Prenatal **nutrition** and early childhood **behaviour**

Jolien Steenweg-de Graaff

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Acknowledgements

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Prenatal Nutrition and Early Childhood Behaviour

Prenatale voeding en gedrag van jonge kinderen

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof.dr. H.A.P. Pols

en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op

woensdag 17 juni 2015 om 15.30 uur

door

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geboren te Geldrop

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PROMOTIECOMMISSIE

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Submitted for publication.

Chapter 1

General introduction

The worldwide prevalence of impairing psychiatric disorders among children and adolescents is estimated between 4-8%. Prevalence rates even increase up to 20-25% if psychopathological symptoms are taken into account as well.[1-5] The most common disorders are anxiety and mood disorders (emotional problems) and attention deficit hyperactivity disorder and disruptive behaviour disorders (behavioural problems). Less frequent developmental disorders, such as autism spectrum disorders (ASD), affect about 1-2% of children.[6] All these disorders not only place an instant but also a long-term burden on these children, their families, and society, for it is known that children with psychopathology are at greater risk for diagnosis in adulthood.[7] In total, mental disorders are estimated to account for 7.4% of the global burden of disease.[8] In order to decrease this burden, we need to understand the aetiology of psychopathology in early childhood.

The development of emotional and behavioural problems is assumed to be a multi-causal phenomenon, influenced by genetic and environmental factors and their interaction.[9] Many of these factors, however, do not lend themselves easily to intervention. In addition, interventions can sometimes only be aimed at treatment of symptoms, whereas prevention of the development of emotional and behavioural problems should perhaps be the primary focus. Considering the neurodevelopmental origin of various childhood emotional and behavioural disorders[10] and the rapid development of the brain during gestation, foetal life may be an important window of opportunity for the prevention of mental health problems later in life.

Over the past decades, both animal and human studies have provided evidence for the concept of foetal 'programming', which signifies the developmental adaptations due to an adverse foetal environment. These adaptations permanently program the foetus' structure, physiology, and metabolism.[11] One of the environmental factors with potential for intervention that have been found to contribute to foetal brain development is maternal nutrition during pregnancy.[12] To date, perhaps the most well-known nutritional intervention to favourably contribute to early neurodevelopment is supplementation with folic acid (a synthetic form of the B-vitamin folate) during the periconceptional period to prevent neural tube defects.[13] Effects, however, do not stop after closure of the neural tube: folic acid deficiency has also been demonstrated to decrease cell division and increase cell death in mouse foetal forebrain.[14] Both animal and human studies have demonstrated that changes in various dietary nutrients during gestation can alter morphology of the brain, as well as its biochemical functions and physiology.[12] This, in turn, may affect subsequent offspring mental health.

Indeed various studies have provided evidence for the hypothesised association between maternal prenatal nutrition and child emotional and behavioural development.[15] Most research has focused on individual foods or nutrients, with little attention for the effects of the prenatal diet as a whole, whereas most positive results of supplementation

interventions come from interventions that involve multiple micronutrients.[16] One study that did investigate the maternal prenatal diet with regard to child emotional and behavioural development found higher adherence to a dietary pattern consisting of unhealthy foods to predict behavioural problems in children from 18 months to 5 years of age.[17] Among the many individually studied nutrients, folic acid supplementation during pregnancy has been found to be associated with fewer emotional and behavioural problems, better social competence, and lower risk of ASD in young children[18], although one study found supplementation to be unfavourable for the personal-social domain.[19] Promising results have also been reported for fish - a major source of omega-3 (n-3) fatty acids - and dietary n-3 and omega-6 (n-6) fatty acid intake: low maternal fish intake was found to be associated with suboptimal prosocial behaviour and increased risk of hyperactivity in childhood[20,21], and increased maternal dietary intake of n-6 fatty acids has been associated with reduced risk of ASD in the offspring.[18] Fish intake and fish oil supplementation were, however, not associated with ASD risk[18] and fish oil supplementation has also not been found to be associated with general child emotional and behavioural development.[16]

Support for the potential relationship between prenatal nutrition and offspring problem behaviour has primarily been provided by animal studies through prenatal deficiency and enrichment studies. For example, both prenatal folate and n-3 fatty acid deficiency have been shown to lead to higher levels of anxiety in mice and rats[22,23] and a pre- and postnatal diet rich in n-6 fatty acids until weaning has a negative effect on social behaviour in mice.[24] In humans, this kind of support is sought by linking actual nutrient status as measured in red blood cell membranes or plasma to child emotional and behavioural problems. One study found lower maternal folate concentrations during pregnancy to increase risk of hyperactivity and peer problems in childhood.[25] Another study did not find maternal folate concentrations to be associated with autistic behaviours in children.[26] As for the n-3 and n-6 fatty acids, there is some indication that perinatal n-3 and n-6 status (either measured in cord blood or maternal blood during gestation) is associated with general child problem behaviour. However, results are inconsistent, ranging from null findings to associations with both emotional and behavioural problems.[27-30]

The aim of this thesis is to extend existing knowledge on maternal nutrition during pregnancy and its association with offspring emotional and behavioural development by exploring whether the maternal prenatal diet as a whole, as well as maternal blood concentrations of specific nutrients (folate and n-3 and n-6 fatty acids) affect early childhood risk of general emotional and behavioural problems, and autistic traits more specifically, within the general population.

The research is imbedded within the Generation R Study, a population-based cohort from early foetal life onwards in Rotterdam, the Netherlands.[31] It was designed

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to identify early biological and environmental determinants of growth, development and health. Pregnant women living in the study area with an expected delivery date between April 2002 and January 2006 were invited to participate. Within the Generation R Study, the effect of maternal nutrition on the development of the young child can be thoroughly studied by means of extensive questionnaire data on nutritional intake early in pregnancy and the availability of blood biomarkers from the first and second trimester of pregnancy. Child emotional and behavioural problems were measured by questionnaires at various ages using different informants.

Outline

In chapter 2, we identify maternal dietary patterns during pregnancy and examine their impact on general child emotional and behavioural problems. In chapter 3, we explore the associations of maternal folic acid supplementation and blood folate concentrations in pregnancy with foetal head circumference growth, general emotional and behavioural problems, and the development of autistic traits in the offspring. In chapter 4, we study the associations of maternal n-3 and n-6 fatty acid status in mid-pregnancy with general emotional and behavioural problems as well as autistic traits in the offspring. In chapter 5, the main findings of these studies are discussed, together with their methodological considerations and clinical implications.

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1

Chapter 2

Dietary patterns

Chapter 2.1

Maternal dietary patterns during pregnancy and child emotional and behavioural problems

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ABSTRACT

Background: Maternal nutritional factors during pregnancy have been linked to foetal brain development and subsequent offspring behaviour. Less is known about associations between maternal dietary patterns and offspring behaviour.

Methods: Within a population-based cohort, we assessed maternal diet using a food frequency questionnaire. Three dietary patterns were derived by means of Principal Component Analysis. Child emotional (emotionally reactive, anxious/depressed or withdrawn, having somatic complaints) and behavioural problems (inattention, aggression) were assessed with the Child Behavior Checklist at 1.5, 3 and 6 years in 3,104 children. We assessed the association of maternal Mediterranean, Traditionally Dutch and Confectionary dietary pattern during pregnancy with child emotional and behavioural problems.

Results: After adjustment, the Mediterranean diet was negatively associated (OR$_{\text{per SD in Mediterranean score}}$ = 0.90, 95% CI: 0.83-0.97) and the Traditionally Dutch diet was positively associated with child behavioural problems (OR$_{\text{per SD in Traditionally Dutch score}}$ = 1.11, 95% CI: 1.03-1.21). Neither diet was associated with emotional problems.

Conclusions: Both low adherence to the Mediterranean diet and high adherence to the Traditionally Dutch diet during pregnancy are associated with an increased risk of child behavioural problems. Further research is needed to unravel the effects of nutrient interplay during and after pregnancy on child behavioural development.

INTRODUCTION

Over the past decades, both animal and human studies have provided evidence for the concept of foetal 'programming', which signifies the developmental adaptations due to an adverse foetal environment. These adaptations permanently program the foetus' structure, physiology, and metabolism.[1] One of the environmental factors that have been found to contribute to foetal and later human development is maternal nutrition during pregnancy.[2] A large part of the research comprises the impact of maternal prenatal nutrition on birth outcomes, physical development and chronic diseases.[3,4] Nutritional factors also affect (foetal) brain development and subsequent offspring behavioural development.[5] For example, prenatal exposure to famine has been found to predict psychopathology in adulthood[6] and maternal status of several nutrients, such as folate and vitamin D[7,8], has been associated with child behavioural development.

Until now, the majority of studies on associations between maternal nutrition and child behaviour have focused on individual foods or nutrients. However, most positive results of supplementation interventions come from interventions that involve multiple micronutrients.[9] This finding is in line with the fact that people do not eat isolated nutrients. Instead, they eat meals, consisting of strongly correlated and interacting nutrients. As a result, the call for analyses of the diet as a whole has increased and dietary pattern analysis has emerged as an approach for studying diet-related research questions.[3,10] Although some studies investigated the association between child dietary patterns and their mental health[11-13], to our knowledge dietary patterns during pregnancy and their relation with offspring behavioural development have not been studied before.

Our objective was to evaluate the effects of maternal dietary patterns in early pregnancy on child behavioural development. As a first step in this field, we chose to focus on the two global domains of child problem behaviour: emotional problems and behavioural problems.[14] Emotional problems comprise emotionally reactive and anxious/depressed symptoms, as well as somatic complaints and symptoms of being withdrawn. Behavioural problems comprise attention problems and aggressive behaviour. We hypothesised that a healthy prenatal diet would be associated with a reduced risk whereas an unhealthy diet would be associated with an increased risk of emotional and behavioural problems during childhood.

METHODS

Study population
The present study was conducted within the Generation R Study, a population-based cohort from early foetal life onwards in Rotterdam, the Netherlands. The study design

2.1

has been described in detail previously.[15] The study was conducted in accordance with the guidelines proposed in the World Medical Association Declaration of Helsinki and was approved by the Medical Ethics Committee at Erasmus Medical Centre, University Medical Centre Rotterdam, the Netherlands. Written consent was obtained from all participants.

Only women of Dutch national origin were eligible for the current study, since nutrition generally differs between ethnic groups. Overall, 3,486 children and their mothers were eligible at study baseline. Due to loss to follow-up, data for 3,104 children (89%) and their mothers were included in one or more analyses (Figure 2.1.1). Some mothers
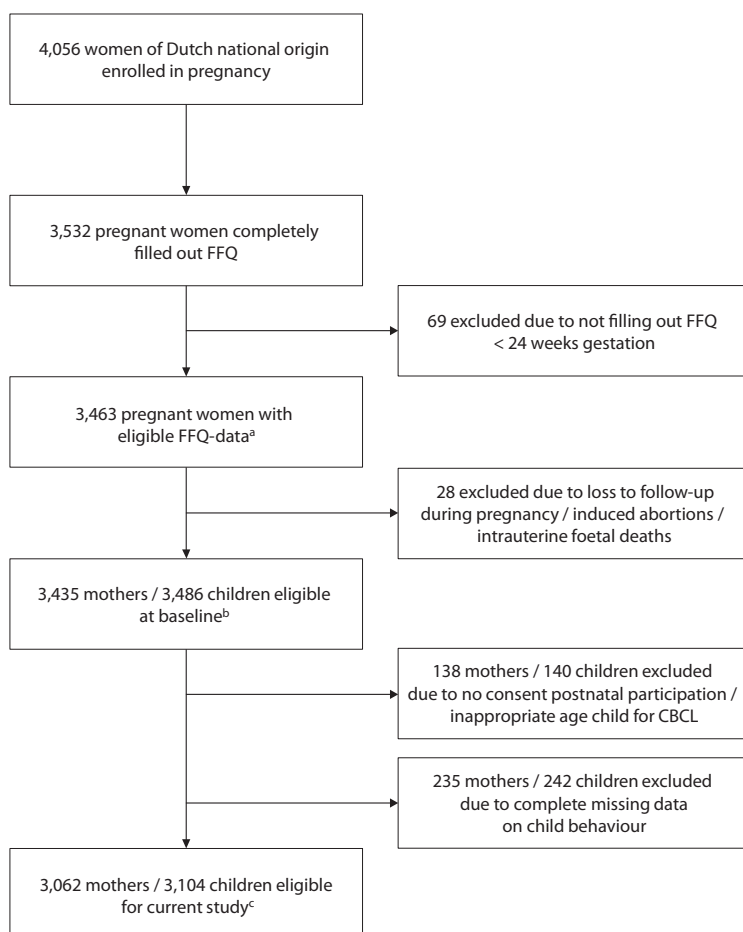

Figure 2.1.1 Flow chart of study population
FFQ – food frequency questionnaire, CBCL – Child Behavior Checklist
[a] Eligible sample for dietary pattern analysis.
[b] 51 mothers participate with twin.
[c] Analytic sample, missing values imputed by multiple imputation.

participated with two (n = 255), or three (n = 3) children. Since results did not differ after random exclusion of one or two of these siblings, they were included in the analyses.

Dietary assessment

Nutritional intake in the past three months was assessed in early pregnancy (median 13.5 weeks, 95% range 10.1-21.8 weeks) by using a modified version of a validated semi-quantitative food frequency questionnaire (FFQ).[16] The FFQ consists of 293 food items and is structured according to meal patterns. Questions in the FFQ assess consumption frequency, portion size, preparation method and additions to the dish. Portion sizes were estimated using Dutch household measures and photographs. Average daily nutritional values were calculated using the Dutch food composition table 2006.[17]

The 293 food items were reduced to 24 predefined food groups, according to The European Prospective Investigation into Cancer and Nutrition (EPIC)-soft classification, based on origin, culinary usage and nutrient profiles.[18]

To derive dietary patterns from food consumption data of women of Dutch origin within the Generation R Study Cohort (n = 3,463), we used Principal Component Analysis (PCA) as previously described by Hu[10] and applied in a number of recent studies of dietary patterns and child development.[12,13] In short, with PCA the variance in dietary data is explained by determining which food groups correlate high and thus together can be interpreted as a dietary pattern. For each individual food group a factor loading was calculated, representing the extent to which the food group relates to a particular factor. Foods with factor loadings of an absolute value ≥ 0.2 on a factor were considered to have a 'strong' association with that factor and were used to describe and label each pattern. Based on the scree plot and interpretability of the patterns, three major dietary patterns with an eigenvalue above 1.5 were identified, explaining in total 21% of the variation in the dietary data (Table 2.1.1). This percentage is comparable to those reported in other studies on dietary patterns.[19] The first pattern has been labelled 'Mediterranean', because of its high loadings on vegetables, fish & shellfish, vegetable oil, fruit, and eggs, and relatively high negative loading on processed meat. The second component was characterised by high intakes of fresh and processed meat and potatoes, a relatively high intake of margarines and a very low intake of soy and diet products. This resembles the traditional Dutch eating pattern, which we labelled as 'Traditionally Dutch'. The third component yielded a dietary pattern high in the consumption of cakes, sugar & confectionary products, tea, cereals, fruit and dairy products. We labelled this pattern 'Confectionary'. Each woman received an individual score for each of the dietary patterns, calculated as the product of the food group value and its factor loadings summed across foods. For convenience we termed this score 'adherence to dietary pattern': a high score represents a high adherence to the particular dietary pattern, whereas a low score represents a low adherence to the pattern. When

Table 2.1.1 Factor loadings of food groups in dietary patterns in women of Dutch national origin (n = 3,463)

Food group	Mediterranean pattern	Traditionally Dutch pattern	Confectionary pattern
Vegetables	**0.68**	0.00	−0.06
Fish and shellfish	**0.67**	−0.12	−0.01
Vegetable oil	**0.65**	0.13	−0.10
Fruit	**0.47**	−0.18	**0.24**
Fresh meat	0.06	**0.76**	−0.10
Miscellaneous (soy & diet)	−0.04	**−0.62**	−0.10
Processed meat	**−0.25**	**0.56**	0.14
Potatoes	−0.14	**0.45**	−0.16
Cakes	0.01	−0.03	**0.70**
Sugar and confectionary	−0.17	0.03	**0.65**
Tea	**0.26**	−0.02	**0.43**
Legumes	0.07	−0.09	−0.09
Cereals and cereal products	**0.23**	0.06	**0.32**
Butter	0.03	0.04	0.17
Margarines	−0.17	**0.20**	0.17
Coffee	0.02	0.02	0.03
Alcoholic drinks	0.12	0.03	0.04
Sauces	0.07	0.03	0.08
Soft drinks	−0.11	0.17	0.02
Eggs	**0.23**	−0.08	0.09
Fruit/vegetable juices	−0.14	0.01	−0.07
Dairy products	0.12	−0.01	**0.22**
Soups and bouillon	0.13	0.04	0.02
Milk	−0.02	0.12	−0.04
Variance explained (%)	8.1	6.9	6.1

Note: Food groups with bold numbers are considered to have a strong association (absolute value of factor loading ≥ 0.2) with a factor.

using PCA to define dietary patterns, participants receive a score on each of the dietary patterns. Although the dietary patterns are statistically uncorrelated, some individuals score high on more than one pattern. This is why we chose to adjust all analyses for the other dietary pattern scores.

Child emotional and behavioural problems

Mothers were asked to fill out the Child Behavior Checklist for toddlers (CBCL 1½-5) when their child was 1.5, 3 and 6 years old. The CBCL is a self-administered parent-report questionnaire to measure the degree of children's problem behaviour. It contains 99 problem items rated on a 3-point scale (0 = not true, 1 = somewhat or sometimes true,

and 2 = very true or often true), based on the preceding two months. Together, these 99 items result in a Total problems score, which can be subdivided in Emotional problems and Behavioural problems. Good reliability and validity have been reported for the CBCL.[14]

Covariates

Several parental characteristics were considered as possible confounding variables, based on previous studies of (maternal) diet and child (emotional and behavioural) development.[7,13] These were: maternal pre-pregnancy BMI, age at enrolment, prenatal smoking and folic acid and multivitamin use, paternal national origin, parental educational level and psychopathology, parity and marital status, and family income. Maternal folate, homocysteine and vitamin B_{12} concentrations in early pregnancy were considered as possible mediators. Assessment of these covariates has been described in detail previously.[20,21] To adjust for the effect of energy intake during pregnancy on the association between maternal diet and child problem behaviour, we also considered prenatal average daily caloric intake, calculated by means of the FFQ-data and the Dutch food composition table 2006.[17]

Child characteristics that were considered comprised gestational age and weight at birth, gender, breastfeeding status at 6 months, and age of the child at the time of assessment, which have been described in detail previously.[7] We also considered child consumption of snacks and sugar containing beverages at age 4/6, which was obtained from parental questionnaires at the age of 4 and 6 years.

All analyses of maternal dietary patterns and child problems were controlled for gender of the child and the age of the child at the time of the measurement (CBCL). Other covariates were included in the models if they changed the effect estimates meaningfully (defined as more than 5%). Following this criterion maternal age, pre-pregnancy BMI and parity, prenatal smoking, vitamin use and caloric intake, paternal national origin, parental educational level and psychopathology, family income, and marital status as well as child consumption of snacks and sugar containing beverages at the age of 4/6 years were included in the present analyses.

Statistical analyses

We used the CBCL emotional and behavioural problem scores as outcome variables. To facilitate a clinical interpretation of problem behaviour, and because the scores could not be normalised, we analysed the emotional and behavioural scores as dichotomous variables. In line with previous publications, we defined a non-optimal score as the highest 20% of emotional and behavioural item scores.[7]

Multivariable logistic regression analyses using generalised estimating equations (GEE) were used to test for associations between maternal dietary patterns and repeat-

edly measured child emotional and behavioural problems. GEE analysis assesses the association between two variables, correcting for the within-subject dependence of repeated ratings of child emotional and behavioural problems. Such an overall estimate reduces the errors derived from multiple comparisons. A possible time trend is not easily interpretable in such a combined model. Because the number of repeated measures in the current study was small (n = 3 measures of child behaviour) and the data were balanced as well as complete (due to imputation of missing data), an unstructured working correlation structure was used in the GEE analyses as adjustment for the dependency between the repeated measurements.

First, we used the dietary pattern scores as continuous variables in the equation (1 unit increase on a diet score equals an increase of 1 SD). We also added quadratic terms of the dietary pattern variables to test for non-linear associations. All models were adjusted for gender and age of the child. To test the independent effect of each dietary pattern, all models were also mutually adjusted for the other dietary patterns. Subsequent models were additionally adjusted for the covariates mentioned earlier. To verify results, we re-analysed the data using the Mediterranean Diet Score as proposed by Trichopoulou et al.[22], though excluding alcohol consumption from the score.

Next, those dietary patterns that were statistically significant associated with child behavioural outcome in the primary analyses were divided by quintiles for ease of interpretation in secondary analyses.

Missing values on covariates and child outcome data for those children with at least one of three outcome measures available (approximately 0.1-24%) were imputed using the Markov Chain Monte Carlo multiple imputation technique with Predictive Mean Matching for continuous variables. We generated 5 datasets and undertook 10 iteration procedures. Subsequently, GEE analyses were performed separately on each completed dataset and thereafter combined to one pooled estimate. Measures of association are presented with 95% confidence intervals (95% CI). Statistical analyses were carried out using PASW Statistics, version 17.0 for Windows (SPSS Inc., Chicago, Illinois) and Stata for Windows, Release 12 (StataCorp LP., College Station, Texas).

RESULTS

Response analysis

Analyses of missing data showed that, compared with children with behavioural data (n = 3,104), children without behavioural data (n = 382, 9.1%) had a shorter gestation [median of 39.9 weeks (95% range: 32.2–42.0 weeks) compared with 40.1 weeks (95% range: 35.4–42.4 weeks); $P < 0.001$] and, on average, a 193 gram (95% CI: 121;265, t = 5.3) lower birth weight. The mothers of children not included on average had a 0.3 points

(95% CI: 0.2;0.4, t = 5.0) lower score on the Mediterranean diet, a 0.1 points (95% CI: −0.2;−0.02, t = −2.3) higher score on the Traditionally Dutch diet and a 0.3 points (95% CI: 0.2;0.4, t = 5.1) lower score on the Confectionary diet in their first trimester of pregnancy. They were, on average, 2.5 (95% CI: 1.9;3.1, t = 8.5) years younger and less educated [31.6% compared with 62.7%, χ^2 (1) = 132; P < 0.001]. They also more often continued smoking during their pregnancy [34.5% compared with 13.5%, χ^2 (2) = 111; P < 0.001] and less often adequately used vitamins during early pregnancy [58% compared with 81%, χ^2 (2) = 88; P < 0.001]. The income of families not included in the analyses was lower than that of families included in the study [60.2% compared with 83.7%, χ^2 (1) = 83; P < 0.001]. Descriptive characteristics of the participating parents and their children are presented in Table 2.1.2.

Table 2.1.2 Subject characteristics

Child characteristics	n = 3,104
Female (%)	49.5
Gestational age at birth (wk)	40.1 (35.4-42.4)[a]
Birth weight (g)	3,478 ± 570[b]
Breastfeeding until 6 mo (%)	31.8
Age child at assessment (mo)	
at 1.5 yr	18.1 (17.6-21.5)
at 3 yr	36.2 (35.4-40.1)
at 6 yr	71.2 (66.9-82.1)
Snacks at age 4/6 yr (% ≥ 2/day)	13.8
Sugar containing beverages at age 4/6 yr (% > 2/day)	21.1
Parental characteristics	n = 3,062
Maternal age at enrolment (yr)	31.7 ± 4.1
Maternal education (% higher)	62.6
Paternal education (% higher)	58.1
Family income (% > 2,000 €/mo)	82.7
Maternal psychopathology in mid-pregnancy (GSI-score)	0.12 (0.00-0.78)
Paternal psychopathology in mid-pregnancy (GSI-score)	0.06 (0.00-0.59)
Maternal smoking during pregnancy (%)	
Never	78.2
Until pregnancy was known	8.3
Continued throughout pregnancy	13.5
Maternal vitamin use during early pregnancy (%)	
Folic acid only supplement use	52.6
Multivitamin use	29.6
No vitamin use	17.8

Table 2.1.2 (continued)

Maternal prenatal caloric intake (kcal/day)	2,150 ± 503
Maternal pre-pregnancy BMI (kg/m^2)	23.3 ± 3.9
Parity (% primiparae)	61.1
Marital status (% cohabitation)	94.6
Paternal national origin (% Dutch)	80.2

GSI-score – Global Severity Index score
[a] Median; 95% range in parentheses (all such values).
[b] Mean ± SD (all such values).

Maternal dietary patterns and child emotional and behavioural problems

The associations between maternal dietary patterns and child emotional and behavioural problems are presented in Table 2.1.3. In the basic models, both the Mediterranean and the Traditionally Dutch pattern were associated with child behavioural problems ($OR_{per\ SD\ of\ Mediterranean\ score} = 0.88$, 95% CI: 0.82;0.94, $P < 0.001$; $OR_{per\ SD\ of\ Traditionally\ Dutch\ score} = 1.15$, 95% CI: 1.06;1.26, $P = 0.001$). Including quadratic terms of the dietary pattern variables in the model did not add to the results (data not shown).

