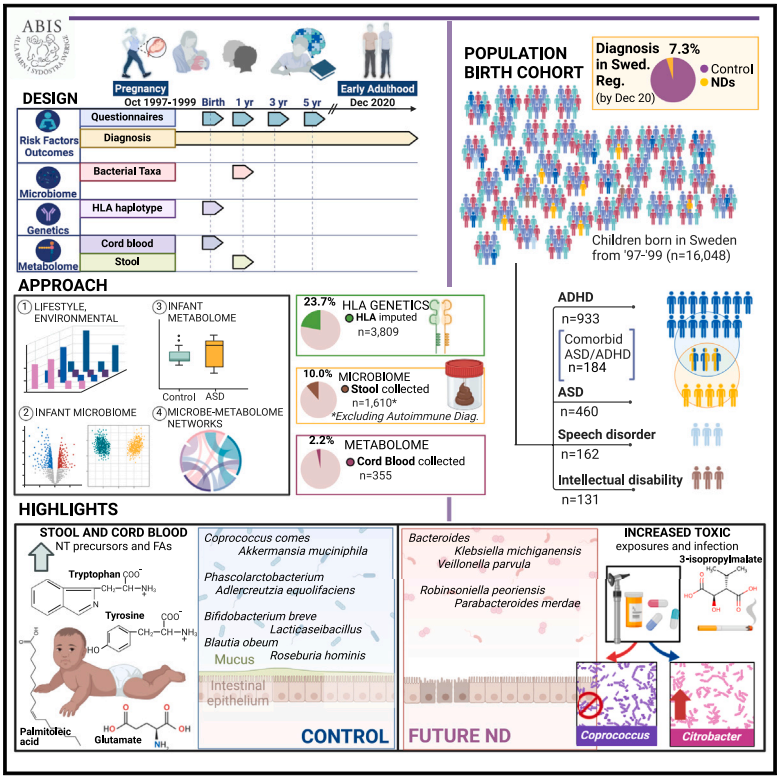
Cell

Infant microbes and metabolites point to childhood neurodevelopmental disorders

Graphical abstract



Highlights

- Infant microbes and metabolites differentiate controls and future NDs
- Early-life otitis lowers *Coprococcus* and increases *Citrobacter* in future NDs
- Preterm birth, infection, stress, parental smoking, and HLA DR4-DQ8 increase ND risk
- Linolenic acid is lower and PFDA toxins higher in the cord serum of future ASD

Authors

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In brief

This prospective, birth cohort multi-omic study reveals disruptions in the infant gut microbiome associated with later-diagnosed neurodevelopmental disorders. Microbial and metabolomic changes, correlating with risk factors, HLA genetics, and mood/gastrointestinal issues, support the hypothesis of early-life origins of neurodevelopmental disorders, underscoring an important role of gut microbiota.

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Article

Infant microbes and metabolites point to childhood neurodevelopmental disorders

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SUMMARY

This study has followed a birth cohort for over 20 years to find factors associated with neurodevelopmental disorder (ND) diagnosis. Detailed, early-life longitudinal questionnaires captured infection and antibiotic events, stress, prenatal factors, family history, and more. Biomarkers including cord serum metabolome and lipidome, human leukocyte antigen (HLA) genotype, infant microbiota, and stool metabolome were assessed. Among the 16,440 Swedish children followed across time, 1,197 developed an ND. Significant associations emerged for future ND diagnosis in general and for specific ND subtypes, spanning intellectual disability, speech disorder, attention-deficit/hyperactivity disorder, and autism. This investigation revealed microbiome connections to future diagnosis as well as early emerging mood and gastrointestinal problems. The findings suggest links to immunodysregulation and metabolism, compounded by stress, early-life infection, and antibiotics. The convergence of infant biomarkers and risk factors in this prospective, longitudinal study on a large-scale population establishes a foundation for early-life prediction and intervention in neurodevelopment.

INTRODUCTION

Neurodevelopmental disorders (NDs) exert profound and lasting impacts on central nervous system maturation, frequently emerging during childhood. They encompass conditions such as autism spectrum disorder (ASD), attention-deficit/hyperactivity disorder (ADHD), intellectual disabilities, and communication disorders. ASD, characterized by social difficulties and restricted and repetitive behaviors and interests, affects 1%–2% of the population, while ADHD's prevalence reaches 7% in children[1] and 3.4% in adults,[2] leading to significant physical and mental health burdens.[3] Communication disorders affect 5%–7% of people[4,5] and can cause problems with speaking, formulating words, and understanding abstract ideas.

Various prenatal and early-life factors[6,7] may contribute to ND etiology, making early diagnosis challenging due to the lack of specific biomarkers. Genetic and environmental influences may disrupt neural adaptability, potentially causing abnormal neuronal homeostasis,[8] while immune dysregulation, inflammation, and autoantibodies may also contribute.[9,10] A substantial subset of individuals with ASD especially experience co-occurring gastrointestinal (GI) symptoms, implicating a gut-brain connection.[11–13]

Gut bacteria play a significant role in metabolism, GI health, neurological health,[14–16] and immune function,[17–21] suggesting their potential involvement in NDs. Gut-brain communication occurs in various ways, including along the vagus nerve and through transport of short-chain fatty acids (SCFAs), cytokines, amino acids, and neurotransmitter precursors.[22] Microbes can locally synthesize neurotransmitters,[23,24] and enteroendocrine cells release hormones and dendritic cells that modulate immune and microglia function, ultimately influencing neuroactive metabolites that impact behavior.[25,26] Bacterial strains can improve social and anxiety-like behaviors[6,27,28] via impacts on gut permeability, the metabolome, and synaptic plasticity. Improvements in GI- and ASD-related symptoms were observed in a small pilot study of ASD-diagnosed children after microbiome transfer therapy,[29] with some long-lasting benefit

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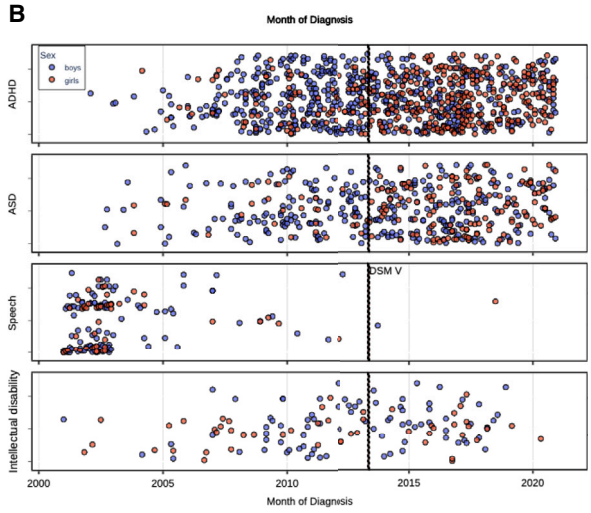
A

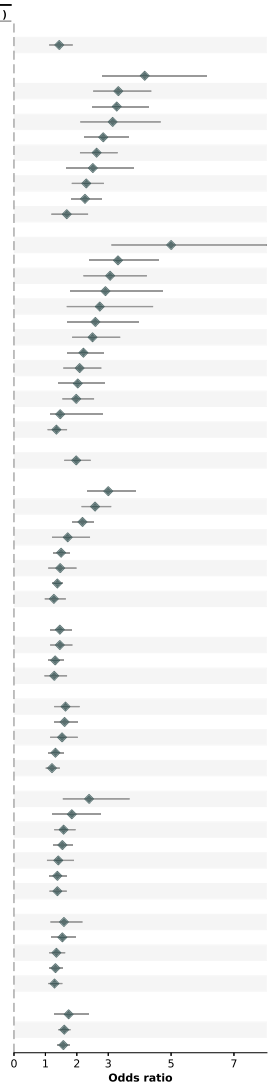
Group	n	sample	mean age	lower	upper
Control	14869	All ABIS			
ASD	460	All ABIS	15.84	15.47	16.21
ADHD	933	All ABIS	15.52	15.26	15.77
Speech Disorder	163	All ABIS	4.59	4.19	4.98
Intellectual Disability	130	All ABIS	13.76	13.02	14.49
Control	1610	Microbiome			
ASD	39	Microbiome	15.41	14.06	16.75
ADHD	85	Microbiome	15.33	14.47	16.20
Speech Disorder	14	Microbiome	6.50	3.68	9.32
Intellectual Disability	8	Microbiome	11.69	9.67	13.71
Control	23	Stool Metabolomics			
ASD	23	Stool Metabolomics	14.90	12.96	16.85
ADHD	13	Stool Metabolomics	15.70	12.95	18.45
Control	304	Cord Blood Polar Metabolomics			
ASD	34	Cord Blood Polar Metabolomics	15.01	13.72	16.31
ADHD	35	Cord Blood Polar Metabolomics	15.64	14.42	16.86
Intellectual Disability	5	Cord Blood Polar Metabolomics	12.04	8.38	15.70
Control	307	Cord Blood Lipidomics			
ASD	34	Cord Blood Lipidomics	15.01	13.72	16.31
ADHD	35	Cord Blood Lipidomics	15.64	14.42	16.86
Intellectual Disability	5	Cord Blood Lipidomics	12.04	8.38	15.70

B



C

Variable	%ND	%not	OR	Est. (95% Conf. Int.)
Birth				
Week of delivery - preterm (less than 37)	5.9%	4.2%	1.44	1.12 to 1.87
Exposures - 12 mo. to 2.5 years				
Mother smoking (more than 15 cigarettes/day vs. none)	6.0%	1.6%	4.16	2.82 to 6.14
Father smoking (more than 10 cigarettes/day vs. none)	12.2%	4.1%	3.32	2.52 to 4.37
Mother smoking (more than 10 cigarettes/day vs. none)	12.6%	4.4%	3.27	2.49 to 4.29
Father smoking (more than 15 cigarettes/day vs. none)	5.5%	2.0%	3.14	2.11 to 4.67
Father smoking (more than 5 cigarettes/day vs. none)	15.6%	6.2%	2.84	2.22 to 3.64
Mother smoking (more than 5 cigarettes/day vs. none)	18.4%	8.0%	2.63	2.1 to 3.3
Someone smoked in the home - daily vs. none	4.7%	1.9%	2.51	1.66 to 3.82
Father smoking - yes	19.5%	9.5%	2.3	1.84 to 2.86
Mother smoking - yes	21.7%	10.9%	2.26	1.83 to 2.79
Someone smoked in the home - yes	6.9%	4.2%	1.68	1.19 to 2.36
Exposures - 2.5 to 5 years				
Mother smoking (more than 15 cigarettes/day vs. none)	5.2%	1.1%	5.0	3.1 to 8.05
Father smoking (more than 10 cigarettes/day vs. none)	11.0%	3.7%	3.31	2.39 to 4.59
Mother smoking (more than 10 cigarettes/day vs. none)	10.9%	3.9%	3.06	2.21 to 4.23
Father smoking (more than 15 cigarettes/day vs. none)	4.6%	1.7%	2.91	1.79 to 4.73
Someone smoked in the home - more than 5 cigarettes daily vs. none	4.5%	1.7%	2.73	1.68 to 4.43
Someone smoked in the home - daily vs. none	5.9%	2.4%	2.59	1.69 to 3.96
Father smoking (more than 5 cigarettes/day vs. none)	12.6%	5.6%	2.5	1.85 to 3.38
Father smoking - yes	17.6%	8.8%	2.21	1.7 to 2.87
Mother smoking (more than 5 cigarettes/day v. none)	14.0%	7.4%	2.09	1.57 to 2.78
Someone smoked in the home - yes	8.4%	4.3%	2.03	1.42 to 2.9
Mother smoking (5 years follow-up)	18.3%	10.2%	1.98	1.54 to 2.55
Penicillin	74.6%	66.7%	1.47	1.17 to 2.83
Accident that required medical care	24.6%	19.5%	1.35	1.07 to 1.69
Exposures - to 5 years				
Difficult life event since birth	32.8%	19.8%	1.98	1.6 to 2.44
Exposures during mother's pregnancy				
Smoked more than 10 cigarettes/day v. no smoking	7.9%	2.8%	3.0	2.33 to 3.87
Smoked more than 5 cigarettes/day v. no smoking	14.8%	6.3%	2.58	2.15 to 3.1
Smoked during pregnancy	20.3%	10.4%	2.18	1.87 to 2.54
Smoked more than 10 cigarettes/day v. less (1-5)	33.9%	25.4%	1.71	1.21 to 2.42
Severe life event during pregnancy	12.9%	9.0%	1.5	1.25 to 1.79
Smoked more than 5 cigarettes/day v. less (1-5)	69.1%	60.3%	1.47	1.09 to 1.99
Analgesics during pregnancy	54.5%	46.5%	1.38	1.21 to 1.56
Cortisone during pregnancy	6.6%	5.3%	1.27	0.98 to 1.65
Family history				
Asthma (mother) - yes	7.7%	5.4%	1.46	1.16 to 1.83
Asthma (father) - yes	6.8%	4.7%	1.46	1.15 to 1.86
T1d (grandparents) - yes	11.2%	8.8%	1.31	1.08 to 1.58
Celiac disease (mother-grandparents) - yes	5.0%	3.9%	1.28	0.97 to 1.69
Infection - 12 mo. to 2.5 years				
Otitis (3 or more times v. never)	18.8%	13.4%	1.64	1.28 to 2.09
Infection that required antibiotics (3 or more times vs. never)	24.5%	17.2%	1.61	1.27 to 2.03
Gastroenteritis (3 or more times v. never)	15.4%	12.2%	1.53	1.15 to 2.03
Otitis	58.1%	51.2%	1.32	1.1 to 1.58
Infection that required antibiotics	64.1%	59.5%	1.21	1.01 to 1.46
Infection - 2.5 to 5 years				
Infection that required antibiotics (6 or more times vs. never)	6.6%	3.7%	2.39	1.55 to 3.68
Infection that required antibiotics (6 or more times vs. never to 2 times)	6.6%	3.7%	1.84	1.23 to 2.77
Infection that required antibiotics (3 or more times vs. never)	20.8%	17.4%	1.58	1.28 to 1.96
Otitis	59.0%	48.2%	1.54	1.26 to 1.88
Otitis (3 or more times v. never)	34.7%	24.5%	1.41	1.05 to 1.91
Gastroenteritis (3 or more times v. fewer)	33.8%	26.9%	1.38	1.13 to 1.7
Influenza	54.6%	46.6%	1.38	1.13 to 1.68
Infections - first 12 months				
Infection requiring penicillin (3 or more times v. never)	12.5%	8.2%	1.59	1.16 to 2.18
Eczema (3 or more times v. never)	13.5%	9.5%	1.54	1.19 to 1.98
Eczema	28.3%	22.6%	1.35	1.12 to 1.63
Infection requiring penicillin	44.3%	37.6%	1.32	1.12 to 1.56
Otitis	31.2%	25.9%	1.29	1.09 to 1.54
Living conditions				
Type of home (2.5 to 5 years) - apartment	32.2%	21.5%	1.74	1.27 to 2.37
Type of home (4-10 months of pregnancy) - apartment	48.0%	36.6%	1.6	1.41 to 1.8
Type of home (first 3 months of pregnancy) - apartment	50.8%	39.6%	1.57	1.39 to 1.78



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observed up to 2 years later.[30] However, the exact contribution of microbiome disruptions to ND etiology and pathophysiology remains unclear, with divergent findings across cross-sectional studies on ASD[31] and ADHD.[32]

The connection between early gut microbiota and cognition remains poorly understood, with only three existing investigations and still no prospective microbiome study of medically documented NDs. These studies explored microbiome diversity and its correlation with Ages and States Questionnaires (ASQ-3) scores[33] and cognitive function in typically developing infants[34] as well as social behavior in 3-year-olds.[35] However, none of these studies made a formal ND diagnosis the primary outcome.

While significant interest surrounds the microbiome, longitudinal prospective studies on early-life microbiomes and future ND diagnoses are lacking.[36] Our study aims to address this gap by investigating early-life factors in the All Babies in Southeast Sweden (ABIS) cohort, collecting biological samples such as cord serum and stool. Health records from birth and comprehensive early-life data were analyzed to identify microbiome and metabolite signatures associated with ND risk.

RESULTS

An array of biomarkers and questionnaires was analyzed to determine factors associated with future ND diagnosis. While some factors were associated with increased risk of ND, other factors were associated with decreased risk. Questionnaire results are presented, followed by biomarker results and integrated results, where we consider their interactions.

Questionnaires revealed many ND risk factors early in life, spanning infection and antibiotic events, chemical exposures, family history of disease, other medical issues, and serious life events

The ABIS questionnaire data were obtained from the parents of participating children at several time periods, starting with pregnancy and continuing throughout childhood. Here, we analyzed the data up until 5 years of age in the broader ABIS material (see Figures 1A and 1B for the cohort and diagnosis dates). The questionnaire data were detailed in scope. Figures 1C and S1 illustrate combined and individual ND outcomes, respectively, and their associations with risk factors, as described below.

Infection and antibiotic events

Infections during early childhood (birth to 5 years) significantly correlated with heightened risks of ADHD or ASD—most notably, otitis and repeated eczema in the first year. Those experiencing three or more penicillin-requiring infections during this period were prone to future NDs, e.g., speech disorder (odds ratio [OR] = 3.89 [2.14–7.05, 95% confidence interval, CI]), ADHD (OR = 3.27 [2.29–4.67]), or intellectual disability (OR = 2.44 [1.18–5.06]). Penicillin use between 1 and 2.5 years resulted in a 1.6-fold (1.2–2.1, 95% CI) higher likelihood of future ASD (p = 0.0030). Children with future ASD were also more likely to have used non-penicillin antibiotics during this period (OR = 1.5 [1.0–2.1], p = 0.0292), while 23.8% of those with future intellectual disability had other antibiotics (OR = 2.2 [1.1–4.4, 95% CI], p = 0.0337) besides penicillin (p = 0.2917). Children experiencing frequent otitis episodes (three or more times from 1 to 2.5 years) were 2.13 (1.1–4.13, 95% CI), 1.74 (1.21–2.51, 95% CI), and 1.75 (1.33–2.30, 95% CI) times more likely to later be diagnosed with intellectual disability, ASD, or ADHD, respectively. From 2.5 to 5 years, increased paracetamol antipyretics (six or more times) raised ASD risk (OR = 1.82 [1.16–2.88, 95% CI]). Penicillin use during this period increased risks of ADHD by 1.54-fold and ASD by 1.76-fold. Children with at least three or six infections requiring antibiotics were 1.58–2.39 times more likely to develop NDs, especially ADHD (OR = 1.62–2.9). Children with future speech disorders were 1.85–2.27 times more likely to have had three or more instances of otitis. This was also seen in future intellectual disability. Frequent gastroenteritis (three or more times) from 2.5 to 5 years was reported in 33.8% of future ND and 35.8% of future ADHD versus 26.9% of controls.

Chemical exposures

Maternal smoking during pregnancy posed risks for NDs cumulatively (OR = 3.0 [2.33–3.87, 95% CI]), as well as for ASD (OR = 3.72 [1.92–7.21, 95% CI]) and ADHD (OR = 3.31 [2.52–4.34, 95% CI]) separately, especially with ten or more cigarettes daily. Likewise, maternal use of analgesics during pregnancy increased the risk of ADHD (OR = 1.41 [1.23–1.62, 95% CI]) and ASD (OR = 1.46 [1.19–1.78, 95% CI]). Exposure to parental smoking from 1 to 2.5 years was dose-dependently associated with increased risk across all NDs except speech disorders. Most striking was ADHD in children whose mothers smoked >15 cigarettes/day (OR = 4.88 [3.23–7.36]) and ASD in those whose fathers smoked >15 cigarettes/day (OR = 3.47 [2.01–6.01]). Toddlers exposed to parental smoking or smoking in general in the home exhibited a dose-dependent, elevated ND risk across all diagnosis groups except speech disorders. For instance, while only 8.8% and 10.2% of controls had a father or mother who smoked, respectively, throughout this period, 20.5% of toddlers with future intellectual disability had a father who smoked and 30% had a mother who smoked. The highest risk was observed in future ADHD, with a 6.05-fold (3.68–9.94, 95% CI) increased likelihood of ADHD if the mother smoked more than 15 cigarettes/day.

