during pregnancy and lactation.

In order to explore the potential molecular and cellular mechanisms underlying the deficits in social behavior displayed by the F_2 generation, we performed an exploratory investigation of the concentration of specific metabolites and cytokines in the host serum whose levels were found dysregulated in either the F_0 or the F_1 generations. Several studies suggest that the interactions between the host and the microbial communities residing in the gut can influence host serum metabolome[995,1003] and circulating cytokine profile[1004].

In particular, microbially-derived, low molecular weight (LMW) bioactive molecules have been implicated in multiple host physiological and pathological processes,[593,1005] including metabolic disorders[828] and cognitive and behavioral dysfunction.[848] In the context of circulating microbially-derived metabolites, we focused our attention on BCAAs, due to the fact that (1) they have been shown to modulate several biochemical processes in the body, including those related to protein synthesis, lipid and glucose metabolism, as well as brain and immune function[1006], (2) recent studies showed that their levels in the blood fluctuates in response to specific changes in the gut microbiota composition[633], (3) their dysregulation has been associated with insulin resistance and ASD, and (4) we showed increased levels of BCAAs in the serum of our MHFD F_1 offspring, normalized by maternal metformin treatment (**Chapter 2**, **Fig. 12a**). Our assessment of circulating BCAAs in MHFD and MRD F_2 offspring, however, did not reveal significant differences between the groups, regardless of sex (**Fig. 8**), suggesting that increased levels of these amino acids might not represent the main mechanism behind the metabolic and social behavioral impairments that we observed in our mouse model. We anticipate that a more in-depth analysis of the offspring metabolome we perform in the near future will provide new insights in the investigation of microbial products influencing F_2 offspring phenotypes.

Since the gut microbiota is implicated in shaping both innate and adaptive immune

response,[889] the next step in the investigation of potential molecular mediators of the deficits observed in the F_2 offspring was focused on the analysis cytokine profiles in the serum of our F_2 mice. In this regard, we initially looked at IL-17a, one of six known members of the IL-17 cytokine family (IL-17a-f). IL-17a is the signature cytokine of Th17 cells, which differentiate from the same precursor of another class of T lymphocytes, Treg cells. The disequilibrium between Treg and Th17 cells may lead to increase inflammation and autoimmune phenomena.[1007] Intriguingly, the gut microbiome has been shown to participate in the balance between these two T cell types[1008], with evidence corroborated by studies performed in germ-free mice suggesting that the absence of the microbial communities of the gut leads to defects in the differentiation of Th17 and Treg cells.[671] In the context of mouse models of environmental ASD, it has been shown that increased activation maternal interleukin-17a pathway in mice following maternal infection induce ASD-like phenotypes in the offspring.[424]

In the light of these previous findings, we hypothesized that MHFD-induced dysbiosis of the gut microbiome might promote Th17 cell differentiation, thus leading to increased inflammatory response which could then impact fetal development and contribute to social deficits in the offspring. Interestingly, we observed a significant increase of IL-17a in F_0 females after one week on HFD (**Fig. 9a**), and at gestational day 14.5, compared to RD-fed mice (**Fig. 9b**), suggesting that HFD regimen *per se* can increase the level of proinflammatory cytokines in the F_0, and that this increase is maintained throughout pregnancy in the dam serum, thus supporting our hypothesis that HFD is capable to alter maternal immune homeostasis. Conversely, we did not find differences in IL-17a levels in the serum of MHFD and MRD F_2 mice, regardless of sex (**Fig. 9c, d**). This finding does not rule out a role for cytokine dysregulation in the onset of social dysfunction in the F_2 mice, but rather suggest the involvement of other cytokines. Alternatively, since the

expression patterns of circulating cytokine are not static, but rather vary dramatically throughout development, it also possible that IL-17a levels are elevated at a different time point–*i.e.*, during pre- and early-post natal development–while normalized in adult mice. C

Finally, in the light of our recent findings showing that treatment with *L. reuteri* rescued both the reduced hypothalamic OT levels and social deficits observed in MHFD F_1 offspring, we investigated whether a similar reduction of hypothalamic OT levels was present in the PVN of our F_2 generation. Our early analyses in a subset of mice showed a significant decrease in the male MHFD F_2 mice compared to the MRD group (**Fig. 10**). Future assessments of OT levels on a larger number of subjects will allow us to provide quantitative comparisons of OT-immunoreactive cells in the PVN of MHFD vs. MRD F_2 cohorts. Nonetheless, these data led us to hypothesize that (*1*) the impairments in the oxytocinergic-dopaminergic social reward circuit observed in the F_1 MHFD offspring persist in F_2, and (*2*) *L. reuteri* treatment may similarly rescue social dysfunction in the F_2 offspring, a hypothesis we are exploring in ongoing work administering *L. reuteri* to MHFD F_2 mice starting at weaning and throughout the behavioral tests, which will be subsequently performed.

