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Original article

ADHD and ASD

Early-life antibiotic use and risk of attention-deficit hyperactivity disorder and autism spectrum disorder: results of a discordant twin study

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Abstract

Background: Development of the gut-brain axis in early life may be disturbed by antibiotic use. It has been hypothesized that this disturbance may contribute to development of neurodevelopmental disorders, including autism spectrum disorder and attention-deficit hyperactivity disorder. We aimed to assess the association between antibiotic use

in early life and the risk of developing attention-deficit hyperactivity disorder or autism spectrum disorder, while controlling for shared genetic and environmental factors in a discordant twin design.

Methods: We conducted a cohort study in twins (7–12 years; 25 781 twins) from the Netherlands Twin Register (NTR) and a replication study in the Childhood and Adolescent Twin Study in Sweden (CATSS; 7946 9-year-old twins). Antibiotic use was recorded before age 2 years. Attention-deficit hyperactivity disorder and autism spectrum disorder were parent-reported in the Netherlands Twin Register and register-based in the Childhood and Adolescent Twin Study in Sweden.

Results: Early-life antibiotic use was associated with increased risk of attention-deficit hyperactivity disorder development [pooled odds ratio (OR) 1.10, 95% confidence interval (CI) 1.02-1.17] and autism spectrum disorder (pooled OR 1.15, 95% CI 1.06-1.25) in a case-control design. When restricting to monozygotic twin pairs discordant for the outcome, associations disappeared for both disorders in both cohorts (attention-deficit hyperactivity disorder OR 0.90, 95% CI 0.48-1.69 and OR 0.80, 95% CI 0.37-1.76, and autism spectrum disorder OR 0.66, 95% CI 0.38-1.16 and OR 0.29, 95% CI 0.02-4.50, respectively).

Conclusions: Our findings suggest that the association between early-life antibiotic use and risk of attention-deficit hyperactivity and autism spectrum disorder may be confounded by shared familial environment and genetics.

Key words: ASD, ADHD, antibiotics, early life, children, discordant twin design, gut-brain axis

Key Messages

- Our cohort study in twins suggests that the association between early-life antibiotic use and risk of attention-deficit hyperactivity disorder (ADHD) and autism spectrum disorder (ASD) may be confounded by shared familial environment and genetics.
- Although benefits and risks of antibiotic use should be considered before start of treatment, this study indicates that there is no association between ADHD and ASD diagnoses and early-life antibiotic use when environmental and genetic family factors are taken into account.

Introduction

Attention-deficit hyperactivity disorder (ADHD) and autism spectrum disorder (ASD) are neurodevelopmental disorders with prevalences in school-aged children of around 5%[1] and 1–2%,[2,3] respectively, albeit with large variations. Coexistence of ADHD and ASD is high; approximately one-quarter of school-aged children with ASD fulfil Diagnostic and Statistical Manual of Mental Disorders, 4th Edition (DSM-IV) criteria for ADHD.[4] The pathophysiology behind both disorders is not fully understood and underlying causes are both genetic and environmental.[5–7] Gastrointestinal differences in microbiota are associated with both disorders and may contribute to neurodevelopmental disorder development. Evidence comes from distinct microbiome profiles observed in children with ADHD and ASD compared with healthy controls.[8–12] For example, in children with ADHD, decrease of *Faecalibacterium* was associated with ADHD diagnosis and parental reports of more severe ADHD symptoms.[12] In children with ASD, faecal flora contained higher incidence of the *Clostridium histolyticum* group of bacteria than in healthy children, increasing risk of gut dysfunction exerting systemic effects.[11] There are at least three hypotheses explaining the link between the gut microbiome and brain function and development, the so-called 'microbiome-gut-brain axis'. First, the vagus nerve is assumed to be affected directly or indirectly by microbiome changes in the gastrointestinal tract. Exposure to bacteria may affect differentiation of signals, inducing anxiogenic and anxiolytic effects.[13,14] Second, the gastrointestinal microbiome may influence brain function

via immune system interactions. Bacteria excrete metabolites, such as short-chain fatty acids, in turn altering the immune system and interacting with nerve cells via sympathetic nervous system stimulation.[15,16] Third, the microbiome-gut-brain axis is developed in early life; over years the microbiome composition may affect central nervous system development by altering neurotransmitter levels.[17,18]

