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# Maternal folate status as a risk factor for autism spectrum disorders: a review of existing evidence

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## Abstract

Emerging evidence from epidemiological studies supports the notion that maternal folate status regulated by dietary and genetic factors early in pregnancy may influence the risk of autism spectrum disorders (ASD). In this review, we provide an overview of what is known about the role of folate in the aetiology of neurodevelopmental disorders; summarise relevant biological, genetic and epigenetic mechanisms; and synthesise the evidence from human observational studies and randomised controlled trials that have examined the relationship between maternal folate and ASD or related traits. Much of the existing literature on this topic is subject to limitations such as potential confounding by healthy behaviours and other dietary factors, and exposure assessed within limited exposure windows. As the existing evidence is inconclusive, further research remains to be conducted in order to verify this hypothesis. Complete assessment of maternal functional folate status through the pre- and peri-conceptual periods requires biological measurement of folate, vitamin B<sub>12</sub> and homocysteine and genetic variants involved in one-carbon metabolism and epigenetic mechanisms. In addition to more complete assessment of maternal functional folate status, careful consideration of potential confounding is warranted.

**Key words:** Autism: Epigenetics: Folic acid: Neurodevelopment: Pregnancy

Autism spectrum disorders (ASD) are neurodevelopmental disorders characterised by impaired social function, abnormal communication and repetitive or unusual behaviour<sup>(1)</sup>. Although the measured prevalence of ASD was no greater than five per 10 000 individuals in the 1980s, estimated rates in US surveillance populations have increased to one in sixty-eight children in 2010<sup>(2)</sup>. Recent genetic and epidemiological studies suggest that the heritability of ASD is approximately 50%<sup>(3,4)</sup>, indicating that non-heritable risk factors contribute to a substantial proportion of ASD risk.

Recent epidemiological studies<sup>(5,6)</sup> have suggested a possible link between maternal folate status during pregnancy and risk of ASD in children. As folate cannot be synthesised by humans and is entirely derived from dietary sources, folate status can be modified through increased intake of folate-rich foods such as leafy green vegetables, folic acid (FA) supplements or fortification of the food supply. This raises the intriguing prospect that, much like with neural tube defects<sup>(7,8)</sup>, the incidence of ASD may be decreased through interventions that enrich maternal folate status. This review aims to summarise the biological, genetic and epidemiological evidence linking folate status and the risk of ASD. In addition, we discuss the research challenges that remain to be addressed before any firm

conclusion can be drawn about the possible link between maternal folate status and risk of ASD.

## Neurodevelopment is influenced by deoxyribonucleic acid methylation

Documentation of problems in ASD core areas such as communication, socialisation and attention in children <12 months of age<sup>(9,10)</sup> supports the notion that disruption of neurodevelopmental processes during the pre- and perinatal period may contribute to ASD. In particular, epigenetic modifications during peri-conception are increasingly recognised as having lasting developmental implications with regard to ASD<sup>(11)</sup>. One especially relevant epigenetic process crucial to neurodevelopment is DNA methylation, which influences gene expression through the methylation of cytosine residues in CpG dinucleotides. DNA methylation depends on the availability of dietary methyl donors such as folate, choline and methionine, which are interrelated through one-carbon metabolism. Changes to the availability of folate or other methyl donors can affect DNA methylation capacity, with potential downstream neurodevelopmental consequences.

**Abbreviations:** ASD, autism spectrum disorders; FA, folic acid; MTHFR, methylene tetrahydrofolate reductase.

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During the peri-conceptual period, methylation patterns of the genome (with the exception of imprinted genes) are first established, beginning with de-methylation of maternal and paternal DNA, followed by re-methylation of the combined genome. De-methylation occurs in the pre-implantation embryo, and it is generally completed by the 16- or 32-cell morula stage, about 4 d after fertilisation. Methylation levels then increase markedly through the blastocyst stage, about 5 d post fertilisation<sup>(12,13)</sup>. Thus, even before a pregnancy can be confirmed, a high concentration of dietary methyl donors is required to establish epigenetic patterns in the cells of the developing embryo<sup>(14)</sup>.

Another critical window occurs during early pregnancy, in which the brain rapidly begins to develop. In the 1st month of gestation, the central nervous system begins to form with neurogenesis and cell migration occurring in the forebrain, midbrain and hindbrain<sup>(15)</sup>. Neurogenesis in most cortical and subcortical structures occurs between 5 and 25 weeks of gestation<sup>(15)</sup>. The development of functional neuron networks<sup>(16)</sup> requires high concentrations of methyl donors for cellular differentiation<sup>(17)</sup>, as DNA methylation is essential for individual cell viability<sup>(18,19)</sup>. Early modifications in DNA methylation that cause developing brain cells to deviate from proper differentiation can result in irreversible reductions or expansions of neuron pools<sup>(16)</sup>. Consequently, epigenetic dysregulation during the period when brain organisation develops could result in alterations in brain connectivity<sup>(20–22)</sup>.