In conclusion, we propose a model in which HFD-induced dysbiosis of the maternal gut microbiota determines alterations in the maternal serum metabolome and/or circulating cytokine expression patterns which reach fetal circulation, thus interfering with proper fetal development. After birth, offspring inherits the at least in part dysbiotic maternal gut microbiome, thus extending the detrimental effects on post-natal development. (**Fig. 11**).

Given that maternal gut microbiota is vertically transmitted to F_1 offspring generation, we hypothesized that a similar transfer might occur from the F_1 to the F_2 generation–meaning that HFD-induced dysbiosis of the gut microbiome, an event initially occurred in the F_0 generation,

can actually persist across generations, thus propagating alterations in metabolism and brain function and behavior in progeny, even in the absence of direct exposure to HFD or MHFD. We showed that the behavioral and body mass composition alterations are still present in the F_2, and that the F2 gut microbiome still carries some of the alterations induced in the F1 generation by the parental HFD. The data obtained by the 16S rRNA gene sequencing provide a rationale for future experiments including WGS sequencing, which allows for highly discriminative comparisons of genetic relatedness between bacteria even on a sub-species level, and functional metagenomic profiling of the maternal gut microbiome ± WD during pregnancy and lactation. Additionally, fecal microbiota transplant studies in germ-free mice could provide causal evidence of the enduring impact of the initial HFD-induced insult on the F_0 dam gut microbiome on the behavioral and metabolic phenotypes of the F_2 generation.

Understanding whether and how diet-induced dysbiosis of the gut microbiome in the maternal lineage drives multi-generational impacts on brain function and metabolism in animal models has the potential to guide translational studies aimed to the identification of new at-risk individuals. Indeed, the concept of multi-generational maternal lineage metabolic dysfunction and obesity as a strong risk factor for NDDs, independently of direct maternal diet, lifestyle, and medical conditions, could help redefine vulnerable populations, and develop both new preventive and treatment strategies with an exponential impact on global health.

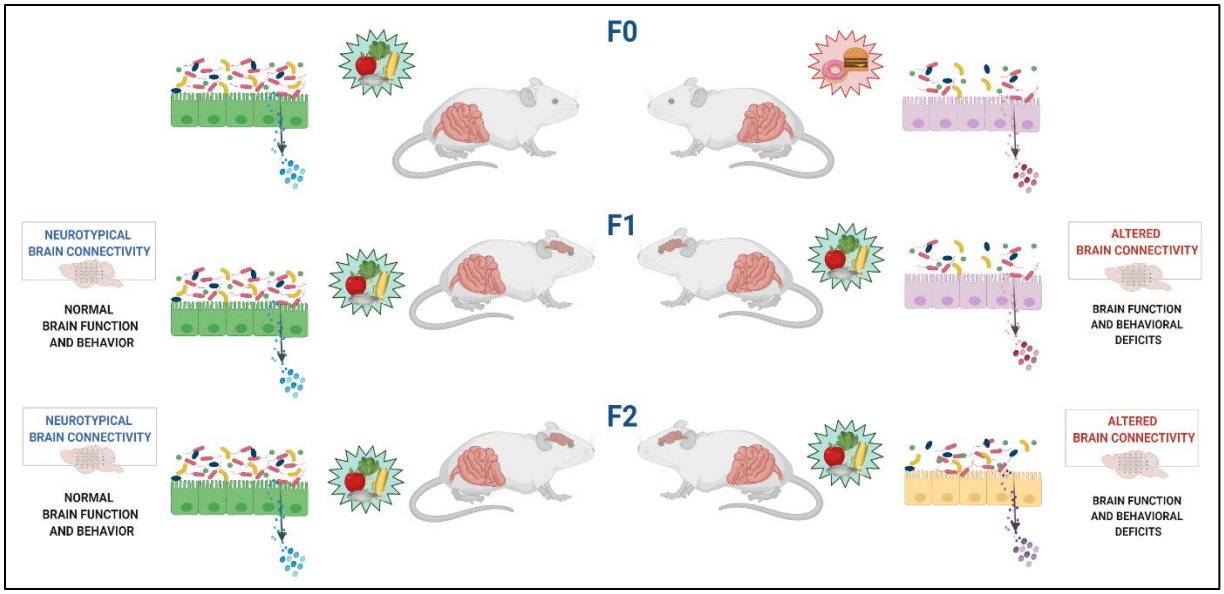
F0
F1
F2
NEUROTYPICAL BRAIN CONNECTIVITY
NORMAL BRAIN FUNCTION AND BEHAVIOR
ALTERED BRAIN CONNECTIVITY
BRAIN FUNCTION AND BEHAVIORAL DEFICITS
NEUROTYPICAL BRAIN CONNECTIVITY
NORMAL BRAIN FUNCTION AND BEHAVIOR
ALTERED BRAIN CONNECTIVITY
BRAIN FUNCTION AND BEHAVIORAL DEFICITS