Family history of disease

Children born to parents with a family history of asthma, celiac disease (CD), or type 1 diabetes (T1D) showed an increased risk of NDs (OR = 1.28–1.46). Paternal asthma notably displayed the strongest link to risk of ASD (OR = 1.71, [1.2–2.42, 95% CI]) as well as ADHD (OR = 1.56, [1.20–2.02, 95% CI]). However,

Figure 1. Early-life environmental and medical risk factors implicated in neurodevelopmental disorders (NDs)

(A) NDs across the ABIS cohort, with mean diagnosis age and 95% confidence interval.

(B) Timeline of all ND diagnoses across ABIS, stratified by condition and biological sex, with red and blue representing female and male, respectively. The *Diagnostic and Statistical Manual of Mental Disorders*, fourth and fifth editions (DSM-IV and DSM-V) guided ICD-10 diagnostic criteria.

(C) Risk factors encompassing lifestyle, family, medical, and environmental factors from birth and 1-, 3-, and 5-year questionnaires. ORs and the 95% confidence interval for each are indicated.

See also Figure S1 for risk factors, separated by condition. OR, odds ratio; T1D, type 1 diabetes.

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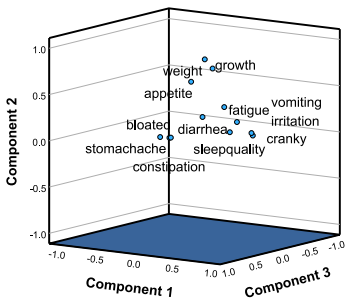
A

	Component 1	2	3
Poor growth?	0.298	0.776	0.090
Poor weight gain?	0.176	0.859	0.060
Poor appetite?	0.095	0.629	0.199
Stomachache?	0.120	0.121	0.809
Bloated / gassy stomach?	0.228	0.120	0.765
Diarrhea 3 times or more / day?	0.356	0.297	0.360
Vomiting 3 times or more / day?	0.583	0.416	0.281
Constipation?	0.108	0.085	0.619
Fatigue?	0.685	0.252	0.190
A general irritation?	0.827	0.137	0.123
A cranky moody or screaming a lot?	0.814	0.102	0.080
Poor sleep quality?	0.576	0.127	0.166
Three-year Symptom Clusters	Mood and unrest	Child growth	GI problems

	Component 1	2	3
Poor growth?	0.291	0.780	0.107
Poor weight gain?	0.160	0.872	0.075
Stomachache?	0.029	0.141	0.811
Poor appetite?	0.100	0.621	0.167
A bloated / gassy stomach?	0.183	0.119	0.773
Diarrhea 3 times or more / day?	0.466	0.280	0.404
Vomiting 3 times or more / day?	0.673	0.388	0.236
Constipation?	0.320	0.065	0.481
Fatigue?	0.745	0.112	0.130
A general irritation?	0.732	0.134	0.121
Poor sleep quality?	0.724	0.151	0.121
Five-year Symptom Clusters	Mood and unrest	Child growth	Stomach pain

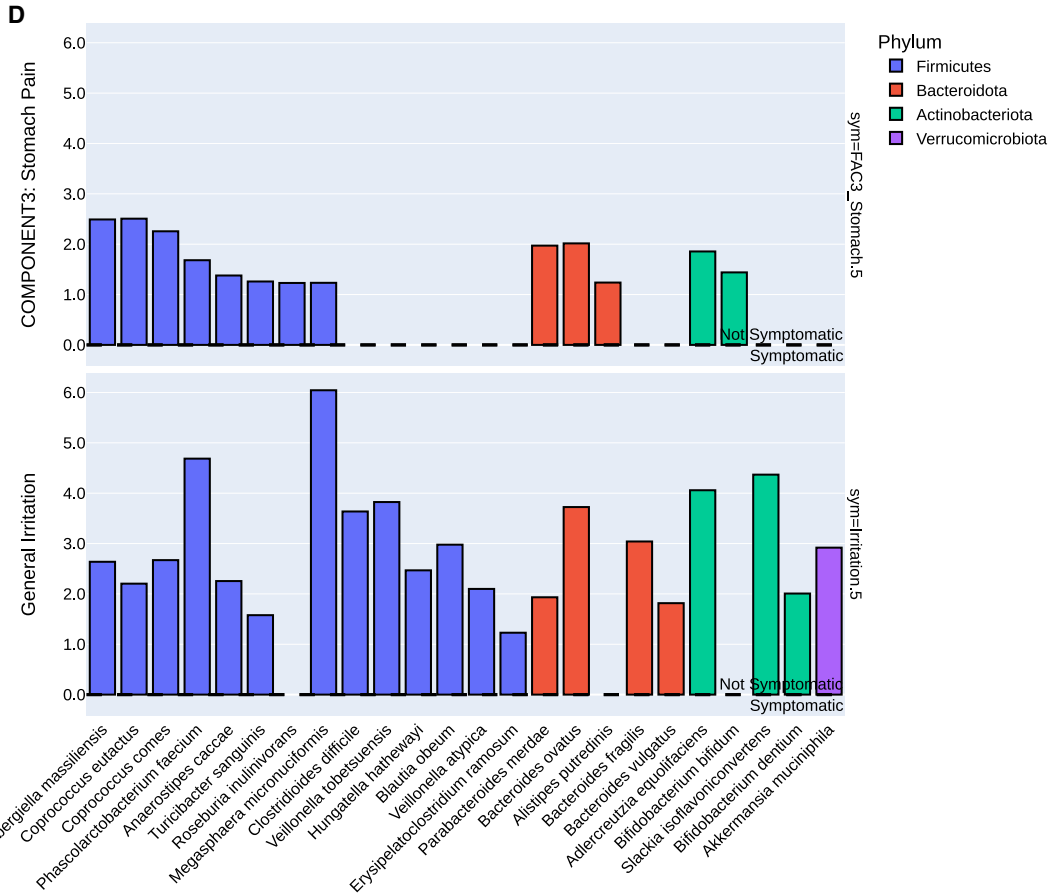
B



C

Variable	%ND	%not	OR	Est. (95% Conf. Int.)	P-value	Diagnosis
3-yr symptom						
=1+ cluster 1 (mood and unrest)	17.9%	5.9%	3.46	1.7381 to 6.9055	0.0004	intellectual disability
=1+ cluster 1 (mood and unrest)	12.9%	5.5%	2.53	1.3670 to 4.6664	0.0031	ASD/ADHD
=1+ cluster 1 (mood and unrest)	11.8%	5.6%	2.27	1.6625 to 3.0919	0.0001	ADHD
=1+ cluster 1 (mood and unrest)	11.4%	5.5%	2.19	1.6508 to 2.8984	0.0001	ND
=1+ cluster 1 (mood and unrest)	11.3%	5.8%	2.05	1.3469 to 3.1084	0.0008	ASD
5-yr symptom						
=1+ cluster 3 (stomach pain)	24.6%	8.6%	3.45	1.9501 to 6.1077	0.0001	comorbid ASD/ADHD
Stomachache	22.7%	8.0%	3.39	1.8942 to 6.0724	0.0001	comorbid ASD/ADHD
General irritation	7.7%	2.5%	3.23	1.8001 to 5.7982	0.0001	ASD
General irritation	5.7%	2.4%	2.47	1.6027 to 3.8086	0.0001	ND
Stomachache	17.0%	8.1%	2.3	1.5313 to 3.4657	0.0001	ASD
=1+ cluster 3 (stomach pain)	18.2%	8.9%	2.29	1.5383 to 3.4093	0.0001	ASD
General irritation	5.0%	2.5%	2.06	1.2346 to 3.4297	0.0056	ADHD
=1+ cluster 3 (stomach pain)	15.2%	8.6%	1.9	1.4403 to 2.5001	0.0001	ND
=1+ cluster 3 (stomach pain)	14.9%	8.7%	1.82	1.3347 to 2.4919	0.0002	ADHD
Stomachache	13.7%	8.0%	1.82	1.3698 to 2.4289	0.0001	ND
Stomachache	13.5%	8.1%	1.77	1.2849 to 2.4506	0.0005	ADHD

D



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these factors did not show significant association for speech disorders or intellectual disabilities.

Other medical issues

Early GI problems, and mood issues to a lesser extent, were evident in children with future NDs. Symptoms at 2.5 and 5 years (Figures 2A and 2B) were reduced by principal-component analysis (PCA) into "mood and unrest," "child growth," and "GI problems" components, explaining a cumulative variance of 57.7% at 2.5 years and 58.3% at 5 years. At 2.5 years, mood and unrest symptoms correlated with future ND (ORs = 2.05–3.46, 95% CI [1.35–1.74 to 2.90–6.91]), which was particularly pronounced in future intellectual disability (OR = 3.46, 95% CI [1.74–6.91], 17.9% cases versus 5.9% controls; Figure 2C). Within the GI problems cluster, symptoms like stomachache, bloated or gassy stomach, or constipation were seen in 21.3% of future intellectual disability (OR = 2.4, 95% CI [1.2–4.9], p = 0.013). Although not significant for future ASD, 5.6% had two or more symptoms, compared to 3.5% of controls (p = 0.077). At 5 years of age, "stomach pain" and "stomachache" were more prevalent in future NDs (13.5%–24.6%), compared to controls (8.0%–8.9%), and were especially prominent in future comorbid ASD/ADHD (OR = 3.39–3.45, p < 0.0001). Irritability or cranky moods were more common in future ASD or ADHD (5.0%–7.7% in future cases versus 2.4%–2.5% in controls), although less frequent than GI symptoms.

Serious life events

Serious life events from birth to 5 years (e.g., separation/divorce, death in the family, serious illness/accident, or unemployment) increased the likelihood of future ND by 1.98 times (1.6–2.44, 95% CI), affecting 32.8% of children, compared to 19.8% of controls. Severe life events during pregnancy were also associated with future ADHD (OR = 1.62 [1.33–1.98, 95% CI]). Remarkably, within the group of children whose mothers smoked during pregnancy, 85% of future ASD experienced such an event by age 5, compared to only 40% of controls. Infants born prematurely exhibited a 1.4-fold (1.12–1.87, 95% CI) increased likelihood of a future ND diagnosis (except ASD). Also, the intellectual disability group showed a substantial association with preterm birth, which occurred in 15.2% versus 4.2% of controls (OR = 4.13 [2.51–6.77, 95% CI]). Children with speech disorders or intellectual disabilities were 1.93–2.02 times more likely to be delivered via cesarean section (C-section).

Biomarker data revealed significant differences in the cord serum metabolome, HLA genotype, infant gut microbiome, and stool metabolome

Biomarker data was collected from cord serum at birth and from stool samples, on average, at 1 year of age. Differences in HLA and the infant gut microbiome were found both by aggregated ND diagnosis as well as by subtypes. The cord serum metabolome, cord serum lipidome, and stool metabolome were explored in ASD only.

Cord serum metabolome and lipidome

To assess cord serum lipidome and metabolome differences (Figures 3A–3K), prenatal risk factors including maternal infections,[37] smoking,[38] stress/severe life events,[39] diet,[40,41] vitamins/supplements, and coffee intake,[42] as well as education of the parents and gestational age, were controlled for using propensity score matching to select matched controls (n = 27) for future ASD cases (n = 27).

Linolenic acid (LA) was the strongest inverse association with future ASD after matching on the prenatal factors (p = 0.00288; Figure 3A). Conversely, perfluorodecanoic acid (PFDA) was the most significant positive association with future ASD (p = 0.0087; Figure 3B). Zealarone was also higher in future ASD (p = 0.01; Figure 3C). Higher in controls were 3-carboxy-4-methyl-5-propyl-2-furanpropanoate, C16:1, decanoic acid, and palmitic acid (Figures 3D–3G). Although approaching significance, octanoic acid (p = 0.054), oleic acid (p = 0.10), and ursodeoxycholic acid (UDCA) were higher in matched controls (Figures 3H, 3J, and 3K), while tauro-β-muricholic acid (TbMCA) was higher in future cases (Figure 3I). Lipidomics revealed eight triglycerides (TGs) enriched in the cord serum of controls (ps < 0.05): TG(14:0/18:2/18:2), mass-to-charge ratio (*m/z*) 822.75; TG(46:2)/(16:1/14:0/16:1); TG(48:3); TG(49:1); TG(49:2); TG(51:1); TG(51:2), *m/z* 862.79; TG(51:3); TG(53:2); TG(53:3), *m/z* 888.80; TG(53:4); and TG(58:1).

HLA genotype

Familial autoimmune disease is more prevalent in individuals with NDs (ASD especially[43–45]) and is, in large part, driven by immune-mediating HLA genetics. HLA also plays important roles in host-microbe interactions. Thus, we performed HLA class II haplotyping on 3,809 children in ABIS, including 327 with future NDs. DR4-DQ8 homozygosity (seen in 2.9% of controls) was more pronounced in future NDs (5.1%; OR = 1.8 [1.03–3.13], p = 0.039) and future ASD, especially (7.6%; OR = 2.8 [1.44–5.25], p = 0.0021). The DR4-DQ8 allele is classically associated with incidence and severity of autoimmune disease,[46] especially T1D and CD.

Infant gut microbiome

Stool was collected from 1,748 infants at 11.9 ± 2.9 months, with an average 61,994 reads/sample. Neither age at stool collection nor copies of 16S rRNA/gram of stool differed between cases and controls, cumulatively or by ND subtype (p's > 0.62). We sought to identify differentially abundant bacteria based on

Figure 2. Symptom clusters at 2.5 and 5 years and their association with microbial species abundance at year 1

A series of gastrointestinal and mood-related symptoms were reported by the participating ABIS parent(s) on the 2.5- and 5-year questionnaires.

(A) Principal components of symptoms at 2.5 and 5 years, with component weights corresponding to each symptom.

(B) Symptom clustering at 3 years.

(C) Impact of symptoms reported at 3 and 5 years on the likelihood of future NDs, represented by odds ratios (ORs) with corresponding 95% confidence intervals and p values. The analysis focuses on individual symptoms as well as symptom clusters, i.e., the presence of at least one symptom within a cluster. The prevalence of symptoms and clusters among diagnosed cases (% ND) and non-diagnosed controls (% not ND) is indicated.

(D) Relative abundance of top microbial species associated with symptoms at 5 years. Symptom groups were dichotomized based on the presence of one or more qualifying symptom, and taxa with base means exceeding 15 included.

See also Figure S2 for associations with symptoms at 2.5 years.

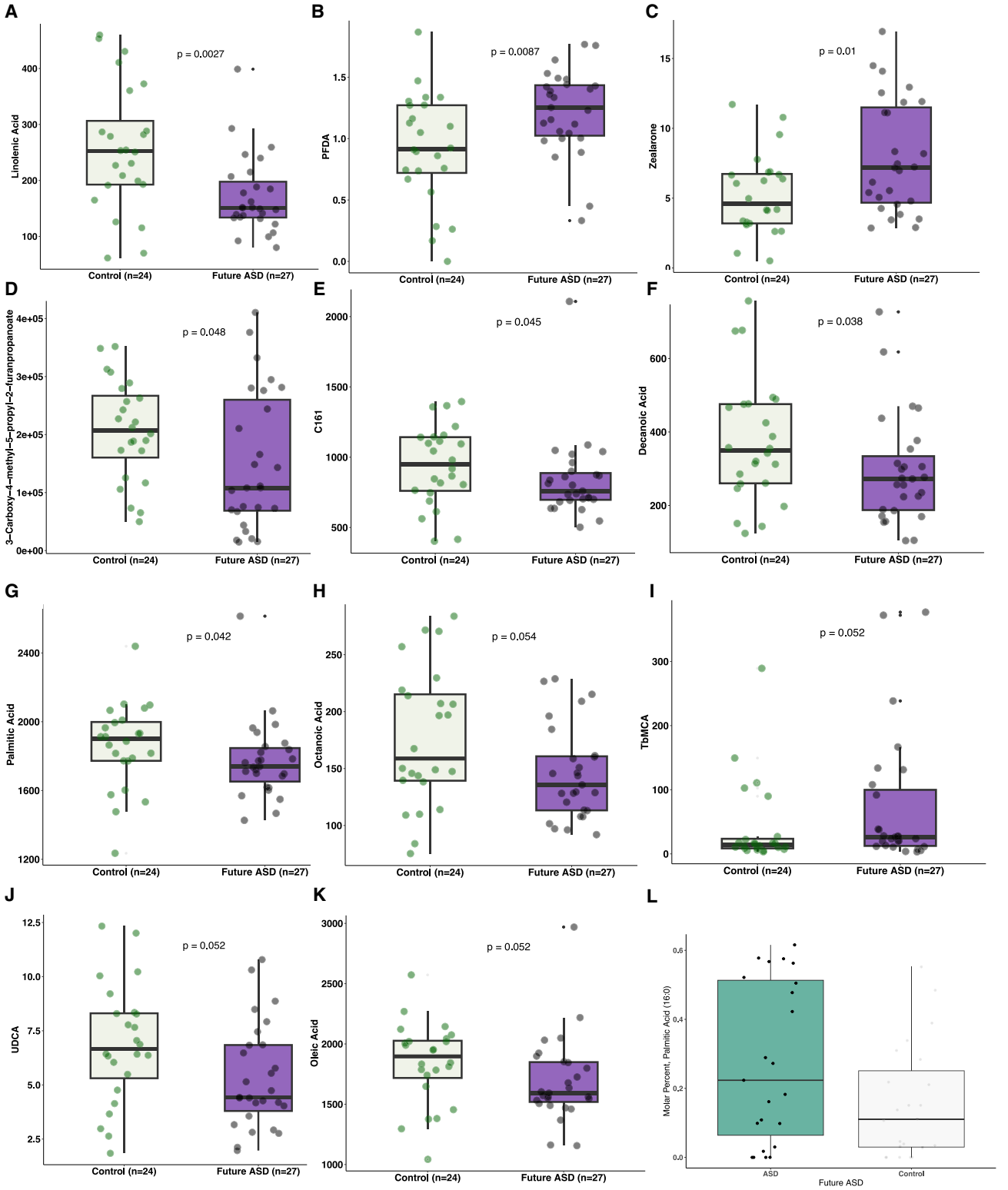

Figure 3. Cord serum metabolomic and stool fatty acid differences in future ASD ($ABIS_{ASD}$)

(A–K) Polar metabolites significantly enriched/depleted in the cord serum of $ABIS_{ASD}$ (n = 27) versus controls (n = 24). Controls were selected for $ABIS_{ASD}$ by propensity score matching (1:1 nearest neighbor) on prenatal factors from the birth questionnaire (encompassing infectious disease or other infection, severe life

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future ND status. For generalizability, first we compared all available controls with future NDs and then matched for risk factors and microbiome diversity confounds, i.e., mode of delivery ($p = 0.003$), geography ($p = 0.001$), first-year antibiotic exposure ($p = 0.005$), and first-year otitis ($p = 0.006$). Psychosocial vulnerability index ($p = 0.08$) and biological sex ($p = 0.10$) approached significance, while other infections and smoking during pregnancy showed no association (Table S1).

Future ND status was differentiable by both individual taxa (Figure 4A) and the common bacterial core[47] (Figure S2). Bacteria enriched in controls included *Akkermansia muciniphila*, *Roseburia hominis*, *Erysipelotrichiaceae UCG-003* spp., *Adlercreutzia equolifaciens*, *Alistipes putredinis*, *Phascolarctobacterium*, *Coprococcus*, and *Bifidobacterium* sp. (Figure 4A). *Veillonella parvula*, *Megamonas funiformis*, ASV-77 *Bacteroides* sp., and ASV-103 *Klebsiella* sp. were higher in future cases. Table S2 reports the greatest differences. Some core species were stable (Figure S2), while others were unique, including *Bacteroides uniformis* and *Bacteroides vulgatus* (all four NDs), *Veillonella parvula* (ASD and ADHD), *Escherichia-Shigella* (ASD, ADHD, and intellectual disability), and *Enterobacter* (ASD and intellectual disability). Differences were not due to sequencing depth and persisted in both rarefied and unrarefied datasets.