The association between gastrointestinal microbiome disturbances and ADHD and ASD might reflect a cause-and-effect relationship. The association could be influenced by intrinsic or external factors such as early-life antibiotic use,[19,20] as antibiotics change the microbiota.[21] Broad spectrum antibiotics are thought to cause more microbiota changes than narrow spectrum antibiotics.[22] However, results regarding early-life antibiotic use and ADHD or ASD development are conflicting, with some studies reporting that antibiotics influence ADHD or ASD development and others not finding any association.[23–29] These studies may have been susceptible to familial confounding, where both the causal factor and the outcome are influenced by either genetic or shared environment.[25,26,29] We aim to investigate the association between early-life antibiotic use and ADHD and ASD, in a co-twin control design to control for confounding by shared genes and shared environment.

Methods

We conducted a cohort study in twins aged 7–12 years in the Netherlands Twin Register (NTR)[30] and tested for replication in 9-year-old twins from the Childhood and Adolescent Twin Study in Sweden (CATSS). All data were pseudonymized. This study was approved by the institutional review boards of the NTR and CATSS.

Discovery cohort

The NTR is a birth cohort[31] initiated in 1987. Twins are registered by their parents after birth, and recruited with help of a commercial organization in The Netherlands called Felicitas and the Dutch Association for Parents of Multiple Births. Data are collected by sending surveys to parents at ages 0, 2, 3, 5, 7, 9/10 and 12 years, and after age 5 also to teachers. The response rate varied from 40% to 75%. Data collected between 1989 and 2016 were analysed.

Replication cohort

The replication cohort CATSS consisted of Swedish twins born between January 2005 and February 2010.[32] Parents were interviewed about their children's health via telephone surveys when twins were 9 years old. Twins were identified via the Swedish Twin Registry (STR), including all Swedish twins. The participation rate was 61%. Data were linked to Swedish national registers (Swedish Prescribed Drug Register, Medical Birth Register and National Patient Register) by personal identity number via the National Board of Health and Welfare.[33]

Outcomes

In the NTR, ADHD was derived from mothers' answers to surveys at ages of 7, 9 and/or 12 years, derived from the short Conners's Parental Rating Scale Revised (CPRS-R: S). The CPRS-R: S consists of 12 items scored on a four-point scale from 0 (not true or never) to 3 (completely true or very often). Cut-offs for probable diagnosis were chosen as presented in Supplementary Table S1, available as Supplementary data at *IJE* online, and based on earlier work.[34–36] An autism scale consisting of 10 items from the Child Behaviour Checklist (CBCL) as described by So *et al.* was used.[35,37] The CBCL is a parent-reported questionnaire for assessing behavioural and emotional problems in children and adolescents, with 118 items scored on a three-point scale; 0: not true, 1: somewhat or sometimes true, and 2: very true or often true.[34,36] We considered t scores of 65 or higher as elevated and indicative of possible clinical diagnosis.[6] Outcomes were based on passing this cut-off in at least one survey.

In CATSS, ADHD and ASD were based on the Autism-Tics, AD/HD and other Comorbidities inventory (A-TAC) from parent-reported questionnaires or from a diagnosis derived from the National Patient Register defined by International Classification of Diseases codes for ADHD (F90) or ASD (F84, but not F84.3 or F84.4) between ages 3 and 9 years. A-TAC consists of 96 items divided by 20 different modules. The modules Concentration & Attention and Impulsiveness & Activity correspond to the ADHD definition. Comparably, Language, Social Interaction and Flexibility represent ASD. The modules contain 19 items for ADHD and 17 for ASD, both about the lifetime presence of symptoms corresponding to ADHD and ASD. Each item is coded as 0= 'no', 0.5= 'yes, to some extent', and 1= 'yes'. Cut-offs were chosen specific for the instrument and population, based on previous work.[38] For ADHD, the low cut-off was 6.0 and high cut-off was 12.5. For ASD, the low cut-off was 4.5 and high cut-off was 8.5. The A-TAC has been validated to correspond with clinical diagnoses and correlates with the CBCL.[39,40] We used the low cut-off to determine ADHD and ASD (Supplementary Table S2, available as Supplementary data at *IJE* online).