Next, the associations were adjusted for confounders. Since pregnant women are advised to use vitamin supplements, the use of supplements was examined in more detail

Table 2.1.3 Associations of maternal prenatal dietary patterns with child emotional and behavioural problems up to 6 years (n = 3,104)[a,b,c]

	Emotional problems			
	Basic[d]		Adjusted for environmental covariates[e]	
Maternal dietary pattern (per SD)	OR (95% CI)	P	OR (95% CI)	P
Mediterranean	0.94 (0.88;1.01)	0.089	0.95 (0.88;1.02)	0.132
Traditionally Dutch	1.04 (0.97;1.12)	0.292	1.05 (0.97;1.13)	0.244
Confectionary	1.03 (0.96;1.10)	0.392	1.00 (0.91;1.09)	0.956
	Behavioural problems			
	Basic[d]		Adjusted for environmental covariates[e]	
Maternal dietary pattern (per SD)	OR (95% CI)	P	OR (95% CI)	P
Mediterranean	0.88 (0.82;0.94)	< 0.001	0.90 (0.83;0.97)	0.006
Traditionally Dutch	1.15 (1.06;1.26)	0.001	1.11 (1.03;1.21)	0.011
Confectionary	1.04 (0.97;1.13)	0.251	0.99 (0.90;1.10)	0.912

[a] Values are odds ratios from logistic generalised estimating equations (GEE) analyses.
[b] Child measures at 1.5, 3 and 6 yr.
[c] Child outcome data imputed for those children with at least one of three outcome measures available.
[d] Model 1: adjusted for the other dietary patterns, gender and age child at time of measurement.
[e] Model 2: model 1, additionally adjusted for parental educational level, income and psychopathology, maternal smoking, vitamin use and average daily caloric intake during pregnancy, age, parity, marital status, and pre-pregnancy BMI, paternal national origin and child consumption of snacks and sugar containing beverages at age 4/6 yr.

for each dietary pattern. Only the Mediterranean pattern was associated with supplement use (dietary pattern divided by tertiles; χ^2 (4) = 12.5, P = 0.014). After adjustment for confounders, the associations remained statistically significant ($OR_{per\ SD\ of\ Mediterranean\ score}$ = 0.90, 95% CI: 0.83;0.97, P = 0.006; $OR_{per\ SD\ of\ Traditionally\ Dutch\ score}$ = 1.11, 95% CI: 1.03;1.21, P = 0.011). When re-analysing the association using the Mediterranean Diet Score, we found very similar results (OR = 0.94, 95% CI: 0.90;0.98, P = 0.004). There were no associations between these dietary patterns and child emotional problems ($OR_{per\ SD\ of\ Mediterranean\ score}$ = 0.95, 95% CI: 0.88;1.02, P = 0.132; $OR_{per\ SD\ of\ Traditionally\ Dutch\ score}$ = 1.05, 95% CI: 0.97;1.13, P = 0.244). However, as can be seen by the overlap in the confidence intervals, the associations between the dietary patterns and emotional problems and behavioural problems did not differ statistically.
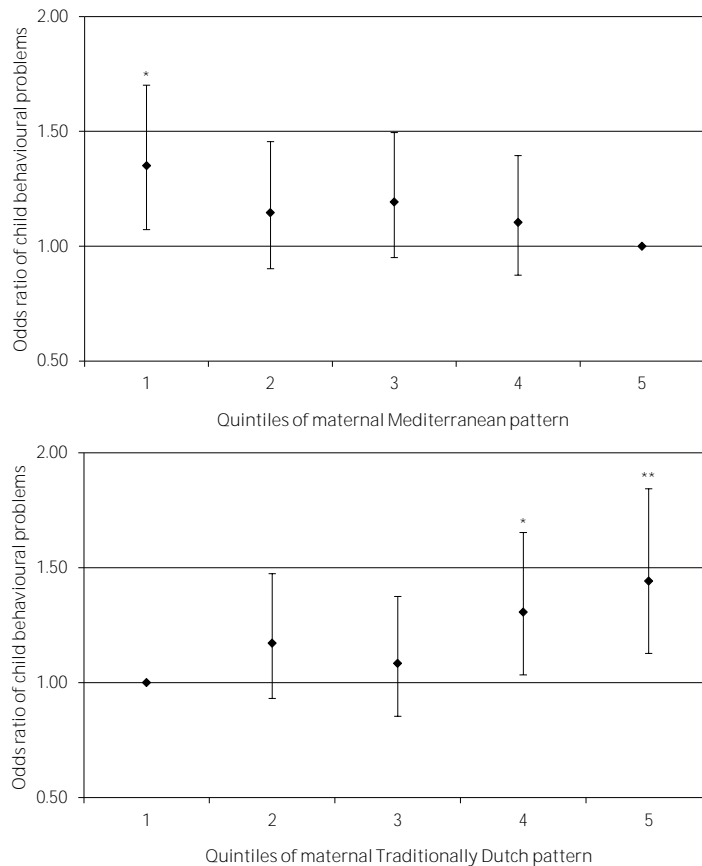

Figure 2.1.2 Quintiles of maternal Mediterranean and Traditionally Dutch dietary pattern and odds ratio of child behavioural problems
Note: Error bars are 95% confidence intervals.
* P < 0.05
** P < 0.01

Additional adjustment for the maternal nutritional biomarkers folate, homocysteine and vitamin B_{12} by adding these variables to the model did not meaningfully change the results. We did not find any association between the Confectionary pattern and child emotional or behavioural problems (see Table 2.1.3).

Next, for ease of interpretation and to illustrate the associations, we examined the quintiles of the maternal Mediterranean and Traditionally Dutch pattern in relation to child behavioural problems. We used the highest quintile (high adherence) of the Mediterranean pattern and the lowest quintile (low adherence) of the Traditionally Dutch pattern as the reference categories. With regard to the previous analyses, we found a similar pattern of results (Figure 2.1.2). Those children of mothers in the lowest quintile of adherence to the Mediterranean pattern had a 35% increased odds (OR = 1.35, 95% CI: 1.07;1.70, P = 0.011) of scoring in the high levels of the CBCL-scale of behavioural problems, compared with children of mothers in the highest quintile of adherence to the Mediterranean pattern (P for trend = 0.017). For children of mothers in the highest and second highest quintile of adherence to the Traditionally Dutch pattern, these odds were increased by respectively 44% (OR = 1.44, 95% CI: 1.13;1.84, P = 0.004) and 31% (OR = 1.31, 95% CI: 1.03;1.65, P = 0.026), compared with children of mothers in the lowest quintile of adherence to the Traditionally Dutch pattern (P for trend = 0.003).

DISCUSSION

In this population-based study, both a low adherence to the Mediterranean diet and a high adherence to the Traditionally Dutch diet during pregnancy were associated with increased risk of behavioural problems in the offspring.

Although there is no single 'Mediterranean' diet, common characteristics of a Mediterranean-type or 'healthy' diet include moderate to high intakes of fruit, vegetables, vegetable oil, and fish, and lower intake of meat. In contrast, the more 'Traditional' patterns in Western countries often exist of higher consumption of meat and potatoes, and lower intake of fruit and vegetables.[19] Similar to our current findings, associations of prenatal Mediterranean and Traditional diets with foetal and child characteristics have been reported. For example, higher adherence to a Mediterranean-type diet during pregnancy has been found to reduce the risk of neural tube defects and orofacial clefts whereas adherence to a Traditional-like diet was found to increase this risk.[23] Likewise, the Mediterranean diet has been found to be beneficial for foetal growth, in contrast to the Traditional-type diet.[21] Several potential mechanisms may underlie the observed effect of the maternal diet on child behavioural problems in the current study.

A first explanation for this observation is the hypothesised neurodevelopmental origin of psychiatric disorders. Neuroimaging studies have shown altered brain structure and function in children with mental disorders such as attention-deficit/hyperactivity disorder, major depressive disorder and conduct disorder.[24] Although the human brain continues to develop throughout childhood, many alterations in the brains of children with mental disorders, such as aggressive behaviour and attention problems, might arise prenatally. An example may be found in maternal status of unsaturated fatty acids during pregnancy. The omega-3 (n-3) and omega-6 (n-6) long-chain polyunsaturated fatty acids (LC-PUFAs) have been found to influence development and maturation of neuronal structures and to be involved in numerous neuronal processes, ranging from effects on membrane fluidity to gene expression regulation.[25] In particular the imbalance between maternal n-3 and n-6 fatty acids has been associated with subtle negative effects on child mental development.[26] During pregnancy, LC-PUFAs are transferred to the foetus across the placenta. The concentration of fatty acids in the foetus is dependent on maternal fatty acid status and the mother's dietary intake. N-6 LC-PUFAs are mainly found in common vegetable oils (sunflower and corn oil), meat, eggs and lean fish. Fatty fish and vegetable oils such as linseed and soybean oil are the major suppliers of n-3 LC-PUFAs.[26] As a consequence, mothers with a high adherence to the Mediterranean pattern may have a better n-3:n-6 ratio than mothers who highly adhere to the Traditionally Dutch pattern, which mainly provides them with n-6 fatty acids. Another example of the neurodevelopmental perspective of psychiatric disorders is a pathway through increased prenatal oxidative stress which may provide a possible explanation for the association of maternal dietary patterns and child behavioural problems. Oxidative stress represents an imbalance in the body, characterised by an excessive production of free radicals and inadequate antioxidant defence mechanisms. A diet high in saturated fat, such as the Traditionally Dutch diet, increases the level of oxidative stress. Antioxidants, mainly found in vegetables and fruits as in the Mediterranean diet, decrease the level of oxidative stress.[27] Increased levels of oxidative stress have been found to reduce the level of Brain Derived Neurotrophic Factor (BDNF) in the rat brain.[27] BDNF is necessary for neuronal growth and differentiation in the nervous system during foetal development. The Confectionary pattern is high in sugar and fat intake. However, fruit intake is also considerably high in this pattern, which may compensate the negative effects of high sugar and fat intake on the level of oxidative stress. Although we were able to additionally adjust for nutritional biomarkers, known to be related to oxidative stress, the number of biomarkers was limited, which may clarify why the association was not further explained.

Second, growth of the foetal body may mediate the observed association. Foetal growth has been found to be inversely related to child behavioural problems.[28] Although birth weight did not change the association between maternal diet and child behaviour in our study, other characteristics of foetal growth may explain the association.

Finally, in observational studies, this type of associations is highly sensitive to effects of confounding factors. A decline in the effect of maternal dietary patterns on the offspring's behavioural problems was noticeable after adjustment for several parental and child characteristics, which stresses the substantial role of confounding factors. For example, Waylen et al., in their study of early intake of dietary n-3 and childhood behavioural problems, found any association to be completely confounded with sociodemographic factors.[29] Diet is a proxy for lifestyle, which in itself is difficult to disentangle. Although we accounted for many sociodemographic factors, further (residual) confounding cannot be excluded. For example, we were not able to completely adjust for the diet of the child itself. Also, other behavioural or lifestyle factors which are associated with both better maternal dietary habits and child behaviour may have gone unnoticed. Possibly, not only high socioeconomic status, but also better home environment or parenting style, account for the association of maternal diet with child behavioural problems.

We found an effect of maternal diet almost exclusively on behavioural problems. Possibly, specific brain structures, involved in the aetiology of behavioural problems such as inattention and aggression, are specifically affected through biological mechanisms as described before. However, this contrast must be interpreted cautiously as the effect of maternal diet on child emotional and behavioural problems was not different. Also, it is known that there is comorbidity of emotional and behavioural problems in individuals.[14]

Strengths and limitations

The strengths of our study were its large sample size, the repeated measures of child problem behaviour throughout early childhood, and the ability to adjust for considerable numbers of covariates. An advantage of using PCA to define dietary patterns is that participants get a score on each of the dietary patterns. This allows for more realistic scenarios in which individuals eat according to a mixture of patterns.

The study also has its limitations. Selective attrition may have influenced our results. Mothers of children not included generally had less favourable dietary habits and socioeconomic circumstances. This selective attrition can only lead to bias if 'exposed' children not included in the study had different levels of behavioural problems than exposed children who were included in the study. Next to this, maternal report of child emotional and behavioural problems may have introduced information bias. However, for young children, mothers are still primarily the main caregivers and may thus also be the best reporters of their child's behaviour.

A point of critique in using PCA is that the obtained patterns are population-specific. Newby and Tucker have, however, shown in their review that a 'Healthy', 'Traditional' and 'Sweets' pattern are fairly reproducible across populations.[30] Furthermore, when using PCA to determine dietary patterns one has to make choices. For example, researchers

have to decide on the number of food groups to include in the analysis and the number of factors to extract. However, no single method of dietary pattern analysis is considered the best and PCA is now widely used in nutritional epidemiology. To reduce possible subjectivity, we applied an existing food group classification (EPIC) and used common criteria to select the factors. The amount of variance (21%) explained by the dietary patterns is quite low, but nevertheless comparable to those reported in other studies on dietary patterns.[19] Moreover, we verified our results by re-analysing the association using the Mediterranean Diet Score and found very similar results.

2.1

Further, the use of an FFQ to capture dietary intake can be questioned. First, the FFQ we used was originally designed for elderly and has not been validated for use in a pregnant population. However, the dietary patterns we found are comparable to those found in other studies that used an FFQ specifically designed or validated for use in pregnant women.[31,32] Next, for this particular population, one could question whether nutrient intake comes from natural foods or is provided in large quantities by supplements. Indeed we found the Mediterranean dietary pattern to be associated with supplement use. Nevertheless, we found statistically significant effects of the maternal dietary patterns after adjusting for supplement use. Finally, the use of an FFQ to measure dietary intake limits the possibility of making clear recommendations about the optimal prenatal nutrient intake as a way to favourably contribute to foetal neurodevelopment and further child behavioural development. To that extent, FFQ-data should be combined with more accurate alternatives to measure nutrient intake, such as automated multiple 24-h recalls, food records, dietary biomarker measurements, and doubly labelled water measurements to estimate energy expenditure.[33] However, in large prospective cohort studies, these alternatives are not feasible because of inconvenience and costs. Yet for the purpose of gaining insight in the overall composition of the diet and ranking individuals according to their usual consumption, the FFQ seems to be well suited.[34] Signs of diet-disease associations may be detected in FFQ-based studies[33] and, moreover, combined effects may indeed be greater than the sum of individual nutrient effects.[23] This does not imply that studying single nutrients is not important. Rather, dietary pattern analysis is complementary to the analysis of individual nutrients or foods. After dietary patterns analysis, further research into foods actually consumed is needed to unravel nutrient interplay and its effect on foetal development.

Relevance

The current study presents novel associations between maternal prenatal dietary patterns and child behavioural problems. In a multi-causal phenomenon such as development of the brain and behaviour, large effects of nutrition are not expected. Indeed the effect sizes presented here are very small. Nevertheless, each small effect contributes to development and should therefore be considered as an opportunity to improve pre-

natal conditions in such a way that the foetus can optimally develop. In contrast to, for example, genetic traits and socioeconomic status, nutrition is a factor that is relatively easy to modify for pregnant women. Therefore, the effects of prenatal food consumption and nutrient interplay on child behavioural development should be further explored.

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Chapter 3

Folic acid and folate

Chapter 3.1

Associations of maternal folic acid supplementation and folate concentrations during pregnancy with foetal and child head growth

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ABSTRACT

Background: Folic acid supplementation during pregnancy has been associated with a reduced risk of common neurodevelopmental delays in the offspring. However, it is unclear whether low folate status has subtle effects on the developing brain. We evaluated the associations of maternal folic acid supplementation and folate concentrations during pregnancy with repeatedly measured prenatal and postnatal head circumference in the offspring.

Methods: Within a population-based cohort, we measured maternal plasma folate concentrations in early pregnancy and assessed folic acid supplementation by questionnaire. Up to 11 repeated measures of head circumference were obtained during foetal life (20 and 30 weeks of gestation) and childhood (between birth and age 6 years) in 5,866 children.

Results: In unadjusted models, foetal head growth was 0.006 SD (95% CI: 0.003;0.009, *P* < 0.001) faster per week per 1-SD higher maternal folate concentration. After adjustment for confounders, this association was attenuated to 0.004 SD per week (95% CI: 0.001;0.007, *P* = 0.01; estimated absolute difference at birth of 2.7 mm). Maternal BMI, smoking during pregnancy, ethnicity and income were the strongest confounders. No associations were found between maternal folate concentrations and child postnatal head growth. Preconceptional start of folic acid supplementation was associated with larger prenatal head *size*, but not with prenatal or postnatal head *growth*.

Conclusions: Our results suggest an independent, modest association between maternal folate concentrations in early pregnancy and foetal head growth. More research is needed to identify whether specific brain regions are affected and whether effects of folate on foetal head growth influence children's long-term functioning.

INTRODUCTION

Supplementation with folic acid during embryonic development reduces the risk of neural tube defects in infants.[1,2] This protective effect has led to recommendations for daily supplementation with folic acid starting preconceptionally in women planning to become pregnant. Additionally, some countries have adopted fortification of flour with folic acid.[3]

Emerging evidence has shown that maternal folic acid supplementation during pregnancy is associated with reduced risk of several other neurodevelopmental disorders in offspring, including language delays[4], autism spectrum disorders[5,6], and problem behaviour.[7] However, the underlying neurobiology of low folate and these neurodevelopmental problems remains unclear. Folic acid supplement use is strongly related to higher socioeconomic status and is a marker of good health literacy[8,9], which increases the susceptibility for confounding.

To further support a potential relationship between folic acid supplement use and neurodevelopmental disorders, nutritional biomarkers and biological intermediates can be examined. Blood folate concentration serves as a key nutritional biomarker. This can be measured in maternal blood and can serve as the endpoint of the determinants of folate status, i.e. after physiological absorption of folic acid supplements and folates from natural (or fortified) foods.[10] Unlike folic acid supplementation, folate concentrations are unaffected by recall bias, thus serving as a better proxy for actual folate status.

Biological intermediates include structural alterations in the brain throughout pregnancy and early postnatal development. Head circumference [HC] can serve as a non-invasive and inexpensive proxy for foetal and early postnatal brain growth and development. HC is closely related to brain volume, particularly in the period before the skull is fully developed.[11,12] Several neurodevelopmental disorders are associated with subtle changes in head growth. Children with autism for instance, have normal to slightly smaller HC at birth, followed by accelerated head growth in the first year of life when compared to non-autistic children.[13]

Folate is involved in a number of essential processes of growth and development, such as DNA synthesis and gene expression. Consequently, folate requirements are increased in periods of rapid growth, such as pregnancy. Low maternal folate concentrations directly limit the availability of folate to the foetal cells, which results in impairment of cell division and potentially impairs growth.[14]

Earlier research provides inconsistent evidence for an association between maternal folate concentrations and HC at birth, and no evidence for an association between maternal folic acid supplementation and HC at birth.[15] In a previous report of the current cohort, maternal folic acid supplementation was associated with head size in prenatal life, but foetal head *growth* was not considered as a pregnancy outcome.[16] Whether

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prenatal folate concentrations or maternal folic acid supplementation during pregnancy has sustained or additive effects on postnatal head growth is unknown.

We used the Generation R cohort to evaluate the associations of both maternal folic acid supplementation and folate concentrations during pregnancy with repeatedly measured offspring prenatal and postnatal head size. We hypothesised that folic acid supplementation as well as higher folate concentrations are associated with increased head growth in the offspring.

METHODS

Study population

The present study was conducted within the Generation R Study, a population-based cohort from early foetal life onwards in Rotterdam, the Netherlands.[17] The study was conducted in accordance with the guidelines proposed in the World Medical Association Declaration of Helsinki and was approved by the local Medical Ethics Committee. Written consent was obtained from all participants.

Out of 8,782 women with a singleton pregnancy, 6,993 women (80%) enrolled in the study before 18 weeks of gestation. We measured plasma folate concentration in early pregnancy in 5,965 (85%) of these women. We excluded 37 women since their measure of folate was above the assay range, resulting in valid folate concentrations at baseline for 5,928 mothers. Twenty children died during the neonatal period and one was lost to follow-up before birth; these were excluded from this study. In total, at least one measure of foetal or child HC was obtained in 5,866 children.

Maternal folic acid supplement use

Before 18 weeks of gestation, pregnant women were asked by questionnaire whether they used folic acid supplements or multivitamin preparations and when they started supplementation. In the Netherlands, both folic acid supplements and multivitamin preparations for pregnant women contain 400-500 µg folic acid; there is no mandatory food fortification with folic acid. In line with previous publications, we categorised folic acid supplement use in four groups: 1) preconceptional start (as recommended[18]) (n = 2,153), 2) start within the first 10 weeks of pregnancy (n = 1,602), 3) start after the first 10 weeks of pregnancy (n = 753), and 4) no use (n = 1,358).[19] To distinguish between effects of folic acid and multiple micronutrients, we separated preconceptional start into 'folic acid only use' (n = 1,396) and 'multivitamin use' (n = 757) in additional analyses.

Maternal folate concentrations

At 13 weeks of gestation, venous blood samples were drawn, centrifuged and stored at −80°C, as previously described.[20] After thawing, folate concentrations were analysed in 2008, using an immunoelectrochemoluminence assay. The between-run coefficients of variation for plasma folate varied between 1.5% and 8.9%, with an analytic range of 1.8-45.3 nmol/L (0.8-20.0 ng/mL).

Folate concentration was analysed:

a) as a continuous variable (standard deviation scores (SDS)), and

b) as a dichotomous variable, categorised into 'folate deficient' (< 7 nmol/L = 3.1 ng/mL, 8.4%)[10] or 'normal folate concentration'.

Additional analyses were performed using a different cut-off (based on normative concentrations determined by the Erasmus MC laboratory: cut-off < 8 nmol/L (3.5 ng/mL); 13.8%).

Foetal and child head circumference

Foetal ultrasound measurements were performed at prenatal visits during each trimester of pregnancy.[17,21] First trimester ultrasounds were primarily used for pregnancy dating.[21,22] The intra- and interobserver reliabilities of foetal biometry in early pregnancy were excellent (all intraclass correlation coefficients greater than 0.98).[23]

HC was subsequently measured at birth and in up to seven visits at child health care centres at the ages of 0-2, 2, 3, 4, 5-10, 10-13, and 13-17 months. Gender of the child and gestational age at birth were extracted from medical records. HC was again measured at the Generation R research centre (mean age of 6.2 years).

Gender and gestational age adjusted SD scores were constructed for all measures of HC based on growth reference curves.[21,24] This approach enables linear analyses of (non-linear) growth patterns.[25]

Covariates

Several maternal and child characteristics were selected as possible confounding variables, based on previous studies of maternal folate or foetal development.[15,26] These were: maternal age and body mass index (BMI) at enrolment, national origin, educational level, smoking and alcohol consumption during pregnancy, psychopathology in mid-pregnancy, parity, and family income, foetal/child gender, and gestational age at time of the HC measurements. Assessment of these covariates has been described in detail previously.[19,26,27]

Statistical analyses

Folic acid supplementation and folate concentration were used as the independent variables in the analyses. In the analyses of folic acid supplementation, the group of

mothers who did not use folic acid supplements comprised the reference group. In the analyses of folate deficiency, the group of mothers with normal folate concentrations comprised the reference group. We additionally used linear regression models to evaluate the associations between maternal folate concentrations with offspring head *size* at all separate time points of HC measurement.

To test the associations with offspring head *growth*, we performed longitudinal analyses using unbalanced repeated measures analysis (mixed models). These analyses were performed separately for prenatal and postnatal head growth, since growth patterns as well as measurement methods differ between these developmental stages. Using maternal folate concentration as the independent variable, the model can be written as follows:

$$\text{SD score of HC} = \beta_0 + \beta_1 \times \text{gawks} + \beta_2 \times \text{folate} + \beta_3 \times \text{gawksxfolate} + \varepsilon$$

(β_0 = intercept; gawks = gestational age in weeks; ε includes additional covariates for the adjusted model and error; similarly, a formula for folic acid supplementation as the independent variable can be defined). The term '$\beta_2$xfolate' tests the difference in intercept for each 1-SD higher folate concentration or comparing the folate-deficient group to the reference group. Gestational age was centered at 20 weeks of gestation in the prenatal models.

Coefficient β_3 reflects the slope (interaction of gestational age and folate) and represents the average decline or increase in head growth per gestational week for each 1-SD higher folate concentration or in children of folate-deficient mothers versus the reference group. A random effects model was applied for the intercept and gestational age to account for within- and between-individual variation. All other covariates were fitted as fixed effects. Longitudinal analyses were additionally adjusted for the effects of maternal ethnicity, smoking and educational level on head growth per week.[28-31]

Missing values of covariates were imputed using the Markov Chain Monte Carlo multiple imputation technique with Predictive Mean Matching for continuous variables (0.1-21.4% missing data). Five imputed datasets were generated. Subsequently, analyses were performed separately on each completed dataset and thereafter combined to one pooled estimate.