Microbial differences after confound adjustment (Figures 4C and 4D) demonstrated distinct core microbial communities in $ABIS_{ND\text{-}Match}$ ($n = 82$) and matched controls ($n = 163$). At 60% microbial prevalence, separation between matched groups was observed (Figure 4C; Table S4). *Lactobacilliaceae*, *Akkermansiaceae*, *Christensenellaceae*, *Erysipelotrichaceae UCG-003*, AV-81 *Bifidobacterium breve*, and *Phascolarctobacterium faecium*, among others, were enriched in matched controls, while *Bacteroidota*, *Enterobacteriaceae*, *Prevotella 9*, *Parabacteroides merdae*, and *Barnesiellaceae* were enriched in $ABIS_{ND\text{-}Match}$ (Figure 4D).

Infant gut microbiome (by ND subtype)

Given the spectrum of severity and diverse characteristics of NDs, we explored differences by subtype, i.e., ASD, ADHD, speech disorder, and multiple NDs. The core microbiome diverged significantly by subtype, although some differences were shared (Figure 4E). Principal components separating subtypes ($n = 114$) demonstrated that while the core microbiota of infants with multiple future NDs ($ABIS_{multiple}$) overlapped with future ASD ($ABIS_{ASD}$), both ADHD ($ABIS_{ADHD}$) and speech disorder ($ABIS_{Speech}$) differed substantially at 65% prevalence. Taxa significantly more abundant across NDs included *Megamonas funiformis* (all NDs, $ABIS_{ADHD}$) and ASV-86 *Enterobacter* sp. ($ABIS_{ASD}$) and in $ABIS_{Speech}$, included *Sutterella*, ASV-252 *Clostridium sensu stricto 1 neonatale*, ASV-184 *Blautia* sp., and ASV-155 *Bacteroides* sp. (Table S5).

To address core variability, controls were separately compared with future ASD, ADHD, speech disorders, or multiple NDs (Figures 5A–5D). Across all subtypes, 178 bacteria, mainly *Clostridia*, were more abundant in controls (Figure S3). Notably, *Akkermansia muciniphila*, *Phascolarctobacterium faecium*, *Roseburia hominis*, *Coprococcus eutactus*, *Coprococcus comes*, *Bacteroides ovatus*, *Bifidobacterium breve*, and *Alistipes putredinis* were consistently less abundant in future NDs, irrespective of subtype (Figure S3). ASV-184 *Blautia* sp. and ASV-155 *Bacteroides* sp. were higher in $ABIS_{Speech}$. *Carnobacteriaceae* and ASV-86 *Enterobacter* sp. were higher in $ABIS_{ASD}$ and *Megamonas funiformis* and ASV-77 *Bacteroides* sp. in $ABIS_{ADHD}$. Neither *Adlercreutzia* nor *Christensenella* was observed in $ABIS_{Speech}$. *Coprococcus eutactus* and *Bacteroides stercoris*, while lower in prevalence in all ABIS, were never observed in $ABIS_{ASD}$ or in comorbid ASD/ADHD. Interestingly, *Bifidobacterium* prevalence differed in future speech disorder, seen in 85.7% of $ABIS_{Speech}$ compared to 98.1% of controls ($OR = 0.11$, $p = 0.0059$), but not in other NDs.

$ABIS_{ASD}$ was further subdivided, considering potential differences in severity and a higher prevalence of ASD among males[49] (Figure S4). Males with late diagnoses ($n = 12$) had higher *Veillonellales-Selenomonadales*, *Coprococcus*, *Akkermansia muciniphila*, and *Ruminococcus gauvreauii*, compared to males with an early diagnosis ($n = 11$). Males with early diagnoses had higher *Parabacteroides distasonis*, *Sutterella wadsworthensis*, *Prevotella 9 copri*, ASVs-18 and 82 *Bacteroides* sp., ASV-86 *Enterobacter* sp., and ASV-103 *Klebsiella* sp. In females, early diagnosis of ASD ($n = 8$) was associated with increased abundance of ASVs belonging to *Blautia*, *Bacteroides* sp., *Citrobacter*, *Enterococcus* sp., *Veillonella* sp., and *Subdoligranulum* sp., while late diagnosis ($n = 8$) was associated with higher *Bacteroides* ASVs 77, 82, and 155. However, differences here may be due to small sample sizes and should not be overinterpreted.

Infant stool metabolome

Differences in the stool metabolome were observed in a subset of future ASD ($n = 23$) versus controls ($n = 23$), selected by propensity score matching on biological sex and municipality (Figures 6A–6C). The positive mode revealed 19 significantly different metabolites, and the negative mode identified 124 (p's < 0.05) by fold change (FC) analysis. Future ASD exhibited higher levels of 3-isopropylmalate ($FC = 6.3$, $p = 0.0024$) and quinate ($FC = 4.0$, $p = 0.035$), while controls showed higher levels of 2-hydroxyphenylacetic acid ($FC = 0.41$, $p = 0.032$), L-lysine ($FC = 0.69$, $p = 0.016$), and glutarate ($FC = 0.4$, $p = 0.032$). Other significant differences are in Table S6 (p's < 0.05), the most notable being various ion formations of glutarate and L-arginine, L-serine, L-cysteine-S-sulfate, nicotinate, and picolinic acid (higher in controls) as well as 3-dehydroshikimate, pyridoxamine, N-acetyl-DL-serine, glutaric acid, α-aminoadipate-N-methyl-L-glutamate, (R,R)-tartaric acid, proline, N-BOC-L-aspartic acid, and 5-hydroxymethyl-2-furaldehyde (higher in future ASD).

Investigating the abundance of key compounds, we observed intriguing patterns that highlight distinct trends in equol and

event, smoking, caffeine intake, vitamins/minerals, and iron supplements as well as consumption of milk, dairy, and eggs during pregnancy and education level of the mother and father) and gestational age in weeks. Significance was determined by Kruskal-Wallis in R.

(L) Comparison of palmitic acid (16:0) molar percentage in stool samples at 1 year of age between $ABIS_{ASD}$ ($n = 23$) and matched $ABIS_{Controls}$ ($n = 23$). Targeted metabolomics employed mass spectrometry with selected reaction monitoring (SRM) for 22 fatty acids. Controls were selected for stool metabolomics via propensity score matching on biological sex and municipality, with no difference in age at stool collection (controls, 10.89 ± 3.51 months; future ASD, 11.19 ± 2.37; $p = 0.726$).

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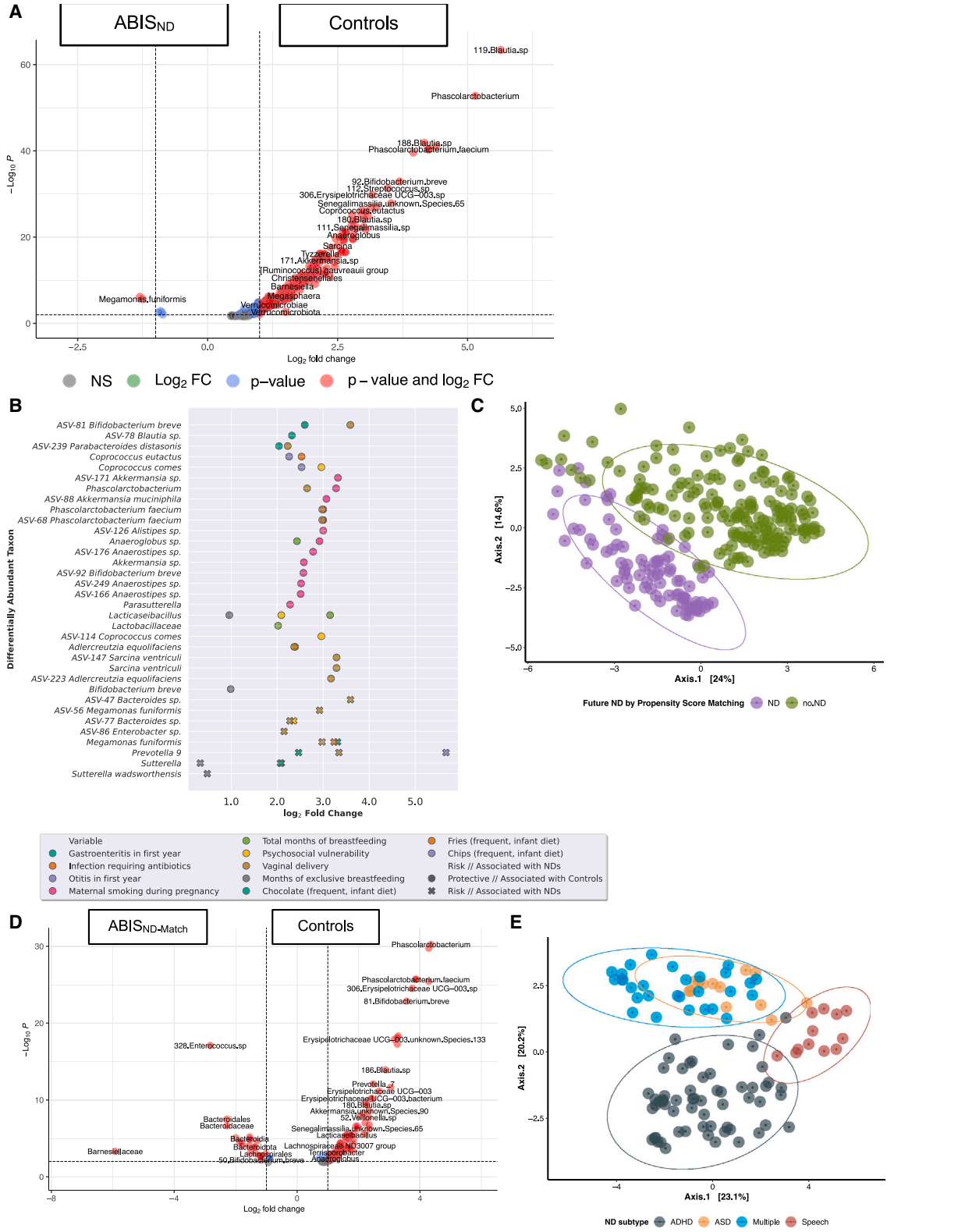

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butyrate production. Given that several producers of equol (a known antioxidant) were consistently higher in controls, we sought to identify potential equol signals. A potential *m/z* for equol was identified (*m/z* 241.0866). Albeit a low signal, the ppm mass error (1.66) was in line with our instrument accuracy. The decrease in this compound from future cases, compared to controls, was significant (4,168.98 ± 743.38 and 4,814.55 ± 984.03, p = 0.008).

Shifting our focus to SCFAs and associated metabolites, we found that despite an increase in butyrate producers, butyrate signals were not substantially different between groups (p = 0.38). This contrasted with the observed equol trend, suggesting that the increase in butyrate producers did not translate into a corresponding rise in butyrate levels.

Partial least squares-discriminant analysis (PLS-DA) identified 24 and 75 significant features in the positive- and negative-ion modes, respectively, with variable importance in projection (VIP) scores ≥2 (Figure S5). Noteworthy metabolites higher in controls in the positive mode included *m/z* 171.1490, *m/z* 144.1018 (likely N-methylpipecolic acid), *m/z* 185.1283 (likely N-(3-acetamidopropyl) pyrolidin-2-one), *m/z* 167.0563 (likely 1-methylxanthine), *m/z* 148.0967 (likely N-(2-hydroxyethyl)-morpholine N-oxide), *m/z* 144.1018 (likely N-methylpipecolic acid), and *m/z* 159.0440 (likely 1,2-napthoquinone). Conversely, *m/z* 149.0446 (likely 3-hydroxyglutaric acid), Ser-Pro (*m/z* 203.1026), and *m/z* 253.093 (likely galactosylglycerol) were higher in future ASD. Among notable negative-mode metabolites elevated in controls are *m/z* 241.1193 (likely pyroglutamylleucine), His-Pro (*m/z* 251.1147), *m/z* 132.0666 (likely N-methyl-threonine or 3-hydroxyvaline), and *m/z* 182.0126 (likely homocysteic acid or 8-hydroxythioguanine). Glutarate, L-glutamine, and riboflavin were higher in controls, while N-methyl-L-histidine, 3-aminoisobutanoate, 3-isopropylmalate, and N-acetyl-DL-glutamic acid were higher in future ASD.

Of the 22 fatty acids detected, two notable differences were observed. $ABIS_{ASD}$ was more likely to lack palmitoleic acid (16:1), X^2 (1, n = 46) = 3.9, p = 0.049, observed in 43.5% of controls but absent in 87.0% of future ASD. Conversely, the molar percentage of palmitic acid (16:0) in stool was 42.6% higher in $ABIS_{ASD}$ (27.4% ± 2.2% $ABIS_{ASD}$; 15.8% ± 16.5% controls, p = 0.056; Figure 3L), which is intriguing since it was lower in the cord serum.

Integration of questionnaire and biomarker data revealed bacterial connections with early-emerging GI and mood issues, environmental factors, and HLA genotype

We next explored microbial interactions associated with early symptoms, risk factors, and observed metabolomic differences.

Early-emerging GI and mood issues

Proteobacteria were 2.0–2.2 times higher in children with GI problems at age 2.5 (q = 0.011, q = 0.048). *Coprococcus* and *Slackia isoflavoniconvertens* exhibited a protective trend (Figure 2D) with log_2 FC ranging from 4.22 to 6.10, inversely correlating with the GI problems cluster and mood and unrest cluster. *Akkermansia muciniphila* and *Alistipes fingoldi*i inversely correlated with mood and unrest symptoms, and *Coprococcus eutactus* correlated negatively with the GI problems cluster. *Adlercreutzia equolifaciens*, *Erysipelotrichaceae UCG-003* spp., and *Roseburia* were higher, and *Robinsoniella peoriensis* and *Megasphaera micronuciformis* lower, in infants without future stomachache or diarrhea.

Similarly to associations with mood at 2.5 years, *Akkermansia*, *Adlercreutzia*, *Coprococcus*, and *Roseburia* spp. were more abundant in infants without mood or GI symptoms at age 5 (Figure 6E). *Akkermansia muciniphila* (FC = 2.9), *Blautia obeum* (FC = 3.0), and *Turicibacter sanguinis* (FC = 1.6) were inversely associated with general irritation or cranky mood. *Coprococcus comes* and *Coprococcus eutactus* inversely correlated with both stomach pain cluster and mood symptoms (FCs = 2.2–2.7). Other taxa inversely correlated with both clusters included

Figure 4. Taxonomic and core microbiome differences between all $ABIS_{Controls}$ (n = 1,456) and future ND, $ABIS_{ND}$ (n = 116), before and after matching for microbial confounds and ND risk factors ($ABIS_{ND\text{-}Match}$)

(A) Differential abundances for all $ABIS_{Controls}$ (n = 1,456) and $ABIS_{ND}$ (n = 116). Log_2 fold change (FC) > 0 corresponds to increased abundance in $ABIS_{Controls}$. Taxa with normalized base means ≥10 are shown, with p values adjusted for false discovery rate (FDR). See also Table S1 for confounds of microbial diversity, Table S2 for statistics, and Table S7 for sequences.

(B) Associations of species and amplicon sequence variants (ASVs) differential abundance with risk factors from the year 1 or birth surveys, after FDR correction. Infant diet features were dichotomized based on frequency (daily or 3–5 times weekly versus seldom or 1–2 times weekly). Months of breastfeeding (total/exclusive) were dichotomized as 1–4 months compared to 5 or more. Psychosocial vulnerability was dichotomized for high/low risk based on the total index. Risk factors included gastroenteritis; infection requiring antibiotics; otitis in the first year; increased psychosocial vulnerability; maternal smoking during pregnancy; fewer months of total/exclusive breastfeeding; more frequent chocolate, fries, and chips; and birth by cesarean section, while protective factors represented the inverse. Log_2 FC values were determined in DESeq2. See also Table S3 for comprehensive statistics across ND models.

(C) Gut microbial communities at 60% prevalence across $ABIS_{ND\text{-}Match}$ (n = 82) and matched controls (n = 163), selected in a 2:1 ratio using nearest-neighbor propensity score matching with the matchIt[48] R package on confounds affecting ND risk or gut microbiome composition, as described. This encompassed biological sex, mode of delivery, geography, toxic exposures, psychosocial vulnerability, and infant diet. From the 3,847 unique ASVs identified in these individuals, ASVs distinguishing NDs from matched controls were extracted using the random forest method implemented in the Prevalence Interval for Microbiome Evaluation (PIME) R package based on microbial prevalence. This process resulted in an out-of-bag (OOB) error of 2.86%. Principal components derived from the top 17 ASVs ranked by mean decrease accuracy (MDA) collectively represented a unique set of 4,129,332 sequences. See also Table S4.

(D) Differential abundances for $ABIS_{ND\text{-}Match}$ (n = 82) and a subset of matched controls (n = 163). Log_2 FC > 0 corresponds to increased abundance in matched controls. Taxa with normalized base means ≥10 are shown, with p values corrected for FDR.

(E) Gut microbial communities at a 65% prevalence threshold across neurodevelopmental disorder (ND) subtypes, encompassing ADHD, ASD, speech disorder, or multiple NDs (n = 114). From the 2,553 ASVs identified in these individuals, ASVs distinguishing subtypes were extracted using the PIME R package, with OOB error of 4.39%. Principal components derived from the top 20 ASVs ranked by MDA collectively represented a unique set of 1,928,573 sequences. See also Table S4.

See also Figure S3.

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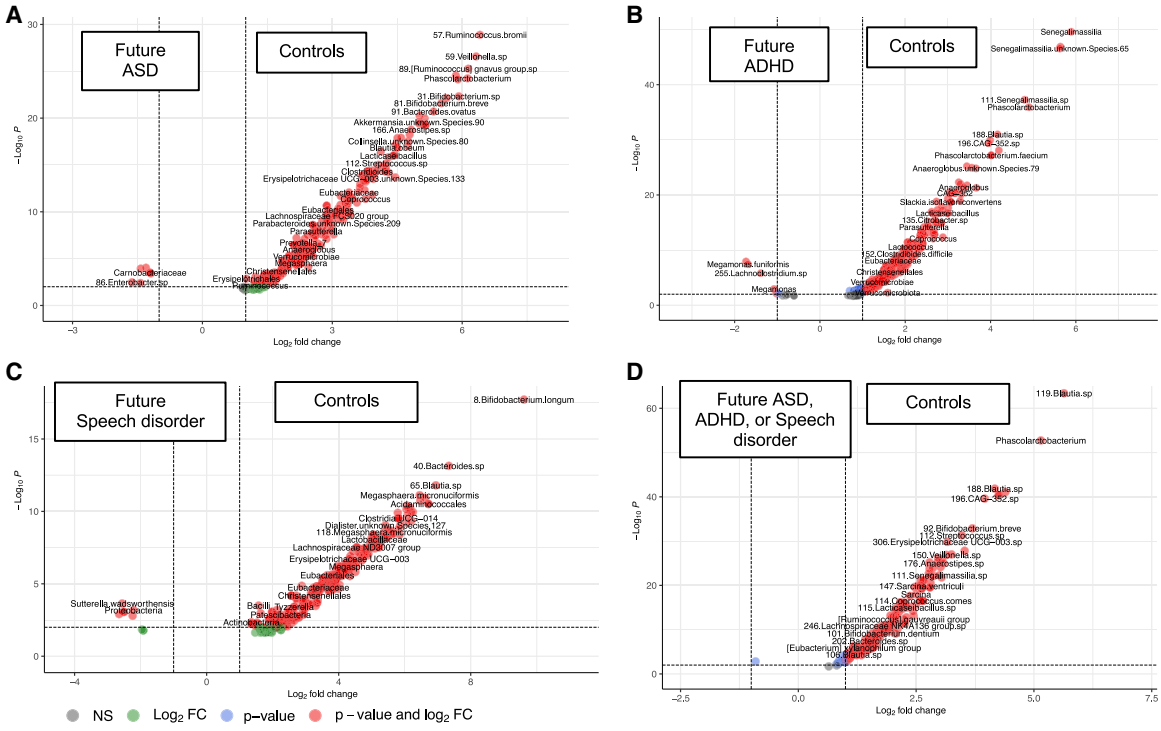

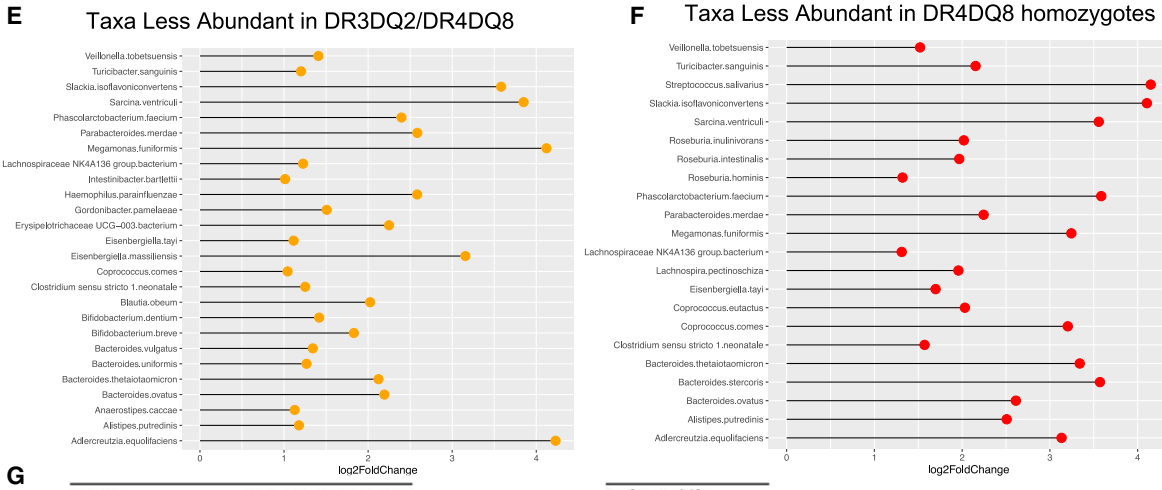

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Variable	ORadj	Est. (95% Conf. Int.)	P-value	Variable
Model 1 - ASD				
Smoking - mother	1.65	1.398 to 1.947	0.0001	Pregnancy
Type of home (1-3 months)	1.31	1.019 to 1.684	0.035	Pregnancy
Father	0.76	0.678 to 0.847	0.0001	Level of education
Mother	0.65	0.58 to 0.731	0.0001	Level of education
Model 2 - ASD				
SREB	6.09	2.218 to 16.721	0.0001	Microbiome
Akkermansia	0.4	0.191 to 0.835	0.015	Microbiome
Model 3 - ND				
SREB	2.67	1.086 to 6.579	0.032	Microbiome
Smoking - mother	2.24	1.267 to 3.967	0.006	Pregnancy
Akkermansia	0.56	0.362 to 0.872	0.01	Microbiome
Mother	0.5	0.337 to 0.736	0.0001	Level of education
Model 4 - ADHD				
SREB	3.23	1.302 to 8.018	0.011	Microbiome
Smoking - mother	2.5	1.38 to 4.523	0.003	Pregnancy
Akkermansia	0.55	0.343 to 0.886	0.014	Microbiome
Mother	0.47	0.312 to 0.722	0.001	Level of education

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Anaerostipes caccae, *Adlercreutzia equolifaciens*, and *Roseburia inulinivorans*.