### Dietary and genetic determinants of folate status affect deoxyribonucleic acid methylation capacity in the brain

Embryonic DNA can become hypomethylated if maternal plasma does not contain sufficient levels of methyl donors such as folate and cofactors such as vitamin B<sub>12</sub> at critical periods in development<sup>(23)</sup>. Dietary deficiency of methyl donors has been associated with DNA hypomethylation in the brains<sup>(24)</sup> of rats and in genes controlling brain development in rat fetuses<sup>(20)</sup>. For example, a maternal diet low in choline, another dietary methyl donor, resulted in global DNA hypomethylation and increased expression of genes that turn off cell cycling and promote early differentiation in the hippocampus of fetal mice<sup>(20)</sup>.

Although environmental exposures (e.g. decreased dietary folate intake) can result in embryonic hypomethylation, hypomethylation also can occur via genetic regulation of processes relevant to both folate metabolism and DNA methylation. For example, functional hypomethylation can result from polymorphisms in the offspring gene coding for protein readers of DNA methylation patterns, namely methyl-CpG-binding protein 2 (MeCP2), a methyl-binding domain protein. This can have important structural and functional consequences, as neurons without functional MeCP2 in the cortex have been found to have significantly smaller dendritic arbours in both humans<sup>(21)</sup> and mice<sup>(22)</sup>.

Another example of genetic regulation is with 5-methyltetrahydrofolate (5-MTHF), the functional methyl donor form of folate, which is synthesised from a folate derivative by the enzyme methylene tetrahydrofolate reductase (MTHFR). Human

carriers of the *MTHFR* C677T and A1298C polymorphisms have lower enzyme activity, reducing the production of 5-MTHF and the ability of folate to function as a methyl donor in the production of methionine and subsequent DNA methylation<sup>(25)</sup>. Interestingly, a meta-analysis of eight studies on the relationship between child *MTHFR* polymorphisms and ASD<sup>(26)</sup> reported that the C677T polymorphism was only associated with ASD in children living in countries without food fortification. The dependence of a genetic association on a background of the dietary environment is suggestive evidence that gene–environment interactions are instrumental in pathways linking folate status with child ASD.

Collectively, the existing human and animal research on one-carbon metabolism suggests that maternal folate status could be a biologically plausible risk factor for ASD through its impact on DNA methylation.

### Other pathways by which folate influences neurodevelopment

In addition to the effects on DNA methylation, folate status can influence neurodevelopment through multiple other pathways. Mutations in the folate receptor 1 (*FOLR1*) gene coding for folate receptor  $\alpha$  (FR $\alpha$ ) have been associated with severe hypomyelination in the brains of affected patients<sup>(27)</sup>. Because cellular uptake of metabolised folate (MTHF) is mediated by FR $\alpha$  (along with other proteins), functional mutations in *FOLR1* also result in systemic folate deficiency<sup>(27)</sup>. Moreover, the folate cycle is peripherally involved in the creation of essential phospholipids (e.g. phosphatidylcholine) and neurotransmitters (e.g. serotonin)<sup>(28)</sup>. Therefore, reduced availability of dietary methyl donors during key exposure windows could interfere with the production of these critical neurodevelopmental elements. Children with ASD have been found to exhibit dysregulated serotonergic function<sup>(29)</sup>, as well as lower levels of phosphatidylcholine in plasma, in comparison with typically developing children<sup>(30)</sup>. Finally, as folate is also involved in DNA synthesis, folate deficiency may result in deficient DNA repair, inducing DNA damage and neuronal death<sup>(31)</sup>. This is consistent with work demonstrating that dietary maternal FA deficiency in mice affects the development of the neocortex and other regions of the brain by reducing the number of progenitor cells through its influence on cell mitosis and apoptosis<sup>(32)</sup>.

Although a comprehensive review examining folate and altered neurodevelopment is beyond the scope of this paper, other authors have reviewed such literature in detail<sup>(33,34)</sup>.

### Human studies linking maternal folate and autism spectrum disorders and related traits

#### Methods

To identify studies examining maternal folate status and autism or related traits specified as such, PubMed was first searched for the terms (folic acid or folate) (autis\*) on 21 January 2014, with no date range specified. This search was repeated periodically through 15 April 2015, to ensure inclusion of new relevant studies. As of 15 April 2015, this search produced 136 citations.

Criteria for inclusion in the review were original research articles examining FA or folate exposure during pregnancy, with autism or related traits as outcomes in the offspring. Studies (or aspects of manuscripts<sup>(35)</sup>) examining multivitamins without specific mention of FA were excluded. Additional exclusion criteria were biochemical studies and/or those focused on understanding biological mechanisms, animal studies and ecological studies, the lattermost because of the lack of rigour and inability to draw causal inference. Of the search results, four citations remained after application of inclusion and exclusion criteria (Fig. 1).