Figure 11. Model for the multi-generational impacts of diet-induced dysbiosis of the gut microbiome in the maternal lineage. HFD consumption in mice, a model of diet-induced obesity, induces alterations in the gut microbiome. This shift in the maternal gut microbiome determines a vast range of rearrangements in the host physiology, such as changes in the composition of serum metabolome and cytokine dysregulation among others, which during pregnancy are reflected in the fetal compartment and have the potential to alter the developmental trajectories of the fetus. At birth and during early postnatal development maternal gut strains colonize the offspring gut, and the alterations in the maternal gut microbiome are at least in part transferred to the first generation of offspring, thus potentially also impacting the postnatal development. Partial recovery in the number of OTUs present in the F2 gut is not sufficient to prevent deficits in social behavior. The maternal diet-driven dysbiosis gut microbiome acquired by F_1 generation persists throughout adulthood, and these 'scars' in the gut microbiome can then influence the pre- and early-postnatal development of the F_2, thus propagating the negative effect on brain function and metabolism of F_0 maternal diet across multiple generations.

Chapter IV – Conclusions and future directions.

As we extensively discussed in **Chapter 1**, NDDs, such as ASD, are characterized by a complex and diversified etiology which is the result of the combination of various both genetic and nongenetic factors. The extensive clinical heterogeneity and phenotypic variability in patients with NDDs has made identification of causal mechanisms contributing to their onset difficult. Yet, given that NDDs affect >3% of children worldwide and their prevalence is increasing, it is critical to identify modifiable risk factors to decrease disease prevalence and reduce the personal and societal costs associated with NDDs.

One of the most common nongenetic, and therefore more easily modifiable, risk factors for the development of NDDs is maternal obesity. Alarmingly, the prevalence of obesity is escalating throughout the world, especially among women of childbearing age. Several epidemiological studies showed that BMI is positively correlated with risk of developing a wide range of diseases, such as diabetes, stroke, cardiovascular disease, and cancer in affected individuals. However, obesity not only threatens the health of the individual, but also increases the risk for metabolic, cognitive, and behavioral outcomes in their offspring.

In Chapter 1, we also presented the notion of pregnancy exposome[319] and the fetal programming (DOHaD) hypothesis[320], which provides a conceptual framework for the design of this study. Maternal diet and metabolic status are crucial components of the pregnancy exposome, and their pathological alterations, as in the case of obesity and diabetes, are thought to strongly impact fetal and early postnatal development, potentially leading to permanent impairments in offspring brain function and behavior, metabolism, and among other essential host functions.[875] This hypothesis is supported by human epidemiological studies suggesting that maternal obesity and its functional consequences on metabolic homeostasis predispose offspring to develop obesity, T2D, hearth disease, asthma, non-alcoholic fatty liver disease (NAFLD), neuropsychiatric and

neurodevelopmental disorders, as well as other chronic diseases. In recent decades, animal models of diet-induced obesity have been extensively used to investigate the effects of maternal obesity and metabolic dysfunction on offspring health and development. However, our knowledge of the cellular and molecular mechanisms underlying the detrimental ramifications of maternal obesity on subsequent generations remains limited and warrants further investigation.