Exposure

Exposure to antibiotics (any pharmaceutical formulation, e.g. oral or intravenous) was defined as any parent-reported antibiotic use in Dutch twins aged 0–2 years in the survey sent out around the second birthday of the twins and as any prescription claim for antibiotics (collected as ATC-codes) between ages 0 and 2 years in Swedish twins (Supplementary Table S2).

Covariates

A directed acyclic graph defined covariates to adjust for (Supplementary Figure S1, available as Supplementary data at *IJE* online). Data describing educational attainment of parents, child's gender, birthweight, delivery mode, breastfeeding and asthma were available from NTR surveys at ages 0, 2, 3, 5, 7, 9/10 and 12 years. Perinatal covariates in CATSS were derived from the Medical Birth Register and educational attainment was obtained by questionnaire. Data about breastfeeding were not available in CATSS. All analyses were adjusted for birthweight and asthma.

Statistical analysis

We used three steps to examine whether early-life antibiotic use was associated with ADHD or ASD.[41,42] The first step was an unmatched, cohort study to test whether there was an association between ADHD or ASD and early antibiotics exposure at population level. Children with ADHD or ASD were compared with non-related individuals without ADHD or ASD. Individuals without ADHD or ASD from twin pairs discordant for ADHD or ASD were excluded from this step. All children with data about antibiotic use and at least one questionnaire (A-TAC, CBCL or CPRS-R: S) answered or a registered diagnosis were included.

Step two was a matched co-twin control analysis restricted to all twin pairs [monozygotic (MZ) and same-sex dizygotic (DZ)] discordant for either ADHD or ASD. In a separate analysis, only same-sex DZ twin pairs discordant for either ADHD or ASD were included. These analyses adjust for shared familial environment and partially for shared genes, as DZ twins share on average 50% of their segregating genes.

Step three was a matched co-twin control analysis restricted to MZ twins discordant for ADHD or ASD, controlling for contributions of shared environmental and all genetic factors as MZ twins share the same genotype.

We used logistic regression models with a generalized estimating equation approach for unmatched analysis to account for twin relatedness, adjusted for all measured covariates. In the matched twin analyses, conditional logistic regression models were applied to account for shared genetic and/or environmental factors. Analyses were performed in Statistical Packages of Social Sciences version 25 (IBM, New York, USA).

To examine robustness of findings, we performed sensitivity analyses in the NTR assessing a more strict definition for controls (a t score of lower than 55), and in CATSS assessing: (i) high cut-offs (to increase outcome sensitivity, see Supplementary Table S11, available as Supplementary data at *IJE* online, for summary characteristics of this group); (ii) antibiotic use per spectrum (broad spectrum and narrow spectrum antibiotics)[43]; and (iii) antibiotic use per antibiotic group (anti-aerobic versus anti-anaerobic antibiotics) (Supplementary Table S2).[44]

Results

Summary characteristics of the Dutch and Swedish population are described in Table 1. We included 25 781 individuals in the NTR and 7946 in CATSS. In the NTR, at age 7–12 years the probable ADHD prevalence was 12.7%, and in CATSS at age 9 was 13.5%. The ASD prevalences were 11.4% and 4.4%, respectively. Exposure to at least one antibiotic prescription was 35.2% in the NTR and 44.9% in CATSS, reflecting population specific antibiotic use. The number of children with a gestational age of 37 weeks or lower was higher in CATSS compared with the NTR: 53.9% compared with 40.4%. More children in CATSS were delivered via caesarean section compared with the NTR: 52.1% vs 26.9%, respectively.