Because of the existence of relevant literature on ASD traits not designated as such, literature for review was also selected from references of included (five additional studies) and excluded search results (one additional study). As many references examined numerous outcomes, autism outcomes experts were consulted to identify the most ASD-relevant outcomes (executive function, communication and social competence) within the ten articles selected for review. Included studies were evaluated based on quality of measured exposure and outcome, control for relevant confounders, biases and other analytical issues.

The human evidence linking maternal folate status with ASD and related traits comes from various studies distinguished by study design (observational *v.* randomised), how folate status is measured (e.g. self-reported folate or supplement intake *v.* biomarker measurement) and study outcome (clinical diagnoses of ASD *v.* ASD-related traits). We discuss this evidence in the following sections, organised by these characteristics. All studies are summarised in Table 1.

### Observational studies of maternal folate and autism spectrum disorders

To date, there are only two published epidemiological studies examining maternal folate status during pregnancy and diag-

noses of ASD. Despite differences in size, populations and study designs, an American case-control study by Schmidt *et al.*<sup>(5)</sup> and a Norwegian cohort study by Suren *et al.*<sup>(6)</sup> reported similar protective associations for maternal FA intake in the peri-conceptional period and early pregnancy, and children's risk of ASD. The case-control study featured 429 cases and 278 controls, whereas the cohort study featured 85 176 children, of whom only 270 had been diagnosed with ASD. The OR for the case-control study<sup>(5)</sup> was 0.62 (95% CI 0.42, 0.92) for a mean daily FA intake of  $\geq 600$  *v.*  $< 600$   $\mu\text{g}$  in the 1st month of pregnancy, whereas the cohort study<sup>(6)</sup> estimated an OR of 0.61 (95% CI 0.41, 0.90) for FA supplementation in the month before and first 2 months of pregnancy compared with no supplementation. Furthermore, the case-control study found that the reduced risk for FA intake  $\geq 600$   $\mu\text{g}$  was only evident for mothers and/or children having the *MTHFR* C677T variant<sup>(5)</sup>.

Although these studies support that maternal folate status may influence ASD risk, there are important considerations. For example, the ASD prevalence in the Norwegian study was 0.3%. Given that 1% is the generally accepted figure for worldwide ASD prevalence<sup>(43)</sup>, this disparity is a strong indication of case under-ascertainment that may influence results. The potential for ascertainment bias is supported by evidence that mothers of severely affected children had lower response rates.

Another limitation is that both studies relied on self-reported maternal intake of FA. Self-reports can introduce recall or reporting bias, although the concern is less in the prospectively designed Norwegian study, which assessed peri-conceptional FA use at 18 weeks of gestation, well before the birth of the child<sup>(6)</sup>. To what degree such self-reports reflect actual folate exposure is unclear, as no biomarkers were available in these studies. In addition, social, economic, behavioural and environmental factors can influence self-reported vitamin supplementation<sup>(44,45)</sup>. For instance, in a study of childbearing-age women in the USA,

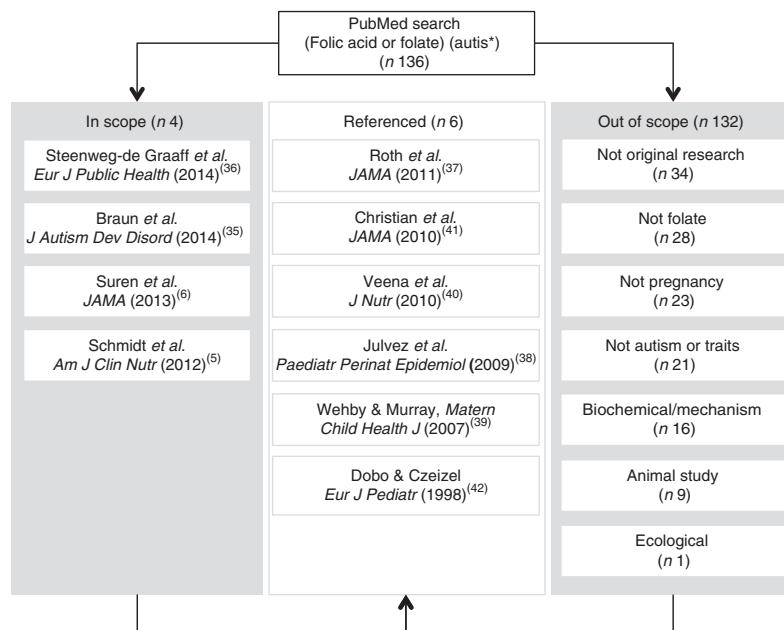


Fig. 1. Derivation of reviewed literature.