Environmental factors

We next investigated differentially abundant bacteria in the context of early-life factors carrying decreased (log_2 FCs $\geq$ 2; Figure 4B; Table S3) or increased risk of future NDs (Figure 7B; Table S3). Protective early-life factors correlated with bacteria higher in controls, while those higher in $ABIS_{ND}$ correlated with C-section birth and with antibiotic use, infection, and infant diet. *Akkermansia muciniphila*, *Bacteroides ovatus*, *Phascolarctobacterium faecium*, *Alistipes*, and *Bifidobacterium breve* were higher in infants whose mothers did not smoke during pregnancy. Higher *Coprococcus comes*, *Adlercreutzia equolifaciens*, *Lacticaseibacillus*, and *Bifidobacterium* were associated with lower psychosocial vulnerability scores. Only five taxa associated with controls were higher in infants never exposed to antibiotics, including *Ruminococcus CAG-352*, *Clostridia UCG-014*, and *Coprococcus eutactus*. *Coprococcus euctatus* was also higher in infants who never had otitis, as were *Coprococcus comes* and *Ruminococcus gauvreauii*. *Bacteroides eggerthii* and ASV-81 *Bifidobacterium breve* were higher in infants who never had gastroenteritis. *Lacticaseibacillus* and members of *Anaeroglobus* were higher in infants breastfed for at least 5 months.

Several taxa were associated with risk factors. More abundant in those delivered by C-section were *Megamonas funiformis*; *Parabacteroides merdae*; *Prevotella* 9; *Sutterella*; ASVs-47, -77, and -155 *Bacteroides*; and ASV-86 *Enterobacter*. ASV-77 *Bacteroides* sp. was more abundant in infants with the highest psychosocial vulnerability scores, while ASV-25 *Bacteroides fragilis* was more abundant in those whose mothers smoked during pregnancy. *Anaerostipes caccae*, *Oscillospiraceae UCG-002* spp., and *Clostridium sensu stricto 1 neonatale* appeared largely driven by diet in the first year, negatively associated with frequent consumption of fried potatoes/fries and chips, especially (FCs = 4.0–5.0). *Anaerostipes caccae* and *Sutterella wadsworthensis* were higher in abundance in infants who exclusively breastfed for the shortest period (1–4 months).

HLA genotype

Bacteria associated with controls were more abundant in individuals without DR4-DQ8 or DR3-DQ2. DR3-DQ2/DR4-DQ8 heterozygotes showed higher *Acidaminococaccaeae* and *Christensenellaceae*. Detailed species-level analysis (Figure 5E) revealed significant differences, with *Adlercreutzia equolifaciens* (FC = 4.2), *Phascolarctobacterium faecium* (FC = 2.4), *Blautia obeum* (FC = 2.0), and *Coprococcus comes* (FC = 1.0) among the notable species. Similarly, in DR4-DQ8 homozygotes, we observed lower abundances of bacteria associated with controls, including *Roseburia* spp., *Phascolarctobacterium faecium*, *Coprococcus eutactus* and *Coprococcus comes*, *Alistipes putredinis*, *Alistipes finegoldii*, and *Adlercreutzia equolifaciens* (Figure 5F).

Integration of questionnaire and biomarker data also connected significant differences in bacterial prevalence with antibiotic use and chemical exposures

Two *Klebsiella michiganensis* strains (ASVs-120 and -318) were more prevalent in $ABIS_{multiple}$ (48.3%) and $ABIS_{ASD}$ (43.6%) versus controls (21.7%), particularly common in controls with frequent antibiotic use (27.1%). Infants with either ASV had higher odds of developing ASD or multiple NDs (OR = 2.8 [95% CI, 1.46–5.30], p = 0.0019, and OR = 3.4 [95% CI, 1.71–6.44], p = 0.0004, respectively), which increased when comparing infants breastfeeding for at least 8 months with minimal antibiotic use (none or 1–2 regimens). The presence of either ASV was associated with a 3.60 times (1.60–8.08, 95% CI) higher risk of comorbid ASD/ADHD, further heightened by exposure to antibiotics (OR = 5.52 [95% CI, 1.74–17.54]) or nicotine/alcohol (OR = 5.69 [95% CI, 2.20–14.71]). Presence of both ASVs increased the risk of comorbid ASD/ADHD (23.1% $ABIS_{ASD}$, 24.1% $ABIS_{multiple}$, 8.3% controls). *Salmonella*-related enteric bacteria were notably higher in future NDs (21% comorbid ASD/ADHD versus 3% controls, p = 1.07e−7; Figure 5G). While *Akkermansia* was found in 48.7% of controls, it was only present in 25.0% of future comorbid ASD/ADHD (OR = 2.84, 1.12–7.20 95% CI, p = 0.027) and 28.2% of future ASD (OR = 2.41, 1.19–4.88 95% CI, p = 0.014). These correlations held in logistic regression considering parental education and maternal smoking during pregnancy (adjusted ORs = 3.2 and 0.55, respectively). Environmental exposures and genetic factors further increased risk.

In exploring the link between early otitis and ND outcomes, *Citrobacter*, *Coprococcus*, and *Phascolarctobacterium* exhibited prominent disparities, even after multiple discovery corrections. Infants with *Citrobacter* were 2.35 times (1.12–4.94, 95% CI) more likely to be $ABIS_{ND}$ with early otitis than a control

Figure 5. Gut microbiome differences between future neurodevelopmental disorder (ND) subgroups and across the HLA risk haplotypes

Differences in prevalence of *Salmonella*-related enteric bacteria (SREB) and *Akkermansia*.

(A–C) Differential abundance results of future cases versus controls (n = 1,545) across ND subtypes. Significant bacteria were filtered to those with normalized base mean counts $\geq$10. (A) Future ASD (n = 39); (B) ADHD (n = 85); (C) speech disorders (n = 14). See also Figure S3 for core taxa differences, Table S3 for statistics by ND subtype, and Table S7 for sequences.

(D) Differences shared across all ND subtypes. See also Figure S4 and Table S5.

(E) Species strongly associated (log_2 fold change > |1|) with absence of DR3DQ2/DR4DQ8, comparing heterozygotes to individuals without either risk allele (base mean $\geq$ 10), after FDR adjustment. All taxa presented were higher in those without DR3DQ2/DR4DQ8.

(F) Top species differentially abundant between DR4DQ8 homozygotes and those children without DR4-DQ8 (absolute values of log_2 fold change > 1.3 and base mean $\geq$ 10), after FDR-adjustment. All significant taxa presented were higher in those without DR4-DQ8.

(G) Adjusted odds ratios (ORadj) after regressing risk factors (smoking of the mother during pregnancy, type of home that the mother resided in during months 1–3 of pregnancy, and parents' levels of education) and prevalence of *Akkermansia* or SREB, using multinomial logistic regression in Python 3.11.4. Significance and 95% confidence intervals are shown for each model.

See also Figure S5.

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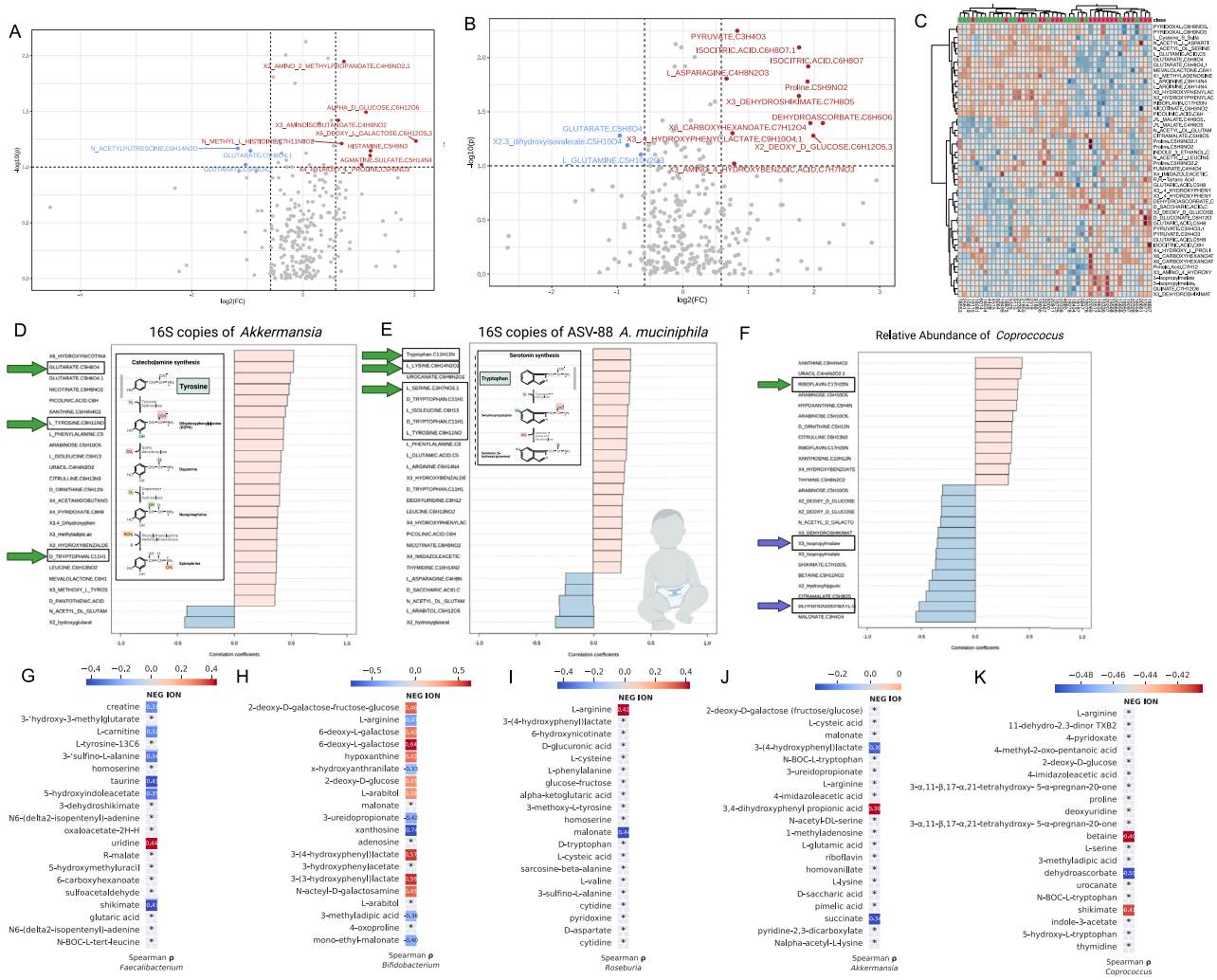

Figure 6. Stool metabolites strongly correlated with future ASD and with *Akkermansia* and *Coprococcus* abundances at 1 year

(A–C) Metabolites significantly enriched or depleted in future ASD in the positive (A) and negative (B and C) modes. Metabolites higher in expression in controls correspond to $\log_2$ FC > 0. See also Table S6.

(D and E) Correlations of stool metabolites with the total 16S rRNA gene copies of *Akkermansia* (D) and ASV-88 *Akkermansia muciniphila* (E).

(F) Stool metabolites correlated with relative abundance of *Coprococcus*.

(G–K) Spearman correlations (ρ) between stool metabolite concentrations with the relative abundance of five genera more abundant in ABIS controls (*Faecalibacterium*, *Bifidobacterium*, *Roseburia*, *Akkermansia*, and *Coprococcus*). Stool metabolites were identified using machine learning with the random forest regressor in Python to extract the top 20 metabolites predicting the relative abundance of the respective genus, followed by Spearman correlations. Metabolites with significant ($p < 0.05$) correlations are noted with the coefficient. Instances within plot of multiple metabolites with the same name demonstrate adducts with distinct *m/z* ratios and retention time (generally the loss of H_2O).

without otitis (p = 0.0235; Figure 7A). Conversely, *Coprococcus* was present in only 10% of future ND with otitis but in 34% of controls without otitis (Figure 7A). Further, *Coprococcus* was observed in only 15.7% of $ABIS_{ND}$ overall, with 78.6% of these children having experienced otitis in their first year. Adjustments for variables, including mode of delivery, prenatal antibiotics and smoking exposures, and breastfeeding, confirmed these associations (Figures 7A and 7C–7E).

Similarly, infants with *Citrobacter* showed a 3.99 times (1.66–9.61, 95% CI) higher likelihood of being $ABIS_{ND}$ with early otitis than a control without otitis (p = 0.002; Figure 7A). In the future ND group, 52% of those with past otitis had *Citrobacter*, compared to only 31% of those without infection (OR = 2.71 [1.08–6.80], p = 0.0336). Among controls, otitis in the first year corresponded to lower *Coprococcus* prevalence (17% versus 28%). Interestingly, *Citrobacter* was found in 61% of $ABIS_{ND}$ who had otitis in the first year but only in 28%–34% of $ABIS_{Control}$ (Figure 7E). Furthermore, *Phascolarctobacterium* (Figure 7D) and *Coprococcus* (Figure 7F) abundances were significantly higher in those without otitis (μ_{mean} = 0.60 versus μ_{mean} = 0.10 and μ_{mean} = 0.34 versus μ_{mean} = 0.10, respectively).

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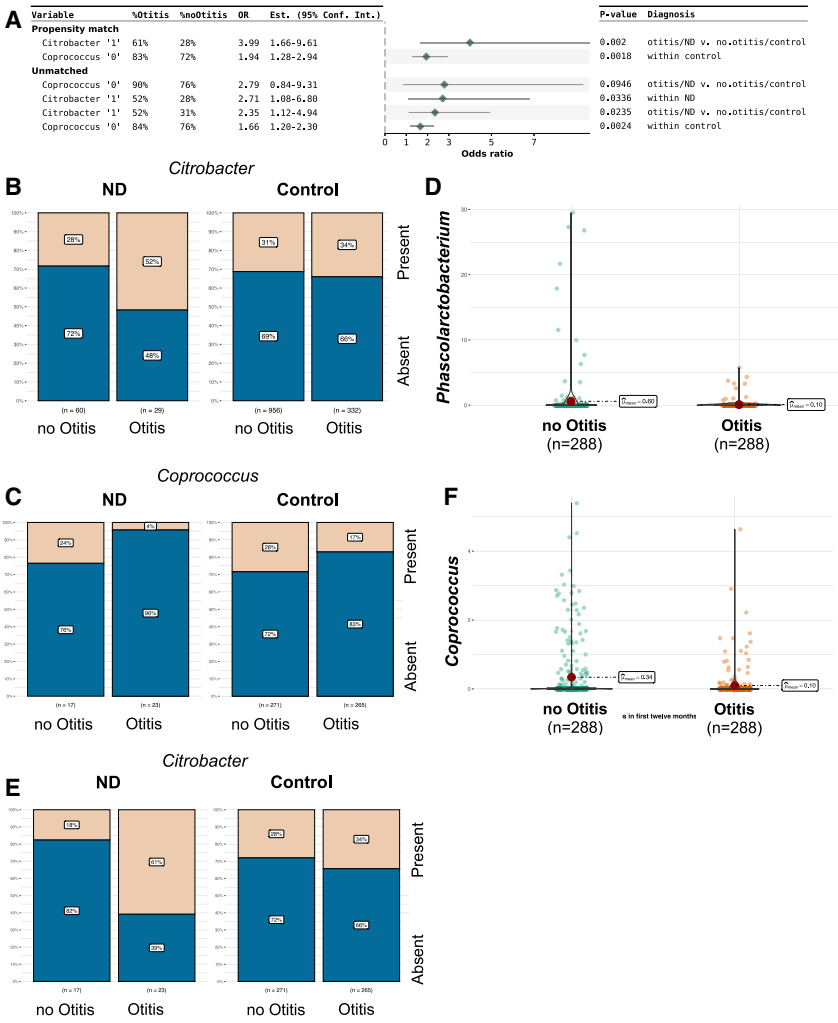

Figure 7. Association of *Citrobacter* and *Coprococcus* prevalence, and *Phascolarctobacterium* abundance, with early otitis infection and future neurodevelopmental disorder (ND) outcomes

(A) Odds ratios for the prevalence of *Citrobacter* and *Coprococcus* by early otitis and future NDs, with corresponding p values and 95% confidence intervals.

(B) Prevalence of *Citrobacter* in the full dataset, by history of otitis and future NDs.

(C and E) Prevalence of *Coprococcus* (C) and *Citrobacter* (E) in the matched dataset, considering early otitis and future NDs.

(D and F) Difference in relative abundance of *Phascolarctobacterium* (D) and *Coprococcus* (F) in the matched dataset, based on early-life otitis infection.

Integration of biomarker metabolomic and microbiome data revealed significant associations with TGs and polar metabolites at birth as well as vitamin and neurotransmitter precursors in infants' stools

Cord serum

To establish a connection between microbes and the cord serum metabolome, polar metabolites and relative abundances of taxa most consistently associated with NDs were examined using correlation analysis across all samples with cord serum and microbiome data (n = 114). ASV-81 *Bifidobacterium breve* correlated with LA, oleic acid, and UDCA, while ASV-17 *Anaerostipes hadrus* correlated with 3-carboxy-4-methyl-5-propyl-2-furanpropanoate. PFDA and zealarone, higher in the cord serum of future ASD, positively correlated with *Citrobacter* (p = 0.007) and ASV-86 *Enterobacter* sp. (p = 0.016), respectively. *Lacticaseibacillus* correlated with eleven of the fifteen TGs higher in concentration in controls and ASV-81 *Bifidobacterium breve* with four. While TG(46:2)/(16:1/14:0/16:1) levels positively correlated with ASV-31 *Bifidobacterium breve*, they also correlated with ASV-120 and ASV-318 *Enterobacter* sp., which were higher in future ASD. *Roseburia inulinivorans* negatively correlated with four TGs.