Diet plays a crucial role in inducing obesity and metabolic dysfunction: the western pattern diet (WPD)–characterized by high intake of animal protein and sugar and decreased complex carbohydrate and fiber consumption–in particular, is associated with higher risk of obesity. Notably, we and others have found that WDP in rodents, non-human primates, as well as humans induces significant modifications in gut microbiome composition, characterized by a decrease in overall microbial diversity and is thus poised to induce long-term impacts on host physiology.

Maternal gut microbiota plays a crucial role in prenatal and early postnatal offspring development; consequently, maternal lifestyle-induced alterations in gut microbiome composition and microbial community structure can impact both pre- and early postnatal offspring development, thus triggering fetal programming and increasing the risk of chronic disease burden in offspring. However, if dysbiosis of the maternal gut microbiome determines fetal programming and can predispose the offspring to a wide range of diseases, then therapeutic modulation of the maternal gut microbiome during pregnancy represents an unrealized opportunity for preventing or reducing the risk of negative effects on fetal programming, thus improving the long-term health of children affected by maternal obesity[856] (and, potentially, that of their children–see Chapter 3).

In previous work, we showed that eight weeks maternal HFD consumption prior to pregnancy induces persistent, functional changes in the offspring gut microbiome that are causally related to offspring autism-like social impairment and related deficits in synaptic plasticity in the

ventral tegmental area (VTA), a dopaminergic reward circuit locus in the brain. Intriguingly, treatment with *L. reuteri*, the species most reduced by MHFD consumption, rescued autism-like social deficits, and the underlying neuropathology, in MHFD offspring.

Here we showed that (1) dysbiosis of the maternal gut microbiota is achieved as early as four weeks on HFD, (2) vertical transmission of the dysbiotic maternal gut microbiome is causally associated with behavioral, cognitive, and metabolic impairments in offspring, (3) HFD-induced increased abundance of opportunistic pathogens in the gut microbiome may drive offspring deficits, (4) maternal pharmacological treatment positively impact offspring health by altering both the maternal and offspring gut microbiome, and (5) behavioral and metabolic impairment in the F_1 generation are seen in the F_2 generation, even in the absence of direct exposure to MHFD. The coexistence of impairments in social behavior, cognition, and metabolism in our F_1 cohorts provides an interesting overlap with a similar occurrence in ASD children, who are often also diagnosed with several comorbidities, including GI disturbances and ID,[32,1000] and metabolic dysregulation[1009], and attests to the translational impact of this work.

We previously showed that treating MHFD F_1 offspring with *L. reuteri*, the species most reduced in the F_1 gut microbiome, was sufficient to rescue F_1 social dysfunction. Here, we began to explore the impact of species whose abundance is *increased* by MHFD, such as the opportunistic pathogen *B. vulgatus*, showing that RD mice treated with this species display cognitive and metabolic phenotypes similar to those observed in the MHFD group. These findings might appear to be inconsistent with each other, however together they support the idea of a vast impact of maternal diet on the composition and community structure of the gut microbiota. Hypothesizing a scenario in which the diet, or any other host factor, selectively targets a single species in the gut microbiome is rather unlikely, since it would not take into account the highly dynamic and

diversified relationships within the gut microbiome and between the microbiome and the host. Indeed, our WGS sequencing of the MHFD F_1 gut microbiota revealed that other species are either increased or decreased in the relative abundance as a result of diet perturbations. In future studies, we will perform an in-depth, WGS sequencing-based screening of bacterial species altered in the F_1 and F_2 MHFD offspring that could provide new insights in the relationship between host diet and microbial communities living in the gut.

While our previous work highlighted the association between *L. reuteri* abundance in the gut and OT production in the PVN via the VN[765], here we focused on bacterial contribution to the host serum metabolome, hypothesizing that opportunistic pathogen-induced increase in the concentration of circulating BCAAs might be one the mechanisms underlying the cognitive and metabolic impairments observed in the MHFD offspring. In future studies, we aim to further elucidate the role BCAAs in inducing alterations in neuronal proteostasis and synaptic function and assess whether the upregulation of BCAA catabolism rescues translational control pathway dysregulation leading to behavioral dysfunction in MHFD offspring. However, BCAAs represent only one of the many metabolites included in the vast repertoire of microbial products in the host serum, which varies across time and in relation to host factors, thus reflecting the high plasticity of the gut microbiome.[1010] In this light, we plan to perform a longitudinal study of MHFD serum metabolome which, together with WGS sequencing of gut microbiota composition and KEGG-based analyses of microbial metabolic pathways, may inform future gain- and loss-of-function studies to determine the precise mechanism by which bioactive serum metabolites that differ between MRD and MHFD offspring impact host physiology.