**Table 1.** Epidemiologic studies assessing maternal folate status and autism spectrum disorders (ASD) or ASD-related traits

Reference (country)	Study design and number of children	Exposure assessment	Outcome	Results
<b>Self-reported maternal folate status and ASD diagnosis</b>				
Suren <i>et al.</i> <sup>(6)</sup> (Norway)	Cohort study of 85 176 (114 AD; 56 Asperger; 100 PDD-NOS)	FA supplementation 4 weeks before to 8 weeks after conception; questionnaire at 18 weeks of gestation	AD, with or without language delay at 36 months; Asperger syndrome; PDD-NOS	Children of FA users v. non-users had 0.61 (95 % CI 0.41, 0.90) times the odds of AD; protective association only in AD with language delay (OR 0.49; 95 % CI 0.25, 0.99) but not in AD without language delay (OR 0.91; 95 % CI 0.46, 1.81); FA use not associated with Asperger or PDD-NOS
Schmidt <i>et al.</i> <sup>(5)</sup> (USA)	Case-control study of 429 ASD; 278 TD; 130 DD	Dietary and vitamin FA intake 3 months before pregnancy and in the 1st month of pregnancy; questionnaire at 2–5 years after birth	ASD defined by Autism Diagnostic Interview-Revised and the Autism Diagnostic Observation Schedule–Generic	Children of mothers taking $\geq 600$ v. $< 600$ $\mu\text{g}$ FA in the 1st month of pregnancy had reduced odds of ASD (OR 0.62; 95 % CI 0.42, 0.92). In stratified analyses, the association was only evident when the mother and/or child had the <i>MTHFR</i> C677T variant genotype
<b>Self-reported maternal folate status and ASD-related traits</b>				
Steenweg-de Graaff <i>et al.</i> <sup>(36)</sup> (The Netherlands)	Cohort study of 3893 children	FA supplement use and when initiated; questionnaire at $< 18$ weeks of gestation	Autistic traits at 6 years – Social Responsiveness Scale (SRS) short form, Pervasive Developmental Problems subscale of the Child Behavior Checklist	Use of FA associated with lower SRS scores: 'pre-conception start' v. 'no use': $-0.042$ , 95 % CI $-0.068$ , $-0.017$ 'Start $< 10$ week' v. 'no use': $-0.041$ , 95 % CI $-0.066$ , $-0.016$ 'Start $> 10$ week' v. 'no use': $-0.057$ , 95 % CI $-0.089$ , $-0.025$
Roth <i>et al.</i> <sup>(37)</sup> (Norway)	Cohort study of 38 954 children (204 severe language delay)	FA supplementation 4 weeks before to 8 weeks after conception; questionnaire at 17 weeks of gestation	Severe language delay at 3 years – MacArthur Communication Development Inventory UK short form	Maternal FA only (OR 0.55; 95 % CI 0.35, 0.86) or in combination with other supplements (OR 0.55; 95 % CI 0.39, 0.78) associated with reduced risk of severe language delay. Supplements without FA not associated with severe language delay (OR 1.04; 95 % CI 0.62, 1.74)
Julvez <i>et al.</i> <sup>(38)</sup> (Spain)	Cohort study of 420 children	FA supplementation at the end of the first trimester; questionnaire at a median of 12.4 weeks of gestation	Verbal competence and executive function at 4 years (McCarthy Scales of Children's Abilities) Social competence at 4 years (California Preschool Social Competence Scale)	FA use associated with higher verbal (3.98 pt, 95 % CI 0.66, 7.31) and social competence (3.97 pt, 95 % CI 0.81, 7.14) scores. Use of vitamins without FA associated with improvements in verbal (6.52 pt, 95 % CI 0.46, 12.58), but not social competence scores (3.73 pt, 95 % CI $-1.86$ , 9.32). FA use not associated with executive function scores
Wehby & Murray <sup>(39)</sup> (USA)	Cohort study of 6774 children	FA supplementation for at least 3 d/week during the 3 months before becoming aware of pregnancy through first trimester; questionnaire at $\geq 6$ months after birth	Personal-social and language domains at 3 years (Denver developmental screening)	FA use associated with somewhat poorer performance within the personal-social domain, compared with non-use of FA (OR 1.78; 95 % CI 0.94, 3.38). No associations observed between FA use and language development (OR 0.91; 95 % CI 0.55, 1.50)
<b>Biomarker-assessed maternal folate status and ASD-related traits</b>				
Braun <i>et al.</i> <sup>(35)</sup> (USA)	Cohort study of 209 children	Maternal whole blood folate at a mean of 16 weeks of gestation (range: 11–21 weeks)	SRS scores at 4–5 years	Folate concentrations associated with slightly higher SRS scores (0.6/sd, 95 % CI 0.3, 1.5) and SRS scores $\geq 60$ (OR 1.42; 95 % CI 0.81, 2.49)