Antibiotics and ADHD

Early-life antibiotic use was associated with increased risk of ADHD in both NTR [odds ratio (OR) 1.08, 95% confidence interval (CI) 1.003-1.158, $n = 24\,451$] and CATSS (OR 1.14, 95% CI 1.00-1.30, $n = 7249$) populations (Table 2). The pooled estimated OR (random effects) was: 1.10 (1.02-1.17).

In the co-twin control analyses, effect estimates for developing ADHD were different between the two cohorts. In the NTR, there was no association between ADHD and early-life antibiotic use (OR 0.80, 95% CI 0.59-1.08, $n = 2196$). In CATSS, the OR increased to 1.73, 95% CI 1.02-2.92; $n = 878$ (Table 2). When limiting to MZ twins, thereby controlling for shared genotype and shared familial environment, risk for developing ADHD attenuated for both CATSS (OR 0.80, 95% CI 0.37-1.76, $n = 322$) and NTR (OR 0.90, 95% CI 0.48-1.69; $n = 696$), pooled estimated OR (random effects): 0.82 (0.62-1.08).

Table 1 Summary characteristics of Dutch (NTR) and Swedish (CATSS) twin cohorts and subgroups with ASD and ADHD

	All individuals NTR (n = 22 041)	ADHD (n = 3053)	All individuals CATSS (n = 7946)	ADHD (n = 1070)	All individuals NTR (n = 24 282)	ASD (n = 3008)	All individuals CATSS (n = 7946)	ASD (n = 350)
Gender								
Male	10 549 (47.9%)	1611 (52.8%)	3978 (50.1%)	700 (65.4%)	11 860 (48.8%)	1516 (50.4%)	3978 (50.1%)	243 (69.4%)
Delivery mode								
Caesarean section	6272 (30.5%)	922 (32.0%)	3265 (52.1%)	447 (52.8%)	6528 (26.9%)	852 (28.3%)	3265 (52.1%)	140 (54.7%)
Gestational age								
<37 wk	1727 (56.5%)	13 052 (59.2%)	4283 (53.9%)	607 (56.7%)	9834 (40.4%)	1366 (45.4%)	4283 (53.9%)	214 (61.1%)
Birthweight (g)	2492 (720)	2452 (760)	2670 (720)	2635 (828)	2493 (721)	2410 (790)	2670 (720)	2635 (817)
Breastfeeding			NA	NA			NA	NA
None	9084 (41.4%)	1223 (40.1%)			10 267 (42.4%)	1214 (40.5%)		
<2 wk	2210 (10.1%)	321 (10.5%)			2377 (9.8%)	341 (11.4%)		
2-6 wk	3381 (15.4%)	512 (16.8%)			3696 (15.3%)	467 (15.6%)		
6 wk–3 mths	2895 (13.2%)	427 (14.0%)			3125 (12.9%)	383 (12.8%)		
3-6 mths	2223 (10.1%)	284 (9.3%)			2434 (10.1%)	297 (9.9%)		
> 6mths	2171 (9.9%)	280 (9.1%)			2300 (9.5%)	298 (9.9%)		
Educational attainment, mother								
≤9 years	877 (4.0%)	190 (6.3%)	166 (2.3%)	42 (4.5%)	1109 (4.6%)	159 (5.3%)	166 (2.3%)	17 (5.5%)
10-12 years	5513 (25.4%)	818 (27.2%)	1934 (27.2%)	347 (37.2%)	6468 (27.0%)	826 (27.7%)	1934 (27.2%)	118 (38.2%)
<2 years tertiary	9175 (42.2%)	1303 (43.4%)	962 (13.6%)	164 (17.6%)	9943 (41.5%)	1268 (42.6%)	962 (13.6%)	51 (16.5%)
≥2 years tertiary	6167 (28.4%)	691 (23.0%)	4036 (56.9%)	381 (40.8%)	6436 (26.9%)	727 (24.4%)	4036 (56.9%)	123 (39.8%)
Educational attainment, father	1226 (5.8%)							
≤9 years		230 (1.1%)	324 (5.0%)	66 (8.4%)	1458 (6.2%)	218 (7.5%)	324 (5.0%)	17 (5.5%)
10-12 years	5643 (26.3%)	885 (29.9%)	2806 (43.2%)	388 (49.4%)	6417 (27.1%)	838 (28.7%)	2806 (43.2%)	118 (38.1%)
<2 years tertiary	7550 (35.2%)	1060 (4.9%)	682 (10.5%)	98 (12.5%)	8267 (34.9%)	1019 (34.9%)	682 (10.5%)	51 (16.5%)
≥2 years tertiary	7048 (32.8%)	778 (3.6%)	2682 (41.2%)	233 (29.7%)	7524 (31.8%)	842 (28.9%)	2682 (41.2%)	123 (39.8%)