Our investigation of the potential impact of MHFD on generations beyond F_1 provides the first evidence of the multigenerational impact of diet-induced dysbiosis of the gut microbiome in

the maternal lineage. We reported significant behavioral and body mass composition alterations in the F_2 offspring, with increased severity in the male sex, which recapitulates the gender bias for ASD prevalence in humans. Our metagenomic analysis of the F_2 composition of the gut microbiome provided important insights into the effect of a dysbiotic gut microbiome across generation. Importantly, given that even MHFD F_0 and F_1 cohorts showed a certain degree of divergence in the composition of their gut microbial ecosystems, we did not expect that the F_2 generation carried exactly the same alterations in the gut microbiome observed in the F_1. Indeed, we rather hypothesize that maternal diet, as well as any other change in the maternal physiology, is capable of disrupting the global equilibrium between the microbial species coexisting in the host gut, even in presence of a partial recovery in the number of observed OTUs.

This idea is supported by human studies suggesting that disease states are indeed often associated with dysbiosis of the gut microbiome, intended as an altered composition or structure when compared with unaffected controls; however, dysbiotic patterns are not fixed, but vary in relation to the specific disease, the characteristic of the individuals, etc. Similarly, defining what constitutes a '*healthy gut microbiome*' or a state of '*eubiosis*', is extremely difficult due to the existence of multiple individual variables. Yet, identifying common disease-associated traits of gut microbiota dysbiosis, as well as pathologically-relevant alterations in the abundance of a subset of microbial species or genera, has the potential to provide valuable insight into the mechanisms by which MHFD impacts host physiology and to aid in the development of both preventive and treatment strategies.

The F_2 generation study here presented included the assessment of BCAA and proinflammatory cytokine IL-17a levels in the offspring serum to evaluate whether F_2 mice might carry the same molecular dysregulations observed in the F_0 and F_1 generations. We did not find

significant differences for either BCAAs or IL-17a between MRD and MHFD F_2 group, suggesting that other molecular mechanisms might underlie the pathological phenotypes observed in the F_2 cohort. These negative findings are not entirely surprising, due to the fact that communication between the gut microbiota and the host, in particular the brain in the context of the MGBA, is mediated by multiple channels, including immune mediators, LMW microbially-derived metabolites, the VN and other components of the PNS, and therefore similar phenotypes may be achieved via different mechanisms, not to mention in relation to specific changes in the gut microbiome and timepoints of brain development. Additionally, the complexity of brain function and behavior, which is the result of sophisticated interactions between diverse brain regions and the neural circuits in which they participate, support the hypothesis that different microbially-driven alterations of host physiology may target different brain circuits and neurotransmitters, thus producing distinct outcomes.

Similar to what we proposed as a future direction for the investigation of the cognitive and metabolic deficits in the F_1 generation, we plan to perform longitudinal study of the F_2 serum metabolome and to then match this information with the data acquired by WGS sequencing to identify the cellular and molecular mechanisms underlying the observed deficits in social behavior. Furthermore, we found interesting similarities between the F_1 and F_2 generations regarding OT levels in the PVN of the hypothalamus. Indeed, the same decrease in OT immunoreactivity that we previously observed in the PVN of MHFD F_1 offspring, was reproduced in the MHFD F_2 group. This finding led us to explore the possibility that *L. reuteri* administration to MHFD F_2 mice might actually improve OT levels in the PVN and rescue social behavior deficits in future experiments.

Probiotic administration is one of the potential therapeutic strategies that could be adopted to ameliorate behavioral and metabolic phenotypes that we observed via the direct manipulation

of the gut microbiota composition. Intriguingly, in our previous work[765] we showed that *L. reuteri* administration improved social deficits in genetic, environmental, and idiopathic ASD models, regardless of the initial perturbation triggering the disorder. Here, we tested whether maternal pharmacological intervention (*e.g.*, metformin administration) could alter offspring gut microbiome composition and rescue MHFD F_1 offspring cognitive, social and metabolic phenotypes. Previous studies showed that metformin induces changes in host gut microbiome that are causally related to its therapeutic function in T2DM. The first effect that we observed in metformin-treated HFD F_0 dams was the improvement of pregnancy rate, which was strongly decreased by the HF regimen and resulted in the limited the availability of MHFD F_1 offspring in our experimental approach. The reduced reproductive success of HFD-fed mice mirrors the lower fertility rate and increase miscarriage risk found among obese women.[1011] Consistently, metformin treatment has been shown to improve fertility in women with polycystic ovary syndrome (PCOS), an endocrine disorder often associated with obesity.[1012]