Table 1 Continued

Reference (country)	Study design and number of children	Exposure assessment	Outcome	Results
Steenweg-de Graaff <i>et al.</i> <sup>(36)</sup> (The Netherlands)	Cohort study of 3893 children	Maternal plasma folate at a median of 13.2 weeks of gestation (90 % range: 10.5–17.2 weeks)	Autistic traits at 6 years – SRS short form, Pervasive Developmental Problems subscale of the Child Behavior Checklist	Folate concentrations not associated with SRS scores (–0.004 pts/1 <i>sd</i> increase; 95 % CI –0.013, 0.004) or odds of probable ASD (OR 1.03; 95 % CI 0.76, 1.39)
Veena <i>et al.</i> <sup>(40)</sup> (India)	Cohort study of 536 children	Maternal plasma folate at a mean of 30 ( <i>sd</i> 2) weeks of gestation	Language production at 9–10 years – A Developmental Neuropsychological Assessment	Folate concentrations not associated with language production scores
Randomised trials of FA supplementation and Christian <i>et al.</i> <sup>(41)</sup> (Nepal)	Cohort follow-up of 676 children	ASD-related traits Assignment of daily supplement use at a mean of 11 weeks ( <i>sd</i> 5.1) of gestation: FA + iron + vitamin A, FA + iron + zinc + vitamin A, FA + iron + zinc + vitamin A + multiple micronutrients, control of vitamin A only	Executive functioning at 7–9 years: Stroop numbers test, backward digit span from the Wechsler memory scale, go/no-go test	Executive function scores improved in the FA + iron group relative to controls for Stroop test (failure proportion –0.14, 95 % CI –0.23, –0.04) and backward digit span (0.36, 95 % CI 0.01, 0.71), but not for go/no-go test
Dobo & Czeizel <sup>(42)</sup> (Hungary)	Cohort follow-up of 625 children	Assignment of multivitamin with FA or placebo-like trace element at least 1 month before conception through 2 months of gestation	Speaking, communication and sociability at 2 years – Developmental Quotient Speech and sociability at 6 years – Goodenough man drawing test	No differences in developmental scores in children of mothers assigned to take a multivitamin with FA compared with trace element controls

AD, autistic disorder; PDD-NOS, pervasive developmental disorder not otherwise specified; FA, folic acid; TD, typically developing; DD, developmental delay.

women of normal weight were more likely to use supplements with FA, compared with women who were overweight or obese<sup>(46)</sup>. However, this difference was not statistically significant. In a study of the Kaiser Permanente Medical Care Program in Northern California, alcohol users were less likely to take multivitamins than non-alcohol users during pregnancy<sup>(47)</sup>. Data from the National Maternal and Infant Health Survey indicate that regular multivitamin users before and after pregnancy were more likely to be older, white, married, have a wanted pregnancy, less likely to consume six or more drinks per week during pregnancy and have greater levels of education and income than mothers not using multivitamins regularly<sup>(48)</sup>. In another study maternal FA use in the peri-conceptional period was associated with increased socio-economic status<sup>(49)</sup>. These factors can be difficult to completely adjust for in observational studies<sup>(44)</sup>.

Exposure assessment in the Norwegian study was of FA as a single nutrient formulation. Although this study had information on multivitamin usage available, it did not explicitly account for this in analyses, indicating that FA content of multivitamins was <400 µg (the amount in single nutrient formulations) in Norway at the time of the study. The exclusion of multivitamin usage from statistical analyses leaves the possibility of confounding by other dietary factors, as FA and multivitamin use may be positively correlated. For example, increased maternal intake of iron<sup>(50)</sup> or *n*-3 fatty acids<sup>(51)</sup> has been associated with a reduced risk of ASD. In a recent study, FA use in the peri-conceptional period was associated with a higher intake of other B vitamins<sup>(49)</sup>. However, the Norwegian study found that fish oil supplementation was not similarly associated with a reduced risk of

ASD, suggesting that maternal healthy behaviours, such as multivitamin use, were not responsible for the observed protective effect observed for FA<sup>(6)</sup>.

The American case-control study considered both supplementary FA and dietary folate from select high concentration sources, but not total dietary folate intake. This study did examine potential confounding by other nutrients and found that adjustment for total amounts of other nutrients from dietary and supplementary sources actually strengthened the reduction in risk between FA and ASD in the 1st month of pregnancy<sup>(5)</sup>.

It is important to emphasise the exposure windows assessed by these two studies. The protective association reported by the prospective Norwegian study was of FA use in the month before conception and in the first 2 months of pregnancy. This study also examined FA use at 22 weeks of gestation and found no association. Meanwhile, the American case-control study assessed exposure in the 1 month of pregnancy only. It is difficult to ascertain how the use of different exposure windows influenced study results because of their other methodological differences. However, despite their limitations, the studies' similar OR were strong and robust to confounder control and sensitivity analyses.

#### Observational studies of self-reported maternal folate and autism spectrum disorder traits

In contrast to the limited evidence concerning maternal folate status and ASD diagnoses, four prospective cohort studies have linked self-reported maternal folate with traits related to autism, such as social competence and verbal abilities, but not to

executive function. However, results are not wholly in agreement.