Categorical characteristics are denoted as frequencies (proportions). Continuous characteristics are denoted as median (inter quartile range).
ASD, autism spectrum disorder, ADHD, attention-deficit hyperactivity disorder, NA, data not available; wk, weeks; mths, months.

Table 2 Early-life antibiotics use and subsequent risk of ADHD in Dutch (NTR) and Swedish (CATSS) twin cohorts

		n AB/ADHD (%)	*n* AB/without ADHD(%)	OR adjusted (95% CI)
NTR	Unmatched	1100/2972 (37.0%)	7520/21 479 (35.0%)	1.08 (1.00-1.16)[a]*
	MZ and same sex DZ	406/1098 (37.0%)	422/1098 (38.4%)	0.80 (0.59-1.08)[b]
	Same sex DZ	287/750 (38.3%)	300/750 (40.0%)	0.74 (0.52-1.05)[b]
	MZ	119/348 (34.2%)	122/348 (35.1%)	0.90 (0.48-1.69)[b]
CATSS	Unmatched	541/1070 (50.6%)	2718/6179 (44.0%)	1.14 (1.00-1.30)[c]*
	MZ and same sex DZ	207/439 (47.2%)	194/439 (44.2%)	1.73 (1.02-2.92)[b]*
	Same sex DZ	133/278 (47.8%)	117/278 (42.1%)	1.71 (0.98-2.98)[b]
	MZ	74/161 (46.0%)	77/161 (47.8%)	0.80 (0.37-1.76)[b]
Pooled result unmatched analyses				1.10 (1.02-1.17)[a]*
Pooled result MZ				0.82 (0.62-1.08)

ADHD, attention-deficit hyperactivity disorder; AB, users of any antibiotics; MZ, monozygotic twin pair level; DZ, dizygotic twin pair level; OR, odds ratio; CI, confidence interval.

[a] Adjusted for gender, delivery mode, educational attainment, birthweight, breastfeeding, asthma.

[b]Adjusted for birthweight, asthma.

[c]Adjusted for gender, delivery mode, educational attainment, birthweight, asthma.

*p <0.05; **p <0.01; ***p <0.001.

Table 3 Early-life antibiotics use and subsequent risk of ASD in Dutch (NTR) and Swedish (CATSS) twin cohorts

		n AB/ASD (%)	*n* AB/without ASD (%)	OR adjusted (95% CI)
NTR	Unmatched	1131/2976 (38.0%)	7089/20 876 (34.2%)	1.14 (1.05-1.23)[a]**
	MZ and same sex DZ	490/1250 (39,2%)	503/1250 (40.2%)	0.88 (0.66-1.17)[b]
	Same sex DZ	314/780 (40.3%)	312/780 (40.0%)	0.98 (0.70-1.36)[b]
	MZ	178/470 (37.9%)	189/470 (40.2%)	0.66 (0.38-1.16)[b]
CATSS	Unmatched	170/350 (48.8%)	3334/7466 (44.7%)	1.29 (0.96-1.74)[c]
	MZ and same sex DZ	44/81 (54.3%)	43/81 (53.1%)	1.43 (0.52-3.89)[b]
	Same sex DZ	30/49 (61.2%)	26/49 (53.1%)	2.23 (0.65-7.57)[b]
	MZ	14/32 (43.8%)	15/32 (46.9%)	0.29 (0.02-4.50)[b]
Pooled result unmatched analyses				1.15 (1.06-1.25)[a]**
Pooled result MZ				0.64 (0.37-1.10)

ASD, autism spectrum disorder; AB, users of any antibiotics; MZ, monozygotic twin pair level; DZ, dizygotic twin pair level; OR, odds ratio; CI, confidence interval.