RESULTS

Descriptive characteristics of the participating mothers and their children are presented in Table 3.1.1.

Maternal folic acid supplementation and offspring head circumference

Table 3.1.2 shows the analyses of the association of maternal folic acid supplementation with offspring head size and growth. In the basic model, foetuses of mothers who started folic acid supplementation before conception or within 10 weeks of pregnancy had a slightly larger head *size* at 20 weeks of gestation compared with children of mothers who did not use supplements during pregnancy. After adjustment for confounders, this association was only observed in foetuses of mothers who started supplementa-

Table 3.1.1 Subject characteristics by maternal folate deficiency[a] in early pregnancy[b]

	Folate deficient (n = 493)	Normal folate concentrations (n = 5,373)	P^c
Child characteristics			
Gender (% girl)	49.5	47.8	0.50
Gestational age at birth (wk)[d,e]	39.7 ± 2.2	39.9 ± 1.7	< 0.01
Maternal characteristics			
Age at enrolment (yr)	27.0 ± 5.5	30.0 ± 4.9	< 0.001
Gestational age at enrolment (wk)[f]	14.3 ± 2.2	13.5 ± 2.0	< 0.001
Ethnicity (%)			
Dutch	28.9	53.9	
Other Western	7.1	9.0	
Turkish or Moroccan	24.8	13.7	< 0.001
Surinamese or Antillean	21.1	10.6	
Other non-Western	18.1	12.8	
Education (%)			
Higher	14.5	45.8	
Secondary	62.7	44.5	< 0.001
Primary	22.8	9.7	
Family income (%)			
> 2,000 €/mo	23.9	60.7	
1,200 – 2,000 €/mo	25.5	19.6	< 0.001
< 1,200 €/mo	50.6	19.7	
BMI at intake (kg/m²)	25.5 ± 5.0	24.4 ± 4.3	< 0.001
Smoking during pregnancy (%)			
Never	62.3	75.4	
Until pregnancy was known	6.0	8.5	< 0.001
Continued throughout pregnancy	31.7	16.1	

3.1

Table 3.1.1 (continued)

	Folate deficient (n = 493)	Normal folate concentrations (n = 5,373)	P^c
Alcohol consumption during pregnancy (%)			
Never	62.2	43.7	
Until pregnancy was known	13.5	14.1	< 0.001
Continued throughout pregnancy	24.3	42.2	
Parity (% primiparae)	42.8	58.4	< 0.001
Psychopathology in mid-pregnancy[g]	0.42 ± 0.47	0.28 ± 0.37	< 0.001
Folic acid supplement use during pregnancy (%)			
No use	72.5	18.6	< 0.001
Start > 10 wk pregnancy	11.0	13.0	
Start ≤ 10 wk pregnancy	10.0	28.9	
Preconceptional start	6.5	39.5	

[a] Folate deficiency was defined as a folate concentration < 7 nmol/L.
[b] Descriptives on imputed data.
[c] Derived from linear regression analysis for continuous variables or logistic regression analysis for categorical variables; on imputed data.
[d] Means ± SD (all such values).
[e] Median (95% range): deficient group: 39.9 (34.4-42.3); non-deficient group: 40.1 (35.8-42.3).
[f] Median (95% range): deficient group: 14.4 (9.8-17.8); non-deficient group: 13.2 (9.6-17.5).
[g] Median (95% range): deficient group: 0.25 (0.00-1.92); non-deficient group: 0.15 (0.00-1.39).

tion before conception, similar to the previous report.[16] Maternal ethnic background, income, BMI at enrolment and smoking during pregnancy were the main confounders.

We found no evidence for an association between maternal folic acid supplementation during pregnancy and prenatal or postnatal head *growth* in the offspring (Table 3.1.2).

The effect of supplement use could not entirely be attributed to other nutrients in the supplement: foetuses of mothers who started using any supplement before conception had a larger HC *size* at 20 weeks of gestation than those whose mother did not use supplements (fully adjusted models: $B_{\text{'preconceptional start folic acid' vs 'no use'}}$ = 0.131, 95% CI: 0.031;0.232, *P* = 0.01; $B_{\text{'preconceptional start multivitamins' vs 'no use'}}$ = 0.115, 95% CI: 0.009;0.221, *P* = 0.03).

Maternal folate concentrations and offspring head circumference

The first part of Table 3.1.3 shows the analyses of the associations of maternal folate concentrations with offspring head *size*. At 20 weeks of gestation, neither folate SD scores nor folate deficiency were associated with head size.

In the basic linear regression models, higher maternal folate concentration was associated with a larger child head size at 20 and 30 weeks of gestation, at birth, at age 5-10 months and at 6 years (Supplementary table 3.1.1; note: size here in mm). Only at

Table 3.1.2 Maternal folic acid supplementation during pregnancy and offspring head size at 20 weeks of gestation, prenatal head growth and postnatal head growth[a]

Head size (intercept (SDS) at 20 wk gestation) and folic acid supplementation (n = 5,832; 11,323 observations)	Head circumference size (SDS)					
	Basic[b]			Adjusted for covariates[c]		
	SDS[d]	B[e] (95% CI)	P	SDS[d]	B[e] (95% CI)	P
No use[f]	−0.163	Reference		−0.003	Reference	
Start > 10 wk	−0.135	0.028 (−0.069;0.126)	0.57	−0.004	−0.001 (−0.104;0.101)	0.98
Start ≤ 10 wk	−0.075	0.088 (0.011;0.166)	0.03	0.028	0.031 (−0.054;0.115)	0.48
Preconceptional start	0.058	0.221 (0.147;0.296)	< 0.001	0.123	0.126 (0.037;0.215)	< 0.01

Period of head growth and folic acid supplementation	Head circumference growth (SDS)				
	Basic[b]			Adjusted for covariates[c]	
	B[g] (95% CI)	P		B[g] (95% CI)	P
2nd & 3rd trimester (n = 5,832; 11,323 observations)					
No use[f]	Reference			Reference	
Start > 10 wk	0.002 (−0.008;0.012)	0.72		0.001 (−0.009;0.011)	0.84
Start ≤ 10 wk	0.004 (−0.004;0.012)	0.31		0.002 (−0.007;0.010)	0.69
Preconceptional start	0.008 (−0.000;0.016)	0.05		0.002 (−0.007;0.012)	0.65
Birth through 6 yr (n = 5,515; 29,976 observations)					
No use[f]	Reference			Reference	
Start > 10 wk	−0.000 (−0.000;0.000)	0.32		−0.000 (−0.000;0.000)	0.26
Start ≤ 10 wk	−0.000 (−0.000;0.000)	0.45		−0.000 (−0.000;0.000)	0.39
Preconceptional start	−0.000 (−0.000;0.000)	0.15		−0.000 (−0.000;0.000)	0.26

SDS – standard deviation score

[a] Gender and gestational age adjusted foetal and child head circumference SDS.

[b] Model 1: no adjustments.

[c] Model 2: model 1, additionally adjusted for maternal age, ethnicity, education, income, parity, BMI, and psychopathology, smoking and alcohol consumption during pregnancy and the interactions between gestational age and: maternal smoking, ethnicity, and education.

[d] Values represent offspring head size in SDS at 20 wk of gestation (intercept) per group of folic acid supplementation.

[e] Values represent B (95% CI) for the difference in offspring head size (intercept) for each group of folic acid supplementation compared with the reference group of 'no use' from mixed model regression analyses.

[f] Reference group of primiparae women who never smoked or drank alcohol during pregnancy, with mean age of 29.8 yr and mean BMI of 24.5 at enrolment, Dutch national origin, higher education, family income > € 2,000/mo, and median Global Severity Index score of 0.17 in mid-pregnancy.

[g] Values represent B (95% CI) for the interaction between maternal folic acid supplementation and gestational age (in wk) from mixed model regression analyses, i.e. the difference in offspring head circumference growth (in SDS) per wk for each group of folic acid supplementation compared with the reference group of 'no use'.

30 weeks of gestation this association was not accounted for by confounders. When using folate as a dichotomous variable, no associations were found between folate deficiency and offspring head size. Similar results were found using 8 nmol/L to define low maternal folate status.

3.1

Table 3.1.3 Maternal folate concentration[a]/deficiency[b] during pregnancy and offspring head size at 20 weeks of gestation, prenatal head growth and postnatal head growth[c]

Head size (intercept (SDS) at 20 wk gestation) and folate concentration (n = 5,832; 11,323 observations)	Head circumference size (SDS)					
	Basic[d]			Adjusted for covariates[e]		
	SDS[f]	B[g] (95% CI)	P	SDS[f]	B[g] (95% CI)	P
Mean folate[h,i]	−0.055	Reference		0.074	Reference	
Folate (per SD)[j]	−0.021	0.034 (0.007;0.061)	0.01	0.081	0.007 (−0.022;0.037)	0.63
Normal folate[i]	−0.051	Reference		0.077	Reference	
Folate deficient	−0.100	−0.049 (−0.148;0.049)	0.32	0.110	0.033 (−0.068;0.134)	0.52

Period of head growth and folate concentration	Head circumference growth (SDS)				
	Basic[d]			Adjusted for covariates[e]	
	B[k] (95% CI)	P		B[k] (95% CI)	P
2nd & 3rd trimester (n = 5,832; 11,323 observations)					
Folate (per SD)[h,i]	0.006 (0.003;0.009)	< 0.001		0.004 (0.001;0.007)	0.01
Folate deficient	−0.013 (0.023;−0.002)	0.02		−0.007 (−0.018;0.004)	0.19
Birth through 6 yr (n = 5,515; 29,976 observations)					
Folate (per SD)[h,i]	−0.000 (−0.000;0.000)	0.28		−0.000 (−0.000;0.000)	0.66
Folate deficient	0.000 (−0.000;0.001)	0.06		0.000 (−0.000;0.000)	0.29

SDS – standard deviation score

[a] Folate concentration in SDS.

[b] Folate deficiency was defined as a folate concentration < 7 nmol/L. Subjects without folate deficiency comprised the reference group 'Normal folate'.

[c] Gender and gestational age adjusted foetal and child head circumference SDS.

[d] Model 1: adjusted for gestational age at venipuncture.

[e] Model 2: model 1, additionally adjusted for maternal age, ethnicity, education, income, parity, BMI, and psychopathology, smoking and alcohol consumption during pregnancy and the interactions between gestational age and: maternal smoking, ethnicity, and education.

[f] Values represent offspring head size in SDS at 20 wk of gestation (intercept) per type of folate concentration from mixed model regression analyses.

[g] Values represent B (95% CI) for the difference in offspring head size (intercept) for each type of folate concentration compared with its reference ('mean folate' or 'normal folate') from mixed model regression analyses.

[h] Mean folate concentration: 0 SDS ≡ 17.4 nmol/L.

[i] Reference group of primiparae women who never smoked or drank alcohol during pregnancy, with mean age of 29.8 yr and mean BMI of 24.5 at enrolment, Dutch national origin, higher education, family income > € 2,000/mo, and median Global Severity Index score of 0.17 in mid-pregnancy.

[j] Standard deviation folate concentration: 8.8 nmol/L.

[k] Values represent B (95% CI) for the interaction between maternal folate concentration/deficiency and gestational age (in wk) from mixed model regression analyses, i.e. the difference in head circumference growth (in SDS) per wk for each SD increase in folate concentration/between children of mothers with and without folate deficiency.

The associations of maternal folate concentrations with offspring head *growth* are presented in Table 3.1.3. Higher maternal folate concentrations were associated with increased foetal head growth and folate deficiency was associated with decreased foetal head growth in the basic models. These associations were attenuated when fully adjusted for confounders (main confounders: maternal ethnic background, income, BMI at enrolment and smoking during pregnancy). However, foetuses exposed to higher maternal folate concentrations in early pregnancy (continuous measure) showed increased head growth between the second and third trimester of pregnancy: for each 1-SD higher folate concentration, foetal head growth was 0.004 SD per week faster. This results in an average estimated increase of 0.16 SD at birth, which translates into approximately 2.7 mm difference in HC at birth.

No associations were found between maternal folate concentrations or folate deficiency and postnatal head growth. These results did not change after exclusion of the birth-measure of HC, which can be highly imprecise due to the moulding that takes place during the birth process.

DISCUSSION

In this population-based study, we found small effects of maternal folic acid supplementation and folate concentration on head growth in the offspring. Maternal preconceptional start of folic acid supplementation was associated with larger prenatal head *size* at 20 weeks of gestation, but not with prenatal head *growth* after week 20 or postnatal head *growth*. Maternal folate concentrations - serving as nutritional biomarkers - were related to a faster foetal head growth from the second to the third trimester of pregnancy, resulting in a larger head size at 30 weeks of gestation. Although the direction of the association between folate deficiency and foetal head growth was in line with that from analyses with the continuous measure of folate, our results suggest a linear effect of folate on HC rather than extremely low folate concentrations driving the association. No effect of maternal folate on HC was observed after birth.

Folic acid supplementation versus folate concentrations

Folic acid supplement use and folate concentrations are correlated, but their correlation is moderate; the quantitative relationship between supplement and biomarker is influenced by various physiological and environmental factors.[32] Folate concentrations are more directly related to the biological processes in the human body than self-reported folic acid use. As such, they are a more precise measure of actual folate status, allowing for better detection of potential subtle effects. This may explain why maternal folate concentrations were associated with offspring head growth, whereas folic acid supple-

mentation was not. Alternatively, in contrast to the categorical approach of folic acid supplementation, a continuous measure of folate concentration has statistically more power to detect differences.

In addition, although we measured folic acid supplementation retrospectively early in pregnancy, it covers the preconceptional period, whereas folate concentrations were measured around 13 weeks of gestation. Our measurements of HC started at 20 weeks of gestation. Since foetuses of mothers who preconceptionally started folic acid supplementation had a larger HC at 20 weeks of gestation compared to those whose mothers did not use folic acid, the effect of supplementation may already have occurred before 20 weeks of gestation.

Prenatal versus postnatal head growth

We observed an association between maternal folate concentrations and offspring head growth prenatally, but not postnatally. Schlotz et al. reported an association between maternal folate concentration measured at the same gestational age as in the current study (13.5 weeks) and HC at birth, but not 9 months after birth, concluding that maternal folate specifically affects prenatal head growth.[33] Our findings support and extend this conclusion. A plausible explanation may be the relatively close proximity of foetal HC measures to the measure of folate concentrations early in pregnancy, in contrast to the more distant measures of child HC. Additionally, small prenatal effects on head size may be compensated in postnatal life, when other factors can modulate neuro-development and brain plasticity, such as child nutrition and enriched environments. Further, animal studies have shown that folate can affect both embryonic and foetal brain development at various stages, including cell proliferation and differentiation, through epigenomic effects.[34] While the effect sizes we observed are marginal, small effects on head growth during prenatal life may have, for example, an exponential effect on the number of synaptic connections, which can influence the overall connectivity and plasticity potential of the developing brain. Each cortical neuron has on average between 1,000 and 10,000 synapses. Thus, a small reduction in neurons can have a very large effect on the number of connections within the brain. This in turn may predispose the child to cognitive and behavioural impairment later in life. Such subtle changes in brain development, however, cannot be accurately measured postnatally with a simple proxy like HC. To improve measurement accuracy alternative measures, such as brain MRI, will need to be used.

Residual confounding

Residual confounding can never be excluded as an explanation for observed associations in epidemiological studies. A decline in the effect of maternal folate concentration on foetal HC size and growth was noticeable after adjustment for confounders. Biomarkers

providing information about supplement use can be confounded although they do not rely on self-report data and are thus less prone to information bias. Further (residual) confounding, for example in terms of other nutrients in the diet, cannot be excluded.

Strengths and limitations

The strengths of our study are its large sample size, availability of plasma folate concentrations as opposed to dietary intake of folate, having multiple measures of offspring HC (starting in pregnancy) and the ability to adjust for a considerable number of covariates.

   The study also has its limitations. First, our assessment of maternal folate status was based on a single measure in blood plasma. One measurement, although indicative, is not a reliable reflection of a mother's long-term folate status. Additionally, red blood cell instead of plasma folate would be a better measure of long-term folate status. On the other hand, plasma folate can be more reliably measured than red blood cell folate.[35] To identify the most folate-sensitive window of foetal head growth, repeated measures of folate concentrations throughout pregnancy are needed. Further, ultrasound measures provide a global measure of brain growth and development and do not have the contrast to determine differences in cortical and subcortical development, development of the cortical sheet, or myelination. Prenatal MRI measurements could identify whether growth differences are global or involve specific brain regions.

Relevance

Higher maternal folate concentrations were associated with slightly increased foetal head growth from the second to the third trimester. Additionally, preconceptional start of folic acid supplementation was associated with a larger head size at 20 weeks of gestation. In the Netherlands, about half of the women do not use folic acid supplements during the conceptional period.[18,36,37] Median folate intake for women in reproductive age in the Netherlands ranges from 249-282 µg/day.[38] Although folic acid fortification could improve folate status, it would not provide the full requirement. It thus remains important to better educate women of childbearing age about the benefits of folic acid supplementation. Additionally, more research is needed to identify 1) the most folate-sensitive period of foetal head/brain growth, 2) whether specific brain regions are affected and 3) whether the effects of folate on foetal head growth influence children's long-term functioning.

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3.1

Supplementary table 3.1.1 Maternal folate concentration[a]/deficiency[b] during pregnancy and foetal/child head circumference size[c]

Time of HC measurement and folate concentration	Head circumference size (mm)			
	Basic[d]		Adjusted for covariates[e]	
	B (95% CI)	P	B (95% CI)	P
20 wk (n = 5,670)				
Folate (per SD)	0.20 (0.04;0.36)	0.02	0.00 (−0.18;0.18)	0.99
Folate deficient	−0.37 (−0.94;0.21)	0.21	0.22 (−0.38;0.82)	0.47
30 wk (n = 5,653)				
Folate (per SD)	0.94 (0.70;1.18)	< 0.001	0.47 (0.21;0.74)	< 0.001
Folate deficient	−1.84 (−2.72;−0.97)	< 0.001	−0.47 (−1.36;0.41)	0.30
Birth (n = 3,226)				
Folate (per SD)	0.62 (0.06;1.18)	0.03	0.19 (−0.42;0.81)	0.53
Folate deficient	−2.64 (−4.71;−0.58)	0.01	−1.41 (−3.51;0.69)	0.19
3 mo (n = 3,287)				
Folate (per SD)	0.16 (−0.23;0.54)	0.42	0.09 (−0.32;0.51)	0.66
Folate deficient	−0.95 (−2.43;0.53)	0.21	−0.53 (−2.05;0.99)	0.50
5-10 mo (n = 4,132)				
Folate (per SD)	0.47 (0.11;0.84)	0.01	0.37 (−0.03;0.78)	0.07
Folate deficient	−1.48 (−2.91;−0.06)	0.04	−0.83 (−2.31;0.65)	0.27
6 yr (n = 4,036)				
Folate (per SD)	0.69 (0.20;1.17)	< 0.01	0.33 (−0.20;0.85)	0.22
Folate deficient	−0.81 (−2.69;1.07)	0.40	0.18 (−1.76;2.11)	0.86

Note: Values at the 20 week head circumference (HC) measurement differ from those reported in Table 3.1.3, first part: size here in mm instead of standard deviation scores (SDS).

[a] Folate concentration in standard deviation scores, mean folate concentration: 0 SDS ≡ 17.4 nmol/L, 1 SD = 8.8 nmol/L.

[b] Folate deficiency was defined as a folate concentration < 7 nmol/L. Subjects without folate deficiency comprised the reference group 'Normal folate'.

[c] Values represent B (95% CI) for the difference in offspring head size for each type of folate concentration compared with its reference ('mean folate' or 'Normal folate') from linear regression analyses, head circumference in mm.

[d] Model 1: adjusted for gestational age at venipuncture, gender and gestational age of the foetus/child.

[e] Model 2: model 1, additionally adjusted for maternal age, ethnicity, education, income, parity, BMI, and psychopathology, smoking and alcohol consumption during pregnancy.

Chapter 3.2

Maternal folate status in early pregnancy and child emotional and behavioural problems

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ABSTRACT

Background: Maternal prenatal folate status has been linked to neurodevelopmental disorders, but the association with child emotional and behavioural problems is unclear. We assessed the association of maternal folate status during pregnancy with child emotional and behavioural problems. Also, we examined whether any association between folate status and child problems is a consequence of maternal folic acid supplement use or variation in maternal *MTHFR* genotype.

Methods: Within a population-based cohort we measured maternal plasma folate concentrations in early pregnancy and assessed folic acid supplement use by questionnaire. Mothers of European descent were genotyped for the *MTHFR* 677 C>T polymorphism. Child emotional and behavioural problems were assessed with the Child Behavior Checklist at 3 years in 3,209 children.

Results: Children of mothers with prenatal folate deficiency were at higher risk of emotional problems (OR = 1.57, 95% CI: 1.03;2.38), but not behavioural problems (OR = 1.00, 95% CI: 0.64;1.56) after adjusting for confounders. A higher risk of emotional problems was also found in children whose mothers started using folic acid supplements late or did not use supplements at all (OR = 1.45, 95% CI: 1.14;1.84) than in children whose mothers started periconceptionally. However, low plasma folate concentrations only partly explained this association (OR = 1.38, 95% CI: 1.08;1.78). Although related to plasma folate concentrations, maternal *MTHFR* genotype did not explain the association of folate status with offspring emotional problems.

Conclusions: Low maternal folate status during early pregnancy is associated with a higher risk of emotional problems in the offspring.

INTRODUCTION

Folate, a water-soluble B-complex vitamin, is involved in growth and development through two distinct metabolic cycles.[1,2] In one cycle, it is involved in one-carbon transfer reactions for the purpose of DNA synthesis. In the other, folate acts as a methyl donor to the amino acid homocysteine, necessary for methylation of various compounds including DNA, which in turn regulates gene expression. Consequently, homocysteine is inversely related to folate concentration. The enzyme methylenetetrahydrofolate reductase (*MTHFR*) plays a key role in these cycles: it channels one-carbon units away from DNA synthesis and into the methylation cycle. The activity of the enzyme is influenced by the *MTHFR* 677 C>T polymorphism. Carriers of the TT variant have a reduced enzymatic activity. At any level of folate or even folic acid supplement intake these persons have lower folate concentrations than do those carrying the CT or CC variant.[3,4]

During pregnancy, a time of rapid growth, folate demand increases. Reduced maternal folate status, increased homocysteine status and *MTHFR* TT genotype have been associated with increased risk of neural tube defects in foetuses.[5-7] Periconceptional supplementation with folic acid, a synthetic form of folate, has been found to reduce this risk.[8,9]

Prenatal folate status may also affect child behavioural development beyond closure of the neural tube. Few studies examined the effects of prenatal folic acid supplementation on child behavioural development and the findings have been inconsistent. Whereas Julvez et al. found a positive effect on social competence, Wehby and Murray found supplementation to be unfavourable for development of the personal-social domain.[10,11] Previously, we showed in the present cohort, that children of mothers, who took folic acid supplements during pregnancy, were less likely to develop problem behaviour at 18 months.[12] Veena et al.[13] found that higher folate concentrations during pregnancy predicted better childhood cognitive ability. So far, only Schlotz et al.[14] studied the effect of maternal folate concentrations on child problem behaviour. They found that lower prenatal folate status increased the risk of hyperactivity and peer problems in childhood.

Recently, associations between maternal *MTHFR* genotype and prenatal folate and folic acid intake with young children's cognitive[15,16] and behavioural development have been reported. Schmidt et al.[17] found that children of mothers carrying the TT genotype were at higher risk of autism if their mothers did not take vitamins prenatally.

Our objective was to evaluate the effects of maternal plasma folate and homocysteine concentrations in early pregnancy on child emotional and behavioural development. We hypothesised that low maternal folate status in early pregnancy is associated with a higher risk of child emotional and behavioural problems. Also, we examined whether any observed association between folate status and child behaviour can be attributed to maternal folic acid supplement use or explained by variation in maternal *MTHFR* genotype.

3.2

METHODS

Study population

The present study was conducted within the Generation R Study - a population-based cohort from early foetal life onwards in Rotterdam, the Netherlands. The study design was described in detail previously.[18,19] The study was conducted in accordance with the guidelines proposed in the World Medical Association Declaration of Helsinki and was approved by the Medical Ethics Committee at Erasmus Medical Centre, University Medical Centre Rotterdam, the Netherlands. Written consent was obtained from all participants.

Overall, 6,783 women were enrolled in the study during early pregnancy (< 18 weeks gestation). Eligible for the present study were children whose mother enrolled during early pregnancy, gave birth to a liveborn and gave consent for postnatal participation (5,420 women). We measured plasma folate concentration in early pregnancy for 4,934 of these women. We excluded 34 women because their measure of folate was above the assay range. This resulted in 4,900 eligible mothers at baseline.