These cohort studies differ in their selection of FA exposure windows. Three studies assessed whether FA use had commenced before pregnancy (4 weeks before conception<sup>(37)</sup>, 3 months before becoming aware of pregnancy<sup>(39)</sup> or pre-conception<sup>(36)</sup>), whereas the remaining study limited assessment of the exposure window to after pregnancy had been established – at the end of the first trimester<sup>(38)</sup>.

Studies assessing FA use before pregnancy reported a reduced risk of severe language delay at 3 years<sup>(37)</sup>, lower autistic trait scores (less autistic traits) at 6 years<sup>(36)</sup>, but also unfavourable development of the personal-social domain at 3 years of age<sup>(39)</sup>. The research investigating FA use only during pregnancy reported improved verbal and social competence, but not executive functioning scores at 4 years<sup>(38)</sup>. Measures used to assess these outcomes are provided in Table 1.

A concern of many of the studies examining folate and ASD is that self-report of folate may be a poor surrogate for biological folate status. To that end, in the Generation R Study, although FA users had higher plasma folate concentrations at a median of 13 weeks of gestation<sup>(36)</sup>, associations between maternal plasma folate and ASD traits did not persist as they had for self-report of FA supplementation. These discordant results could be related to the timing of biological sampling, given that the most crucial period for dietary methyl donor availability is likely in the peri-conceptual period. Therefore, if folate were indeed protective against ASD, peri-conceptual folate levels would be more relevant to measure. This also may indicate that residual confounding may be partly responsible for the observed protective effect, in that health-related behaviours are more common among women who take supplements.

In particular, one health-related behaviour that could confound observed protective effects of FA on developmental outcomes is dietary intake of nutrients other than FA<sup>(49)</sup>. This is a possibility in the study in which improvements in verbal and social competence scores were noted for children of mothers using multivitamins both with ( $n$  244) and without FA ( $n$  28)<sup>(52)</sup>. A second study reported significant associations between multivitamin and/or mineral use and reduced risk on the personal-social and language scales, but suggested a relationship between maternal FA use and unfavourable development on the personal-social scale<sup>(39)</sup>. This may indicate that a component of multivitamins other than FA may be responsible for the observed protective association. Alternatively, as 83% of individuals in this latter study reported multivitamin and/or mineral use and only 3.2% of the total sample in the study reported FA use, this is perhaps suggestive of confounding by indication among the small sample of FA users. Conversely, the Norwegian study<sup>(37)</sup> that examined severe language delay reported results both for mothers taking FA only and those taking it in combination with other supplements, and reported similar OR (FA only: OR 0.55; 95% CI 0.35, 0.86; FA with other supplements: OR 0.55; 95% CI 0.39, 0.78). A fourth study did not consider the use of other vitamins in their analyses<sup>(36)</sup>.

Results from these studies were inconsistent, but overall tended to support a protective effect. Self-report of FA supplementation in early pregnancy, either alone or in combination

with other vitamins, was associated with reduced risk of ASD-related traits in three of the four studies, whereas a harmful association was noted in one of the studies. However, because of potential confounding by other nutrients, two of the three studies reporting protective associations cannot specifically ascribe this protection to FA. In addition, if health-related behaviours are incompletely controlled in these studies, observed associations may be biased because of residual confounding.

### *Observational studies of maternal folate biomarker and autism spectrum disorder traits*

Three epidemiological studies of biologically ascertained maternal FA status and ASD traits were identified, one of which<sup>(36,52,53)</sup> was discussed in the preceding section. This study reported no association between maternal serum folate concentrations at a median of 13 weeks of gestation and child autistic traits<sup>(36)</sup>. Another study of 209 children reported a weak positive association between maternal whole blood folate concentrations at a mean of 16 weeks of gestation and Social Responsiveness Scale scores at 4–5 years<sup>(55)</sup>. Within the domain of language production, an Indian study of 536 births reported that significant associations with maternal serum folate at  $30 \pm 2$  weeks of gestation did not persist after adjustment<sup>(40)</sup>.

One advantage of these studies is that use of a biomarker for folate status mitigates the measurement error associated with subjective, self-reported measures. However, as previously described, assessment at different time periods may affect findings. As folate concentrations might be expected to be most similar within short time intervals, folate biomarkers measured later in pregnancy might be less correlated with peri-conceptual values than those measured earlier in pregnancy. Studies of FA usage patterns during pregnancy indicate that FA use is typically commenced within the first trimester<sup>(54–56)</sup>. It is thus not likely that maternal folate even at 13 weeks of gestation, the earliest of biomarker measurement of these three studies, is highly correlated with concentrations during the peri-conceptual period. Results of these studies may therefore not be as meaningful as those measuring maternal folate by the time of neural tube closure at 6 weeks of gestation<sup>(6,55)</sup> or by development of basic brain structures at 5–10 weeks of gestation<sup>(57)</sup>.

In summary, the studies utilising biomarker measures of folate status do not consistently support an overall effect. A weak association was reported in the cohort assessing folate at 16 weeks of gestation, but reduced risks of ASD-related traits were not shown in the cohorts assessing maternal folate biomarkers at 13 and 30 weeks of gestation. However, given the timing of maternal folate biomarker measurement, these findings should be interpreted with caution.