[a]Adjusted for gender, delivery mode, educational attainment, birthweight, breastfeeding, asthma.

[b]Adjusted for birthweight, asthma.

[c]Adjusted for gender, delivery mode, educational attainment, birthweight, asthma.

*p <0.05; **p <0.01; ***p <0.001.

Antibiotics and ASD

Table 3 shows adjusted ORs and 95% CIs for ASD in relation to early-life antibiotic use. There was an association for ASD and early-life antibiotic use in the NTR (OR 1.14, 95% CI 1.05-1.23, $n = 23\,852$). In CATSS the same direction was seen (OR 1.29, 95% CI 0.96-1.74, $n = 7816$). The pooled estimated OR (random effects) was: 1.15 (1.06-1.25). Similar null results as reported for ADHD were shown in NTR and CATSS after restriction to MZ and same-sex DZ twins (OR 0.88, 95% CI 0.66-1.17, $n = 1560$ and OR 1.43, 95% CI 0.52-3.89, $n = 162$, respectively) and after restriction to MZ twins (OR 0.66, 95% CI 0.38-1.16, $n = 940$ and OR 0.29, 95% CI 0.02-4.50, $n = 64$, respectively), pooled estimated OR (random effects): 0.64 (0.37-1.10).

Sensitivity analyses

Results of sensitivity analyses in which we only included twins with a high cut-off value for ADHD or ASD in CATSS were consistent with the results described above, although controlling for shared environment (same-sex DZ

and MZ twins) attenuated ADHD risk (Supplementary Tables S3 and S4, available as Supplementary data at *IJE* online). Restriction to MZ or same-sex DZ twins was not possible for high-cut off ASD. The results of sensitivity analyses in the NTR with a more strict definition for controls were consistent with the results described above (Supplementary Tables S5 and S6, available as Supplementary data at *IJE* online).

The results of the narrow spectrum antibiotics analysis in CATSS showed no association with either ADHD or ASD; however, numbers of twins for these analyses were low (Supplementary Table S7, available as Supplementary data at *IJE* online). Broad spectrum antibiotics showed an association with ADHD in unmatched analyses which attenuated in matched analyses (Supplementary Table S8, available as Supplementary data at *IJE* online). No association was seen for ASD with broad spectrum antibiotics. Controlling for environment and genes (MZ) was null.

Anti-anaerobic antibiotics are not associated with ADHD or ASD. However, there was an association between anti-aerobic antibiotics and ADHD in unmatched analyses which remained, although the 95% CI crossed the null in matched analyses (same-sex DZ and MZ). Controlling for environment and genes (MZ) was null (Supplementary Tables S9 and S10, available as Supplementary data at *IJE* online).

Discussion

In this large co-twin study performed in two countries, early-life antibiotic use was associated with increased risk of ADHD and ASD, but the results suggest that the association disappeared when controlled for shared familial environment and genetics, indicating that this association may be susceptible to confounding.

The increased risk of a high ADHD score in our unmatched analysis after antibiotic use corresponds with increased risk of high Connors ADHD index scores reported after antibiotic use in the first year of life in 871 European children; however, controlling for confounding by shared environment and genetics was not undertaken in this study.[45] Our results indicated an attenuated risk of ADHD after correction for shared familial environment, but please note that the confidence intervals in CATSS were wide. This finding is in line with Danish and Canadian population-based, prospective cohort studies including 671 592 and 67 671 children, respectively, that both were based on a sibling design to correct for unobserved familial factors. Both these studies did not observe an association for increased risk of ADHD after antibiotic use (broader spectrum than penicillin) within the first 2 years in an adjusted within-family survival model [hazard ratio (HR) 0.99 (95% CI 0.92-1.06)[25] and after antibiotic use in the first year (HR 0.96, 95% CI: 0.89-1.03].[29]