Infant stool metabolites

Stool metabolome profiles of infants with and without future ASD were similarly analyzed. Total 16S rRNA copies of *Akkermansia* and ASV-88 *Akkermansia muciniphila* positively correlated with stool metabolites including glutarate, arabinose, picolinic acid, L-phenylalanine, L-isoleucine, L-serine, leucine, and serotonin and catecholamine precursors D-tryptophan and L-tyrosine (Figures 6D and 6E). *Coprococcus* positively correlated with riboflavin, xanthine, uracil, arabinose, thymine, and 4-hydroxybenzoate and negatively correlated with 3-isopropylmalate and dehydroascorbate, with the latter also more highly expressed in future ASD (Figure 6F).

To link metabolomic and microbial differences derived from stool, machine learning was used to extract the top twenty metabolites in the negative-ion mode predicting bacterial abundance for five of the most salient genera in this investigation and correlations with the relative abundance determined (*Bifidobacterium*, *Roseburia*, *Faecalibacterium*, *Akkermansia*, and *Coprococcus*, Figures 6G–6K). Many positive correlations were observed with *Bifidobacterium*, including 2-deoxy-D-galactose-fructose, 3,4-hydroxy-phenyllactate, 3-hydroxyphenylacetate, N-acetyl-D-galactosamine, L-arabitol, 2-deoxy-D-glucose, and 6-deoxy-L-galactose, while xanthosine and mono-ethyl-malonate negatively correlated. L-arginine positively correlated with *Roseburia*, while malonate negatively correlated. Shikimate negatively correlated with both *Faecalibacterium* and *Coprococcus*. *Faecalibacterium* was also positively correlated with uridine and negatively correlated with 3-suflino-L-alanine, taurine, 5-hydroxyindoleacetate, creatine, and L-carnitine. *Akkermansia* positively correlated with 3,4-dihydroxy-phenyl-propionic acid.

DISCUSSION

Our study observed an inverse correlation of certain commensal bacteria with ND progression, early-life GI symptoms, mood

issues, and HLA alleles linked to autoimmune disease, proposing a strong link between neurodevelopment, gut barrier function, and the immune system. Collectively, our findings suggest that an inflammatory stage, mediated by gut bacteria, may contribute to ND risk very early in life.

Microbial and metabolomic imbalances that we observed at birth and 1 year have several important implications. First, cord serum metabolome differences' persistence after controlling for immunostimulatory events and psychosocial stressors underscores inherent biological variations at birth rather than just external influences. Notably, a decrease in crucial lipids, like LA and α-linolenic acid (ALA), bile acids, and TGs in neonates with future ASD, together suggests pro-inflammatory events present at birth. LA and ALA are precursors to long-chain polyunsaturated fatty acids (PUFAs) like docosahexaenoic acid (DHA, 22:6n-3), which have anti-inflammatory effects on the brain[50,51] and regulate autophagy, neurotransmission, and neurogenesis.[52] Their modulation of the endocannabinoid system, through inhibition of neurotransmitter release, e.g., γ-aminobutyric acid (GABA), influences synaptic function and plasticity.[53] We observed cord serum TG depletion in infants with future ASD, and these levels were found to be correlated with *Bifidobacterium* abundance at 1 year. During the last trimester, the fetal brain rapidly accumulates PUFAs, particularly DHA,[54] implicated in many brain disorders.[55,56] Regarding bile acids, UDCA, also lower in future ASD, has shown therapeutic promise in conditions spanning metabolic disease, autoimmune disease, chronic inflammatory disease, and neuropathology.[57–60] Its ability to cross the blood-brain barrier is notable,[61] with anti-inflammatory and anti-apoptotic mechanisms possibly linked to dopamine and mitochondrial regulation.[58] Moreover, the environmental pollutant PFDA was significantly higher in future ASD. Per- and polyfluoroalkyl substances including PFDA exert significant influence on immune responses.[62] These substances are known to be associated with chronic inflammation, oxidative stress,[63] immune suppression, and possible involvement in autoimmune diseases.[64]

Second, the onset of gut microbial dysbiosis occurs very early in life (in infancy) and is significantly correlated with the disruption of essential metabolites (e.g., vitamins, fatty acids, and neurotransmitter precursors). Although acute, chronic GI inflammation and increased intestinal permeability have been observed in ASD[65,66] and first-degree relatives,[65] microbial differences were evident here in ABIS years before diagnosis, characterized by a depletion of anti-inflammatory microbes and fortifiers of the gut barrier. Recent research has demonstrated the remarkable potential of *Akkermansia muciniphila* for promoting intestinal health,[67] although it has not been studied in neurodevelopment. *Akkermansia muciniphila* was absent in ABIS infants later diagnosed with ASD or comorbid ASD/ADHD and inversely correlated with GI and mood symptoms in early childhood. Intriguingly, *Akkermansia* was not associated with future ADHD, suggesting that disruptions in mucin health have a more robust connection to ASD. *Akkermansia muciniphila* facilitates mucin and produces folate,[68] propionate, and acetate[69,70]; is known for enhancing enterocyte monolayer integrity and fortifying compromised gut barriers[71]; and possesses immunomodulatory properties.[72] Microbiome-derived 3,4-dihydroxy-phenylpropionic acid, an epigenetic modifier that downregulates interleukin-6 (IL-6) cytokine production,[73] showed a correlation with *Akkermansia. Bifidobacterium* was also depleted in future NDs, across conditions. *Bifidobacteria* promotes healthy immune responses and enhances dopamine production by elevating phenylalanine.[32] In stool, *Bifidobacterium* abundance correlated with 4-hydroxyphenyllactate, which is a metabolite of tyrosine—a catecholamine precursor associated with cognitive function.[74,75] Species of *Coprococcus*,[76] *Akkermansia*, *Roseburia*, and *Turicibacter* were inversely associated with mood symptoms at age 5. *Coprococcus* has potent anti-inflammatory properties and, in ABIS, was inversely associated with NDs and linked to protective factors, including lower vulnerability scores, fewer antibiotics (none in the first year), and infant diets with fewer snacks. *Roseburia* also possesses potent anti-inflammatory properties, playing roles in colonic motility and the immune system.[77] *Turicibacter sanguinis* plays roles in serotonin utilization[78] and pathways of steroid and lipid metabolism.

Although several key butyrate producers[69] were inversely associated with future NDs (*Faecalibacterium prausnitzii*,[76,79] *Roseburia*,[69,80] *Anaerostipes*,[80] and *Acidaminococcales*), no marked difference in butyrate levels was observed in stool ($p = 0.38$). However, it is not known whether butyrate turnover rates differed between controls and future cases. Citrate levels were higher in future ASD ($p = 0.038$), consistent with a cross-sectional study of children with ASD finding increased succinate and citrate in urine,[81] while uridine levels in stool positively correlated with *Faecalibacterium*. Treatment with uridine can promote tissue regeneration and repair by metabolic adaptation, improving mitochondrial activity,[82] and may reduce inflammation and oxidative stress.[83] *Acidaminococcales* suppresses inflammation, and its low abundance aligns with a cross-sectional study of ASD.[84]

Several known equol producers were consistently higher in controls, including *Slackia* and *Adlercreutzia equolifaciens*. *Coriobacteriaceae*, a family involved in lipid metabolism[85] and equol production, was also depleted in infants with future NDs. *Eggerthella* and *Slackia* positively influence host lipid and xenobiotic metabolism.[86] We observed a significant decrease in a potential equol signal in future ASD cases. Equol has been studied for anti-inflammatory effects and estrogenic activity. In preclinical models, equol exhibits blood-brain barrier permeability capability,[87] anti-neuroinflammatory properties, and neuroprotective effects, protecting microglia against lipopolysaccharide (LPS)-induced inflammation[87] and neurotoxins.[88]

Third, the dysbiosis that we observed persists even after adjusting for environmental factors, like infant diet, psychosocial vulnerability (i.e., maternal smoking during pregnancy), and antibiotic use. While diets can vary during the transition from breast milk to solid foods, it is improbable that diet alone can account for the substantial differences we observed, as the microbial DNA was derived from 1-year-old infants and findings persisted in light of diet differences. Thus, collectively, our findings address a critical gap in literature studying children already diagnosed with NDs.[89] They indicate that dysbiosis is not solely a result of post-diagnosis dietary changes but rather existed prior to diagnosis, providing valuable insights into the early development of these conditions. In many cases, the infant microbiome

differences pointed to early mood and GI symptoms as well at 2.5–5 years. Both adjusted and unadjusted approaches showed enrichment of some bacteria in controls (e.g., *Coprococcus eutactus*, *Akkermansia muciniphila*, and *Blautia obeum*) and cases (e.g., *Corynebacterium variabile*). *Corynebacterium* spp. are generally considered pathobionts, some susceptible only to vancomycin or aminoglycosides,[90] and have been linked to increased respiratory infection in newborns delivered by C-section.[91] Certain *Bifidobacterium breve* ASVs—as well as *Ruminococcaceae CAG-352*, *Adlercreutzia equolifaciens*, and *Roseburia* spp.—lost significance after adjustment, suggesting their role in NDs is heavily mediated by environmental factors.

Fourth, the extent of the microbial dysbiosis seen in ABIS infants may be linked to the effects of HLA, stress, and infection/repeated antibiotics in childhood. Immunostimulatory events and psychosocial stressors have lasting effects on fetal health, development, and immunity, with the former disrupting cell differentiation, migration, and synaptic maturation.[37] In ABIS, prenatal and early-life stress and psychosocial adversity heightened ND risk, aligning with other studies.[92,93] In preclinical models, stress-induced prenatal events activates GABAergic delay, mediated by pro-inflammatory cytokine IL-6 and impacting adult microglia.[94] Repeated infections and antibiotic exposures in early life were also a significant risk factor across the first several years of life. C-section was a risk for speech disorder and intellectual disability. Although ABIS lacks intrapartum antibiotic data, we suspect all C-section births (11.9% of births in ABIS) entailed antibiotics as well.[95] HLA, which significantly impacts the immune system and especially microbial interactions,[96,97] is often overlooked in neurodevelopmental research. Its contribution to synaptic function, central nervous system development, and neurological disorders is increasingly demonstrated.[98] Nearly 30 years ago, DRβ1*0401 was first implicated in ASD.[99] Here, we found further evidence of this: DR4-DQ8 homozygosity (linked to autoimmune diseases including CD and T1D) increased ND and ASD risk by 1.8- and 2.8-fold. Children heterozygous for DR3-DQ2/DR4-DQ8 or homozygous for DR4-DQ8 shared deficits in *Roseburia*, *Phascolarctobacterium faecium*, and *Coprococcus* species, as did infants with future NDs.

Young children later diagnosed with ASD or exhibiting significant autistic traits tend to experience more ear and upper respiratory symptoms.[100] In ABIS, infants who had otitis in their first year were found to be more prone to acquiring NDs if they lacked detectable levels of *Coprococcus* or harbored *Citrobacter*. The absence of *Coprococcus*, despite comparable levels in controls irrespective of otitis, raises questions about microbial community recovery. This potential failure of the microbiome to recover following such events may serve as a mechanism connecting otitis media to ND risk. Moreover, antibiotic-resistant *Citrobacter*[101] was more prevalent in these infants. The presence of strains related to *Salmonella* and *Citrobacter*, labeled in this investigation as SREB, was significantly higher in infants who later developed comorbid ASD/ADHD (21%), compared to controls (3%). This disruption may have consequences on neurodevelopment during a critical period. *Salmonella* and *Citrobacter* have shown the ability to upregulate the Wingless (Wnt) signaling. The Wnt pathway is vital for immune dysregulation and brain development, and its disruption has been implicated in ASD pathogenesis.[102–105]

Last, early dysbiosis points to disruption of several metabolites in stool, including amino acids, fatty acids, vitamins, and neurotransmitter precursors. In ABIS, significant depletions of semi-essential amino acid L-arginine and essential amino acid lysine were observed in infants with future ASD, aligning with amino acid disruptions in children already diagnosed.[106,107] In ABIS, positive correlations between L-arginine and *Roseburia*, *Coprococcus*, and *Akkermansia* abundances were observed. L-arginine's association with improved innate immune response and barrier function has been documented.[108] Both lysine and arginine are critical for growth metabolism, immune function, histone modifications, and the production of nitric oxide. Lysine is involved in fatty acid metabolism, calcium absorption, immune function, and protein deposition, with high rates of metabolism in splanchnic tissues.[109] Its degradation pathway is closely linked to that of an important neurotransmitter precursor, tryptophan.[110] Arginine, a semi-essential amino acid, is vital for infant health, contributing to cardiovascular, neurological, and intestinal functions,[111] including neurogenesis regulation.[112] Depletion of arginine, especially during bacterial challenges, substantially hampers neonates' capacity to generate an adequate immune response, thereby elevating susceptibility to infections, particularly those originating in the GI tract.[113] Promising effects of amino acid supplementation have been demonstrated in premature neonates.[114,115]

Two fatty acid differences were notable in the stool of future ASD versus controls: omega-7 monounsaturated palmitoleic acid, (9Z)-hexadec-9-enoic acid (below the level of detection in 87.0% of future ASD but present in 43.5% of controls), and palmitic acid (elevated in future ASD). Palmitoleic acid has been associated with a decreased risk of islet and primary insulin autoimmunity.[116] Conversely, palmitic acid, a saturated fatty acid, has been linked to neuronal homeostasis interference.[117] Its effects are partially protected by oleic acid,[117] which although approaching significance, was lower in the cord serum of future ASD.

Few metabolites were higher in stool of infants with future ASD, but there are a few notable examples: α-d-glucose, pyruvate, and 3-isopropylmalate. *Coprococcus* inversely correlated with 3-isopropylmalate, suggesting gut-brain connections[118] and a possible imbalance in branched-chain amino acid (BCAA) pathways given the role of 3-isopropylmalate dehydrogenase in leucine and isoleucine biosynthesis.[119] An increase in dehydroascorbate suggests potential disruptions in vitamin C metabolism, crucial for neurotransmitter synthesis and antioxidant defense, while elevated pyruvate suggests disturbance of neurotransmitter synthesis or energy production early in life. Pimelic acid elevation, found in disorders of fatty acid oxidation,[120] suggests disruption of mitochondrial pathways for fatty acid oxidation.

Akkermansia and *Coprococcus*, absent or reduced in infants with future NDs, positively correlated with signals in stool representing neurotransmitter precursors and essential vitamins in stool. Specifically, *Akkermansia* correlated with tyrosine and tryptophan (i.e., catecholamine and serotonin precursors, respectively) and *Coprococcus* with riboflavin. Disruption of

BCAA metabolism in ASD has been documented,[121,122] involving coding variants in large amino acid transporters (LATs) and reduced utilization of trypotphan and large aromatic amino acids[121] along with increased glutamate and decreases in tyrosine, isoleucine, phenylalanine, and tryptophan in children with ASD.[122] Oxidative stress, a diminished capacity for efficient energy transport,[122] and deficiencies in vitamins (like vitamin B2) essential for neurotransmitter synthesis and nerve cell maintenance have been implicated.[123] Riboflavin as an antioxidant reduces oxidative stress and inflammation,[124] demonstrating neuroprotective benefits in neurological disorders,[125] possibly through maintenance of vitamin B6,[126] which is necessary for glutamate conversion to glutamine and 5-hydroxytryptophan to serotonin.

Together, these findings support a hypothesis of early-life origins of NDs, mediated by gut microbiota. This provides a foundation for research and for developing early interventions for NDs.

Limitations of the study

Neuropsychological testing was not performed, but groupings are robust, based on physician assessments using International Classification of Diseases (ICD)-10 criteria. Generalizability is unknown, as participation was restricted to children born in Sweden from 1997 to 1999. Microbial DNA at 1 year was analyzed in only 10.8% of the ABIS cohort, potentially affecting representation. Despite efforts to control variables, unmeasured confounders might impact results. While ABIS provides rich metadata through extensive questionnaires, some crucial data, such as antibiotics delivered intrapartum, were unavailable. Strains of bacterial species can vary significantly in physiological properties. Temporal dynamics of dysbiosis in relation to inflammation, continuity of microbial features, and whether dysbiosis acts as a trigger or outcome remain uncertain. Our study emphasizes the need for further research on dysbiosis persistence and functional consequences across early childhood, crucial for developing optimal interventions and understanding causality.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cell.2024.02.035.

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AUTHOR CONTRIBUTIONS

Conceptualization, A.P.A., E.W.T., K.I., T.H., M.O., and J.L.; methodology, A.P.A., T.H., M.O., E.W.T., J.R.P., K.I., T.J.G., and J.L.; formal analysis, A.P.A., T.H., and J.R.P.; investigation, A.P.A., T.H., J.R.P., and C.D.G.; data curation, A.P.A., T.H., J.R.P., T.J.G., M.O., E.W.T., and J.L.; writing – original draft, A.P.A.; writing – reviewing and editing, A.P.A., E.W.T., K.I., T.H., T.J.G., J.R.P., and J.L.; visualization, A.P.A., T.H., J.R.P., T.J.G., and E.W.T.; funding acquisition, J.L., M.O., and E.W.T.; resources, E.W.T., M.O., T.J.G.,

and J.L.; project administration, J.L.; supervision, E.W.T., M.O., T.J.G., and J.L. J.L. created and still leads the ABIS study, including collection of data and biological samples.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Cord blood from ABIS participants	All Babies in Southeast Sweden, Linköping, Sweden	https://www.abis-studien.se/hem/english-11100423
Stool samples from 1-year-old ABIS participants	All Babies in Southeast Sweden, Linköping, Sweden	https://www.abis-studien.se/hem/english-11100423
Chemicals, peptides, and recombinant proteins		
16S rRNA amplification primers (customized in Triplett lab[127])	Invitrogen, USA	341F: NNNNCCTACGGGAGGCAGCAG; 806R: GGGGACTACVSGGGTATCTAAT
Resuspension buffer	Illumina	Cat#15026770
Sodium Hydroxide Solution, 5N	Fisher Scientific	Cat#SS256-500
2-diheptadecanoyl-sn-glycero-3-phosphoethanolamine (PE(17:0/17:0))	Avanti Polar Lipids	Cat#830756
1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine (LPC(18:0))	Avanti Polar Lipids	Cat#855775
N-heptadecanoyl-D-erythro-sphingosylphosphorylcholine (SM(d18:1/17:0))	Avanti Polar Lipids	Cat#121999-64-2
2-diheptadecanoyl-sn-glycero-3-phosphocholine (PC(17:0/17:0))	Avanti Polar Lipids	Cat#850360
1-heptadecanoyl-2-hydroxy-sn-glycero-3-phosphocholine (LPC(17:0))	Avanti Polar Lipids	Cat#855676
2-Dioctadecanoyl- -sn-glycero-3-phosphocholine (PC(18:0/18:0))	Avanti Polar Lipids	Cat#850333
1-Hexadecanoyl-2-oleoyl-sn-glycero-3-phosphocholine (PC(16:0/18:1)	Avanti Polar Lipids	Cat#850457
1-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine (LPE(18:1))	Avanti Polar Lipids	Cat#850456
1-Palmitoyl-2-Hydroxy-sn-Glycero-3-Phosphatidylcholine (LPC(16:0))	Avanti Polar Lipids	Cat#846725
Triheptadecanoylglycerol (TG(17:0/17:0/17:0))	Larodan	Cat#33-1700
Trihexadecanoalglycerol (TG(16:0/16:0/16:0))	Larodan	Cat#33-1610
1-stearoyl-2-linoleoyl-sn-glycerol (DG(18:0/18:2))	Avanti Polar Lipids	Cat#855675
3-trioctadecanoylglycerol (TG(18:0/18:0/18:0))	Larodan	Cat#33-1810
3β-Hydroxy-5-cholestene-3-linoleate (ChoE(18:2))	Larodan	Cat#64-1802
1-hexadecyl-2-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine (PC(16:0e/18:1(9Z)))	Avanti Polar Lipids	Cat#800817
1-(1Z-octadecanyl)-2-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine (PC(18:0p/18:1(9Z)))	Avanti Polar Lipids	Cat#878112
1-oleoyl-2-hydroxy-sn-glycero-3-phosphocholine (LPC(18:1))	Larodan	Cat#38-1801
1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (PE(16:0/18:1))	Avanti Polar Lipids	Cat#852467