### *Folate supplementation and autism spectrum disorder characteristics: randomised trials*

Only two randomised trials have examined relationships between maternal FA supplementation and ASD traits. A Nepali study of 676 mother–child pairs reported higher executive functioning scores among 7- to 9-year-old children of women

assigned to take daily iron/FA at a mean of 11 weeks of gestation, as compared with a control group assigned to take daily vitamin A supplements<sup>(41)</sup>. Similar findings were not reported among children of women assigned to take iron/FA/zinc or iron/FA/zinc and micronutrients. Conversely, a Hungarian study of 625 mother–child pairs reported no meaningful differences in developmental scores assessing speech, communication and sociability at 2 and 6 years between children of mothers assigned to take multivitamins containing FA at least 1 month before conception through 2 months gestation, compared with children of mothers taking trace elements only<sup>(42)</sup>.

Although both studies were randomised, were of similar size and had a high rate of follow-up, the potential effect of FA could not be isolated in either study, as FA was taken with other vitamins. The multivitamin supplement in the Hungarian study<sup>(42)</sup> contained zinc, which may have an inhibitory role, as evidenced by the Nepali<sup>(41)</sup> study and as described in the literature<sup>(58,59)</sup>. As the Hungarian study most likely examined the most critical exposure window, results produced by this study may have been more meaningful had this study examined FA use independent of other vitamins.

### Trends in maternal folate status

Although maternal folate status may be a biologically plausible risk factor for ASD, the collective evidence is not conclusive. Similar to ASD prevalence, maternal folate status has undergone a large change in recent decades, especially for countries that have adopted fortification. In the USA, the recommendation of 400 µg of daily supplemental FA for women of childbearing age was put forth by the Centers for Disease Control and Prevention (CDC) in 1992<sup>(60)</sup> and cereal fortification with folate began in 1996<sup>(61)</sup>. These actions resulted in an elevation in median serum folate from 12.6 µg/l in 1994 to 18.7 µg/l in 1998<sup>(61)</sup>. However, one possible misconception is that if maternal folate status was indeed causal for ASD, then the introduction of FA fortification in the 1990s should have resulted in a decline in ASD prevalence. Given the apparent rise in ASD prevalence in the USA over the past two decades, this would appear to contradict the possible role of maternal folate status.

Such ecologic thinking can be misleading, as it is more likely that changes in diagnostic practices, increased awareness and secular trends in other modifiable risk factors (e.g. advanced parental age) would offset any potentially beneficial effects of higher folate. In a California study<sup>(62)</sup>, approximately 26% of the increase in autism between 1992 and 2005 could be directly attributed to changes in diagnostic criteria. In particular, a separate study noted that higher autism prevalence was significantly associated with corresponding declines in the prevalence of mental retardation and learning disabilities between 1994 and 2003<sup>(63)</sup>. Within the California sample, it was estimated that 16% of the increase in autism prevalence over time was because of social influence and increased awareness<sup>(64)</sup> and that 11% was attributable to the increase in parental age over time<sup>(65,66)</sup>. Thus, much of the increase in autism diagnoses within this California sample could be explained by changes in diagnostic practices, increased awareness and advanced parental age.

It has been suggested that the coincident timing of FA fortification with the beginning of the increase in measured autism prevalence<sup>(67)</sup> is not random, but rather the reflection of altered natural selection. The natural selection theory is that increased maternal folate status arising from these FA policy changes increased survival rates of infants with the *MTHFR* C677T polymorphism, who in the absence of increased FA *in utero* may have been miscarried. For example, there has been an increase in the frequency of the C677T allele and its homozygous genotype in individuals born in the last quarter of the twentieth century, as compared with the first three quarters<sup>(68)</sup>, with a greater frequency of this polymorphism in autistic individuals<sup>(69,70)</sup>. Whether this hypothesis carries weight remains to be examined.

### Recommendations for future research

In this review, we examined the evidence that maternal folate status, especially early in pregnancy, might be involved in the development of ASD. The evidence to date is inconclusive, and highlights future research needs.

#### *More complete assessment of maternal functional folate status*

As folate intake is not the single determinant of functional folate status, studies with more complete assessment of this measure would allow for improved exposure assessment and clearer understanding of this aetiology.

With regard to folate measures, much of the epidemiological data on this topic use self-reported dichotomous data on multivitamin/FA intake as a surrogate for prenatal folate exposure. In contrast to self-reported measures, biological measures of folate status are not subjective, and therefore they are not subject to recall bias, as self-report may be. The use of serum or plasma folate as an indicator of folate status enables the exploration of potential dose–response effects, which is critical in establishing causality. Furthermore, given that one folate biomarker measurement is not necessarily indicative of a mother's folate status throughout pregnancy, serum folate measured at multiple time points before conception and throughout the first trimester would provide a more complete view of maternal folate status during this critical period of development. Repeated folate measurements may also help elucidate a critical window for adequate methyl donor availability, revealing potential mechanisms of ASD development.