Our results indicated increased risk of ASD after early-life antibiotic use in the unmatched case-control analysis. After restriction to discordant MZ twin pairs, the association attenuated in both cohorts, suggesting confounding by shared environment and genetics in the unmatched analysis. However, also for ASD, it should be noted that the confidence intervals of the results in the CATSS cohort were wide. An earlier published Canadian population-based cohort study including 214 834 children reported no association between antibiotic use in the first year of life and risk of ASD, after controlling for unobserved familial factors.[24] Also, a Danish population-based prospective cohort study including 671 606 children found that early-life antibiotic use increased risk of ASD, but no causal relation after controlling for unobserved familial factors.[26] The sibling design could not correct for all genetic factors, as siblings share on average 50% of their segregating genes; we performed discordant twin analyses in MZ pairs, thereby controlling for shared environment and also completely for genes.

We note that twins are more likely to be delivered via caesarean section [30.5% (NTR, all individuals ADHD analysis), 26.9% (NTR, all individuals ASD analysis) and 52.1% (CATSS)] compared with singletons (17.5% in the Danish studies).[25,26,46] Despite the possible consequences for the microbiome of the twins due to the high proportion of caesarean sections in the twin population, our findings seem to be similar to the studies performed in singletons.[25,26]Within-family studies comparing twins with singletons did not show a higher prevalence of ADHD or ASD in twins compared with singletons, despite the possible consequences of the mode of delivery.[47,48]

In our study, increased risk of ADHD after use of broad spectrum and anti-aerobic antibiotics attenuated after restriction to DZ same-sex and MZ twin pairs, showing that the association with ADHD and broad spectrum/anti-aerobic antibiotics is likely to be confounded by shared familial environment and genetics, but please note that confidence intervals were very wide in these analyses. Our finding that broad spectrum and anti-aerobic antibiotics do not seem to increase risk of ADHD corresponds with findings from the previously discussed Danish population-based study, even though the definition for broad spectrum was slightly different and only penicillin use, indicated as an anti-aerobic antibiotic, was considered. Results indicated an attenuated risk for ADHD after correction for unobserved familial factors.[25] To our knowledge, our study is the first investigating small spectrum and anti-(an)-aerobic antibiotics in relation to ADHD development.

After performing the analysis per antibiotic spectrum or anti-aerobicity, we did not observe an association between early-life antibiotic use and ASD development. Due to reduced statistical power it is not possible to state with certainty that there is no association. However, results of broad spectrum antibiotic use are in correspondence with the Danish study, showing that penicillin and antibiotics broader than penicillin were not associated with increased risk of autism after controlling for unobserved familial factors in a between-within Cox model.[26] Use of narrow spectrum and anti-(an)aerobic antibiotics in association with ASD development has not been investigated by others.

The strength of our study is the large discovery cohort including 25 781 twins and replication in 7946 twins, with prevalence rates of ADHD and ASD similar to those reported elsewhere.[2,49,50] Note that the overall percentage of antibiotic use in our populations is consistent with national data describing antibiotic exposure in young children in The Netherlands (31% in 2019)[51] and Sweden (26–61% depending on the region in 2010).[52] These rates are considered to be among the lowest in the world. Furthermore, we investigated risk of ADHD and ASD per spectrum and anti-aerobicity of antibiotics extensively. Low-cut off results were validated with a high cut-off scored population in CATSS for both ADHD and ASD, and NTR results were validated with a more strict definition of ADHD and ASD, showing robustness of our results. The twin design controls for shared genetic predisposition and familial environment, such as exposure to certain bacteria, rural area, parental upbringing, health-seeking behaviours.