Mothers' reports on child emotional and behavioural problems at the age of 3 years were available for 3,210 children at follow-up. We excluded 1 child with a Child Behavior Checklist (CBCL) score > 4 SDs in our analytic sample. In total, data for 3,209 children (65%) with maternal folate and behavioural data were included in one or more analyses (Figure 3.2.1).

Folate and homocysteine concentrations

In early pregnancy (median 13.1 weeks of gestation, 90% range 10.5-16.9 weeks) venous samples were drawn and stored at room temperature before being transported to the regional laboratory for processing and storage for future studies. Processing was aimed to finish within a maximum of three hours after venipuncture. The samples were centrifuged and thereafter stored at −80°C.[20] To analyse folate and homocysteine concentrations, EDTA plasma samples were picked and transported to the Department of Clinical Chemistry of the Erasmus Medical Centre, Rotterdam in 2008. After being thawed, folate and homocysteine concentrations were analysed by using an immunoelectrochemoluminence assay on the Architect System (Abott Diagnostics B.V.). The between-run CV depending on folate and homocysteine concentration varied between 1.5%-8.9% and 2.1%-3.1% respectively. Analytic ranges for folate and homocysteine concentration were 1.8-45.3 nmol/L and 1-50 µmol/L respectively.

In the study population of 3,209 mothers and children, plasma homocysteine concentrations and information on folic acid supplementation were available in 98.7% and 90.0% of the mothers, respectively.
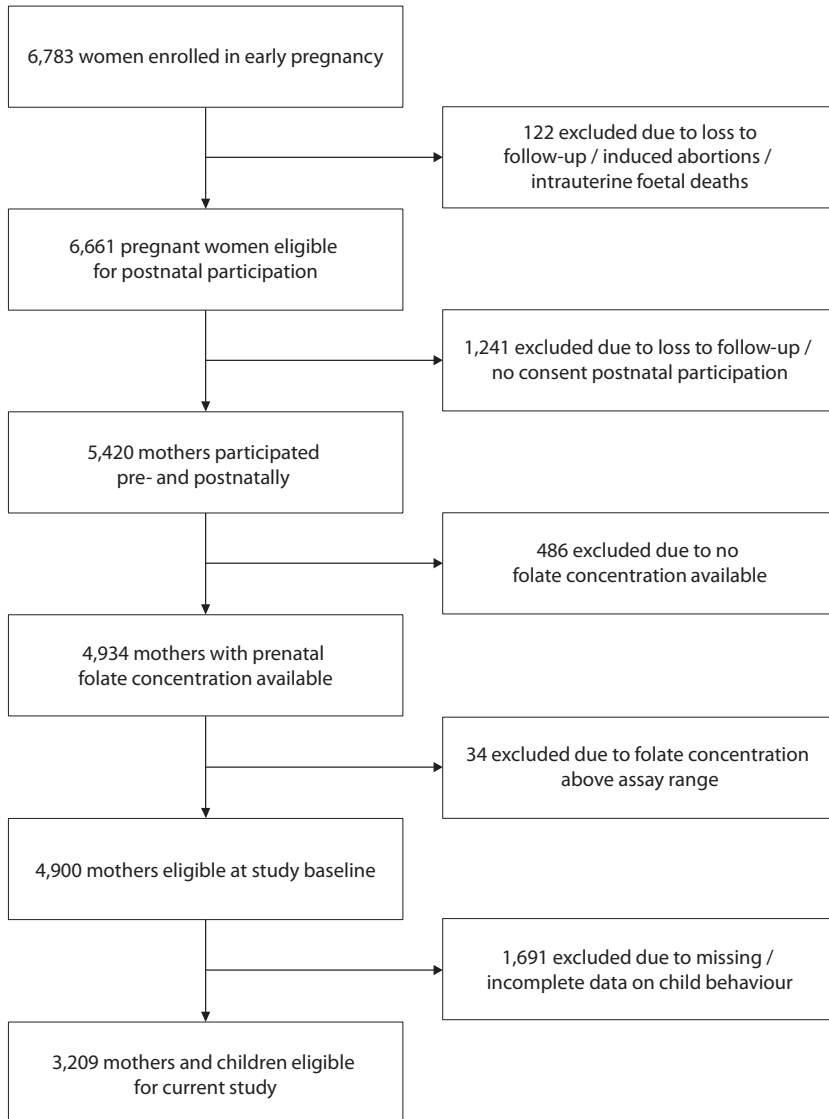
Figure 3.2.1 Flow chart of study population

Maternal folic acid supplement use

Early in pregnancy (gestational age < 18 weeks), pregnant women were asked by questionnaire whether they used folic acid supplements or multivitamin preparations and when supplementation was started. In the Netherlands, supplements for pregnant women contain 0.4–0.5 mg of folic acid. According to the advice of the Health Council of the Netherlands[21], we categorised folic acid supplement use in the following groups: 1) adequate use (preconceptional start or start within the first 10 weeks of pregnancy)

and 2) inadequate use (start after the first 10 weeks of pregnancy or no use during pregnancy). Also, categorisation was in line with two previous publications from the Generation R Study, but we retained the group 'start after the first 10 weeks of pregnancy' in the analyses.[12,22]

MTHFR genotyping

Maternal DNA was derived from blood samples in early pregnancy. Genotyping of the *MTHFR* 677 C>T polymorphism (rs1801133) was performed by using TaqMan allelic discrimination assay (Applied Biosystems) and Abgene QPCR ROX mix (Abgene). The genotyping reaction was amplified using the GeneAmp® PCR system 9600 (95°C (15 min), then 40 cycles of 94°C (15 s) and 60°C (1 min)). The fluorescence was detected on the 7900HT Fast Real-Time PCR System (Applied Biosystems), and individual genotypes were determined by using SDS software (version 2.3; Applied Biosystems). Child DNA and *MTHFR* genotype data were available but not analysed, because we expected no relation with maternal plasma folate concentrations.[23] Only data from women of European descent (n = 2,348) were used in the current study, because of the differences in both *MTHFR* allele frequencies and prevalence of child emotional and behavioural problems among women of various national origins. European descent was based on self-report and defined as having 2 parents born in a European country.[18] *MTHFR* genotype was available for 89.6% of women of European descent.

Child emotional and behavioural problems

Mothers were asked to fill out the Child Behavior Checklist for toddlers (CBCL 1½-5) when their child was 3 years of age. The CBCL is a self-administered parent-report questionnaire to measure the degree of emotional and behavioural problems of children. It contains 99 problem items rated on a 3-point scale (0 = not true, 1 = somewhat or sometimes true, and 2 = very true or often true), based on the preceding two months. In the current analyses, we focused on emotional problems and behavioural problems. Emotional problems comprise emotionally reactive and anxious/depressed symptoms, and somatic complaints and symptoms of being withdrawn. Behavioural problems comprise attention problems and aggressive behaviour. Good reliability and validity have been reported for the CBCL.[24]

Covariates

Several maternal characteristics were considered as possible confounding variables, based on studies of folate concentration, folic acid supplement use, and child behavioural development.[25-29] Maternal BMI (in kg/m^2) in early pregnancy was calculated from length (m) and weight (kg) measured at enrolment. Information on maternal age, national origin, educational level, psychopathology, parity, marital status, pregnancy plan-

ning, smoking and alcohol consumption was obtained by questionnaire. We classified national origin into five categories: 1) Dutch, 2) other Western, 3) Turkish or Moroccan, 4) Surinamese or Antillean, or 5) other non-Western. Educational level was assessed by the highest completed education and classified into three categories: 1) primary school; 2) secondary school; and 3) higher education. We used the Brief Symptom Inventory to assess maternal psychopathology in mid-pregnancy (gestational age 18-25 weeks).[30,31] The scores on the Global Severity Index (overall psychopathology) and on depressive symptoms were used as continuous variables. Maternal smoking in early pregnancy was categorised into 'yes' or 'no use'. Alcohol consumption in early pregnancy was defined as 'one or more glasses per week' compared with 'less than one alcoholic beverage per week'. The latter group includes both non-drinkers and women who drank alcoholic beverages very occasionally.

Date of birth, birth weight, and gender of the infant were obtained from midwife and hospital registries at birth. Foetal ultrasound examinations were carried out in early pregnancy to establish gestational age at the time the venous samples were drawn and at birth.

3.2

Statistical analyses

We used the CBCL emotional and behavioural problem scores as outcome variables. To facilitate a clinical interpretation of problem behaviour, and because the CBCL scores were not normally distributed, we analysed these scores as dichotomous variables. As in previous analyses, we defined a non-optimal score as the highest 20% of emotional and behavioural item scores.[32,33]

Multiple logistic regression analyses were used to test for associations between maternal plasma folate concentration and child emotional and behavioural problems. First, we used plasma folate concentration as a continuous variable in the equation (nmol/L per SD). To illustrate a possible dose response relationship for any association with child emotional and behavioural problems, we then divided plasma folate concentrations by quintiles. Next, we tested for a threshold effect of folate deficiency, defined as plasma folate concentrations < 7 nmol/L (n = 131).[34] Persons with a plasma folate concentration above this cut-off were classified in the reference group, further referred to as 'normal folate concentration'.

In a similar way, we tested for associations between maternal plasma homocysteine concentration and child emotional and behavioural problems. We tested a threshold effect at a cut-off of 11 µmol/L (2.6% of the study population)[35], and as a lower threshold of 9.1 µmol/L (10% of the study population), using multiple logistic regression analyses.

Logistic regression analysis was also used to test for an association between prenatal folic acid supplement use and child emotional and behavioural problems, with mothers who started folic acid supplementation periconceptionally as the reference category.

Finally, we used a Mendelian Randomisation approach, that is, we aimed to test whether maternal plasma folate concentration is causally associated to child emotional and behavioural problems, using the maternal *MTHFR* genotype as a proxy for maternal plasma folate concentration. If a low plasma folate concentration during pregnancy is a cause of emotional and behavioural problems, then children of mothers, who have genetic variants associated with lower plasma folate concentrations (T allele), are at higher risk of emotional and behavioural problems. Logistic regression analyses were used to test for a main effect of maternal *MTHFR* genotype (CC genotype as reference) on child emotional and behavioural problems and for possible mediation of this effect by maternal plasma folate concentration.

Next, we tested an interaction effect and stratified the sample by genotype to test whether any association between maternal plasma folate concentration and child emotional and behavioural problems depended on maternal *MTHFR* genotype.

All analyses of maternal plasma folate concentration and child emotional and behavioural problems were controlled for gestational age at the time of venipuncture, gender of the child and the age of the child at the time of the measurement (CBCL). Other covariates were selected and included in the models if they changed the effect estimates meaningfully (defined as > 5%).[36] After this change-in-estimate criterion, maternal age, national origin, educational level, psychopathology, parity, marital status and pregnancy planning were included in the present analyses. In contrast, child birth weight and maternal smoking, alcohol consumption and BMI in early pregnancy were not included in the final models. Percentages of missing values on covariates ranged from 0.03% to 11.4% (average: 4.4%). Given these low levels of missingness, the mean value was imputed for missing values on continuous variables. For missing values on a categorical variable, a separate 'missing' category was included in the regression analyses. Measures of association are presented with 95% CIs. Statistical analyses were carried out by using PASW Statistics, version 17.0 for Windows (SPSS, Inc.).

Response analyses

Analyses of missing data showed that, compared with children with behavioural data (n = 3,209), children without behavioural data (n = 1,757) had a shorter gestation [median of 40.0 weeks (95% range: 35.0–42.3 weeks) compared with 40.1 weeks (95% range 35.6–42.4 weeks); *P* < 0.001] and, on average, a 113 gram (95% CI: 80;146, t = 6.7) lower birth weight.

The mothers of children not included used folic acid supplements less often during early pregnancy [52.7% compared with 76.2%, χ^2 (1) = 249, *P* < 0.001] and on average had a 4.0 nmol/L (95% CI: 3.5;4.5, t = 15.3) lower folate and 0.2 µmol/L (95% CI: 0.1;0.4, t = 3.3) higher plasma homocysteine concentration in their first trimester of pregnancy. They were, on average, 2.9 (95% CI: 2.6;3.2, t = 19.6) years younger, less often of Dutch

origin [35.7% compared with 66.4%, χ^2 (4) = 485, P < 0.001], and less educated [15.5% compared with 5.2%, χ^2 (2) = 431, P < 0.001].

RESULTS

Descriptive characteristics of the participating mothers and their children are presented by maternal folate deficiency in Table 3.2.1. Children of mothers with folate deficiency in early pregnancy had a lower birth weight than did children of mothers with normal folate status. Folate-deficient mothers were younger, were more often of non-Dutch origin, were less educated, and had higher psychopathology and depression scores than did mothers with a normal plasma folate concentration. They also differed on alcohol consumption in early pregnancy, pregnancy planning, marital status and parity. Folate-deficient mothers used folic acid or multivitamin supplements less often. In total, 2,485 (78%) of mothers in the study used supplemental folate. Of these, 766 (31%) used folate as a multivitamin supplement.

3.2

Table 3.2.1 Subject characteristics by maternal folate deficiency[a] in early pregnancy[b]

	Folate deficient (n = 131)	Normal folate concentrations (n = 3,078)	P
Child characteristics			
Female (%)	45.0	50.6	0.22
Gestational age at birth (wk)	40.1 (34.2-42.2)	40.1 (35.7-42.4)	0.77
Birth weight (g)	3,267 ± 600	3,464 ± 562	< 0.001
Age at 3 yr assessment (mo)	36.4 (35.5-44.4)	36.2 (35.5-40.2)	0.05
Maternal characteristics			
Age at enrolment (yr)	28.5 ± 5.5	31.3 ± 4.3	< 0.001
National origin (%)			
Dutch	40.6	67.3	
Other Western	8.6	8.9	
Turkish or Moroccan	18.8	8.1	< 0.001
Surinamese or Antillean	12.5	6.4	
Other non-Western	19.5	9.3	
Education (%)			
Primary	15.9	4.8	
Secondary	54.8	35.1	< 0.001
Higher	29.4	60.1	
Psychopathology in mid-pregnancy	0.21 (0.00-1.25)	0.15 (0.00-1.04)	0.02

Table 3.2.1 (continued)

	Folate deficient (n = 131)	Normal folate concentrations (n = 3,078)	P
Depression in mid-pregnancy	0.08 (0.00-1.30)	0.00 (0.00-1.17)	< 0.01
Smoking in early pregnancy (% yes)	22.9	21.0	0.63
Alcohol consumption in early pregnancy (% ≥ 1 glass/wk)	14.3	25.8	< 0.01
BMI at enrolment (kg/m^2)	24.3 ± 3.7	24.1 ± 4.0	0.56
Pregnancy planned (% yes)	66.1	81.9	< 0.001
Marital status (% cohabitation)	82.4	92.5	< 0.001
Parity (% primiparae)	46.9	61.2	0.001
Folic acid only supplement use (% yes)	30.6	61.3	< 0.001
Multivitamin use (% yes)	3.6	27.7	< 0.001

[a] Folate deficiency is defined as a plasma folate concentration < 7 nmol/L.
[b] Values are means ± SD for continuous, normally distributed variables, medians (95% range) for continuous non-normally distributed variables, and percentages for categorical variables. *P*-values are derived from independent t-tests for continuous normally distributed variables, Mann-Whitney U tests for continuous non-normally distributed variables, or chi-square tests for categorical variables.

Maternal plasma folate concentration and child emotional and behavioural problems

The associations between maternal plasma folate concentration and child emotional and behavioural problems are presented in Table 3.2.2. We found no linear association of maternal plasma folate concentration with child emotional (OR = 0.94, 95% CI: 0.85;1.05) or behavioural (OR = 1.02, 95% CI: 0.92;1.13) problems after confounders were accounted for. The association is illustrated by dividing plasma folate concentration by quintiles. The odds of developing emotional and behavioural problems is only increased in children of mothers with a plasma folate concentration in the lowest quintile compared with children of mothers with a plasma folate concentration in the highest quintile; however, after adjustment for confounders the associations were no longer statistically significant. Maternal national origin, educational level and psychopathology were the most important confounders.

Next, we tested the association of maternal folate deficiency (< 7 nmol/L; 4% of the study population) during pregnancy with child emotional or behavioural problems. The higher risk of emotional problems in the children of folate-deficient mothers remained significant after adjustment for confounders (OR = 1.57, 95% CI: 1.03;2.38). In a post hoc analysis, we also defined the lowest 10% plasma folate concentrations (≤ 8.5 nmol/L) as the low folate concentration to further test a threshold effect. We found consistent results for the association with child emotional problems in the fully adjusted model (OR = 1.39, 95% CI: 1.04;1.86), which is in line with a dose-response effect in the lower tail of the folate distribution.

No associations were found between maternal plasma folate concentration and child behavioural problems (Table 3.2.2).

As expected, plasma folate and homocysteine concentrations were negatively correlated (r = −0.21, P < 0.001). However, the plasma homocysteine concentration was, neither as a continuous nor as a dichotomous variable, related to child emotional and behavioural problems (data not shown). We therefore did not further examine any

Table 3.2.2 Associations of maternal, prenatal folate concentration with child emotional and behavioural problems at 3 years[a] (n = 3,196)

	Emotional problems			
	Basic[b]		Adjusted[c]	
Folate concentration[d]	OR (95% CI)	P	OR (95% CI)	P
Linear				
Folate (per SD)	0.82 (0.75;0.90)	< 0.001	0.94 (0.85;1.05)	0.26
Quintiles (Q)				
Q5 (≥ 27.41; n = 640)	Reference		Reference	
Q4 (21.41-27.40; n = 639)	0.96 (0.71;1.30)	0.81	0.97 (0.71;1.33)	0.84
Q3 (16.11-21.40; n = 636)	0.93 (0.69;1.27)	0.66	0.94 (0.69;1.29)	0.71
Q2 (11.01-16.10; n = 637)	1.19 (0.89;1.60)	0.24	1.00 (0.74;1.37)	0.99
Q1 (≤ 11.00; n = 644)	1.70 (1.28;2.25)	< 0.001	1.13 (0.83;1.55)	0.43
Folate deficient				
No (≥ 7; n = 3,065)	Reference		Reference	
Yes (< 7; n = 131)	2.06 (1.41;3.03)	< 0.001	1.57 (1.03;2.38)	0.03
	Behavioural problems			
	Basic[b]		Adjusted[c]	
Folate concentration[d]	OR (95% CI)	P	OR (95% CI)	P
Linear				
Folate (per SD)	0.92 (0.84;1.01)	0.09	1.02 (0.92;1.13)	0.69
Quintiles (Q)				
Q5 (≥ 27.41; n = 640)	Reference		Reference	
Q4 (21.41-27.40; n = 639)	1.13 (0.84;1.50)	0.42	1.13 (0.84;1.51)	0.43
Q3 (16.11-21.40; n = 636)	0.88 (0.65;1.18)	0.39	0.87 (0.64;1.18)	0.36
Q2 (11.01-16.10; n = 637)	1.14 (0.86;1.52)	0.37	1.02 (0.76;1.37)	0.90
Q1 (≤ 11.00; n = 644)	1.38 (1.04;1.83)	0.03	1.03 (0.76;1.40)	0.86
Folate deficient				
No (≥ 7; n = 3,065)	Reference		Reference	
Yes (< 7; n = 131)	1.30 (0.85;1.97)	0.22	1.00 (0.64;1.56)	0.99

[a] Values were derived from linear and non-linear logistic regression analyses.
[b] Model 1: adjusted for gestational age at venipuncture, gender and age child at time of measurement.
[c] Model 2: adjusted as for model 1 plus maternal national origin, educational level and age, psychopathology in mid-pregnancy, parity, marital status and pregnancy planning.
[d] Folate concentrations in nmol/L.

3.2

mediating effect of plasma homocysteine concentrations in the association between maternal plasma folate concentrations and child emotional problems.

Maternal folic acid supplementation and child emotional problems

Next, we studied whether maternal plasma folate concentrations mediate the association between folic acid supplementation in pregnancy and child emotional problems. An overview of plasma folate and homocysteine concentrations for each folic acid supplementation category is shown in Table 3.2.3. As expected, the mean plasma folate concentration was lower if folic acid supplementation started later in pregnancy (overall $P < 0.001$). Plasma folate concentrations were lowest for mothers who did not use folic acid supplements at all. Similarly, but less clear, plasma homocysteine concentrations were higher if supplementation started later in pregnancy.

We found a higher risk of emotional problems at age 3 years in children, whose mothers reported any form of inadequate folic acid supplement use (OR = 1.45, 95% CI: 1.14;1.84), than in children of mothers who reported adequate use. Adjustment for plasma folate concentration attenuated the association, but results remained significant (OR = 1.38, 95% CI: 1.08;1.78).

Table 3.2.3 Maternal folate and homocysteine concentrations in early pregnancy per folic acid supplementation category[a] (n = 2,860)

Folic acid supplementation[b]	Folate concentration in nmol/L[c]	Homocysteine concentration in µmol/L[c]
Adequate use[d]		
Start preconceptionally (n = 1,358)	23.8 ± 0.96	6.6 ± 0.24
Start ≤ 10 wk (n = 822)	20.2 ± 0.96	6.8 ± 0.24
Inadequate use		
Start > 10 wk (n = 305)	18.7 ± 1.02	7.1 ± 0.26
No use (n = 375)	12.0 ± 0.98	7.8 ± 0.25
Overall P	< 0.001	< 0.001

[a] Values are means ± SDs from univariate ANOVA.
[b] Mean gestational age at venipuncture = 13.3 wk.
[c] Adjusted for gestational age at venipuncture, maternal national origin, education and age.
[d] Based on Dutch recommendations.

Maternal *MTHFR* and child emotional and behavioural problems

Furthermore, we examined whether genetic factors are at the root of the association of maternal folate status with child emotional and behavioural development. An overview of mean plasma folate and homocysteine concentrations per *MTHFR* genotype for women of European descent is shown in Table 3.2.4. *MTHFR* genotype and allele frequencies were in Hardy Weinberg equilibrium (*P* = 0.49). Plasma folate concentrations decreased

Table 3.2.4 Maternal folate and homocysteine concentrations in early pregnancy per *MTHFR* genotype[a] (n = 2,103)

MTHFR genotype[b]	Folate concentration in nmol/L[c]	Homocysteine concentration in μmol/L[c]
CC (n = 945)	20.9 ± 0.27	7.0 ± 0.06
CT (n = 919)	20.4 ± 0.27	7.2 ± 0.06
TT (n = 239)	18.8 ± 0.53	7.4 ± 0.12
P for trend	0.001	0.02

MTHFR – methylenetetrahydrofolate reductase
[a] Values are means ± SDs from univariate ANOVA.
[b] Mean gestational age at venipuncture = 13.2 wk. *MTHFR* genotype for women of European descent only.
[c] Adjusted for gestational age at venipuncture.

and homocysteine concentrations increased in an allele-dependent dose response (*P* for trend = 0.001).

The results of a logistic regression analysis of maternal *MTHFR* genotype on child emotional as well as behavioural problems, with use of the CC genotype as the reference group, are shown in Table 3.2.5. We found no evidence of an association between maternal *MTHFR* genotype and the offspring's behaviour (*P* > 0.10 for all comparisons). Similarly, we found no evidence that the effect of plasma folate concentrations on child behaviour depended on maternal genotype (*P*-interaction = 0.14, detailed data not shown). However, the latter analysis was underpowered because only 19 children were exposed to low plasma folate concentrations in the group of mothers carrying the TT variant.

3.2

Table 3.2.5 Associations of maternal *MTHFR* genotype with child emotional and behavioural problems at 3 years[a] (n = 2,128)

	Emotional problems				
	Basic[b]			Adjusted[c]	
MTHFR genotype[d]	OR (95% CI)	*P*		OR (95% CI)	*P*
CC (n = 956)	Reference			Reference	
CT (n = 930)	0.88 (0.71;1.09)	0.24		0.88 (0.70;1.09)	0.23
TT (n = 242)	1.02 (0.73;1.43)	0.89		1.02 (0.73;1.42)	0.92
	Behavioural problems				
	Basic[b]			Adjusted[c]	
MTHFR genotype[d]	OR (95% CI)	*P*		OR (95% CI)	*P*
CC (n = 956)	Reference			Reference	
CT (n = 930)	0.91 (0.73;1.15)	0.43		0.91 (0.73;1.15)	0.43
TT (n = 242)	1.16 (0.83;1.63)	0.39		1.16 (0.83;1.63)	0.40

MTHFR – methylenetetrahydrofolate reductase
[a] Values were derived from linear and non-linear logistic regression analyses.
[b] Model 1: adjusted for child gender and age at time of measurement.
[c] Model 2: adjusted as for model 1 plus plasma folate concentration.
[d] *MTHFR* genotype for women of European descent only.