Our metagenomic 16S rRNA gene amplicon sequencing experiments revealed a significant effect of metformin on the fecal microbial community structure of both the maternal (F_0) and offspring gut microbiome (F_1). Interestingly, the gut microbiome structure of metformin treated dams and their offspring does not perfectly overlap with that of their RD-fed counterparts, suggesting that pharmacological manipulations of the gut microbiome produce distinct effects, including an increased divergence with the alleged '*eubiotic*' gut microbiome of control mice. However, maternal metformin treatment did decrease the abundance of *Bacteroides* and *Parabacteroides* (*P*.) in MHFD offspring, consistent with our hypothesis that the cognitive and metabolic phenotypes observed in the MHFD F_1 mice could be related to the increased proportion opportunistic pathogens advantaged by the maternal HFD. This idea is further support by

behavioral assessments showing that maternal metformin treatment rescues MHFD offspring long-term memory. Finally, we found that maternal metformin treatment corrected MHFD offspring social deficits, which is reminiscent of the effects of postweaning *L. reuteri* treatment of offspring behavior. In ongoing experiments, we are investigating the reciprocal effect of *L. reuteri* on *B. vulgatus* abundance and vice versa in order to determine whether an indirect effect of *L. reuteri* on microbiome composition could be causally related to the observed improvements in host phenotypes. In future experiments we will explore the molecular mechanisms underlying the positive effect of metformin on brain function and behavior.

Studies based on mouse model of diet-induced obesity, like the one that we performed here, are an indispensable tool in elucidating the long-term multigenerational effects of maternal nutritional state on offspring brain function, behavior, and metabolism across multiple generations and to identifying novel, innovative therapies. Indeed, the investigation of transgenerational effects of maternal conditions in human populations presents a variety of epidemiological challenges, including long-generation time as well as heterogeneity within populations. Nonetheless, animal models of human pathological conditions come with several limitations, including a partial ability to faithfully recapitulate disease features and outcomes due to physiologic and anatomic differences between mice and humans.[1013]

In this regard, humanized gnotobiotic mouse models have emerged as a powerful tool for the investigation of human gut microbial communities on a wide range of diseases. These models, obtained through the transplantation of human fecal microbiota into GF mice, have helped to reduce the gap existing between murine and human gut physiology and pathology since their first application in 1980s.[1014] More recently, new approaches have been established to overcome the limitations of GF mice as FMT recipients, including antibiotic-treated conventional mice.

Regardless of the specific protocol used to perform the FMT, human microbiota (HM) colonized mice are considered relevant translational model systems especially in the context of testing the efficacy of microbiota-targeted therapeutics prior to initiating large-scale clinical trials.

In this light, one fascinating future direction being pursued by our lab is transplantation of gut microbiota obtained from human donors affected by ASD or TD control into GF mice, an experimental approach already proven successful in reproducing autism-like phenotypes in mice with microbiota from individuals diagnosed with ASD.[1015] Combined with a multi-omics approach encompassing metagenomics, proteomics and metabolomics, this procedure will allow us to investigate the efficacy of microbiota-targeted therapeutic strategies in a controlled environment while taking into account the effects of the heterogenous, highly diversified nature of human ASD. Furthermore, we are expanding our efforts and have begun exploring the relationship between T2D and dementia (Alzheimer's Disease) risk in the context of the gut microbiome.

In conclusion, our study contributed to increase the understanding of the pathophysiological events associated with maternal obesity-associated long-term health risks in offspring. In current and future experiments, we will apply our expertise to assess the potential for antenatal targeting of the maternal gut microbiome via dietary (prebiotic), pharmacological, probiotic, or combination therapies aimed to prevent the detrimental effects of MHFD-induced obesity on offspring health outcomes. In particular, prenatal probiotic treatment with commensal bacterial species could represent an effective and easy-to-implement way to improve maternal health status by rescuing dysbiosis of the gut microbiome, and therefore promote normal fetal development.

We believe that our animal study has a strong translational potential and could drive a shift in the practice of antenatal care for women of overweight and obese status and have a lifelong, positive impact on the health of children affected by maternal obesity.

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