(*Continued on next page*)

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
3β-hydroxy-5-cholestene-3-stearate (ChoE(18:0))	Larodan	Cat#64-1800
1-palmitoyl-d31-2-oleoyl-sn-glycero-3-phosphocholine (PC(16:0/d31/18:1))	Avanti Polar Lipids	Cat#850757
2-diheptadecanoyl-sn-glycero-3-phosphoethanolamine (PE(17:0/17:0))	Avanti Polar Lipids	Cat#830756
N-heptadecanoyl-D-erythro-sphingosylphosphorylcholine (SM(d18:1/17:0))	Avanti Polar Lipids	Cat#860585
1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine (LPC(18:0))	Avanti Polar Lipids	Cat#855775
beta-Muricholic acid	Steraloids	Cat#C1895-000
Chenodeoxycholic acid	Sigma-Aldrich	Cat#C1050000
Cholic acid	Sigma-Aldrich	Cat#C2158000
Deoxycholic acid	Sigma-Aldrich	Cat#700197P
Glycochenodeoxycholic acid	Sigma-Aldrich	Cat#700266P
Glycocholic acid	Sigma-Aldrich	Cat#700265P
Glycodehydrocholic acid	Steraloids	Cat#C2020-000
Glycodeoxycholic acid	Sigma-Aldrich	Cat#361311
Glycohyocholic acid	Steraloids	Cat#C1860-000
Glycohyodeoxycholic acid	Steraloids	Cat#C0867-000
Glycolitocholic acid	Sigma-Aldrich	Cat#700268P
Glycoursodeoxycholic acid	Sigma-Aldrich	Cat#06863
Hyocholic acid	Steraloids	Cat#C1850-000
Hyodeoxycholic acid	Steraloids	Cat#C0860-000
Litocholic acid	Sigma-Aldrich	Cat#700218P
alpha-Muricholic acid	Steraloids	Cat#C1891-000
Tauro-alpha-muricholic acid	Steraloids	Cat#C1893-000
Tauro-beta-muricholic acid	Steraloids	Cat#C1899-000
Taurochenodeoxycholic acid	Sigma-Aldrich	Cat#700249P
Taurocholic acid	Sigma-Aldrich	Cat#T9034
Taurodehydrocholic acid	Sigma-Aldrich	Cat#700242P
Taurodeoxycholic acid	Sigma-Aldrich	Cat#700250P
Taurohyodeoxycholic acid	Sigma-Aldrich	Cat#700248P
Taurolitocholic acid	Sigma-Aldrich	Cat#700252P
Tauro-omega-muricholic acid	Steraloids	Cat#C1889-000
Tauroursodeoxycholic acid	Sigma-Aldrich	Cat#580549
Trihydroxycholestanoic acid	Avanti Polar Lipids	Cat#700070P
Fumaric acid	Sigma-Aldrich	Cat#47910
Glutamic acid	Sigma-Aldrich	Cat#G0355000
Aspartic acid	Sigma-Aldrich	Cat#A1330000
Serine	Sigma-Aldrich	Cat#S4500
Threonine	Sigma-Aldrich	Cat#PHR1242
Glutamine	Sigma-Aldrich	Cat#G3126
Proline	Sigma-Aldrich	Cat#V0500
Valine	Sigma-Aldrich	Cat#PHR1172
Lysine	Sigma-Aldrich	Cat#L5501
Methionine	Sigma-Aldrich	Cat#M0960000
Syringic acid	Sigma-Aldrich	Cat#63627
Isoleucine	Sigma-Aldrich	Cat#I2752

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Leucine	Sigma-Aldrich	Cat#L8000
Malic Acid	Sigma-Aldrich	Cat#PHR1273
Phenylalanine	Sigma-Aldrich	Cat#P2126
Ferulic acid	Sigma-Aldrich	Cat#Y0001013
Citric acid	Sigma-Aldrich	Cat#C7129
Tryptophan	Sigma-Aldrich	Cat#93659
3-Indoleacetic acid	Sigma-Aldrich	Cat#I3750
3-Hydroxybutyric acid	Sigma-Aldrich	Cat#52017
Isovaleric acid	Sigma-Aldrich	Cat#78651
Indole-3-propionic acid	Sigma-Aldrich	Cat#57400
Salicylic acid	Sigma-Aldrich	Cat#247588
Isocaproic acid	Sigma-Aldrich	Cat#277827
Decanoic acid	Sigma-Aldrich	Cat#C1875
Myristic acid	Sigma-Aldrich	Cat#70079
Linolenic acid	Sigma-Aldrich	Cat#62160
Palmitoleic acid	Sigma-Aldrich	Cat#76169
Linoleic acid	Sigma-Aldrich	Cat#62230
Eicosapentaenoic acid	Sigma-Aldrich	Cat#44864
Palmitic acid	Sigma-Aldrich	Cat#P0500
Oleic acid	Sigma-Aldrich	Cat#75090
Stearic acid	Sigma-Aldrich	Cat#S4751
Arachidic acid	Sigma-Aldrich	Cat#39383
[D4]- Glycoursodeoxycholic acid	Bionordica	Cat#31309
[D4]- Glycocholic acid	Bionordica	Cat#21889
[D4]- Ursodeoxycholic acid	Bionordica	Cat#21892
[D4]- Glycochenodeoxycholic acid	Bionordica	Cat#21890
[D4]- Cholic acid	Bionordica	Cat#20849
[D4]- Glycolitocholic acid	Bionordica	Cat#31308
[D4]- Chenodeoxycholic acid	Bionordica	Cat#20848
[D4]- Deoxycholic acid	Bionordica	Cat#20851
[D4]- Litocholic acid	Bionordica	Cat#20831
Valine-d8	Sigma-Aldrich	Cat#486027
Glutamic acid-d5	Sigma-Aldrich	Cat#631973
Succinic acid-d4	Sigma-Aldrich	Cat#293075
Heptadecanoic acid	Sigma-Aldrich	Cat#H3500
Lactic acid-d3	Sigma-Aldrich	Cat#616567
Citric acid-d4	Sigma-Aldrich	Cat#485438
Arginine-d7	Sigma-Aldrich	Cat#776408
Tryptophan-d5	Sigma-Aldrich	Cat#615862
Glutamine-d5	Sigma-Aldrich	Cat#616303
Critical commercial assays		
qPCR assays for QuantStudio 3 system	Applied Biosystems, Life Technologies, USA	Cat#A28567
Reaction mixture for qPCR (PowerUp SYBR Green 2X Master Mix)	Applied Biosystems, Life Technologies, USA	Cat#25742
E.Z.N.A Stool DNA Kit	Omega Bio-Tek, USA	Cat#D4015-02
E.Z.N.A Cycle-Pure Kit	Omega Bio-Tek, USA	Cat#D6492-02

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Nextera XT DNA Library Preparation Kit	Illumina	Cat#FC-131-1096
MiSeq Reagent Kit v3 (600-cycle)	Illumina	Cat#MS-102-3003
PhiX Control v3	Illumina	Cat#FC-110-3001
Deposited data		
Processed data	This paper	Dryad: https://doi.org/10.5061/dryad.ghx3ffbwj

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Prof. Eric Triplett (ewt@ufl.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Processed data reported in this paper have been deposited as the "All Babies in Southeast Sweden - Neurodevelopment and Early Life Factors" dataset (Dryad: https://doi.org/10.5061/dryad.ghx3ffbwj) in Dryad and are publicly available as of the date of publication. Curated data were selected in compliance with necessary, ethical human subjects protections for our child participants.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Human Participants

ABIS is a prospective, general population-based cohort study to which parents of all children born in southeast Sweden during the period 1 October 1997–1 October 1999 were invited.[128] Of the 21,700 families asked, 17,055 agreed to participate (78.6%), giving informed consent after receiving oral and written information. The original motivation behind ABIS was to prospectively study the etiology of immune-mediated diseases and the effects of environmental and genetic factors. The children have been followed from birth. Participating parents completed questionnaires at birth and at 1, 2-3, 5, 8-10, 14-16, and 17-19 years, with diaries kept during the first year of life.[129] ABIS children also completed questionnaires at 8, 10-12, and 17-19 years. Data include, but are not limited to, family medical history, antibiotic use, medications, diet, lifestyle, mood and disposition, home environment, environmental exposures, and psychosocial vulnerability.

For this investigation, we analyzed the earliest questionnaires derived from parents of ABIS children at one, three, and five years of age. The latest diagnoses considered in this investigation were obtained when the children were 21 to 23 years of age. The ABIS cohort showed a relatively balanced distribution of biological sex (48.2% female, 51.8% male). Although data on race, ancestry, and ethnicity were not collected, we anticipate homogeneity given that 89.2% of ABIS children had both parents born in Sweden, 7.7% had one parent born outside of Sweden, and only 3.1% had both parents born outside of Sweden. In the late 1990s, the majority of the population consisted of Ethnic Swedes, followed by Finns.

Regarding socioeconomics, 6.6% of ABIS children exhibited the greatest psychosocial vulnerabilities (vulnerability index scores >2). Disposable household income for ABIS families in 2000, 2006, and 2012 was reported as 305,360.7 SEK (95% CI: 297,279-313,462.5), 411,253.2 SEK (408,306.9-414,199.5), and 554,889.7 SEK (549,889.1-559,890.3), respectively.

Given the influence of biological sex on diagnosis, especially in boys, we explored microbiome associations stratified by sex and age of diagnosis (ASD only). Caution is warranted due to the limited sample size after this stratification. The compounded influence of biological sex on cohort-wide risk factors was not explicitly considered and may be a limitation to generalizability. It is important to note that, by design, the ABIS cohort consists of individuals from Sweden, and therefore, the findings may not be directly applicable to more diverse or non-European populations. Further research in populations with varied demographics is warranted to enhance the external validity of these findings.

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Diagnoses

International Classification of Diseases diagnoses (ICD-10) were derived from the National Patient Register (NPR),[130] capturing diagnoses through December 2020. Psychiatric diagnoses were set according to the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV[131]) or fifth edition (DSM-V[132]), depending on diagnosis date (Figure 1B). Qualifying diagnosis codes included ASD (F84.0, F84.1, F84.2, F84.3, F84.4, F84.5, F84.8, F84.9), ADHD (F90.0, F90.0A, F90.0B, F90.0C, F90.0X), speech disorders (F80.0, F80.0A, F80.0B, F80.0C, F80.1, F80.1A, F80.1B, F80.1C, F80.2, F80.2A, F80.2B, F80.2C, F80.3, F80.8, F80.8A, F80.8B, F80.8C, F80.8D, F80.8W, F80.9), and intellectual disability (F70.0, F70.1, F70.8, F70.9, F71.0, F71.1, F71.8, F71.9, F72.0, F72.1, F72.8, F72.9, F73.0, F73.1, F73.8, F73.9, F78.0, F78.1, F78.8, F78.9, F79.0, F79.1,F79.8,F79.9, F83.9).

Ethics approval and consent to participate

All families provided informed consent to be included in this study. The ABIS study was approved by the Research Ethics Committee, Linköping University and Lund University, approval numbers Dnr 03-092; LiU 287.96, LU 83-97, LiU 321-99.and Linköping University Dnr 36287, Dnr 03-513, Dnr 2018/380-32. The analysis of de-identified biospecimens used in this study was conducted under the approval of the University of Florida's Institutional Review Board (IRB). This study was approved under IRB201800903 and IRB202301239, ensuring that it met the criteria for minimal risk to participants.

METHOD DETAILS

Acquisition of cord serum metabolites

Cord serum samples were obtained from maternal donations, with a total of 120 samples from mothers whose infant also donated stool at one year. The analysis encompassed two methods: lipidomics and hydrophilic (water-soluble) metabolite profiling. The latter included examination of free fatty acids, bile acids and amino acids. Bile acids measured included 12-oxo-litocholic acid, 7-oxo-deoxycholic acid, 7-oxo-hyocholic acid, beta-muricholic acid, chenodeoxycholic acid, cholic acid, deoxycholic acid, dihydroxycholestanoic acid, glycochenodeoxycholic acid, glycocholic acid, glycodehydrocholic acid, glycodeoxycholic acid, glycohyocholic acid, glycohyodeoxycholic acid, glycolitocholic acid, glycoursodeoxycholic acid, hyocholic acid, hyodeoxycholic acid, litocholic acid, omega/alpha-muricholic acid, perfluorooctanoic acid, tauro-alpha-muricholic acid, tauro-beta-muricholic acid, taurochenodeoxycholic acid, taurocholic acid, taurodehydrocholic acid, taurodeoxycholic acid, taurohyodeoxycholic acid, taurolitocholic acid, tauro-omega-muricholic acid, tauroursodeoxycholic acid, trihydroxycholestanoic acid, and ursodeoxycholic acid. Free fatty acids included decanoic acid, myristic acid, linolenic acid, palmitoleic acid, linoleic acid, eicosapentaenoic acid, palmitic acid, oleic acid, stearic acid, and arachidic acid. Polar metabolites included fumaric acid, glutamic acid, aspartic acid, malic acid, phenylalanine, ferulic acid, citric acid, tryptophan, 3-indoleacetic acid, 3-hydroxybutyric acid, isovaleric acid, indole-3-propionic acid, salicylic acid, isocaproic acid, and succinic acid, serine, threonine, glutamine, proline, valine, lysine, methionine, syringic acid, and isoleucine/leucine.

Lipidomic analysis of cord serum

A total of 360 cord serum samples were randomized and analyzed as described below. 10 μl of serum was mixed with 10 μl 0.9% NaCl and extracted with 120 μl of $CHCl_3$: MeOH (2:1, v/v) solvent mixture containing internal standard mixture (c = 2.5 μg/ml; 1,2-diheptadecanoyl-sn-glycero-3-phosphoethanolamine (PE(17:0/17:0)), N-heptadecanoyl-D-erythro-sphingosylphosphorylcholine (SM(d18:1/17:0)), N-heptadecanoyl-D-erythro-sphingosine (Cer(d18:1/17:0)), 1,2-diheptadecanoyl-sn-glycero-3-phosphocholine (PC(17:0/17:0)), 1-heptadecanoyl-2-hydroxy-sn-glycero-3-phosphocholine (LPC(17:0)) and 1-palmitoyl-d31-2-oleoyl-sn-glycero-3-phosphocholine (PC(16:0/d31/18:1)) and, triheptadecanoylglycerol (TG(17:0/17:0/17:0)). The samples were vortexed and let stand on the ice for 30 min before centrifugation (9400 rcf, 3 min). 60 μl of the lower layer of was collected and diluted with 60 μl of $CHCl_3$: MeOH. The samples were kept at -80 ℃ until analysis.

The samples were analyzed using an ultra-high-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UHPLC-QTOFMS from Agilent Technologies; Santa Clara, CA, USA). The analysis was carried out on an ACQUITY UPLC BEH C18 column (2.1 mm × 100 mm, particle size 1.7 μm) by Waters (Milford, USA). Quality control was performed throughout the dataset by including blanks, pure standard samples, extracted standard samples and control plasma samples. The eluent system consisted of (A) 10 mM NH_4Ac in H_2O and 0.1% formic acid and (B) 10 mM NH_4Ac in ACN: IPA (1:1) and 0.1% formic acid. The gradient was as follows: 0-2 min, 35% solvent B; 2-7 min, 80% solvent B; 7-14 min 100% solvent B. The flow rate was 0.4 ml/min.

Quantification of lipids was performed using a 7-point internal calibration curve (0.1-5 μg/mL) using the following lipid-class specific authentic standards: using 1-hexadecyl-2-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine (PC(16:0e/18:1(9Z))), 1-(1Z-octadecenyl)-2-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine (PC(18:0p/18:1(9Z))), 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine (LPC(18:0)), 1-oleoyl-2-hydroxy-sn-glycero-3-phosphocholine (LPC(18:1)), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (PE(16:0/18:1)), 1-(1Z-octadecenyl)-2-docosahexaenoyl-sn-glycero-3-phosphocholine (PC(18:0p/22:6)) and 1-stearoyl-2-linoleoyl-sn-glycerol (DG(18:0/18:2)), 1-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine (LPE(18:1)), N-(9Z-octadecenoyl)-sphinganine (Cer(d18:0/18:1(9Z))), 1-hexadecyl-2-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine (PE(16:0/18:1)) from Avanti Polar Lipids, 1-Palmitoyl-2-Hydroxy-sn-Glycero-3-Phosphatidylcholine (LPC(16:0)), 1,2,3 trihexadecanoalglycerol (TG(16:0/16:0/16:0)), 1,2,3-trioctadecanoylglycerol (TG(18:0/18:0/18:)) and 3β-hydroxy-5-cholestene-3-stearate (ChoE(18:0)), 3β-Hydroxy-5-cholestene-3-linoleate

(ChoE(18:2)) from Larodan, were prepared to the following concentration levels: 100, 500, 1000, 1500, 2000 and 2500 ng/mL (in CHCl3:MeOH, 2:1, v/v) including 1250 ng/mL of each internal standard.

The International Lipid Classification and Nomenclature Committee (ILCNC) introduced the LIPID MAPS,[133] a comprehensive classification framework for lipids. This chemically based system organizes lipids into eight classes: fatty acyls, glycerolipids (GL), glycerophospholipids (GP), sphingolipids (SP), saccharolipids (SL), polyketides (PK), prenol lipids (PR) and sterol lipids (ST). Classification is based on lipid class, fatty acid composition, carbon count, and double bonds content. Specific lipid species, such as PCs, Pes, Pis, SMs and ceramides, possess two fatty acyl groups attached to their head group, while lysoPCs and lysoPEs are characterized by a single fatty acyl group. Classes including CEs, DGs, and TGs feature varying numbers of fatty acyl groups (one, two and three, respectively). Furthermore, lipids are also classified into classes like phophatidylcholines (PC), lysophophatidylcholines (lysoPC), phosphatidylethanolamines (PE), di- and triacylglycerols (DG, TG), sphingomyelins (SM), ceramides (Cer), phosphatidylinositols (PI), phosphatidylglycerols (PG), monohexosylceramides (HexCer), lactosylceramides (LacCer). Additional categorization of TGs is based on the fatty acid composition, distinguishing between saturated, monounsaturated, and polyunsaturated species. Notably, PCs and PEs exhibit subcategories, including alkylether PCs or PEs (plasmalogens), each possessing slightly different structure and biological function. In cases where detailed structure has not been determined, naming is based on carbon and double bond sums.

Data were processed using MZmine 2.53.[134] The identification was done with a custom data base, with identification levels 1 and 2, i.e. based on authentic standard compounds (level 1) and based on MS/MS identification (level 2) based on Metabolomics Standards Initiative. Quality control was performed by analysing pooled quality control samples (with an aliquot pooled from each individual samples) together with the samples. In addition, a reference standard (NIST 1950 reference plasma), extracted blank samples and standards were analysed as part of the quality control procedure.

Analysis of polar and semipolar metabolites

40 μl of serum sample was mixed with 90 μl of cold MeOH/H2O (1:1, v/v) containing the internal standard mixture (Valine-d8, Glutamic acid-d5, Succinic acid-d4, Heptadecanoic acid, Lactic acid-d3, Citric acid-d4. 3-Hydroxybutyric acid-d4, Arginine-d7, Tryptophan-d5, Glutamine-d5, 1-D4-CA,1-D4-CDCA,1-D4-CDCA,1-D4-GCA,1-D4-GCDCA,1-D4-GLCA,1-D4-GUDCA,1-D4-LCA,1-D4-TCA, 1-D4-UDCA) for protein precipitation. The tube was vortexed and ultrasonicated for 3 min, followed by centrifugation (10000 rpm, 5 min). After centrifuging, 90 μl of the upper layer of the solution was transferred to the LC vial and evaporated under the nitrogen gas to the dryness. After drying, the sample was reconstituted into 60 μl of MeOH: H_2O (70:30).