DISCUSSION

This population-based study showed that low maternal folate status in early pregnancy, but not high homocysteine status, is associated with increased risk of emotional problems in the offspring. Low maternal plasma folate concentrations partly explained the association of folic acid supplementation with child emotional problems. Even though plasma folate concentration was related to *MTHFR* genotype, we found no association between maternal *MTHFR* genotype and child emotional and behavioural problems.

Maternal plasma folate concentration and child emotional and behavioural problems

Several potential mechanisms may underlie the effect of low folate status during pregnancy on child emotional problems.

First, low maternal plasma folate concentrations directly limit the availability of folate to the foetal cells. This affects de novo DNA synthesis, which results in impairment of cell division.[1] Moreover, neurodevelopment continues after closure of the neural tube. Craciunescu et al.[37] reported that folate deficiency in pregnant dams decreased mitosis and increased apoptosis in mouse foetal forebrain during late gestation.

Second, folate is a methyl donor. Low folate availability allows homocysteine status to increase. Possible biological effects of high homocysteine concentration include reduced methylation reactions, such as DNA hypomethylation. Research in epigenetics has shown that DNA methylation is a candidate mechanism by which inutero conditions affect later health[38], particularly the risk of neural tube defects.[39,40] We did not find an effect of increased maternal plasma homocysteine concentrations on child behaviour. However, usually concentrations > 11-15 μmol/L are used to define hyperhomocysteinemia.[35] Plasma concentrations in our study sample were, even among mothers who did not use folic acid supplements, only 7.8 μmol/L on average, and thus were probably not high enough to find an effect.

We found an effect of low plasma folate concentrations almost exclusively on emotional problems. If not a chance finding, it may reflect that low socioeconomic status and poverty play a more important role in determining behavioural as compared to emotional problems in children.[41] Alternatively, this reflects the fact that emotional problems are more reliable at young ages. Emotional problems in toddlers have been found to be more stable as compared with behavioural problems.[24]

Maternal folic acid supplementation and child emotional problems

We previously reported a protective effect of folic acid supplementation on child emotional and behavioural problems at 18 months. Our current findings confirm these results in the same cohort of children for emotional problems at age 3 years. Above

all, by including prenatal plasma folate concentrations in our analyses, we were able to investigate to what extent folate status accounts for the effect of folic acid supplementation on child behaviour. On the one hand, the results indicate that the use of folic acid supplements helps pregnant women to decrease the risk of emotional problems in the offspring. On the other hand, the effect could certainly not be entirely attributed to maternal folate status. Our assessment of folate status was based on a single measure in early pregnancy in close temporal proximity to the assessment of folic acid supplement use. One measurement, although indicative, is not a reliable reflection of a mother's long-term folate status. Although plasma folate concentrations corresponded to the degree of compliance with supplementation recommendations, fluctuations in concentrations most certainly occurred. Also, about 31% of the women used folic acid as part of a multivitamin supplement. Hence, we cannot rule out that other nutrients contributed to the effect ascribed to folic acid supplements.[42]

Maternal *MTHFR* and child emotional and behavioural problems

We found no effect of maternal *MTHFR* genotype on child emotional and behavioural problems, even though *MTHFR* was related to plasma folate concentration. A first explanation may be that we measured common, nonclinical problems. Previously reported associations for maternal *MTHFR* genotype concerned disorders such as Down syndrome and Schizophrenia[43,44], whereas Glaser et al.[45] found no association between maternal *MTHFR* and adolescent's non-clinical psychotic symptoms.

Second, the current study can probably not be viewed as a prototypical test of Mendelian Randomisation. Genetic variants associated with a difference in intermediate phenotypes such as plasma folate concentrations, typically index lifetime differences in exposure.[46] In the current study, we were interested in the effect of maternal plasma folate concentrations during pregnancy on emotional and behavioural development in preschool children. Possibly, the effect of very low maternal plasma folate concentrations during this crucial period of neurodevelopment cannot be contrasted with the impact of common genetic variations, which typically sort their (small) effect via continuous differences in the intermediate phenotype. Furthermore, the child genotype, which is certainly more relevant than the maternal genotype once the child is born, cannot easily be used to model early intrauterine effects of low plasma folate concentrations.

Possibly, the maternal *MTHFR* genotype plays a less pronounced role in the DNA synthesis pathway through which low maternal folate concentrations may exert an effect on the development of child emotional problems. In contrast, there may be a more prominent role for the *MTHFR* genotype in the methylation pathway, in which it - in combination with low folate concentrations – can expose the embryo to higher homocysteine concentrations.[2,47] Our study, however, was underpowered to detect such an effect because of the small number of TT carriers who were also exposed to low plasma folate concentrations.

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Strengths and limitations

The strengths of our study were its large sample size, the combination of data on plasma folate concentration measured early in pregnancy, folic acid supplementation and *MTHFR* genotype, and the ability to adjust for considerable numbers of covariates. The study also has its limitations. Selective attrition may have influenced our results. Mothers of children not included in general had lower folate concentrations and less favourable socioeconomic circumstances. This selective attrition can only lead to bias if exposed children not included in the study had different levels of behavioural problems. Furthermore, as mentioned earlier, we only had one measure of maternal plasma folate concentration.

Next, in observational studies, residual confounding can never be excluded. A decline in the effect of maternal plasma folate concentration on the offspring's emotional problems was noticeable after adjustment for several maternal characteristics. This shows that biomarkers themselves can be confounded. It also stresses the substantial role of confounding factors for both maternal folic acid supplement use and plasma folate concentration in their relation to emotional and behavioural problems in the offspring. Further (residual) confounding, for example in terms of behavioural or other lifestyle factors, cannot be excluded. Possibly, not only high socioeconomic status, but also better home environment or other indicators of social status, which are associated with higher plasma folate concentrations, account for the association with child emotional problems.

Relevance

Our large population-based study suggests a role of maternal prenatal folate status in the offspring's behavioural development. In the Netherlands, women of reproductive age are advised to take daily folic acid supplements ≥ 4 weeks before conception until 8 weeks after conception.[21] However, although campaigns to promote folic acid intake in the Netherlands increased supplement use, still about half of the women do not use folic acid supplements in the conceptional period.[21,48,49] Folic acid fortification could improve folate status in women of childbearing age. However, fortification alone would not provide the full requirement. It thus remains important to better educate women of childbearing age about folic acid supplement use.

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3.2

Chapter 3.3

Folate concentrations during pregnancy and autistic traits in the offspring

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ABSTRACT

In a population-based study, we examined the associations of maternal plasma folate concentrations at 13 weeks of gestation and prenatal folic acid supplement use with autistic traits in the offspring at age 6 years. Parent-reported autistic traits were assessed using the Social Responsiveness Scale short form. Maternal folate was not associated with autistic traits in the offspring. In contrast, prenatal folic acid use was associated with less child autistic traits. Future research should focus on the timing of the potential effect of prenatal folate on the development of autistic traits in combination with clinical diagnosis of autism in the offspring.

INTRODUCTION

For decades, scientists have been trying to unravel the aetiology of autism spectrum disorders (ASD). Despite advances in genetic research, the causes of ASD remain unclear.[1] As a result, research into environmental risk factors for autism has increased, with particular interest for risk factors during the prenatal period. Recently, strong evidence has been found for an association of periconceptional folic acid supplement use with reduced ASD risk in the offspring.[2] However, to further support a potential relationship between folic acid supplement use and ASD, nutritional biomarkers need to be examined in order to gain insight in the underlying mechanism.

In this prospective population-based study, we addressed this issue by exploring whether folate concentration in pregnancy predicted autistic traits in the offspring at 6 years of age. We hypothesised that higher maternal folate concentrations are associated with less autistic traits in the offspring.

METHODS

Within the Generation R Study, a population-based birth cohort in Rotterdam, the Netherlands[3], we measured maternal plasma folate concentrations in early pregnancy (median: 13.2 weeks of gestation; 90% range: 10.5–17.2 weeks). In line with previous studies, we excluded mothers of multiple births and those of neonates with a gestational age < 32 weeks at birth or a birth weight < 2,500 grams to isolate folate exposure from other exposure reported to increase the risk of ASD.[4] As a result, valid folate concentrations were available in 5,591 mothers of single liveborn neonates. Folate concentration was used as a continuous variable in the analyses (nmol/L per SD) and as a dichotomous variable, dichotomised at the level of deficiency (<7 nmol/L[5], n = 214). Additionally, folic acid supplementation was assessed by questionnaire early in pregnancy. In line with previous publications, folic acid supplement use was categorised in four groups: i) preconceptional start, ii) start within the first 10 weeks of pregnancy, iii) start after the first 10 weeks of pregnancy, and iv) no use (reference category).[6] Folate concentrations in mothers who started supplementation preconceptionally were higher than in non-users (22.6 versus 10.0 nmol/L, t = 46.6, $P < 0.001$). Information on autistic traits was available in 3,893 (70%) children at the age of 6 years.

We assessed parent-reported autistic traits using the Social Responsiveness Scale (SRS) short form (median score: 0.17, 95% range: 0.00-0.88) and the Pervasive Developmental Problems (PDP) subscale (median score: 2.00, 95% range: 0.00-8.74) of the Child Behavior Checklist[7] (mean age = 6.2 ± 0.5 years). SRS scores were transformed by square root and analysed continuously. To facilitate clinical interpretation, we defined a probable autistic

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child using stringent criteria [PDP score > 98th percentile and SRS score in the top 5% of the sample (n = 72, 1.8%)] in line with previous studies.[7]

Multivariate linear regression was used to explore associations of both maternal folate concentrations and folic acid supplementation with children's autistic traits (SRS scores). The odds of being a probable autistic child with increase in maternal folate concentration were calculated using multivariate logistic regression. Basic models were adjusted for gestational age at venipuncture, and gender and age of the child at assessment. Parental ethnicity, education, age and psychopathology during pregnancy, family income, marital status, pregnancy planning, and maternal smoking and alcohol consumption during pregnancy, parity and pre-pregnancy BMI were included as confounders in fully adjusted models. Step-wise forward regression was used to explore to which confounders the attenuation in effect could be attributed.

Missing values on folic acid supplementation (10.2%), covariates (0.2-26.8%) and child outcomes (SRS = 18.3%; PDP = 3.8%) were imputed using the Markov Chain Monte Carlo multiple imputation technique, generating 5 datasets. A complete overview of the population characteristics and percentages of missing values for all used variables is given in Supplementary table 3.3.1. Analyses were conducted using Stata version 12.0 (StataCorp). The study was approved by the Medical Ethics Committee at Erasmus MC, Rotterdam, the Netherlands.

RESULTS

We found that children of mothers who started using folic acid supplements before conception had lower scores on autistic traits than children whose mother did not use folic acid supplements ($B_{\text{'preconceptional start' vs 'no use'}}$ = −0.129, 95% CI: −0.155;−0.102, $P < 0.001$). This association attenuated, but remained statistically significant after adjusting for confounders (fully adjusted model: $B_{\text{'preconceptional start' vs 'no use'}}$ = −0.042, 95% CI: −0.068;−0.017, $P = 0.001$). Similar results were found for the other two groups of folic acid supplement use (fully adjusted model: $B_{\text{'start < 10 weeks' vs 'no use'}}$ = −0.041, 95% CI: −0.066;−0.016, $P = 0.001$; $B_{\text{'start > 10 weeks' vs 'no use'}}$ = −0.057, 95% CI: −0.089;−0.025, $P = 0.001$).

For maternal folate concentrations, no association with child autistic traits was found (fully adjusted model: B = −0.004, 95% CI: −0.013;0.004, $P = 0.30$). The initial association in the basic model disappeared after joint inclusion of maternal prenatal psychopathology, maternal education and family income (Table 3.3.1). Similarly, we did not find evidence for an association of extreme low maternal folate concentrations (folate deficiency) with child autistic traits (fully adjusted model: B = 0.022, 95% CI: −0.014;0.058, $P = 0.22$). Analyses using a different cut-off (8.1 nmol/L, i.e. lowest 10% in study sample) showed similar results.

Table 3.3.1 Maternal folate concentration during pregnancy and child autistic traits at 6 years (n = 3,893)

	Autistic traits[a]	
	Model 1[b] B (95% CI)	Model 2[c] B (95% CI)
Folate (per SD)[d]	−0.026 (−0.035;−0.017)[e]	−0.007 (−0.016;0.001)[f]
Maternal psychopathology		0.092 (0.058;0.127)
Maternal education		
Higher		Reference
Secondary		0.058 (0.038;0.078)
Primary		0.121 (0.082;0.160)
Family income		
> 2,000 €/mo		Reference
1,200-2,000 €/mo		0.035 (0.013;0.057)
< 1,200 €/mo		0.077 (0.037;0.118)

[a] Social Responsiveness Scale, mathematically transformed (square root) to satisfy the assumption of normality in linear regression analysis.
[b] Model 1: adjusted for gestational age at venipuncture, gender and age of the child.
[c] Model 2: model 1, additionally adjusted for maternal psychopathology, education and family income.
[d] Folate concentrations in nmol/L.
[e] $P < 0.001$
[f] $P = 0.09$

Finally, we found that the odds of being a probable autistic child did not decrease with higher maternal folate concentration (fully adjusted model: OR = 1.03, 95% CI: 0.76;1.39).

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DISCUSSION

In this population-based study, we did not find evidence for an association of maternal folate concentrations in early pregnancy with autistic traits in the offspring at the age of 6 years. Similar to earlier studies[2,8], we found that prenatal folic acid supplement use – although strongly confounded – was associated with less child autistic traits. Interestingly, in contrast to previous studies, we did not find this association to be specific for preconceptional start with folic acid supplementation.

The association between prenatal folic acid supplementation and child autistic traits, although repeatedly reported, may be explained by residual confounding. Prenatal folic acid supplement use, a marker of good health literacy, is associated with many health-conscious behaviours that decrease the background risk of autistic traits in the offspring. An alternative explanation for our inconsistent findings between folic acid supplementation and folate concentrations might be the timing of biomarker assessment. Although we did not find an effect of folate concentrations measured at 13 weeks

of gestation, the potential effect of maternal folate on the development of autistic traits in the offspring might occur earlier or later in pregnancy.

Our findings do not provide evidence for a biological pathway between prenatal folic acid supplement use and child autistic traits through higher maternal folate concentrations. Yet, our study was limited by the fact that clinical diagnoses of autism were not at our disposal. Autistic traits are defined as subclinical deficits in socialisation, communication, and restricted/stereotypic behaviours that do not meet formal criteria for an ASD diagnosis.[9] However, although not meeting strict diagnostic criteria, comorbid psychiatric traits and psychosocial difficulties have been reported in individuals with increased levels of autistic traits.[10]

Nevertheless, to gain further insight in the biological underpinnings of ASD, future research should focus on the timing of the potential effect of prenatal folate on the development of autistic traits in combination with clinical diagnosis of autism in the offspring.

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3.3

Supplementary table 3.3.1 Subject characteristics by maternal folate deficiency[a] in early pregnancy[b]

	Folate deficient (n = 214)	Normal folate concentrations (n = 3,679)	P[c]
Child characteristics			
Gender (% girl)	49.5	49.8	0.94
Age at assessment (yr)[d,e]	6.4 ± 0.6	6.2 ± 0.5	< 0.001
Maternal characteristics			
Age at enrolment (yr)	27.6 ± 5.7	30.9 ± 4.5	< 0.001
Ethnicity (%)			
Dutch	32.8	63.1	
Other Western	7.7	9.0	
Turkish or Moroccan	24.4	10.0	< 0.001
Surinamese or Antillean	15.5	7.7	
Other non-Western	19.6	10.2	
Education (%)			
Higher	21.6	53.4	
Secondary	59.8	40.3	< 0.001
Primary	18.6	6.3	
Family income (%)			
> 2,000 €/mo	33.0	70.6	
1,200 – 2,000 €/mo	24.6	17.1	< 0.001
< 1,200 €/mo	42.4	12.3	
Psychopathology in mid-pregnancy (GSI-score)[f,g]	0.37 ± 0.45	0.25 ± 0.32	< 0.001
Pre-pregnancy BMI (kg/m^2)	24.7 ± 4.7	23.5 ± 4.0	< 0.001
Smoking during pregnancy (%)			
Never	65.8	77.8	
Until pregnancy was known	3.3	8.8	< 0.001
Continued throughout pregnancy	30.9	13.3	
Alcohol consumption during pregnancy (%)			
Never	56.8	38.4	
Until pregnancy was known	13.3	14.6	< 0.001
Continued throughout pregnancy	29.9	47.0	
Parity (% primiparae)	44.6	59.8	< 0.001
Pregnancy planning (% yes)	58.6	79.7	< 0.001
Marital status (% cohabitation)	77.2	91.4	< 0.001
Folic acid supplement use during pregnancy (%)			
No use	67.9	13.2	
Start > 10 wk pregnancy	12.7	11.7	
Start ≤ 10 wk pregnancy	11.4	29.6	< 0.001
Preconceptional start	8.0	45.5	

Supplementary table 3.3.1 (continued)

	Folate deficient (n = 214)	Normal folate concentrations (n = 3,679)	P^c
Paternal characteristics			
Age at enrolment (yr)	31.0 ± 5.8	33.5 ± 5.4	< 0.001
Ethnicity (%)			
Dutch	31.4	63.8	
Other Western	5.1	6.6	
Turkish or Moroccan	27.5	10.5	< 0.001
Surinamese or Antillean	16.6	7.9	
Other non-Western	19.4	11.2	
Education (%)			
Higher	31.1	52.8	
Secondary	53.7	40.0	< 0.001
Primary	15.2	7.2	
Psychopathology in mid-pregnancy (GSI-score)[f,h]	0.22 ± 0.32	0.15 ± 0.24	0.06

[a] Folate deficiency was defined as a folate concentration < 7 nmol/L.

[b] Descriptives on imputed data. Data was incomplete in the following variables: age child at assessment (17.0%), maternal ethnicity (0.2%), education (3.3%), psychopathology in mid-pregnancy (13.3%), pre-pregnancy BMI (14.9%), parity (0.4%), and smoking (1.1%), alcohol consumption (6.0%) and folic acid supplement use during pregnancy (10.2%), family income (12.0%), pregnancy planning (8.6%), marital status (4.2%), and paternal age (8.9%), ethnicity (2.6%), education (23.8%) and psychopathology in mid-pregnancy (26.8%).

[c] Derived from linear regression analysis for continuous variables or logistic regression analysis for categorical variables; on imputed data.

[d] Means ± SD (all such values).

[e] Median (95% range): deficient group: 6.2 (5.7-8.1); non-deficient group: 6.1 (5.7-7.6).

[f] GSI: Global Severity Index, scale range 0-4.

[g] Median (95% range): deficient group: 0.21 (0.01-2.06); non-deficient group: 0.14 (0.00-1.24).

[h] Median (95% range): deficient group: 0.11 (0.00-1.17); non-deficient group: 0.06 (0.00-0.81).

3.3

Chapter 4

Fatty acids

Chapter 4.1

Maternal LC-PUFA status during pregnancy and child problem behaviour

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ABSTRACT

Background: Omega-3 (n-3) and -6 (n-6) long-chain polyunsaturated fatty acids (LC-PUFAs) and the n-3:n-6 ratio are important for brain development. Whether maternal LC-PUFA status during pregnancy affects risk of problem behaviour in later childhood is unclear.

Methods: Within a population-based cohort, we measured maternal plasma docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and arachidonic acid (AA) concentrations and n-3:n-6 ratio in mid-pregnancy. Child emotional and behavioural problems at age 6 years of age were assessed by parents (Child Behavior Checklist), teachers (Teacher Report Form) and combined parent/teacher report.

Results: Higher maternal DHA and n-3:n-6 ratio were associated with fewer child emotional problems using parent (OR_{DHA} = 0.82, 95% CI: 0.70;0.96, *P* = 0.02; $OR_{n-3:n-6}$ = 0.83, 95% CI: 0.71;0.96, *P* = 0.01; n = 5,307) and combined parent/teacher scores (OR_{DHA} = 0.79, 95% CI: 0.66;0.95, *P* = 0.01; $OR_{n-3:n-6}$ = 0.77, 95% CI: 0.65;0.92, *P* < 0.01; n = 2,828). Higher AA was associated with more child behavioural problems using teacher (OR = 1.10, 95% CI: 1.00;1.20, *P* = 0.04; n = 3,365) and combined parent/teacher scores (OR = 1.12, 95% CI: 1.02;1.22, *P* = 0.02; n = 2,827). Maternal EPA was not associated with child problem behaviour.

Conclusions: Indications of associations of maternal LC-PUFA status with child emotional and behavioural problems were found. Future research is needed to identify LC-PUFA-sensitive periods of foetal brain development by including multiple assessments of prenatal LC-PUFA status.

INTRODUCTION

Two families of polyunsaturated fatty acids (PUFAs), the omega-3 (n-3) and -6 (n-6) fatty acids (FAs), are nutrients that play a major role in brain development and functioning. They affect numerous processes, including membrane fluidity, neurotransmission, and gene expression.[1] The long-chain PUFAs (LC-PUFAs) eicosapentaenoic acid (EPA; C20:5n-3), docosahexaenoic acid (DHA; C22:6n-3), and arachidonic acid (AA; C20:4n-6) are the main FAs that are metabolically active. In addition, the ratio between n-3 and n-6 FAs is important for optimal brain functioning. During the last century, however, the Western diet has provided a lower n-3 and higher n-6 intake than in previous generations, diminishing the n-3:n-6 ratio.[2]

Because of their role in brain function and development, maternal LC-PUFAs during foetal development have been hypothesised to affect child problem behaviour. Approximately half of the dry weight of the brain is lipid, of which 35% are PUFA, most of which is LC-PUFA.[1] Increase in AA and DHA content of brain tissue starts during the third trimester of pregnancy[3], indicating that the foetal period possibly is a sensitive period. Moreover, the foetus mostly depends on maternal LC-PUFA supply for its development, because foetal LC-PUFA synthesis is limited.[4]

Research in this field is, however, scarce and results are conflicting. Two studies on maternal fish intake, a major source of DHA, during pregnancy and offspring problem behaviour were undertaken. They found low maternal fish intake to be associated with suboptimal prosocial behaviour and increased risk of hyperactivity in childhood.[5,6] Another study on dietary intake of n-3 LC-PUFAs did not find any association with child behaviour.[7] Animal studies have shown that prenatal and not postnatal DHA deficiency was associated with increased depression and anxiety later in life.[8] In humans, there is some indication that perinatal LC-PUFA status (either measured in cord blood or maternal blood during gestation) is associated with child behaviour. However, results are inconsistent, ranging from null findings to associations with both emotional and behavioural problems.[9-12] Most studies were small and probably underpowered to detect subtle effects of nutrition, as expected in a multi-causal phenomenon such as development of the brain and behaviour. Moreover, multiple informants - the gold standard in child psychiatric research to measure a child's core characteristics from different perspectives and in different contexts - were generally not used.

A recent, relatively large study in another birth cohort from the Netherlands that did use multiple informants reported a modest association between maternal DHA level and child emotional symptoms.[12] In a study twice as large, with extended behavioural assessment by multiple informants and the availability of Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) diagnoses of problem behaviour, we aimed to further study the effects of maternal LC-PUFAs during pregnancy on child problem

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behaviour. To this end, we tested the hypotheses that maternal LC-PUFA status is associated with child emotional problems and with behavioural problems at age 6 years. Because of their metabolic role in brain development, we focused on AA, EPA, and DHA, as well as the n-3:n-6 ratio.

METHODS

Study population

The present study was conducted within the Generation R Study, a population-based cohort from early foetal life onwards in Rotterdam, the Netherlands.[13] The study was conducted in accordance with the guidelines proposed in the World Medical Association Declaration of Helsinki and was approved by the Medical Ethical Committee of the Erasmus Medical Centre. Written consent was obtained from all participants.

Out of 8,663 women who enrolled in the study before their third trimester of pregnancy (< 25 weeks gestation), valid FA profiles in plasma were available for 6,999 women (80.8%). Overall, 6,916 mother-child pairs were eligible at study baseline. In total, at least one measure of child behaviour was available for 5,307 children (76.7%) and their mothers (see Figure 4.1.1). Some mothers participated with two (n = 309), or three (n = 6) children. Since results of all analyses using parent report did not differ after exclusion of twins (n = 110) or random exclusion of one or two of the siblings, they were kept in the analyses.

Fatty acid analyses

Between 2001 and 2005, venous samples were drawn in mid-pregnancy (median: 20.5 weeks of gestation; 90% range: 18.8-22.9 weeks) and thereafter stored at room temperature before being transported to the regional laboratory for processing and storage for future studies. Processing was aimed to finish within a maximum of 3 hours after venipuncture. The samples were centrifuged and thereafter stored at −80ºC (without anti-oxidant). To analyse FA concentrations, EDTA plasma samples were picked and transported to the Division of Metabolic Diseases and Nutritional Medicine, Dr. von Hauner Children's Hospital, University of Munich Medical Centre, in 2010. After being thawed, the analysis of plasma glycerophospholipid FA (hereafter referred to as 'plasma FA') composition was performed by a sensitive and precise high-throughput method described recently.[14] The average coefficient of variation was 15.7%.