Our study also has limitations. When we compare the replication with the discovery cohort, the unmatched analyses show generalizability in these two Western European cohorts. However, the confidence intervals of the CATSS same-sex DZ, and MZ and same-sex DZ analyses for ADHD and ASD, are wide, making this conclusion tentative. Parent-reported outcomes might be prone to recall bias. However, antibiotic use between 0–2 years was asked about from parents in the NTR at the child's age of 2 years, and not older, minimizing recall bias. Moreover, CATSS contained medication prescription claims including specific ATC-codes, and overall, results were similar. Our sensitivity analyses and MZ twin analyses in CATSS were susceptible to power issues. Only in CATSS were ATC-codes available and MZ discordant twin numbers are low. Therefore, drawing conclusions from our sensitivity analyses and CATSS MZ twin analyses is difficult. However, the disease-discordant twin design implies greater statistical power over the traditional case-control design.[53] The disease-discordant twin design allows large sample size reduction compared with a case-control design. Second, measurement bias is possible due to use of different parent-reported scores for ASD an ADHD, also shown in a difference in prevalence between CATSS and NTR. However, integration of results from different approaches or different instruments strengthens conclusions.[54] The ages in CATSS for ADHD and ASD were relatively low for diagnosis, and definitions used in the NTR and CATSS differed, possibly explaining differences in prevalences. Third, we cannot exclude the possibility that ADHD and/or ASD-like symptoms might have developed before antibiotic use, i.e. reverse causation, although these neurodevelopmental symptoms are most often diagnosed after the age of 2 years.[18] Fourth, indication for antibiotics could cause potential bias, although we adjusted the within-twin analyses for birthweight and asthma, which may be reasons for greater risk of antibiotic use.

To conclude, our findings suggest that the association between early-life antibiotic use and risk of ADHD and ASD may be confounded by shared familial environment and genetics, despite possible influences of antibiotics on the microbiome-gut-brain axis.[8–12] Although benefits and risks of antibiotic use should be considered before start of treatment, our results indicate that there is no association between ADHD and ASD diagnoses and early antibiotic use, when environmental and genetic family factors are taken into account.

Supplementary Data

Supplementary data are available at *IJE* online.

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Author Contributions

D.I.B. and C.E.vB. were responsible for the NTR data collection and data management and supervising the analyses, and were involved in the design of the current study, interpretation of the data and the write-up of this manuscript. C.V.D. was responsible for statistical methodology and interpretation of the data, and revised the manuscript and supervised the analyses. M.B. was responsible for the NTR data collection and data management, interpretation of the data and write-up of this manuscript. E.M.S. helped the NTR with coding and digitization of the antibiotic data, carried out the analyses in the NTR and CATSS, and was involved in the study design of the current study, the interpretation of the data and the write-up of this manuscript. T.D. helped the NTR with the digitization process of the antibiotic data, NTR analyses, write-up of the manuscript and data management. C.A., B.K.B. and T.G. were responsible for the CATSS data collection and management, supervised the CATSS analyses,and were involved in the design of the current study, the interpretation of the data and the write-up of this manuscript. H.L., S.L. and P.L. were responsible for the CATSS data collection, were involved in the design of the current study, interpretation of the data, revision of the manuscript and CATSS data management. A,D.K., S.J.H.V., G.H.K. and A.H.vdZ. were involved in the design of the current study, the interpretation of the data and the write-up of this manuscript. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

Conflict of Interest

A.H.M.vdZ. received unrestricted research funding from GSK and Boehringer Ingelheim. She participated in advisory boards of Astra Zeneca and Boehringer Ingelheim. G.H.K. reports grants from Lung Foundation of the Netherlands, TEVA the Netherlands, UBBO EMMIUS Foundation, TETRI Foundation, GSK and VERTEX, outside the submitted work, and partipated in a global advisory board on paediatric asthma for GSK. H.L. has served as a speaker for Evolan Pharma and Shire and has received research grants from Shire; all outside the submitted work. S.J.H.V., B.K.B., D.I.B., C.A., H.L., T.G., S.L., T.D., P.L., C.V.D., M.B. and E.M.A.S. report no conflicts of interest relevant to this article.

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