Analyses were performed on an Acquity UPLC system coupled to a triple quadrupole mass spectrometer (Waters Corporation, Milford, USA) with an atmospheric electrospray interface operating in negative-ion mode. Aliquots of 10 μL of samples were injected into the Acquity UPLC BEH C18 2.1 mm × 100 mm, 1.7-μm column (Waters Corporation). The mobile phases consisted of (A) 2 mM NH_4Ac in H_2O: MeOH (7:3) and (B) 2 mM NH_4Ac in MeOH. The gradient was programmed as follows: 0–1 min, 1% solvent B; 1–13 min, 100% solvent B; 13–16 min, 100% solvent B; 16–17 min, 1% solvent B, flow rate 0.3 mL/min. The total run, including the reconditioning of the analytical column, was 20 min.

Quantification of BAs and PFAS were performed using a 7-point internal calibration. The identification was done with a custom data base, with identification levels 1 and 2, based on Metabolomics Standards Initiative. Quality control was performed by analysing pooled quality control samples (with an aliquot pooled from each individual samples) together with the samples. In addition, a reference standard (NIST 1950 reference plasma), extracted blank samples and standards were analysed as part of the quality control procedure.

Human leukocyte antigen genotype and analysis across ND subtypes

Human leukocyte antigen (HLA) class II genotype was determined using sequence-specific hybridization with lanthanide-labelled oligonucleotide probes on blood spots on 3,783 children. Due to the genetic overlap and comorbidities observed in the literature with autoimmune disease, prevalence of risk alleles commonly reported in autoimmunity, specifically DR4-DQ8 and DR3-DQ2, was compared across NDs and controls using odds ratios in Python 3.11.4.

Stool sample collection and preservation

Stool samples were collected from 1,748 participating infants at one year. With a sterile spatula and tube provided by the WellBaby Clinic, samples were obtained from the diaper. Immediate freezing followed collection, either at the infant's home or the clinic. For samples collected at home, the use of freeze clamps facilitated frozen transport to the WellBaby Clinic, where subsequent dry storage at −80°C was maintained. Stool samples were collected at an average of age 11.93 ± 2.94 months, with no significant age differences between the control and ND groups at the time of collection.

Sequencing and quantification of microbial abundances

Extraction and Sequencing

DNA extraction from stool samples and subsequent 16S rRNA-PCR amplification targeting the V3-V4 region were carried out. A total of 1,748 samples were sequenced in ten pools using Illumina MiSeq 2x300 bp at the Interdisciplinary Center for Biotechnology Research (ICBR) at the University of Florida, Gainesville, Florida, USA, following established protocols.[127,129] Amplicons for targeted

V3-V4 16S rRNA sequencing were produced using Standard Illumina Read 1 sequencing/indexing primers 341F (NNNNCC TACGGGAGGCAGCAG) and 806R (GGGGACTACVSGGGTATCTAAT).

Forward primer: 5'- P5 – Adapter – Linker – SBS3 – 16S -3'

5' - AATGATACGGCGACCACCGAGCIWTHTAYGGIAARGGIGGGIATHGGIAA - 3'

Reverse primer: 5' - P7 Adapter – Linker – Barcode – SBS12 – 16S - 3'

5'-CAAGCAGAAGACGGCATACGAGAT-(BARCODE)-GTGACTGGAGTTCAGACGTGTGCTCTTCCGSTCTGGGGACTACVSGGGTATCTAAT - 3'

For pooling, barcodes 11 nucleotides in length were used. Each PCR sample was spin-colum purified and quantified by Qubit prior to pooling.

Paired end Joining and Demultiplexing

Amplicons were first processed in Qiime1,[135] involving paired-ends read joining (join_paired_ends.py), de-multiplexing (split_libraries_fastq.py), and generation of separate fastq files (split_sequence_file_on_sample_ids.py). In the initial QIIME1 processing, we were mindful of the potential impact of filtering on the meaning of downstream quality scores. Therefore, we intentionally set our demultiplexing options in the split_libraries_fastq.py step to be as lenient as possible so that error correction could be carried out using one, consistent method. Specifically, we chose the following parameters: **-q** (maximum unacceptable Phred quality score): Set to "0," allowing the most lenient passage of reads for downstream processing; **-r** (maximum number of consecutive low-quality base calls allowed before truncating a read): Set to "0," meaning no consecutive low-quality base calls were tolerated, however, at a Phred quality score of "0", thus passing reads of all quality scores forward; **-n** (maximum number of ambiguous or undetermined bases, or "N" characters, allowed in a sequence): Set to "100." These lenient parameter choices were made with the intention of conducting most of the filtering and quality control in DADA2[136] R package.

Filtering and Sample Inference

Using DADA2, sequences with poor quality were removed and high-resolution amplicon sequence variants (ASVs) were derived. DADA2 incorporates an error model to estimate error rates at each position in the sequence and thereby distinguish biological variations from sequencing errors.[136] Using this package, initial inspection of the raw, demultiplexed reads (plotQualityProfile), filtering and trimming (filterAndTrim), subsequent inspection of the filtered reads (plotQualityProfile), learning of error rates (learnErrors, plotErrors), and sample inference (dada) were carried out. In the filterAndTrim step, we employed the quality control process with the following parameters: truncation length (truncLen=c(421)) set to discard reads shorter than 421 bases, trimming (trimLeft=21) set to remove the first 21 bases were removed to eliminate barcodes, resulting in reads of 400 bases in length, maxN set to "0" (the default) to remove all ambiguous or undetermined bases (represented as "N" characters), maximum expected error (maxEE) set to "2" to discard reads with expected errors exceeding this threshold (EE = sum(10(-Q/20)), removal of reads matching against the phiX genome, and multithreading enabled to filter the input files in parallel. Subsequently, five samples with very low reads were excluded. Sample inference was conducted using the dada function with multithreading, and sequence tables from the ten pools were merged before applying the consensus-method chimera removal. Taxonomic assignment was determined using the Silva 138[137] database and verified using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST)[138] as needed.

In total, we observed 102,972,740 reads (average number of reads per sample: 61,994.4; median: 57,860; maximum: 776,158; minimum: 10,059), encompassing 12,844 unique ASVs. Among these, 4,938 ASVs were shared across at least two children, and 2,444 were present in five or more children. Table S7 provides complete ASV sequences for reference. For identification of core taxa, our rarefied dataset was employed, where counts were standardized to a sequencing depth of 21,800 reads, resulting in the removal of 27 control samples and 636 corresponding ASVs.

For microbiome analyses investigating ND status, samples with low read counts and samples from infants who later received an autoimmune diagnosis in the absence of a comorbid ND were removed, given the microbial associations that we have seen at this age in infants with future autoimmune disorders, resulting in a dataset of 1,661 samples. Of these, 116 were later diagnosed with an ND ($ABIS_{ND}$), with 87 children acquiring two or more NDs while 1,545 were deemed controls ($ABIS_{Controls}$) in the absence of a future diagnosis. Of the 116 $ABIS_{ND}$, 14 children were later diagnosed with a speech disorder, 7 with intellectual disability, 85 with ADHD, and 39 with ASD. For the analysis of environmental factors, all samples from ABIS were considered, after removing those with low counts (n=1,743).

Global and targeted metabolomics on stool at one year

Untargeted LC-MS metabolomic analysis was performed on stool samples from a subset of 46 individuals (n=23 $ABIS_{ASD}$ and n=23 $ABIS_{C}$), selected by propensity score matching on biological sex at birth and municipality, with no difference in age at stool collection ($ABIS_{Controls}$: 10.89 ±3.51 months, $ABIS_{ASD}$: 11.19 ±2.37, p=0.726). Stool samples, averaging 11.4 mg in weight, underwent cellular extraction and pre-normalization to sample protein content.

Global metabolomics profiling was conducted at the Southeast Center for Integrated Metabolomics (SECIM) at the University of Florida, Gainesville, FL. Full metabolomic methods (chromatography and MS) were performed as described previously.[139] Briefly, a Thermo Q-Exactive Oribtrap mass spectrometer with Dionex UHPLC and autosampler employed positive and negative heated electrospray ionization, each with a mass resolution of 35,000 at m/z 200, as separate injections. Separation was achieved using an ACE 18-pfp 100 x 2.1 mm, 2 μm column with a consisting of 0.1% formic acid in water (mobile phase A) and acetonitrile (mobile phase B).

This polar embedded stationary phase provides comprehensive coverage. It should be noted that there are some limitations of this technique in the coverage of very polar species. The flow rate was 350 μL/min, with a column temperature of 25°C, and injection volumes were 4 μL for negative ions and 2 μL for positive ions.

A total of 4,105 features were detected, with 1,250 in positive mode and 2,855 in negative mode. Feature identification, deisotoping, alignment, and gap filling for features that may have been missed in the first alignment algorithm were performed using MZmine[134] freeware. Data were processed to remove adducts and complexes. Metabolomic features were referenced against SECIM's internal retention time metabolite library of 1,414 compounds for identification based on existing metabolomic databases (Metlin, ChemSpider, pubChem).

Targeted metabolomics was also performed at the SEIC, University of Florida, Gainesville, FL, on fatty acids (22 metabolites) and trytophan (six metabolites), employing selected reaction monitoring (SRM). Fatty acids were quantified and identified using LipidMatch[140] *in silico* libraries.

QUANTIFICATION AND STATISTICAL ANALYSIS

Environmental risk factors from pregnancy to early childhood

The ABIS cohort was stratified into four distinct neurodevelopmental conditions: ASD, ADHD, Speech Disorders, and Intellectual Disabilities. Children with multiple diagnoses were included in each corresponding diagnostic category, accounting for potential overlap. Within each of these conditions, the prevalence of environmental factors (including family medical history, infections, exposures, and living conditions) using IBM SPSS version 29. These environmental factors were derived from ABIS parent questionnaires administered at birth, one year, three years, and five years.

To avoid the potential for inflated false discovery rates, we adopted a targeted approach. First, we identified factors with a minimum of 15% difference in prevalence across the conditions. Subsequently, odds ratio calculations were performed for each selected factor and diagnostic group combination where appropriate using Python 3.11.4. The number of comparisons within each comparative group was deliberately limited to fewer than 50. This deliberate restraint was exercised to maintain the robustness of the findings and obviate the need for false discovery corrections given the focused nature of the comparisons. Likewise, only factors involving family medical history, infections, exposures, and living conditions were considered.

Mood and GI symptom clusters at three and five years

Parents participating in the ABIS study completed an extensive questionnaire at the child's three and five year visits, including a set of 11–12 binary-response questions concerning growth, mood, and gastrointestinal issues. Symptoms were structured around the question, "Do you think that the child suffers from or is affected by..." to assess poor growth, poor weight gain, poor appetite, stomachache, bloated/gassy stomach, diarrhea three times or more per day, vomiting three times or more per day, constipation, fatigue, general irritation, cranky mood/screaming, and poor sleep quality.

Principal component analysis (PCA) was conducted using IBM SPSS version 29 on binary responses to the twelve symptoms reported at the child's 3-year visit and eleven symptoms at the 5-year visit. Components were subjected to Varimax rotation with Kaiser normalization, and component scores were computed through regression. This approach allowed interpretation of the components, with KMO measures indicating satisfactory sampling adequacy (KMO = 0.854 at 3 years and 0.839 at 5 years), and significant Bartlett's tests of sphericity (both p's < 0.001), confirming data suitability for PCA.

To evaluate individual symptoms and cumulative occurrence across the three- and five-year components, odds ratios were calculated using Python 3.11.4.

Otitis and comparative prevalence of microbes

Differences in the microbiome based on otitis infection in the first twelve months of life were sought within a propensity match group (n=576). Children were selected in a 1:1 fashion based on mode of delivery, antibiotics and smoking during pregnancy, and total months of breastfeeding by otitis infection status. The unmatched approach included all ABIS samples with available otitis data (n=1307). Genera were selected for prevalence testing based on DESeq2 results following FDR correction. Based on these results, odds ratios for the prevalence of *Citrobacter* and *Coprococcus* were calculated in Python 3.11.4 to assess association with early otitis and future ND outcomes.

Confounds of the gut microbiome

To examine the influence of confounding factors on gut microbiota composition (β-diversity), a permutational multivariate analysis of variance (PERMANOVA; adonis function, vegan package[141]; R Foundation) was conducted using the Bray-Curtis distance on compositional-transformed counts, employing 1000 permutations. The analysis aimed to evaluate influence of several factors, including biological sex a, mode of delivery, maternal smoking during pregnancy, geographic region (county of Sweden), infant antibiotic usage, early-life respiratory or gastrointestinal infections, and the child's vulnerability index score.

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Differential abundance between $ABIS_{Controls}$ and $ABIS_{ND}$

Differential expression analyses of gut microbiome variations between controls and future NDs were performed using the DESeq2[142] package. Children with future autoimmune conditions, which could also reflect dysbiosis, were excluded from the analysis. Estimations of size factors and dispersion were computed, and negative binomial general linear models (GLM) were fitted using Wald statistics with local type fitting of dispersions to mean intensity.

The GLM analysis yielded $\log_2$ fold change (FC) and standard error (lfcSE) values. To account for multiple comparisons, false discovery rate (FDR)-adjusted p-values were calculated within each analysis by the Benjamini-Hochberg method.[143] Significance was assessed for each analysis across different comparative groups and taxonomic ranks. Comparisons were made between controls and NDs collectively (diagnosis of any qualifying ND) and then stratified by diagnosis types (i.e., ASD, ADHD, speech disorder).

Differential abundance across symptom clusters and risk factors, as well as HLA

We employed the full microbiome cohort (n=1743) to investigate associations involving symptoms, risk factors, and HLA, using the DESeq2[142] package with FDR correction within each comparison. The prospective assessment of microbial taxa at one year of age in relation to symptoms at three and five years involved binary categorizations of symptoms such as fatigue, stomachache, general irritation, diarrhea, and sleep quality, as well as symptom clusters that exhibited the strongest links with development of one or more future NDs. Risk and protective factors from the year one and birth surveys were dichotomized for analysis. Risk factors encompassed gastroenteritis, infection requiring antibiotics, otitis in the first year; increased psychosocial vulnerability; maternal smoking during pregnancy; fewer months of total/exclusive breastfeeding; more frequent chocolate, fries, and chips in the first year; and birth by cesarean section, while protective factors represented the inverse (i.e., no infection; reduced or no psychosocial vulnerability; no smoking; longer periods of total/exclusive breastfeeding; no or fewer servings of chocolate, fries, and chips in the first year; birth by vaginal delivery). Infant diet features were dichotomized based on frequency (daily or 3-5 times weekly, versus seldom or 1-2 times weekly). Months of breastfeeding (total/exclusive) were dichotomized as one to four months compared to five or more. Psychosocial vulnerability was dichotomized for high/low risk based on the total index.

For the HLA investigation, we conducted two separate analyses within DESeq2: first, comparing DR4-DQ8 homozygotes and those without DR4-DQ8, and second, comparing individuals carrying DR3-DQ2/DR4-DQ8 to those lacking either of these risk alleles.

Differential abundance by age of diagnosis within ASD group, stratified by sex at birth

To address the potential impact of early ASD diagnosis on phenotype severity, we also explored differences based on diagnosis age within the $ABIS_{ASD}$ subgroup. These analyses were conducted separately for males and females, considering the average age of diagnosis was much lower in males (13.4 ±4.0 years, males; 17.6 ±2.6 years, females).

Differential abundance after controlling for microbiota confounders and ND risk factors ($ABIS_{ND\text{-}Match}$)

Propensity score matching was applied using the matchIt[48] R package to control for confounds affecting neurodevelopmental risk or gut microbiome composition. The confounds considered were biological sex, mode of delivery, geography (region/county of Sweden), toxic exposure (e.g., smoking during pregnancy), total psychosocial vulnerability index, and infant diet (including total months of breastfeeding and frequency of consumption of beef, chocolate, other candy, chips/cheese doodles in the first 12 months of life). Cases with missing data in these confounds were excluded, leaving 82 ND cases ($ABIS_{ND\text{-}Match}$). The controls (n=163) were selected using nearest neighbor propensity score matching to balance the distribution of these confounds. It was found that other additional confounds including HLA genotype ($p's > 0.162$) and antibiotic use ($p's > 0.203$) were inherently balanced between $ABIS_{ND\text{-}Match}$ and these selected controls. The dataset contained 15,120,951 reads, with an average of 61,718.2 reads per sample (median 57,208, maximum 328,839 and minimum 11,440) spanning 3,847 unique ASVs. 1,573 ASVs were seen in at least two children, and 878 in four children. Within the $ABIS_{ND\text{-}Match}$ group, 26 infants were diagnosed with future ASD, 59 with future ADHD, and 13 with a future speech disorder. Most infants received only one ND diagnosis later in life, with 43 with ADHD, 11 with a speech disorder, eight with ASD, and one with intellectual disability, while 19 received multiple ND diagnoses.

The DESeq2 analysis was repeated on this matched group of infants as previously described. Furthermore, overall microbiome community composition differences were assessed using the PIME[144] R package, which filters taxa based on prevalence intervals to achieve optimal classification. This was run at the genus level for differences between matched NDs and controls and at the ASV level for separating ND subtypes (i.e., multiple diagnoses, ASD, ADHD, and speech disorder).

Selection of core microbiota across ND subgroups

Considering the complexity of the microbiome, our analysis also extended to assess the prevalent and widely shared bacterial species based on ND status. Core taxa, determined for controls and future NDs (ASD, ADHD, speech disorder, and intellectual disability), were assessed using the microbiome R package on compositionally transformed ASV counts.

Our approach encompassed two datasets: the complete and the rarefied dataset (the latter restricted to 21,800 counts for an even sampling depth. For this approach, taxa that never achieved a prevalence exceeding a minimum of 10% (20% for speech and intellectual disability) were excluded. To pinpoint the key microbial taxa consistently present across ND subgroups, we employed the

core_members function from the microbiome R package. The core microbes were defined as those taxa displaying relative abundances surpassing 0.01% within over 50% of the samples belonging to a specific ND group. The resulting core taxa were further visualized using the plot_core R function.

To bolster the reliability of our findings, our analysis encompassed both the original, unrarefied dataset and the rarefied dataset. Notably, the process of rarefaction did not exert any discernible influence on the outcomes of the core taxa identification. For consistency, the rarefied dataset was adjusted to a uniform sequencing depth of 21,800 reads.

Stool metabolomics analysis and correlations with microbial abundances at one year

Metabolite concentrations were assessed for association with future ASD in stool samples (n=23 $ABIS_{ASD}$ and n=23 $ABIS_{Controls}$) using MetaboAnalyst[145] 5.0, focusing on their relationship with ND outcomes, particularly ASD and comorbid ASD/ADHD. Subsequently, selected metabolites were compared against bacterial relative and absolute abundances (the latter, calculated using qPCR). The data underwent normalization to the sum of metabolites per sample, followed by log-10 transformation of metabolite concentrations. Stool metabolites were analyzed separately in positive- and negative-ion mode, including integrated peak height intensities of 571 known metabolites (300 in negative and 271 in positive ionization mode). Metabolites exhibiting enrichment or depletion in cord serum or stool samples from infants diagnosed later with ASD were identified using Kruskal Wallis/Mann-Whitney U, partial least squares-discriminant analysis (PLS-DA), and fold-change analyses. Hierarchical clustering and point biserial correlation were also conducted.

Fatty acid differences in the stool samples were assessed for association with future ASD status using Mann-Whitney U tests in R. The groups did not differ significantly in terms of the sum of peaks (p=0.722). Molar percentages were calculated and compared. Notably, an observation was made that children with future ASD often exhibited a deficiency in palmitoleic acid. To investigate this further, we calculated the difference in the prevalence of palmitoleic acid using a chi square analysis.