Plasma samples had previously undergone one thaw-freeze cycle. In order to explore the impact of this extra thaw-freeze cycle, 105 new plasma samples from the same series of aliquots tubes were randomly selected for re-analysis and intraclass correlation coefficients (ICCs) for all 25 FA concentrations were calculated using a two-way mixed ef-
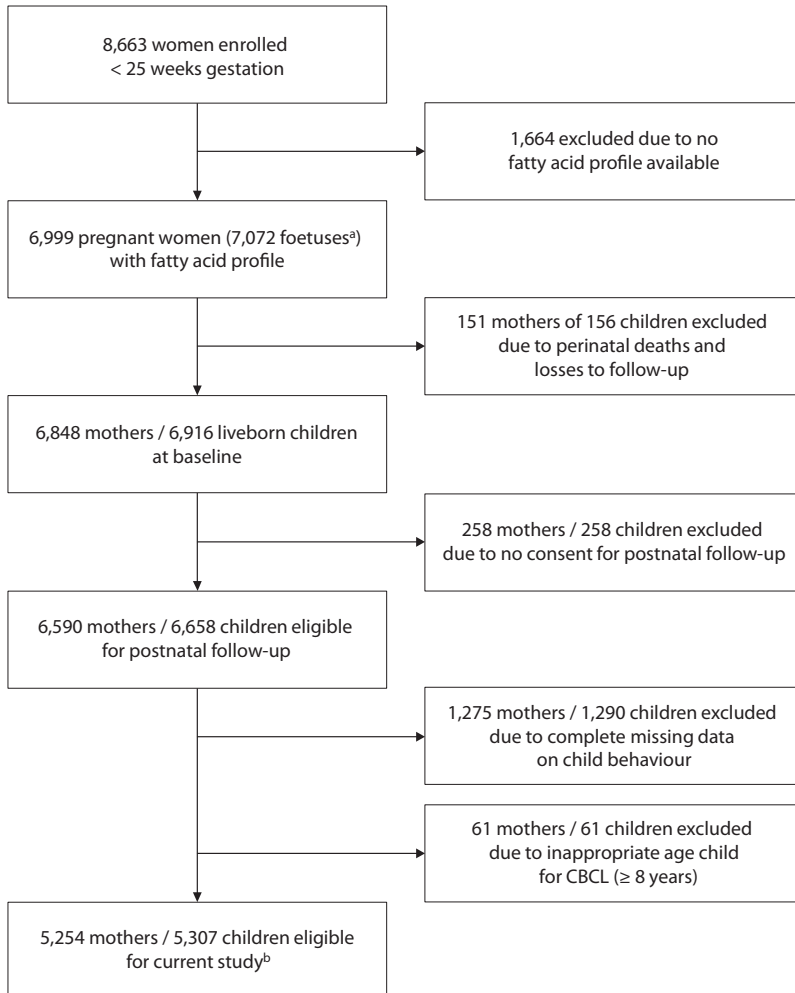
```
┌─────────────────────────────┐
│     8,663 women enrolled     │
│     < 25 weeks gestation     │
└─────────────────────────────┘
               │          ┌─────────────────────────────┐
               ├─────────▶│    1,664 excluded due to no   │
               │          │   fatty acid profile available│
               ▼          └─────────────────────────────┘
┌─────────────────────────────┐
│ 6,999 pregnant women         │
│ (7,072 foetuses[a])          │
│ with fatty acid profile      │
└─────────────────────────────┘
               │          ┌─────────────────────────────┐
               ├─────────▶│ 151 mothers of 156 children   │
               │          │   excluded due to perinatal   │
               │          │ deaths and losses to follow-up│
               ▼          └─────────────────────────────┘
┌─────────────────────────────┐
│ 6,848 mothers / 6,916        │
│ liveborn children at baseline│
└─────────────────────────────┘
               │          ┌─────────────────────────────┐
               ├─────────▶│ 258 mothers / 258 children    │
               │          │ excluded due to no consent for│
               │          │    postnatal follow-up        │
               ▼          └─────────────────────────────┘
┌─────────────────────────────┐
│ 6,590 mothers / 6,658        │
│ children eligible for        │
│ postnatal follow-up          │
└─────────────────────────────┘
               │          ┌─────────────────────────────┐
               ├─────────▶│ 1,275 mothers / 1,290 children│
               │          │ excluded due to complete      │
               │          │ missing data on child behaviour│
               │          └─────────────────────────────┘
               │          ┌─────────────────────────────┐
               ├─────────▶│ 61 mothers / 61 children      │
               │          │ excluded due to inappropriate │
               │          │ age child for CBCL (≥ 8 years)│
               ▼          └─────────────────────────────┘
┌─────────────────────────────┐
│ 5,254 mothers / 5,307        │
│ children eligible for        │
│ current study[b]             │
└─────────────────────────────┘
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Figure 4.1.1 Flow chart of study population
CBCL – Child Behavior Checklist
[a] Due to twin pregnancies.
[b] Analytic sample, missing values imputed by multiple imputation.

fects model for single measures with absolute agreement. Substantial to almost perfect agreement between the two assessments was found (ICCs: 0.61-0.96; ICC for sum of FAs: 0.87), with only one exception for C22:5n-3 (ICC: 0.59).

In the current study, we used AA, EPA and DHA. The n-3:n-6 ratio was calculated by summing the available n-3 PUFAs and dividing this by the sum of the corresponding n-6 PUFAs in the pathway (see Supplementary table 4.1.1 for an overview). FAs are expressed as percentage by weight (wt%) of all glycerophospholipid FAs detected with a chain length between 14 and 22 carbon atoms.

Child emotional and behavioural problems

Mothers were asked to fill out the Child Behavior Checklist (CBCL) when their child was about 6 years old. Since the majority of the children were younger than 6 (58.7%), we used the CBCL 1½-5 for all children. Additionally, children 8 years or older were excluded from the analyses (n = 61). In our sample, for all scales Cronbach's alphas were the same in 5 year-old children and in children older than 5, indicating that problems were also reliably measured in children older than 5.

The CBCL is a self-administered parent-report questionnaire to measure the degree of children's problem behaviour.[15] It contains 99 problem items rated on a 3-point scale (0 = not true, 1 = somewhat or sometimes true, and 2 = very true or often true), based on the preceding two months. A broad-band Emotional problems (i.e. being anxious, withdrawn and emotionally reactive) and Behavioural problems (i.e. inattention and aggression) scale can be computed. Clinical cut-off scores were used to classify children as having emotional or behavioural problems (91st percentile of a Dutch norm group[16]).

The Teacher Report Form (TRF) 6-18 is the teacher version of the CBCL 6-18. Good reliability and validity have been reported for the CBCL and TRF.[15,17] In total, TRF data was available in 3,366 children whose mother had a FA profile. Clinical cut-off scores (91st percentile of the middle norm group from multicultural samples[17,18]) were used to classify children as having emotional or behavioural problems.

Correlations between parent- and teacher-report were modest ($r_{CBCL-TRF}$ = 0.19 for emotional problems; $r_{CBCL-TRF}$ = 0.22 for behavioural problems), which is in line with literature on cross-informant agreement[19] and reflects important variability in behaviour across different contexts.

Parent and teacher scores were also combined by averaging the individual age-standardised sum scores. Since no clinical cut-offs were available from norm populations, we used the 91st percentile in our study sample as a cut-off to define child emotional and behavioural problems in the clinical range.

DSM-IV based diagnosis of problem behaviour was obtained by the Diagnostic Interview Schedule for Children-young child version (DISC-YC) in a subsample of the cohort when children were about 6 years old. The DISC-YC is a highly structured DSM-IV based parent-administered interview that generates DSM-IV diagnoses in four modules: anxiety disorders & mood disorders (emotional), behavioural disorders, and miscellaneous (elimination and tic disorders). Good overall reliability of symptom scales has been published.[20] DISC-YC administration in the total Generation R cohort was aimed at children enriched for problem behaviour (i.e. scoring above the 85th percentile on CBCL Total Problems or above the 98th percentile of CBCL syndrome scales; n = 1,080) plus a random comparison group (n = 330), resulting in oversampling of children at risk for diagnosis. In the current study, interview data were available for 839 children whose

mother had a FA profile. Of these, 98 children were classified with a diagnosis 'Emotional problems with mild impairment' and 221 with 'Behavioural problems with mild impairment'. For each of the cases, four matched (on gender and European origin) controls were selected from the sample with CBCL-data available (children scoring below the 50[th] percentile on both CBCL Emotional and Behavioural Problems). Controls were selected from a total eligible control group of 2,134 children, comprising of 117 children who were not diagnosed with emotional or behavioural problems based on the DISC-YC, and an additional group of 2,017 children without interview data.

Covariates

Several maternal and child characteristics were considered as possible confounding variables, based on previous studies of perinatal LC-PUFA status and child emotional and behavioural development.[9-12] These were: family income, maternal educational level, pre-pregnancy BMI, age at enrolment, smoking and alcohol consumption during pregnancy, national origin, general psychiatric symptoms in mid-pregnancy (highest prevalence for anxious and depressive symptoms), parity and marital status. Child characteristics that were considered comprised gestational age and weight at birth, gender, breastfeeding status at 6 months, daycare attendance during early childhood and age of the child at the time of assessment.

All analyses of maternal LC-PUFA status and child problem behaviour were controlled for gestational age at venipuncture, gender of the child and the age of the child at assessment, as well as the other FAs (to test the independent effect of each LC-PUFA). Other covariates were included in the adjusted models if they changed the effect estimates meaningfully (defined as more than 5%). Following this criterion, maternal pre-pregnancy BMI, and child gestational age and weight at birth and breastfeeding status at 6 months were excluded from the analyses.

We did not have information on child LC-PUFA levels. Instead, we used dietary n-3 and n-6 intake during infancy, as assessed by a 211-item food frequency questionnaire when children were 14 months old.[21] N-3 and n-6 intake were adjusted for total energy intake using the residual method[22] and were available for 2,785 of the children in the current study sample.

Statistical analyses

To minimise bias resulting from rater perspectives, we used the parent (CBCL, DISC-YC), teacher (TRF) and combined parent/teacher scores of child emotional and behavioural problems as outcome variables.

First, multivariable logistic regression analyses were used to test for associations of maternal AA, EPA, and DHA levels (wt%) as well as the maternal n-3:n-6 ratio (per SD) with child emotional and behavioural problems using the CBCL-, TRF- and the combined

4.1

CBCL/TRF-scores. For the DISC-YC data, we used a case-control approach with conditional logistic regression analysis.

We added quadratic terms of the LC-PUFA variables to test for non-linear associations. Analyses were repeated while excluding preterm children (< 37 weeks of gestation) and children with low birth weight (< 2,500 grams) (n = 396), and adjusting for child dietary n-3 and n-6 intake at age 14 months in a subsample with these data available.

Next, we explored potential effect modification by child gender and maternal educational level in the fully adjusted models using parent reports. If the interaction term between maternal n-3:n-6 ratio and the effect modifier was statistically significant ($P <$ 0.05), we performed additional stratified analyses.

Finally, for ease of interpretation, we divided the n-3:n-6 ratio by quintiles in analyses using parent report of child problem behaviour.

Missing values on covariates and child outcome data (when at least one other CBCL measure at 1.5 or 3 years was available) were imputed using the Markov Chain Monte Carlo multiple imputation technique with Predictive Mean Matching for continuous variables (0.1-19.9% missing data) and generating 5 datasets. Statistical analyses were carried out using PASW Statistics, version 20.0 for Windows (SPSS Inc., Chicago, Illinois).

For the non-response analysis, we compared child and maternal characteristics of included participants (n = 5,307; 76.7%) with those from whom we did not obtain behavioural data (n = 1,609). Excluded children on average had a 108 gram (95% CI: 76;140, $P <$ 0.001) lower birth weight compared with included children. The mothers of children not included on average had a 0.2 wt% (95% CI: 0.17;0.29, $P <$ 0.001) lower DHA level in mid-pregnancy, were on average 3.3 (95% CI: 3.0;3.6, $P <$ 0.001) years younger and less educated (22.5% primary education compared with 8.1%, χ^2 (2) = 520; $P <$ 0.001). They also had more severe psychopathological complaints (median 0.25 (95% range: 0-1.98) compared with 0.15 (95% range: 0-1.29), $P <$ 0.001).

RESULTS

Descriptive characteristics of study participants by determinant and by outcome are presented in Tables 4.1.1 and 4.1.2 respectively.

Maternal LC-PUFAs and child emotional problems

The fully adjusted associations between maternal LC-PUFA status and child emotional problems, as reported by different informants and assessed with different instruments, are presented in Table 4.1.3. No associations of maternal AA or EPA with child emotional problems were found. Increased levels of maternal DHA were associated with fewer child emotional problems as reported by parents (CBCL) (OR$_{parent score}$ = 0.82, 95% CI:

Table 4.1.1 Subject characteristics by maternal n-3:n-6 ratio[a] in mid-pregnancy[b]

	Lowest quintile of n-3:n-6 ratio	Other n-3:n-6 quintiles	P[c]
Child characteristics	n = 1,058	n = 4,249	
Gender (% girl)	49.7	50.0	0.83
Gestational age at birth (wk)[d]	40.0 (35.3-42.4)	40.1 (35.6-42.4)	0.03
Weight at birth (g)[e]	3,381 ± 565	3,441 ± 567	< 0.01
Breastfeeding (partial) until 6 mo (%)	36.5	33.4	0.58
Daycare attendance (nr of hrs/wk)	10.4 (0.0-36.3)	20.0 (0.0-39.4)	< 0.001
Age child at assessment (yr)	6.0 (5.6-7.5)	5.9 (5.6-7.1)	< 0.001
	n = 386	n = 2,399	
N-3 intake at 14 mo (g/day)[f]	0.64 ± 0.35	0.63 ± 0.34	0.44
N-6 intake at 14 mo (g/day)[f]	5.3 ± 2.9	4.7 ± 2.4	< 0.001
Maternal characteristics	n = 1,050	n = 4,204	
Age at enrolment (yr)	28.8 ± 5.2	31.0 ± 4.7	< 0.001
Ethnicity (% European)	39.7	70.8	< 0.001
Education (%)			
Higher	27.1	53.5	
Secondary	53.9	40.2	< 0.001
Primary	19.0	6.3	
Family income (%)			
> 2,000 €/mo	38.5	69.8	
1,200 – 2,000 €/mo	28.3	16.0	< 0.001
< 1,200 €/mo	33.2	14.2	
General psychiatric symptoms in mid-pregnancy (GSI-score)[g]	0.24 (0.00-1.55)	0.13 (0.00-1.22)	< 0.001
BMI before pregnancy (kg/m^2)	23.3 (17.9-35.7)	22.5 (18.2-34.5)	< 0.001
Smoking during pregnancy (%)			
Never	72.3	78.0	
Until pregnancy was known	6.1	8.4	< 0.001
Continued throughout pregnancy	21.6	13.6	
Alcohol consumption during pregnancy (%)			
Never	66.1	37.2	
Until pregnancy was known	9.6	14.7	< 0.001
Continued throughout pregnancy	24.3	48.1	
Parity (% primiparae)	46.9	60.3	< 0.001
Marital status (% cohabitation)	89.3	89.1	0.89

n-3 – omega-3, n-6 – omega-6

[a] Participants in the lowest quintile of n-3:n-6 ratio (1:13.7–1:6.5) versus those in the other quintiles (1:6.4–1:1.9).

[b] Descriptives on imputed data.

[c] Derived from linear regression analysis for continuous variables or logistic regression analysis for categorical variables; on imputed data.

[d] Median; 95% range in parentheses (all such values).

[e] Mean ± SD (all such values).

[f] Adjusted for energy intake.

[g] GSI-score – Global Severity Index score.

4.1

Table 4.1.2 Subject characteristics by child total emotional and behavioural problems[a] at 6 years[b]

	Emotional and behavioural problems	No emotional and behavioural problems	P^c
Child characteristics	n = 208	n = 5,099	
Gender (% girl)	38.7	50.5	< 0.01
Gestational age at birth (wk)[d]	39.9 (33.9-42.2)	40.1 (35.6-42.4)	0.05
Weight at birth (g)[e]	3,354 ± 608	3,432 ± 565	0.14
Breastfeeding (partial) until 6 mo (%)	31.5	34.1	0.62
Daycare attendance (nr of hrs/wk)	14.5 (0.0-39.2)	18.0 (0.0-38.7)	0.01
Age child at assessment (yr)	6.0 (5.4-7.2)	5.9 (5.6-7.2)	0.14
	n = 81	n = 2,704	
N-3 intake at 14 mo (g/day)[f]	0.67 ± 0.38	0.63 ± 0.34	0.32
N-6 intake at 14 mo (g/day)[f]	5.1 ± 2.7	4.8 ± 2.5	0.27
Maternal characteristics	n = 208	n = 5,046	
Age at enrolment (yr)	28.9 ± 5.6	30.6 ± 4.8	< 0.001
Ethnicity (% European)	41.2	65.6	< 0.001
Education (%)			
Higher	29.9	49.0	
Secondary	52.1	42.5	< 0.001
Primary	18.0	8.5	
Family income (%)			
> 2,000 €/mo	37.6	64.6	
1,200 – 2,000 €/mo	25.3	18.2	< 0.001
< 1,200 €/mo	37.1	17.2	
General psychiatric symptoms in mid-pregnancy (GSI-score)[g]	0.42 (0.01-2.00)	0.15 (0.00-1.24)	< 0.001
BMI before pregnancy (kg/m^2)	22.6 (17.9-35.6)	22.7 (18.1-34.6)	0.68
Smoking during pregnancy (%)			
Never	67.2	77.2	
Until pregnancy was known	6.8	8.0	< 0.001
Continued throughout pregnancy	26.0	14.8	
Alcohol consumption during pregnancy (%)			
Never	54.4	42.5	
Until pregnancy was known	13.1	13.7	0.01
Continued throughout pregnancy	32.5	43.8	
Parity (% primiparae)	62.0	57.5	0.22
Marital status (% cohabitation)	78.1	89.6	< 0.001

N-3 – omega-3, N-6 – omega-6

[a] Children with problem behaviour (based on clinical cut-off scores for Child Behavior Checklist Total Problems scale from a Dutch norm-population) versus children without problem behaviour.

[b] Descriptives on imputed data.

[c] Derived from linear regression analysis for continuous variables or logistic regression analysis for categorical variables; on imputed data.

[d] Median; 95% range in parentheses (all such values).

[e] Mean ± SD (all such values).

[f] Adjusted for energy intake.

[g] GSI-score – Global Severity Index score.

0.70;0.96, P = 0.02) and using the combined scores of both parents (CBCL) and teachers (TRF) (OR$_{combined\ score}$ = 0.79, 95% CI: 0.66;0.95, P = 0.01). Similarly, a higher n-3:n-6 ratio was associated with a lower risk of emotional problems (OR$_{parent\ score}$ = 0.83, 95% CI: 0.71;0.96, P = 0.01; OR$_{combined\ score}$ = 0.77, 95% CI: 0.65;0.92, P < 0.01). In contrast, no associations of maternal DHA or n-3:n-6 ratio with child emotional problems based on

Table 4.1.3 Associations of maternal prenatal fatty acids with child emotional and behavioural problems at 6 years[a]

| Maternal fatty acid (wt%)[f] | Emotional problems | | | | | | | |
| | Parent report (n = 5,307)[b] | | Teacher report (n = 3,366)[c] | | Combined parent and teacher report (n = 2,828)[d] | | Parent interview, DSM-IV based diagnosis (n = 490)[e] | |
	OR[g] (95% CI)	P	OR[g] (95% CI)	P	OR[g] (95% CI)	P	OR[g] (95% CI)	P
AA	1.03 (0.95;1.13)	0.44	1.03 (0.94;1.14)	0.52	1.05 (0.96;1.16)	0.27	1.08 (0.90;1.29)	0.42
EPA	1.09 (0.65;1.86)	0.74	0.60 (0.28;1.28)	0.19	1.00 (0.53;1.89)	0.99	1.83 (0.64;5.23)	0.26
DHA	0.82 (0.70;0.96)	0.02	1.04 (0.86;1.25)	0.70	0.79 (0.66;0.95)	0.01	0.82 (0.57;1.18)	0.28
N-3:n-6 ratio (per SD)	0.83 (0.71;0.96)	0.01	0.98 (0.82;1.17)	0.84	0.77 (0.65;0.92)	< 0.01	0.94 (0.65;1.38)	0.76

| Maternal fatty acid (wt%)[f] | Behavioural problems | | | | | | | |
| | Parent report (n = 5,307)[b] | | Teacher report (n = 3,365)[c] | | Combined parent and teacher report (n = 2,827)[d] | | Parent interview, DSM-IV based diagnosis (n = 1,105)[e] | |
	OR[g] (95% CI)	P	OR[g] (95% CI)	P	OR[g] (95% CI)	P	OR[g] (95% CI)	P
AA	1.04 (0.91;1.18)	0.60	1.10 (1.00;1.20)	0.04	1.12 (1.02;1.22)	0.02	1.07 (0.95;1.22)	0.25
EPA	1.58 (0.84;2.95)	0.15	0.85 (0.45;1.58)	0.60	1.22 (0.70;2.10)	0.48	1.09 (0.59;2.02)	0.79
DHA	0.93 (0.75;1.16)	0.53	1.23 (1.03;1.46)	0.02	1.07 (0.90;1.26)	0.47	0.89 (0.72;1.11)	0.30
N-3:n-6 ratio (per SD)	1.09 (0.88;1.34)	0.44	1.23 (1.05;1.44)	0.01	1.16 (0.99;1.36)	0.06	0.89 (0.73;1.08)	0.24

DSM-IV – Diagnostic and Statistical Manual of Mental Disorders, AA – arachidonic acid, EPA – eicosapentaenoic acid, DHA – docosahexaenoic acid, N-3 – omega-3, n-6 – omega-6

[a] Values are odds ratios from logistic regression analyses, except for the analyses based on parent interview, which are odds ratios from a case-control approach using conditional logistic regression analyses.

[b] Cases based on clinical cut-off scores from a Dutch norm-population; cases Emotional problems: n = 305; cases Behavioural problems: n = 129.

[c] Cases based on clinical cut-off scores for the middle norm group ('medium scoring societies') from multicultural norms; cases Emotional problems: n = 194; cases Behavioural problems: n = 223.

[d] Cases based on cut-off scores at 91[st] percentile in study population; cases Emotional problems: n = 255; cases Behavioural problems: n = 254.

[e] Cases based on DSM-IV-based diagnosis of Emotional/Behavioural problems with mild impairment; cases Emotional problems: n = 98, controls: n = 392; cases Behavioural problems: n = 221, controls: n = 884.

[f] 1 unit increase equals an increase of 1% of total fatty acid composition for the respective long-chain polyunsaturated fatty acid.

[g] Analyses are adjusted for the other fatty acids (except in case of the n-3:n-6 ratio), gestational age at venipuncture, gender, and age of child at assessment, family income, and maternal educational level, European origin, general psychiatric symptoms in mid-pregnancy, smoking and alcohol consumption during pregnancy, age at enrolment, parity, marital status, and child daycare attendance.

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teacher report only were found (OR_{DHA} = 1.04, 95% CI: 0.86;1.25, P = 0.70; $OR_{n-3:n-6}$ = 0.98, 95% CI: 0.82;1.17, P = 0.84). Also, no associations of maternal fatty acids with offspring emotional problems were found using the parent-administered DISC-YC (OR_{DHA} = 0.82, 95% CI: 0.57;1.18, P = 0.28; $OR_{n-3:n-6}$ = 0.94, 95% CI: 0.65;1.38, P = 0.76).

Including quadratic terms of the FAs in the model did not suggest non-linear associations and results did not change after exclusion of preterm children and children with low birth weight.

The analyses were repeated while adjusting for dietary n-3 and n-6 intake at age 14 months in a subsample with these data available ($n_{parent\ score}$ = 2,785). Dietary n-3 and n-6 data did not change the effect size of DHA nor the n-3:n-6 ratio on child emotional problems (in this subsample, before and after adjustment for child diet: OR_{DHA} = 0.75, 95% CI: 0.59;0.96, P = 0.02; $OR_{n-3:n-6}$ = 0.74, 95% CI: 0.59;0.93, P = 0.01).

With regard to potential effect modification, the interaction term of maternal n-3:n-6 ratio with child gender, but not maternal educational level, was statistically significant (P < 0.05). Analyses stratified by gender showed that the effect of maternal n-3:n-6

Table 4.1.4 Associations of maternal prenatal n-3:n-6 ratio with child emotional and behavioural problems at 6 years[a] (n = 5,307)

Maternal n-3:n-6 ratio quintiles	Emotional problems[b]			
	Basic[c]		Adjusted for covariates[d]	
	OR (95% CI)	P	OR (95% CI)	P
1 (1:13.7-1:6.5)	Reference		Reference	
2 (1:6.4-1:5.6)	0.81 (0.58;1.13)	0.22	1.02 (0.72;1.44)	0.91
3 (1:5.5-1:4.9)	0.58 (0.40;0.86)	< 0.01	0.82 (0.55;1.22)	0.33
4 (1:4.8-1:4.2)	0.39 (0.25;0.59)	< 0.001	0.62 (0.40;0.95)	0.03
5 (1:4.1-1:1.9)	0.35 (0.23;0.53)	< 0.001	0.61 (0.39;0.96)	0.03
Maternal n-3:n-6 ratio quintiles	Behavioural problems[b]			
	Basic[c]		Adjusted for covariates[d]	
	OR (95% CI)	P	OR (95% CI)	P
1 (1:13.7-1:6.5)	Reference		Reference	
2 (1:6.4-1:5.6)	1.24 (0.64;2.42)	0.52	1.35 (0.70;2.60)	0.37
3 (1:5.5-1:4.9)	1.12 (0.60;2.10)	0.71	1.31 (0.70;2.43)	0.40
4 (1:4.8-1:4.2)	0.77 (0.39;1.53)	0.45	1.02 (0.51;2.06)	0.95
5 (1:4.1-1:1.9)	0.86 (0.45;1.65)	0.66	1.28 (0.65;2.54)	0.48

n-3 – omega-3, n-6 – omega-6
[a] Values are odds ratios from logistic regression analyses.
[b] Parent report, cases based on clinical cut-off scores from a Dutch norm population; cases Emotional problems: n = 305; cases Behavioural problems: n = 129.
[c] Model 1: adjusted for gestational age at venipuncture, gender, and age of child at assessment.
[d] Model 2: model 1, additionally adjusted for family income, and maternal educational level, European origin, general psychiatric symptoms in mid-pregnancy, smoking and alcohol consumption during pregnancy, age at enrolment, parity, marital status, and child daycare attendance.

ratio on child emotional problems could be attributed to an effect in girls ($OR_{girls} = 0.71$, 95% CI: 0.56;0.91, $P < 0.01$; $OR_{boys} = 0.91$, 95% CI: 0.74;1.12, $P = 0.36$).

In Table 4.1.4, we show the association of maternal n-3:n-6 ratio divided by quintiles with child emotional problems. The lowest quintile was used as the reference category (quintile 1). Compared to this reference, a maternal n-3:n-6 ratio in the highest two quintiles was associated with fewer emotional problems in the offspring ($OR_{Q4vsQ1} = 0.62$, 95% CI: 0.40;0.95, $P = 0.03$; $OR_{Q5vsQ1} = 0.61$, 95% CI: 0.39;0.96, $P = 0.03$).

Maternal LC-PUFAs and child behavioural problems