It is important to note that only one of the studies reviewed in this paper measured maternal vitamin B<sub>12</sub><sup>(40)</sup>, and none of the studies measured plasma homocysteine status in their analyses. Vitamin B<sub>12</sub> and homocysteine are important components in the functional pathways connecting dietary methyl donors to neurodevelopment. Deficiency of vitamin B<sub>12</sub> results in functional deficiency of folate, as the reaction cannot proceed in the absence of vitamin B<sub>12</sub>. For example, peri-conceptual vitamin B<sub>12</sub> deficiency is linked with abnormal brain development in children<sup>(71)</sup>.

Homocysteine is a more complete indicator of methyl donor status and functionality, as folate is only one of three major dietary methyl donors. Dietary methyl donors are not functionally independent of one another, but rather changes in





concentration of one donor result in compensatory changes in the others. Thus, the folate-ASD hypothesis may be further substantiated if links between other agents in the folate metabolic pathway are reported. Future studies should incorporate vitamin B<sub>12</sub> and total homocysteine concentration measurements into their exposure assessments in the peri-conceptual period and in early pregnancy to account for the complex interdependency between these dietary factors.

### *Incorporating genetic data*

To assess vulnerability to low maternal folate, to examine interactions between dietary and genetic factors and to understand the potential capability of maternal folate intake to offset genetic risk factors, further study is needed in which important genetic factors are sequenced. Most of the previously reviewed studies found in the literature did not explore associations between both dietary folate and genetic risk factors. A notable exception was the case-control study of FA intake and ASD<sup>(5)</sup>.

It is conceivable that if any of the enzymes on the pathway between folate and 5-MTHF acquire functional mutations, it may impair the ability of folate and/or FA to be converted to 5-MTHF and act as an efficient methyl donor. For example, polymorphisms in the *MTHFR* gene, which metabolises folate into a form capable of methyl donation, can reduce enzymatic activity, attenuating the ability of folate to function effectively. Other key enzymes on this pathway include dihydrofolate reductase, which converts synthetic FA and dihydrofolate into trihydrofolate (THF)<sup>(72)</sup>, and serine hydroxymethyltransferase, which converts THF into 5,10-MTHF with vitamin B<sub>6</sub> as a co-enzyme<sup>(73)</sup>. Functional mutations in enzymes related to the other two dietary methyl donors, choline and betaine, could also affect the action of folate because of compensatory changes that may occur through their interrelated metabolic pathways. Last, mutations in the *MeCP2* gene can prevent the MeCP2 protein product from binding to and interpreting DNA methylation marks, resulting in functional hypomethylation by allowing downstream genes to inappropriately escape repression. Such mutations would also be worthwhile to assess in study cohorts.

### **Conclusion**

Animal and human data indicate that maternal folate status could be a biologically plausible risk factor for ASD. Insufficient folate intake can result in DNA hypomethylation, and hypomethylation is associated with neurodevelopment. However, the weight of evidence regarding the role of maternal folate status and the development of ASD is far from unequivocal. Given their limitations in study design, especially with regard to timing of exposure and potential confounding by other vitamins, the randomised trials and studies of maternal folate biomarkers provide little insight into the potential role of FA as a protective factor against ASD traits.

Studies of self-reported maternal FA use and ASD and related traits are inconsistent, providing limited evidence of a protective effect. Nevertheless, methodological limitations exist, including potential confounding by other nutrients and residual confounding by health-related behaviours, and thus these results should be

interpreted with caution. However, the two epidemiological studies of FA intake and ASD diagnoses incorporated various sensitivity analyses or controlled for multivitamin use, thereby suggesting reduced susceptibility to confounding.

In light of an apparent rising prevalence along with the profound individual, familial and societal burden of ASD, there is an urgent need to fill in the gaps in what is currently known of the relationship between folate and ASD. To investigate this aetiology most effectively and efficiently, large historical archives of existing prospective, population-based cohorts should be utilised. Complete assessment of maternal functional folate status requires repeated biological measurement of folate, vitamin B<sub>12</sub> and homocysteine through the first trimester of pregnancy, as well as folate-relevant genetic variants involved in one-carbon metabolism and epigenetic mechanisms. Information on dietary and supplemental intake ideally would be available to relate measures of folate status in a large population, and aid in interpretation of existing studies utilising measures of self-report. Children's ASD diagnoses should be assessed from a clinical source, with high levels of case ascertainment.

As ASD is complex, heterogeneous and multi-causal, exploring environmental influences in conjunction with functionally relevant genes may help identify an additional subset of causes for which genetic contributions may be indirect. If one-carbon metabolism is involved in the aetiology of ASD, this provides a potential route for prevention through nutritional intervention in the pre- and peri-conceptual periods, especially for the subgroup of children having specific genetic risk factors for ASD.

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