Machine learning was utilized to extract the top twenty stool metabolomic features in the negative-ion mode that best predicted the relative abundance of *Bifidobacterium*, *Roseburia*, *Faecalibacterium*, *Akkermansia*, and *Coprococcus*, separately. Random forest regressors were run on each of the genera separately to identify the top twenty metabolites predicting abundance. Subsequently, the metabolites were linked to bacterial abundance using Spearman correlations. This analysis was carried out in Python 3.11.4

Additional associations with stool metabolite concentrations were evaluated for *Akkermansia* and *Coprococcus* using Spearman correlations within the MetaboAnalyst 5.0[146] framework. This analysis incorporated the total counts of 16S copies for *Akkermansia* and ASV-88 *Akkermansia muciniphila*, the relative abundance of *Coprococcus*, and normalized metabolite concentrations.

Cord serum metabolomics and correlations with microbial abundances at one year

Because of the prenatal programming influence of maternal infections,[37] smoking,[38] stress/severe life events,[39] diet,[40,41] and coffee intake[42] during pregnancy on the cord serum and fetus, we controlled for these factors with propensity score matching[48] to select controls (n=27) for future cases of ASD (n=27). For this we employed, a 1:1 nearest neighbor method on the following variables from the birth survey: infectious disease or other infection, severe life event, smoking, caffeine intake, vitamins/minerals and iron supplements, and consumption of milk, dairy, and eggs during pregnancy, as well as education level of the mother and father and birth week of the child. We examined the relationship between concentrations of known, polar metabolites detected in the 114 cord serum samples and the relative abundances of several of the taxa most consistently associated with NDs in this investigation. Pearson correlations were performed in R. Differences between future ASD and controls were determined by Kruskal Wallis/Mann Whitney testing in R.

Supplemental figures

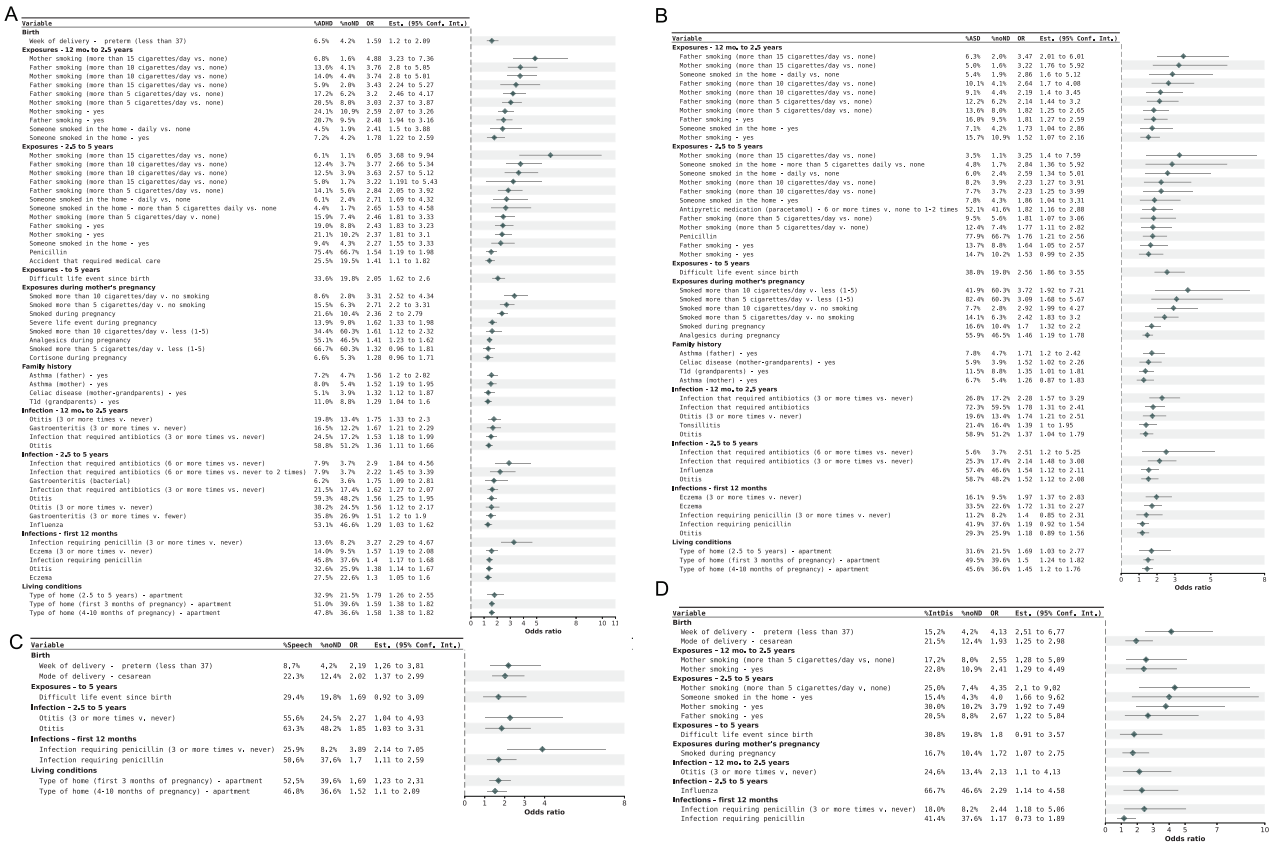

Figure S1. Early-life environmental and medical risk factors implicated in ADHD, speech disorder, and intellectual disability, related to Figure 1

Risk factors encompassing lifestyle, family, medical, and environmental factors across the birth and 1-, 3-, and 5-year questionnaires for ADHD (A), ASD (B), speech disorder (C), and intellectual disability (D), with cumulative ND results presented in Figure 1.

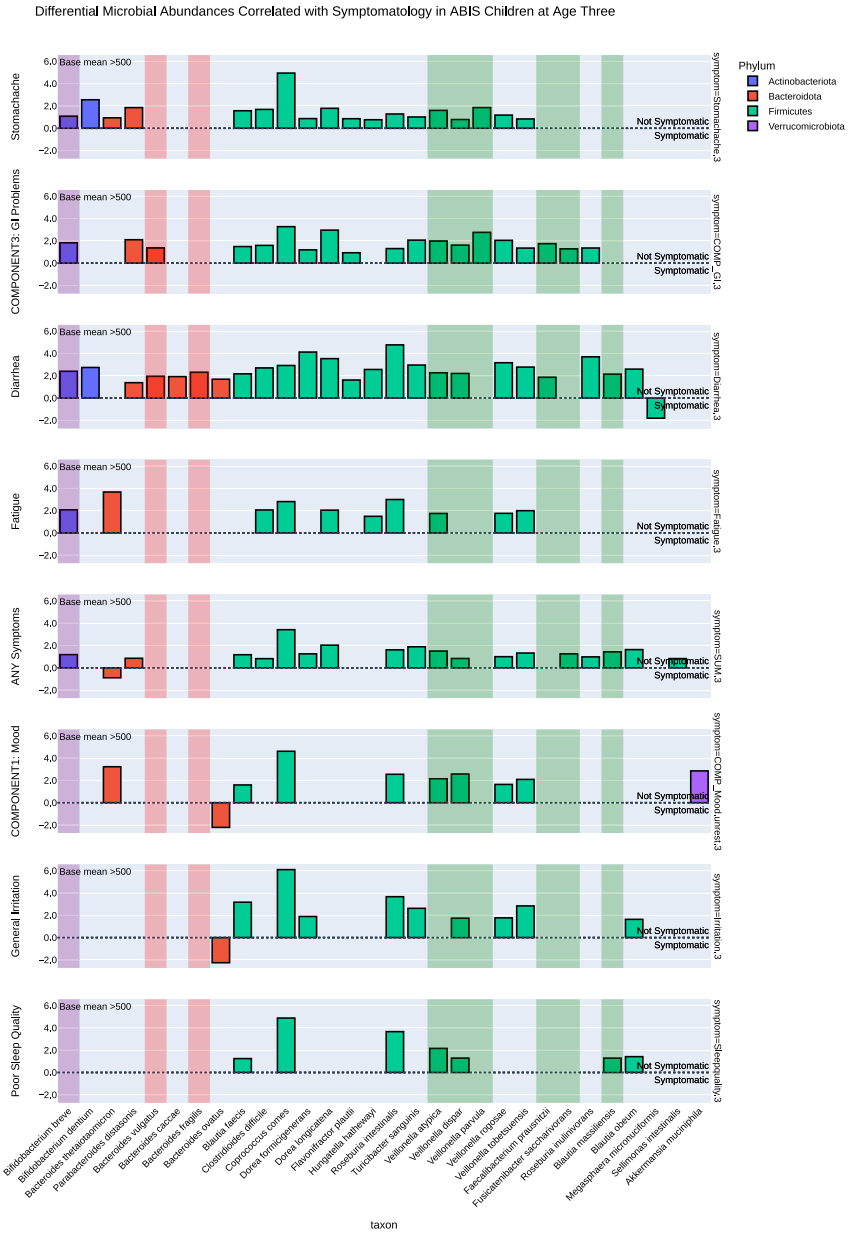

Figure S2. Symptoms at 2.5 years and association with microbial abundance at 1 year, related to Figure 2

Top microbial species associated with prevalence of symptoms at 2.5 years. Symptom groups were dichotomized based on the presence of one or more qualifying symptoms and taxa with base means exceeding 15 included.

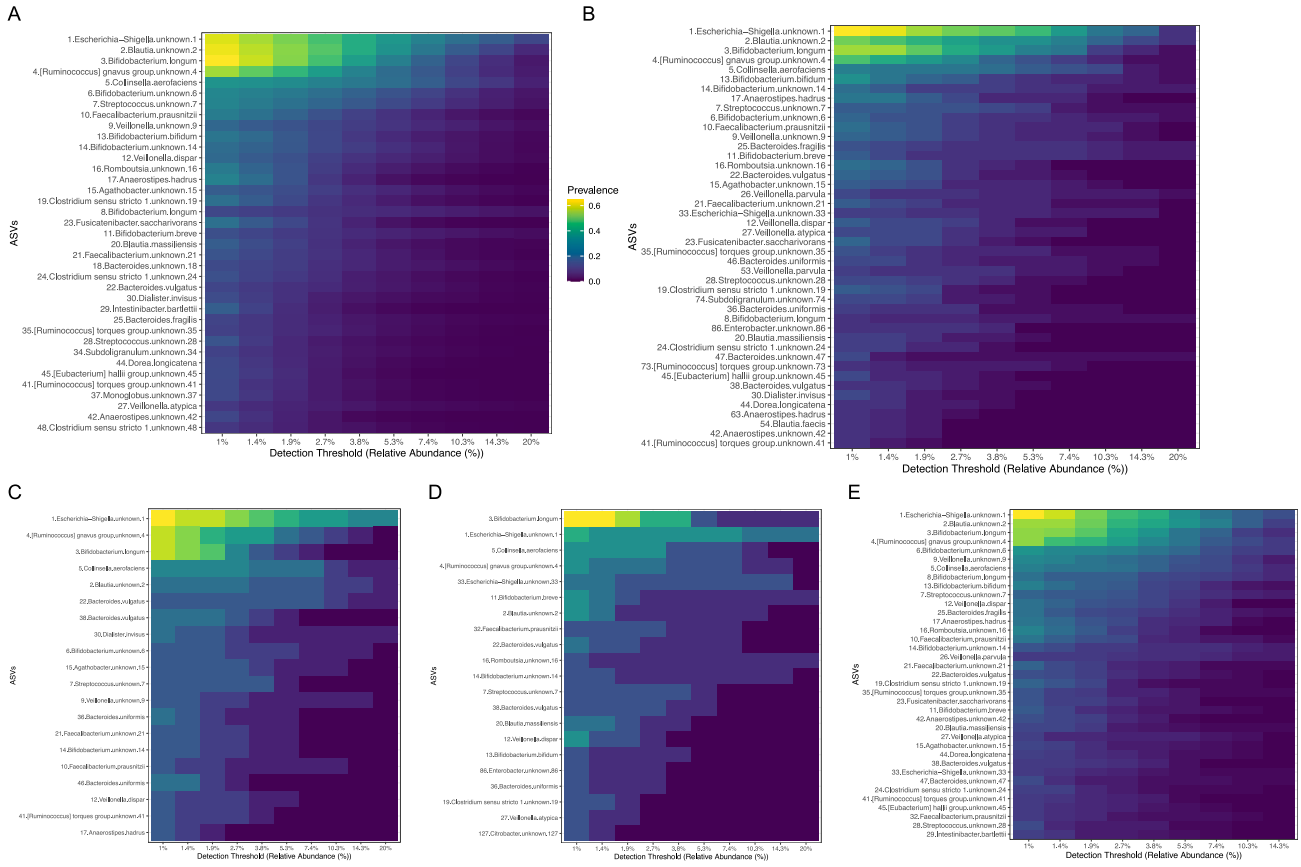

Figure S3. Core microbiota across controls and neurodevelopmental disorders (NDs), related to Figures 4 and 5

(A–E) Core microbiota identified at 0.01% detection and 50% prevalence within each ND subgroup. For speech and intellectual disability subgroups, taxa exceeding 20% prevalence are shown.

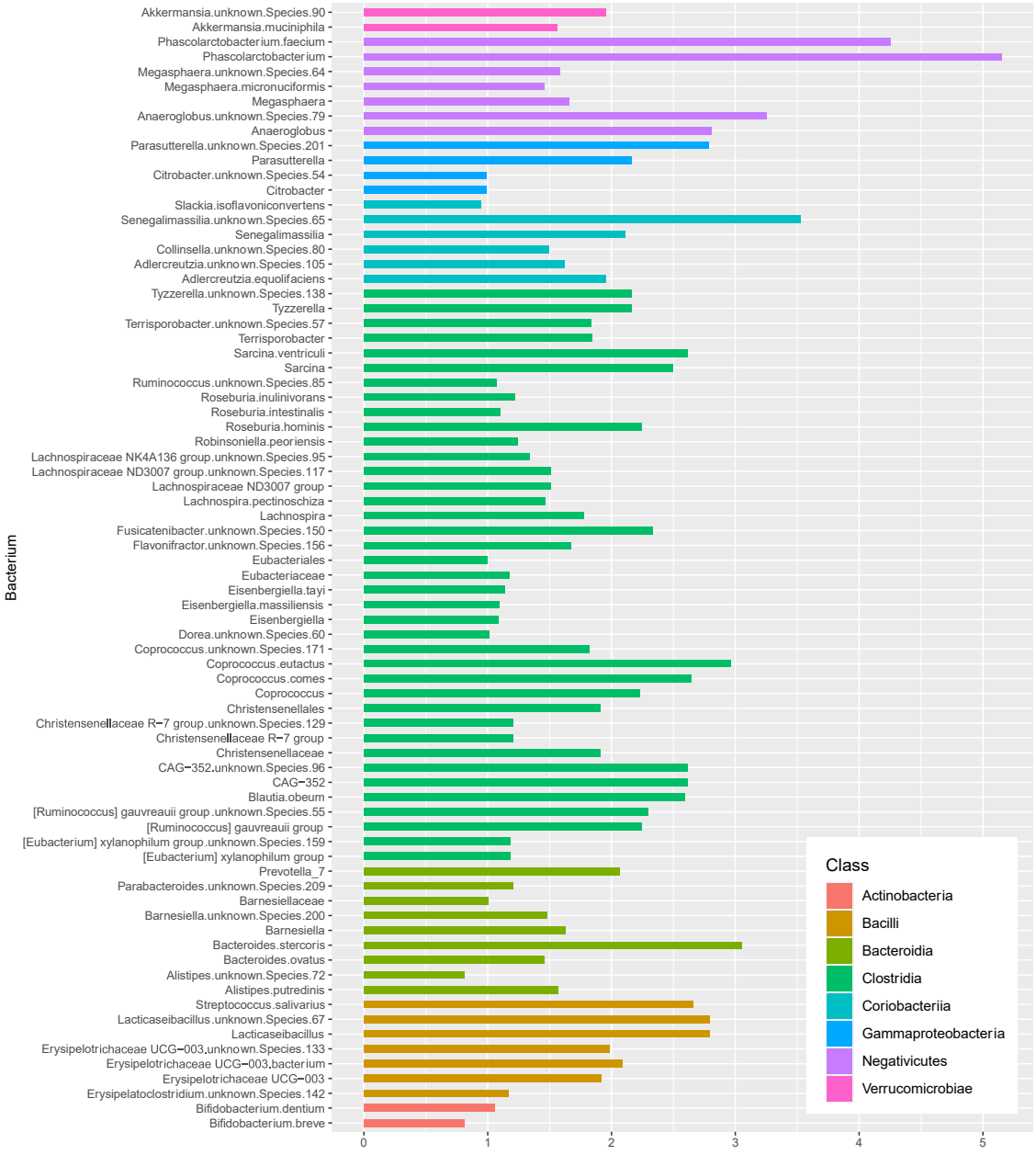

Figure S4. Gut microbiota increased in ABIS controls compared to every ND category, related to Figure 5

Log_2 fold changes of taxa higher in $ABIS_{controls}$ across all three ND subtype comparisons, based on FDR-adjusted q values.

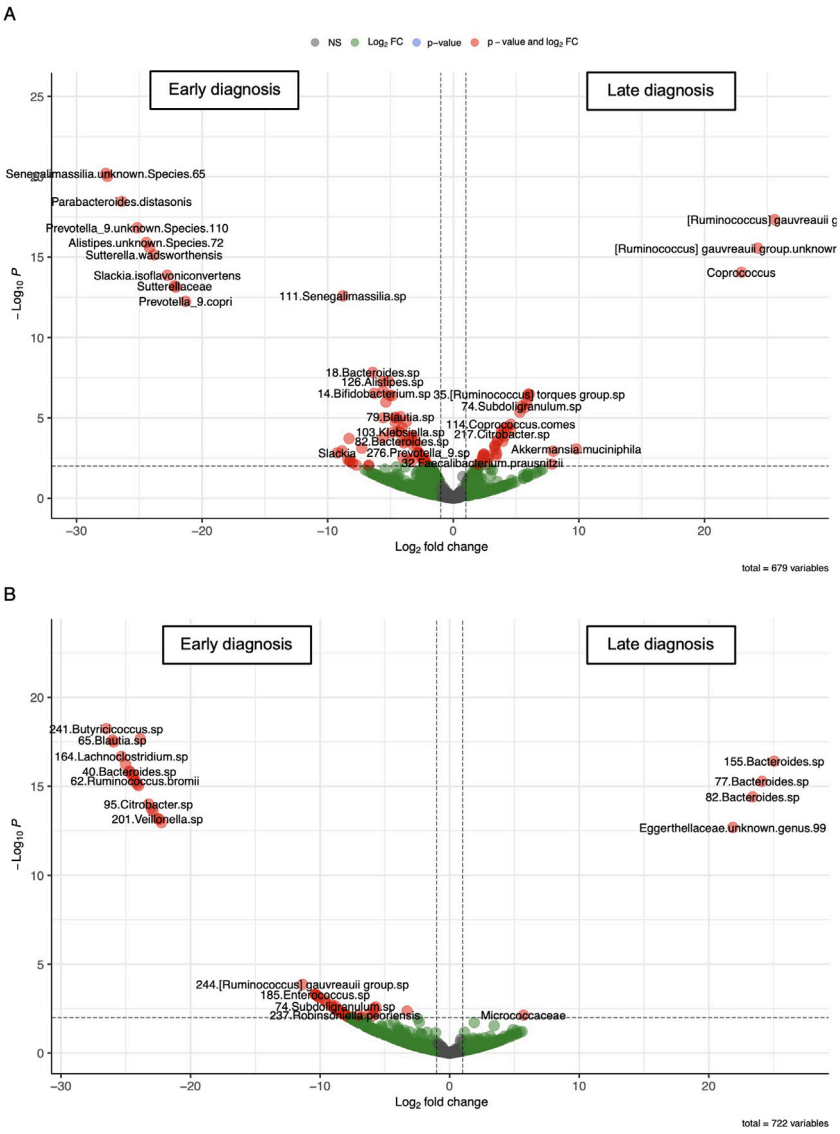

Figure S5. Gut microbiome differences within future autism spectrum disorder (ASD) by age of diagnosis (N = 39), related to Figure 5

Differentially abundant bacteria based on age at diagnosis in males (A) and females (B) after FDR adjustment. Early/late was defined by mean age of diagnosis (13.4 years for males, 17.6 years for females). Early diagnosis: 11 males, 8 females. Late diagnosis: 12 males, 8 females. Positive $\log_2$ fold changes reflect higher abundance in those with a late diagnosis.