The fully adjusted associations between maternal LC-PUFA status and child behavioural problems are presented in Table 4.1.3. No associations were found between maternal EPA and child behavioural problems. Higher maternal DHA level and n-3:n-6 ratio were only associated with more child behavioural problems using the teacher report ($OR_{DHA} = 1.23$, 95% CI: 1.03;1.46, $P = 0.02$; $OR_{n-3:n-6} = 1.23$, 95% CI: 1.05;1.44, $P = 0.01$).

However, for both the teacher score and the combined parent/teacher score, increased levels of maternal AA were associated with more child behavioural problems ($OR_{teacher\ score} = 1.10$, 95% CI: 1.00;1.20, $P = 0.04$; $OR_{combined\ score} = 1.12$, 95% CI: 1.02;1.22, $P = 0.02$). These positive associations were not confirmed in analyses using parent report only (either CBCL or DISC-YC interview).

Again, including quadratic terms of the FAs in the model did not suggest non-linear associations and results did not change after exclusion of preterm children and children with low birth weight.

Adjustment for child n-3 and n-6 intake in a subsample ($n_{teacher\ score} = 1,582$; $n_{combined\ score} = 1,553$), did not change the association of AA with child behavioural problems using the teacher report (in this subsample, before and after adjustment for child diet: $OR_{AA} = 1.22$, 95% CI: 1.04;1.43, $P = 0.02$). The association between AA and child behaviour using the combined parent/teacher report in this subsample did not reach level of statistical significance, either before or after adjustment for child dietary intake ($OR_{AA} = 1.13$, 95% CI: 0.99;1.30, $P = 0.07$).

DISCUSSION

In this population-based study, we found higher maternal DHA levels and n-3:n-6 ratio to be associated with fewer offspring emotional problems using both a parent and combined parent/teacher report of child problem behaviour. This association could not be confirmed using teacher report only or using the DISC-YC interview. If anything, we interpret these findings as an indication of a negative association of maternal DHA levels and n-3:n-6 ratio with offspring emotional problems.

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Similarly, we found higher maternal AA levels to be associated with more child behavioural problems using both teacher and combined parent/teacher report. The associations using parent report and DISC-YC data were statistically null findings. These findings indicate a positive association of maternal AA levels with offspring behavioural problems.

No associations between maternal EPA levels and child emotional or behavioural problems were found.

Interpretation

Our findings closely resemble those from Loomans et al., who also found higher maternal DHA - but not AA or EPA - to be associated with fewer child emotional symptoms at the age of 5-6 years based on combined report of mother and teacher using the Strength and Difficulties Questionnaire (SDQ).[12] We additionally found indications of higher maternal n-3:n-6 ratio and AA levels to be associated with, respectively, fewer emotional and more behavioural problems in the offspring, whereas these associations disappeared after adjustment for confounders in the study by Loomans et al. Our study, however, has more power. Additionally, our measures of behavioural problems also comprise aggressive and rule-breaking behaviours, whereas the SDQ only measures hyperactivity and inattention.

Our results are also compatible with findings from Krabbendam et al., who found higher DHA but not AA cord blood levels to be associated with fewer emotional problems in children at age 7 based on CBCL parent report.[10] Moreover, like Krabbendam et al., who found no association between current child LC-PUFA status and child problem behaviour, we did not find infant n-3 and n-6 intake to affect the associations between maternal LC-PUFAs and child emotional or behavioural problems at age 6. This stresses the potential independent effect of prenatal LC-PUFA status on child emotional and behavioural development.

Our results are generally not in line with those from Kohlboeck et al., who found higher levels of AA in cord blood to be associated with fewer emotional problems at age 10 years based on parent report using the SDQ.[11] Whether statistically significant or not, all associations of maternal AA with child problem behaviour in our study suggest effect estimates in the opposite direction, as do results from other studies.[9,10,12] Kohlboeck et al. also found higher levels of DHA to be associated with less hyperactivity/inattention, whereas we found an indication of higher maternal AA levels to be associated with more behavioural problems. Differences in findings may be attributable to a combination of differences in fatty acid assessment (cord blood serum versus mid-pregnancy plasma sample), instrument and informant of child problem behaviour (parent SDQ versus multi-informant assessment by CBCL, TRF and DISC-YC interview), and child age at assessment (10 versus 6 years of age).

Finally, our results are not consistent with those from Dunstan et al., who reported no significant associations between cord blood LC-PUFAs and problem behaviour at age 2.5 years using CBCL parent report.[9] Our study, however, has a much larger sample size and thus increased power to detect associations. Additionally, our study has a longer follow-up time, during which parents may become better in recognising and thus reporting problem behaviour of their child, and during which other informants of child problem behaviour, such as teachers, become available.

In contrast to the studies mentioned above, we provide an overview of the associations of maternal LC-PUFAs with child problem behaviour as reported by different informants. There is a large body of literature indicating the important variability in behaviour across different contexts, such as home versus school.[19] Our approach increases insight in the effect that the difference in multi-informant agreement on children's problem behaviour may have on associations under investigation. As a consequence, however, this approach makes it harder to make strong statements when results do not all point towards to same conclusion. Therefore, not only findings from studies using one informant/instrument, but also findings from the current study should be interpreted with caution.

Mechanism of risk

Animal studies have shown that maternal dietary LC-PUFA composition may durably alter brain monoamine, dopamine and serotonin concentrations in the offspring.[23,24] Moreover, such modifications in two-months old rats were only reversed by supply of a LC-PUFA-balanced diet that started before weaning[24], when rat brain development is similar to that of a full-term human newborn.[25] It is known in humans that maternal LC-PUFA status declines during pregnancy[26] and LC-PUFAs accumulate in the foetal brain, with a spurt in the last trimester of pregnancy.[1] However, maternal LC-PUFA status also may affect brain development earlier in pregnancy. Gale et al. found oily fish intake during early but not late pregnancy to be associated with less hyperactivity in children.[6] Lower maternal LC-PUFA concentrations directly limit the amount of LC-PUFA available to the foetus[4], and animal studies showed that already early in pregnancy LC-PUFAs promote neurogenesis and then continue to influence synaptogenesis and myelination.[27-29] The developmental stage in which inadequate LC-PUFA status occurs during pregnancy may thus additionally account for different effects of LC-PUFA status.

Alternatively, maternal depression underlies the observed association of maternal LC-PUFAs with child problem behaviour. Antenatal depression is an established cause of child emotional and behavioural problems.[30] Low LC-PUFA status might place women at risk of depression or can result from dietary changes due to depression.[31] However, we adjusted for maternal depressive symptoms during pregnancy to control for such an indirect effect.

Finally, this type of nutritional associations in observational studies is sensitive to effects of confounding and effect-modifying factors. A decline in the effect of maternal

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LC-PUFA status on the offspring's problem behaviour was noticeable after adjustment for confounders. Admitting we were not able to adjust for child LC-PUFA concentrations, we found no evidence that child dietary intake of n-3 and n-6 FAs influenced the associations. Effect modification by gender further points to a complex causal chain, in which many factors exaggerate effects on child emotional and behavioural development. Although we accounted for many sociodemographic factors, further (residual) confounding cannot be excluded.

Strengths and limitations

The strengths of our study were its large sample size, the availability of plasma LC-PUFA concentrations, the multi-informant assessment of child behaviour, and the ability to adjust for several potential confounders.

The study also has its limitations. Selective attrition may have influenced our results. Mothers of children not included generally had less favourable LC-PUFA status and socioeconomic circumstances. This selective attrition can only lead to bias if associations of maternal fatty acids and child emotional/behavioural problems in those mother-child pairs not included in the study differ from the associations found in those that were included in the study. With regard to the hypothesised biological mechanism underlying the association we do not consider this likely. It can however not be inferred from our results. Next, our assessment of LC-PUFA status was based on a single measure in blood plasma in mid-pregnancy. One measurement, although indicative, is not a reliable reflection of a mother's long-term FA status. Furthermore, other nutrients that are associated with brain development and that simultaneously affect FA status, such as iron, were not included in this study.[27]

Conclusion

We found indications of higher maternal DHA levels and n-3:n-6 ratio during second trimester of pregnancy to be associated with fewer emotional, and higher AA levels with more behavioural problems in the offspring at age 6 years. These findings are a step forward for the growing body of literature in this field and underline the importance of the use of multiple informants of child problem behaviour in association studies like these. Further research is needed to identify LC-PUFA-sensitive periods of foetal brain development, optimal intake of LC-PUFAs during pregnancy, and the specific effects of inadequate prenatal LC-PUFA status on emotional and behavioural development in the offspring. These studies should include multiple assessments of FAs during pregnancy, as well as in-depth assessments of child problem behaviour by multiple informants and instruments.

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Supplementary table 4.1.1 Characteristics of available fatty acids in the DHA- and AA-synthesis pathway, included in the n-3:n-6 ratio (n = 5,254)

Fatty acid	Range	Mean (SD)	Median (95% range)
N-3 family (wt%)[a]			
18:3n-3 (α-linolenic acid)	0.1-0.8	0.3 (0.1)	0.3 (0.2-0.5)
20:5n-3 (eicosapentaenoic acid, EPA)	0.1-3.0	0.5 (0.3)	0.5 (0.2-1.5)
22:5n-3 (docosapentaenoic acid)	0.3-2.1	0.8 (0.2)	0.7 (0.4-1.4)
22:6n-3 (docosahexaenoic acid, DHA)	1.9-10.3	4.8 (1.1)	4.7 (2.9-7.2)
N-6 family (wt%)[a]			
18:2n-6 (linoleic acid)	14.2-35.2	22.2 (2.8)	22.1 (17.1-28.1)
20:4n-6 (arachidonic acid, AA)	4.4-16.0	9.6 (1.6)	9.6 (6.6-12.8)
22:4n-6 (adrenic acid)	0.1-1.1	0.4 (0.1)	0.4 (0.2-0.7)
22:5n-6 (osbond acid)	0.1-1.8	0.5 (0.2)	0.5 (0.2-0.9)
N-3:n-6 ratio	0.1-0.5	0.2 (0.1)	0.2 (0.1-0.3)
Total fatty acids (mg/L)	771-2,535	1,630 (223)	1,621 (1,213-2,089)

n-3 – omega-3, n-6 – omega-6
[a] Fatty acids are expressed as percentage by weight of total fatty acid composition.

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MILK

Chapter 4.2

Maternal fatty acid status during pregnancy and child autistic traits

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ABSTRACT

Background: Omega-3 (n-3) and -6 (n-6) polyunsaturated fatty acids (PUFAs) are impor-
tant for brain function and development. We examined whether maternal PUFA status
during pregnancy affects risk of autistic traits in childhood.

Methods: Within the Generation R cohort, we measured maternal plasma PUFA concen-
trations and the n-3:n-6 ratio in mid-pregnancy (2001-2005). Child autistic traits at 6
years were assessed using the Social Responsiveness Scale short form in 4,624 children.

Results: Lower maternal n-3:n-6 ratio during pregnancy was associated with more autis-
tic traits in the offspring (B = −0.009, 95% CI: −0.017;−0.001). In particular, higher total
n-6 and linoleic acid status were associated with more autistic traits (B_{n-6} = 0.004, 95% CI:
0.000;0.007; $B_{linoleic\ acid}$ = 0.004, 95% CI: 0.000;0.008). Associations were independent of
child intelligence, suggesting specific effects of fatty acid distribution on the develop-
ment of autistic traits in addition to general neurodevelopment. Maternal plasma n-3
status was not associated with child autistic traits and, consistently, neither was prenatal
dietary fish intake.

Conclusions: Future research on optimal PUFA intake should include blood PUFA concen-
trations to account for biological processes that determine the bioavailability of PUFAs
in the human body.

INTRODUCTION

Autism Spectrum Disorders (ASD) are neurodevelopmental disorders, characterised by deficits in reciprocal social behaviour, impaired communication and/or repetitive or stereotyped behaviours.[1] Understanding the pathogenesis of ASD has proven to be challenging. Next to insights from genetic epidemiological studies[2], research on environmental risk factors for autism risk has increased and consistently demonstrated associations between environmental factors and autism over the past decade.[3]

Due to their role in brain development and function, the omega-3 (n-3) and -6 (n-6) polyunsaturated fatty acids (PUFAs) have been posited as potential environmental risk factors for autism/autistic traits. N-3 and n-6 PUFAs affect numerous processes, including membrane fluidity, neurotransmission, and gene expression.[4] Whereas both types of PUFAs are important for brain development, they exert different and sometimes opposing effects, for example on the production and activity of various components of the immune system that modulate brain functions.[5] During the last century, the Western diet has provided a lower n-3 and higher n-6 intake than in previous generations, diminishing the n-3:n-6 ratio.[6] Disturbances in the n-3:n-6 ratio have been suggested to induce changes in brain development that are related to ASD.[7] In this respect, maternal PUFA status during pregnancy may be of particular interest, as the prenatal phase is a time of both rapid neurodevelopment and increased foetal demand of PUFAs[8], for which the foetus depends on maternal supply.[9] Indeed, prenatal PUFA status has repeatedly been associated with general child neurodevelopment, expressed by intelligence quotient (IQ) and other scores of global cognitive ability (for an overview see [10]). Whether PUFAs additionally affect the development of ASD is less clear.

Lyall et al. investigated the potential role of maternal PUFAs in the development of ASD in the offspring, using dietary fatty acid intake as a measure of prenatal PUFA status.[11] Higher intake of maternal linoleic acid (LA) – a n-6 PUFA – was found to be associated with reduced risk and a very low total n-3 intake with a higher risk of child ASD. Other, less accurate, proxies of PUFA status such as intake of fish (a major source of n-3 PUFAs) or fish oil supplementation during pregnancy were not associated with child ASD[11,12], although results from one case-control study suggested parent emigration from a place of high fish consumption to be a risk factor for ASD.[13] No studies of dietary intake, however, investigated the ratio between n-3 and n-6 PUFAs. More important though, nutritional biomarkers, which are less prone to information bias, were not incorporated to further support a potential relationship between fatty acid intake and ASD.

In this prospective population-based study, we explored whether maternal plasma PUFA concentrations in pregnancy predicted autistic traits in the offspring at 6 years of age. Given the association of ASD with child IQ[14], we further examined whether found

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associations with child autistic traits were independent of child IQ. We hypothesised that lower maternal n-3:n-6 ratio is associated with more child autistic traits.

METHODS

Study population

The present study was conducted within the Generation R Study.[15] The study was conducted in accordance with the guidelines proposed in the World Medical Association Declaration of Helsinki and was approved by the Medical Ethical Committee of the Erasmus Medical Centre. Written consent was obtained from all adult participants and caregivers of participating children.

Out of 8,663 women who enrolled in the study before their third trimester of pregnancy (< 25 weeks gestation), valid fatty acid (FA) profiles in plasma were available for 6,999 women (80.8%). Overall, 6,611 mothers and their single liveborn were eligible at study baseline. A measure of child autistic traits at the age of 6 years was available for 4,624 children (69.9%) (see Figure 4.2.1). IQ scores were available for 3,838 of these children.
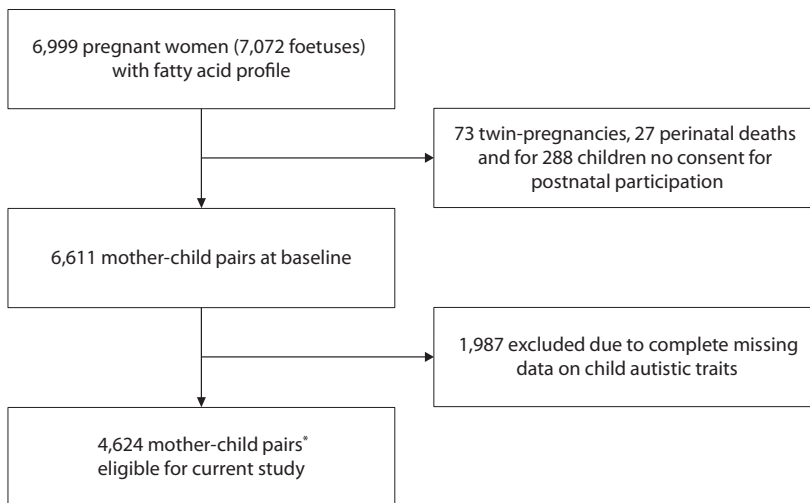

Figure 4.2.1 Flowchart of study population
* Of which 3,838 children have data on intelligence score.

Fatty acid analyses

Between 2001 and 2005, venous samples were drawn in mid-pregnancy (median: 20.6 weeks; 90% range: 18.9-22.8 weeks) and processed as described earlier.[16] Briefly, samples

were stored at room temperature before being transported to the regional laboratory for processing and storage for future studies. The samples were centrifuged and thereafter stored at –80ºC. After being thawed, the analysis of plasma glycerophospholipid FA composition was performed by a sensitive and precise high-throughput method.

In the current study, we used the available n-3 PUFAs in the PUFA synthesis pathway (alpha-linolenic acid – ALA, eicosapentaenoic acid – EPA, docosapentaenoic acid – DPA, and docosahexaenoic acid – DHA) and the corresponding n-6 PUFAs (linoleic acid – LA, arachidonic acid – AA, adrenic acid – AdA, and osbond acid – OA). The n-3:n-6 ratio was calculated by summing the n-3 PUFAs and dividing this by the sum of the n-6 PUFAs, as previously defined[16] (see Supplementary table 4.2.1 for an overview). FAs are expressed as percentage by weight (wt%) of all glycerophospholipid FAs detected with a chain length between 14 and 22 carbon atoms.

Child autistic traits

The Social Responsiveness Scale (SRS) was administered to assess parent-reported autistic traits in children in a population-based setting[17], reported by mothers at mean child age of 6.2 years (SD 0.5). We utilised the 18-item short form of the scale[18] for assessment of autistic behaviours based on parents' observations of children's social behaviour in a naturalistic setting; items selected encompassed all DSM-IV autism domains. Each item on the SRS is rated from 0 (never true) to 3 (almost always true), covering social, language, and repetitive behaviours; higher scores indicate more problems.

Additionally, at mean child age of 6.0 years (SD 0.4) mothers were asked to fill out the Child Behavior Checklist (CBCL), which includes a subscale on Pervasive Developmental Problems (PDP).[19] The PDP subscale is a useful screening instrument to identify children with ASD[20] with good predictive validity identifying preschoolers at risk of ASD.[21] Since SRS and PDP scores correlated well (r = 0.6, $P < 0.001$, n = 3,567), scores on the PDP subscale were included to impute missing values on the SRS (n = 870, 18.8%).

Child IQ

At the age of 6 (mean 6.1 (0.4) years), the children were invited to visit the Generation R research centre. During this visit, children's non-verbal IQ was assessed using two subtests of the validated Dutch test battery 'Snijders-Oomen Niet-verbale Intelligentietest– Revisie' (SON-R 2½-7).[22] These subtests were Mosaics (assesses spatial visualisation abilities), and Categories (assesses abstract reasoning abilities). Raw test scores were converted into non-verbal IQ scores using norms tailored to exact age.

Maternal fish intake

Nutritional intake, including fish consumption, in the past three months was assessed in early pregnancy (median 13.8 weeks, 90% range 10.8-21.4) by using a modified version

of a validated semi-quantitative food frequency questionnaire (FFQ).[23] Assessment of fish intake has been described in detail previously.[24] Total fish intake (in g/day) includes all types of fish consumed and was adjusted for daily total energy intake using the residual method.[25] Fish intake data was available in 3,802 mothers of the current study population.

Covariates

Several parental and child characteristics were included as confounding variables, based on previous studies of the associations of perinatal PUFA status with child autistic traits and IQ.[10-12] These were: maternal IQ, age, educational level, national origin, psychopathology in mid-pregnancy, pre-pregnancy BMI, pregnancy planning, parity, marital status, smoking, alcohol consumption and folic acid supplement use during pregnancy, and family income. Since several recent studies underscored the importance of paternal determinants of ASD, we also included the age, educational level, national origin, psychopathology and BMI of the father.[26] Child characteristics comprised gestational age and weight at birth, gender, breastfeeding status at 6 months, daycare attendance during early childhood and age of the child at the time of assessment.

As no information on child PUFA levels was available, we used dietary n-3 and n-6 intake during infancy, as assessed by a 211-item FFQ when children were 14 months old[27], to adjust for child fatty acid status. N-3 and n-6 intake were adjusted for total energy intake using the residual method[25] and were available for 2,436 of the children in the current study sample.

Statistical analyses

We tested the associations of maternal n-3:n-6 ratio (per SD), as well as total n-3 and n-6 concentrations (wt%) with child autistic traits with multivariable linear regression analyses. The outcome variable, autistic trait scores, was square root-transformed to achieve a more normal distribution. If one or more of the associations between these overall measures of maternal PUFA status and child autistic traits was significant, further association tests of individual maternal PUFAs (wt%) with child autistic traits were performed. In addition, as prenatal nutritional n-3 deficiency has been found to affect offspring behaviour[11,28,29], the lowest 10% and 5% of the distribution of total n-3 status were compared with the remaining 90% respectively 95% of these distributions to examine the effects of very low n-3 status in secondary analyses. Analyses were repeated adjusting for child dietary n-3 and n-6 intake at age 14 months in a subsample with these data available (n = 2,436) to test intrauterine effects. To further illustrate results, we divided the maternal n-3:n-6 ratio by quintiles in analyses with child autistic traits.

Similarly, we explored associations of maternal n-3:n-6 ratio (per SD), and total n-3 and n-6 concentrations with child IQ.

Finally, we examined the associations of fish intake during pregnancy with offspring autistic traits and IQ in a subsample of mothers with prenatal dietary intake data available (n = 3,802). Average daily total fish intake (g/day, per SD) was used as both a continuous and dichotomised (use versus no use) predictor variable in multivariate linear regression models.

All analyses of maternal PUFA status and child outcome were controlled for gestational age at venipuncture, gender of the child and the age of the child at assessment, as well as the other PUFAs (to test the independent association of each PUFA). The other covariates were included in subsequent covariate-adjusted models. Analyses with child autistic traits as an outcome were additionally adjusted for child IQ. Analyses of maternal fish intake were additionally adjusted for daily total energy intake.

Missing values on covariates (0.1-30.4% missing data, average 11.4%), child IQ (17.0%) and autistic traits (18.8%, only imputed if a Pervasive Developmental Problems score was available) were imputed using the Markov Chain Monte Carlo multiple imputation technique with Predictive Mean Matching for continuous variables and generating 5 datasets. All statistical analyses were carried out using PASW Statistics, version 21.0 for Windows (SPSS Inc., Chicago, Illinois).

For the non-response analysis, we compared child and parental characteristics of included participants (n = 4,624; 69.9%) with characteristics of participants of whom we did not obtain child behavioural data (n = 1,987). Excluded children on average had a 99 gram (95% CI: 70;128, $P < 0.001$) lower birth weight compared with included children. The mothers of children not included on average had a 0.4 wt% (95% CI: 0.36;0.51, $P < 0.001$) lower total n-3 level and a 1.1 wt% (95% CI: 0.99;1.28, $P < 0.001$) higher total n-6 level in mid-pregnancy, were on average 3.1 (95% CI: 2.8;3.4, $P < 0.001$) years younger and were less educated (20.6% primary education compared with 7.4%, χ^2 (2) = 537, $P < 0.001$). Mothers of excluded children also had more severe psychopathological complaints (median 0.25 (95% range: 0-1.72) compared with 0.15 (95% range: 0-1.29), $P < 0.001$).

4.2

RESULTS

Descriptive characteristics of study participants by maternal n-3:n-6 ratio are presented in Table 4.2.1.

Maternal PUFAs and child autistic traits

Table 4.2.2 shows the unadjusted and adjusted associations of maternal PUFA status with child autistic traits. Lower maternal n-3:n-6 ratio and higher total n-6 levels were associated with more child autistic traits (B$_{n-3:n-6\ ratio}$ = −0.010, 95% CI: −0.017;−0.002;

Table 4.2.1 Subject characteristics by maternal n-3:n-6 ratio[a] in mid-pregnancy[b]

	Quintile 1 of n-3:n-6 ratio	Other n-3:n-6 quintiles	P[c]
Child characteristics	n = 926	n = 3,698	
Gender (% girl)	50.2	49.5	0.69
Gestational age at birth (wk)[d,e]	39.8 ± 1.8	40.0 ± 1.7	0.002
Weight at birth (g)	3,387 ± 558	3,472 ± 548	< 0.001
Breastfeeding (partial) until 6 mo (%)	43.9	36.6	0.10
Daycare attendance (nr of hrs/wk)[f]	12.6 ± 11.1	18.9 ± 10.7	< 0.001
Age child at assessment (yr)[g]	6.3 ± 0.6	6.2 ± 0.5	< 0.001
	n = 336	n = 2,100	
N-3 intake at 14 mo (g/day)[h]	0.64 ± 0.34	0.62 ± 0.34	0.25
N-6 intake at 14 mo (g/day)[h]	5.4 ± 2.8	4.6 ± 2.4	< 0.001
Maternal characteristics	n = 926	n = 3,698	
Age at enrolment (yr)	28.9 ± 5.1	31.2 ± 4.5	< 0.001
Ethnicity (% Dutch)	33.8	65.4	< 0.001
Education (% higher)	27.7	55.7	< 0.001
Family income (% > 2,000 €/mo)	41.4	72.6	< 0.001
Psychopathology in mid-pregnancy[i]	0.37 ± 0.43	0.24 ± 0.31	< 0.001
IQ-score[j]	91.5 ± 15.5	98.1 ± 14.7	< 0.001
BMI before pregnancy (kg/m²)	24.2 ± 4.5	23.4 ± 4.0	< 0.001
Smoking during pregnancy (% continued)	20.0	13.2	< 0.001
Alcohol consumption during pregnancy (% continued)	25.3	49.7	< 0.001
Parity (% primiparae)	47.2	60.7	< 0.001
Pregnancy planned (% yes)	70.9	78.3	< 0.001
Marital status (% cohabitation)	89.1	90.1	0.38
Folic acid supplement use during pregnancy (%)	67.1	85.3	< 0.001
	n = 690	n = 3,112	
Fish intake (g/day)[h,k]	9.4 ± 11.8	15.3 ± 13.8	< 0.001
Energy intake (kcal/day)	1,983 ± 593	2,082 ± 544	< 0.001
Paternal characteristics	n = 926	n = 3,698	
Age at enrolment (yr)	32.1 ± 6.0	33.8 ± 5.4	< 0.001
Ethnicity (% Dutch)	32.4	66.7	< 0.001
Education (% higher)	31.9	53.8	< 0.001
Psychopathology in mid-pregnancy[l]	0.20 ± 0.28	0.14 ± 0.22	0.006
BMI (kg/m²)	25.6 ± 3.6	25.2 ± 3.3	0.005

n-3 – omega-3, n-6 – omega-6, IQ – intelligence quotient

[a] Participants in the lowest quintile of maternal n-3:n-6 ratio (1:13.4–1:6.5) versus those in the other quintiles (1:6.4–1:1.9).

[b] Descriptives on imputed data.

[c] Derived from linear regression analysis for continuous variables or logistic regression analysis for categorical variables; on imputed data.

[d] Means ± SD (all such values).

[e] Median (95% range): quintile 1: 40.1 (35.6-42.4); other quintiles: 40.3 (36.0-42.4).

[f] Median (95% range): quintile 1: 10.5 (0.0-36.6); other quintiles: 20.0 (0.0-38.5).

[g] Median (95% range): quintile 1: 6.2 (5.7-8.0); other quintiles: 6.0 (5.7-7.6).

[h] Adjusted for energy intake.

[i] Median (95% range): quintile 1: 0.23 (0.00-1.63); other quintiles: 0.13 (0.00-1.19).

[j] Median (95% range): quintile 1: 95.0 (55.0-120.0); other quintiles: 100.0 (69.0-120.0).

[k] Median (95% range): quintile 1: 7.4 (−1.8-36.8); other quintiles: 12.9 (−1.0-51.7).

[l] Median (95% range): quintile 1: 0.10 (0.00-0.96); other quintiles: 0.06 (0.00-0.76).

$B_{\text{total n-6}}$ = 0.004, 95% CI: 0.001;0.007). With regard to the individual n-6 PUFAs, only LA remained associated with child autistic traits after full adjustment for confounders (B = 0.004, 95% CI: 0.000;0.008).

No association was found between maternal total n-3 and child autistic traits (Table 4.2.2). In secondary analyses, the lowest 10% or 5% of the n-3 distribution compared with the remaining 90% respectively 95% of the distribution were also not associated with autistic traits (fully adjusted models: $B_{\text{lowest 10%}}$ = 0.007, 95% CI: −0.022;0.036;

Table 4.2.2 Associations of maternal fatty acids during pregnancy with child autistic traits at 6 years (n = 4,624)

Maternal fatty acid	Autistic traits[a]					
	Basic[b]		Adjusted for covariates[c]		Adjusted for child IQ[d]	
	B (95% CI)	P	B (95% CI)	P	B (95% CI)	P
N-3:n-6 ratio (per SD)	−0.033 (−0.040;−0.025)	< 0.001	−0.010 (−0.017;−0.002)	0.014	−0.009 (−0.017;−0.001)	0.019
Total n-3 (wt%)[e]	−0.008 (−0.014;−0.002)	0.009	−0.002 (−0.008;0.004)	0.58	−0.002 (−0.007;0.004)	0.60
Total n-6 (wt%)[e]	0.012 (0.008;0.015)	< 0.001	0.004 (0.001;0.007)	0.017	0.004 (0.000;0.007)	0.026
N-3 (wt%)[e]						
ALA	−0.052 (−0.131;0.026)	0.19	−0.003 (−0.083;0.077)	0.94	−0.011 (−0.091;0.068)	0.78
EPA	−0.007 (−0.038;0.024)	0.65	0.001 (−0.029;0.031)	0.96	0.005 (−0.024;0.034)	0.75
DPA	−0.011 (−0.053;0.030)	0.58	0.011 (−0.028;0.050)	0.58	0.011 (−0.029;0.051)	0.58
DHA	−0.004 (−0.015;0.007)	0.44	−0.003 (−0.014;0.009)	0.63	−0.003 (−0.015;0.009)	0.59
N-6 (wt%)[e]						
LA	0.012 (0.008;0.016)	< 0.001	0.004 (0.000;0.008)	0.035	0.004 (0.000;0.008)	0.038
AA	0.014 (0.008;0.020)	< 0.001	0.004 (−0.001;0.010)	0.14	0.004 (−0.002;0.010)	0.20
AdA	0.169 (0.051;0.288)	0.006	−0.029 (−0.144;0.086)	0.62	−0.034 (−0.152;0.084)	0.57
OA	−0.025 (−0.097;0.047)	0.50	0.032 (−0.044;0.108)	0.40	0.038 (−0.037;0.113)	0.32

IQ – intelligence quotient, N-3 – omega-3, n-6 – omega-6, ALA – alpha-linolenic acid, EPA – eicosapentaenoic acid, DPA – docosapentaenoic acid, DHA – docosahexaenoic acid, LA – linoleic acid, AA – arachidonic acid, AdA – adrenic acid, OA – osbond acid

[a] Social Responsiveness Scale, mathematically transformed (square root) to satisfy the assumption of normality in linear regression analysis.

[b] Model 1: adjusted for the other n-3 and n-6 fatty acids, gestational age at venipuncture, gender, and age of the child at assessment.

[c] Model 2: model 1, additionally adjusted for maternal IQ, pre-pregnancy BMI, educational level, national origin, age at enrolment, psychopathology score in mid-pregnancy, smoking, alcohol consumption, and folic acid supplement use during pregnancy, parity, marital status, pregnancy planning and family income, and child gestational age and weight at birth, breastfeeding at 6 months and daycare attendance, and paternal educational level, national origin, age, psychopathology score and BMI.

[d] Model 3: model 2, additionally adjusted for child IQ.

[e] Expressed as percentage by weight; 1 unit increase equals an increase of 1% of total fatty acid composition for the respective polyunsaturated fatty acid(s).

4.2

$B_{lowest\ 5\%}$ = 0.000, 95% CI: −0.038;0.038). Consistently, there were no associations of individual n-3 PUFAs with child autistic traits (Table 4.2.2).

Adjustment for child n-3 and n-6 intake in a subsample with these data available (n = 2,436) did not change the associations of n-3:n-6 ratio, total n-6 or LA with child autistic traits meaningfully (in this subsample, both before and after adjustment for child diet: $B_{n-3:n-6\ ratio}$ = −0.011, 95% CI: −0.021;−0.001; $B_{total\ n-6}$ = 0.006, 95% CI: 0.001;0.011; B_{LA} = 0.006, 95% CI: 0.001;0.012).

To further illustrate the association of maternal n-3:n-6 ratio with child autistic traits, we divided the ratio by quintiles. The highest quintile was used as the reference category (quintile 5). Compared to this reference, a maternal n-3:n-6 ratio in the lowest quintile was associated with more autistic traits in the offspring (B = 0.025, 95% CI: 0.001;0.049; Figure 4.2.2).
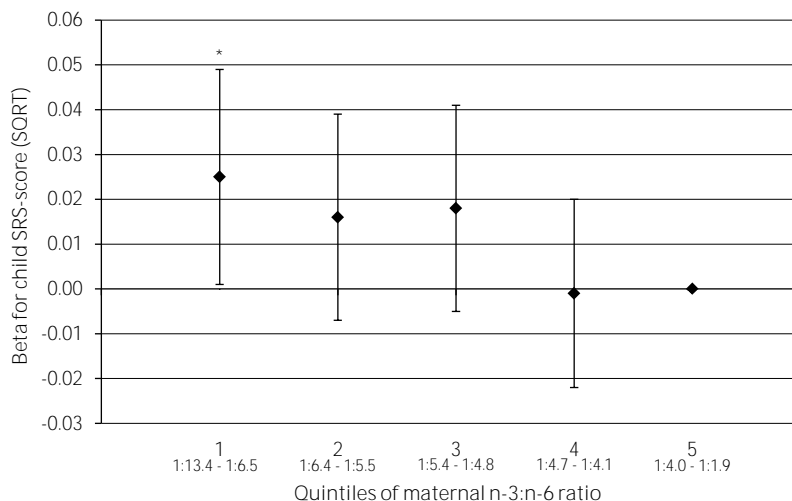

Figure 4.2.2 Quintiles of maternal n-3:n-6 ratio and child autistic traits
SRS – Social Responsiveness Scale, SQRT – square root, n-3 – omega-3, n-6 – omega-6
Note: Error bars are 95% confidence intervals.
* $P < 0.05$

Maternal PUFAs and child IQ

The associations between maternal PUFA status and child IQ are presented in Table 4.2.3. Maternal n-3:n-6 ratio, total n-3 and total n-6 were not associated with child IQ. The associations of maternal n-3:n-6 ratio, total n-6 and LA status with child autistic traits indeed did not materially change after adjustment for child IQ (Table 4.2.2).

Table 4.2.3 Associations of maternal fatty acid status during pregnancy with child IQ at 6 years (n = 3,838)

| | IQ score | | | |
| Maternal fatty acid | Basic[a] | | Adjusted for covariates[b] | |
	B (95% CI)	P	B (95% CI)	P
N-3:n-6 ratio (per SD)	2.65 (2.19;3.11)	< 0.001	0.37 (−0.12;0.86)	0.14
Total n-3 (wt%)[c]	0.83 (0.46;1.19)	< 0.001	0.10 (−0.27;0.47)	0.61
Total n-6 (wt%)[c]	−0.82 (−1.01;−0.62)	< 0.001	−0.15 (−0.36;0.06)	0.17

IQ – intelligence quotient, N-3 – omega-3, n-6 – omega-6

[a] Model 1: adjusted for the other n-3 and n-6 fatty acids, gestational age at venipuncture, and gender of the child.

[b] Model 2: model 1, additionally adjusted for maternal IQ, pre-pregnancy BMI, educational level, national origin, age at enrolment, psychopathology score in mid-pregnancy, smoking, alcohol consumption, and folic acid supplement use during pregnancy, parity, marital status, pregnancy planning and family income, and child gestational age and weight at birth, breastfeeding at 6 months and daycare attendance, and paternal educational level, national origin, age, psychopathology score and BMI.

[c] Expressed as percentage by weight; 1 unit increase equals an increase of 1% of total fatty acid composition for the respective polyunsaturated fatty acids.

Maternal fish intake and child autistic traits/IQ

In a subsample with prenatal fish intake data available, we tested the association of maternal fish intake during pregnancy with child autistic traits (n = 3,802) and IQ (n = 3,162) (Supplementary table 4.2.2). Consistent with results from the analyses with n-3 concentrations, fish intake, a major source of n-3 PUFAs, was not associated with child autistic traits or IQ after adjustment for confounders.

DISCUSSION

In this population-based study, we found that a lower maternal n-3:n-6 ratio during pregnancy was associated with more autistic traits in the offspring at age 6 years. This association was largely accounted for by relatively higher n-6, in particular higher maternal LA. The associations were independent of child IQ. Maternal fish intake - a major source of n-3 - during pregnancy was, consistent with our non-significant findings for n-3 biomarkers, not associated with offspring autistic traits.

Interpretation

Our findings that lower maternal n-3:n-6 ratio and higher maternal total n-6 PUFA status are associated with more autistic traits in the offspring are in line with several studies relating PUFA intake in adults to other health related outcomes such as cardiovascular disease, cancer, and inflammatory and auto-immune diseases. For these diseases, high intake of n-6 PUFAs and a low n-3:n-6 ratio have been hypothesised to promote pathogenesis.[30]

4.2

Blood concentrations of PUFAs reflect both dietary intake and biological processes. One of these processes concerns the endogenous synthesis of ALA and LA to their long chain end products, respectively DHA/EPA and AA. During this synthesis, the n-3 and n-6 PUFAs compete for the same enzyme systems.[6] As a result, higher intake of n-6 PUFAs will decrease the synthesis and thus availability of n-3 PUFAs, and vice versa. Additionally, fatty acid desaturase genotypes are involved in this synthesis pathway and modulate the amounts of PUFA in the blood.[31] The association of maternal n-3:n-6 ratio with child autistic traits seems to be accounted for by the n-6 rather than the n-3 PUFA status. In particular, we found higher maternal LA status to be associated with more autistic traits. It should be kept in mind, however, that LA is the largest contributor to total n-6 status and as such can be measured with more precision than the other n-6 PUFAs. In addition, the limited range of total n-3 status in our study sample may make an association between very low n-3 status and child autistic traits hard to detect. Further, the loss of power due to dichotomisation of the predictor variable might have contributed to the lack of significant findings in these analyses of extremes.

An earlier study reported that higher dietary n-6 and LA-*intake* were associated with a lower risk of autism[11] which may be considered inconsistent with our finding. Yet, although correlated, dietary intake and biomarker concentrations cannot be compared straightforwardly. First, as mentioned earlier, biomarkers also reflect biological metabolism and are a more genetically determined characteristic. Second, dietary intake data are prone to information bias due to over- and under-reporting in FFQs.[32] When the same informant (mother) additionally reports on the study outcome, shared method variance bias is introduced.[33] A potential solution is the use of nutritional biomarkers that are able to objectively assess dietary status without the bias of self-reported dietary intake errors.[34]

We did not find an association of maternal fish intake – a major source of n-3 - during pregnancy with child autistic traits nor with *non-verbal* IQ, in line with most previous studies which only found associations with verbal IQ.[11,35,36] Likewise, we did not find n-3 status to be associated with child autistic traits and IQ.

Mechanism of risk

N-3 and n-6 PUFAs play an important role in various neurodevelopmental processes. The underlying mechanism of the association of maternal n-3:n-6 ratio with child autistic traits may be explained by insights gained from animal studies.[7] Studies in mice have shown that both n-3 and n-6 PUFAs are required for the maturation of nerve growth cones and subsequent synapse formation.[37] Rat studies have shown that maternal dietary fatty acids can alter foetal brain growth cone n-3 and n-6 levels.[38] Impaired synapse formation has been indicated as a core mechanism in ASD pathology.[39] Also, n-3 and n-6 PUFAs have been identified as endogenous ligands for peroxisome proliferator-

activated receptors (PPARs), which are involved in myelination.[40] Indeed, expression of PPARs in young mice was positively related to the dietary n-3:n-6 ratio during maternal pregnancy and lactation[41], and changes in myelination alter brain connectivity, which has been proposed as a mechanism in ASD pathology.[42]

Further, epigenetic factors have been proposed to play a role in the origins of ASDs[43] and there is emerging evidence that PUFAs can modify the epigenome.[44] However, whether epigenetic changes caused by PUFAs can also affect development of autistic traits is yet unknown.

Finally, this type of nutritional associations in observational studies is sensitive to effects of confounding factors. A substantial attenuation in the association of maternal PUFA status with the offspring's autistic traits was noticeable after adjustment for confounders. Although we accounted for many sociodemographic factors, further (residual) confounding cannot be excluded.

Other limitations include selective attrition and possible information bias. Mothers of children not included generally had less favourable PUFA status and socioeconomic circumstances. Whether this selective attrition leads to bias cannot be inferred from our results. Next, our assessment of PUFA status was based on a single measure in blood plasma in mid-pregnancy. One measurement, although indicative as it reflects intake over an 8 week time frame[45], is not a very reliable reflection of a mother's long-term FA status. However, repeated measurements were not feasible in this large study.

Conclusion

In conclusion, we found maternal plasma n-3:n-6 ratio and n-6 PUFA status in mid-pregnancy to be associated with child autistic traits at age 6 years. As the associations were independent of child IQ, the results suggest specific effects of fatty acid distribution on the development of autistic traits in addition to general neurodevelopment. Our study underscores the possibility of a biological pathway to explain the association of maternal dietary fatty acid intake during pregnancy and development of ASD in the offspring. We did not find maternal plasma n-3 status to be associated with child autistic traits and, likewise, maternal fish intake during pregnancy was not associated with child autistic traits. Possibly, the diet as a whole is involved, and focus should not only be on increasing n-3 intake, but also on lowering the intake of food products with high n-6 content. More research is needed to confirm our findings. In addition, future research on optimal n-3 and n-6 PUFA intake should also include blood PUFA concentrations to account for the endogenous biological processes that determine the actual bioavailability of n-3 and n-6 PUFAs in the human body.

4.2

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4.2

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Supplementary table 4.2.1 Characteristics of available fatty acids in the PUFA synthesis pathway, included in the n-3:n-6 ratio (n = 4,624)

Fatty acid	Range	Mean (SD)	Median (95% range)
Total fatty acids (mg/L)	771-2,535	1,632 (223)	1,623 (1,217-2,091)
N-3:n-6 ratio	0.1-0.5	0.2 (0.1)	0.2 (0.1-0.3)
N-3 family (wt%)[a]	2.9-14.5	6.4 (1.5)	6.3 (4.0-9.9)
18:3n-3 (α-linolenic acid, ALA)	0.1-0.8	0.3 (0.1)	0.3 (0.2-0.6)
20:5n-3 (eicosapentaenoic acid, EPA)	0.1-2.9	0.6 (0.3)	0.5 (0.2-1.5)
22:5n-3 (docosapentaenoic acid, DPA)	0.3-2.1	0.8 (0.2)	0.7 (0.4-1.4)
22:6n-3 (docosahexaenoic acid, DHA)	1.9-10.3	4.8 (1.1)	4.7 (2.9-7.3)
N-6 family (wt%)[a]	24.7-42.1	32.7 (2.7)	32.6 (27.8-38.2)
18:2n-6 (linoleic acid, LA)	14.2-35.2	22.2 (2.8)	22.0 (17.0-28.0)
20:4n-6 (arachidonic acid, AA)	4.4-16.0	9.6 (1.5)	9.6 (6.7-12.7)
22:4n-6 (adrenic acid, AdA)	0.1-1.1	0.4 (0.1)	0.4 (0.2-0.7)
22:5n-6 (osbond acid, OA)	0.1-1.4	0.5 (0.2)	0.5 (0.2-0.9)

PUFA – polyunsaturated fatty acid, n-3 – omega-3, n-6 – omega-6
[a] Fatty acids are expressed as percentage by weight of total fatty acid composition.

Supplementary table 4.2.2 Associations of maternal fish intake during pregnancy with child autistic traits and IQ at 6 years

	Autistic traits (n = 3,802)[a]			
	Basic[b]		Adjusted for covariates[c]	
Maternal fish intake	B (95% CI)	P	B (95% CI)	P
Linear (per SD)	−0.003 (−0.011;0.005)	0.48	−0.001 (−0.009;0.007)	0.80
Dichotomised				
No use (n = 319)	Reference		Reference	
Use (n = 3,483)	−0.041 (−0.075;−0.006)	0.022	−0.023 (−0.056;0.010)	0.16
	IQ (n = 3,162)			
	Basic[b]		Adjusted for covariates[c]	
Maternal fish intake	B (95% CI)	P	B (95% CI)	P
Linear (per SD)	0.30 (−0.20;0.79)	0.24	0.18 (−0.31;0.66)	0.48
Dichotomised				
No use (n = 257)	Reference		Reference	
Use (n = 2,905)	2.91 (1.04;4.78)	0.002	1.36 (−0.41;3.13)	0.13

IQ – intelligence quotient
[a] Social Responsiveness Scale, mathematically transformed (square root) to satisfy the assumption of normality in linear regression analysis.
[b] Model 1: adjusted for maternal daily caloric intake and gender of the child. Additional adjustment for child age at assessment in analyses with child autistic traits.
[c] Model 2: model 1, additionally adjusted for maternal IQ, pre-pregnancy BMI, educational level, national origin, age at enrolment, psychopathology score in mid-pregnancy, smoking, alcohol consumption, and folic acid supplement use during pregnancy, parity, marital status, pregnancy planning and family income, and child gestational age and weight at birth, breastfeeding at 6 months and daycare attendance, and paternal educational level, national origin, age, psychopathology score and BMI. Additional adjustment for child IQ in analyses with child autistic traits.

4.2

Chapter 5

General discussion

The main aim of this thesis was to extend existing knowledge on the relation between maternal nutrition during pregnancy and offspring emotional and behavioural development. The first part of this thesis described the association of maternal dietary patterns with child emotional and behavioural problems. The second and third part focused on maternal blood concentrations of two specific nutrients: folate and fatty acids. For both nutrients, we reported how they affect early childhood risk of general emotional and behavioural problems, and autistic traits more specifically. The current chapter provides a summary of the main findings from these series of investigations and discusses major methodological issues with regard to studies of nutritional epidemiology and child psychopathology, after which I conclude with implications for clinical practice and future studies.

MAIN FINDINGS

Maternal diet during pregnancy affects child behavioural problems

Researchers from the Dutch Famine Study were the first to demonstrate that maternal malnutrition during pregnancy may predict psychopathology later in life. Susser and colleagues found that prenatal exposure to famine is related to an increase in schizophrenia risk in adulthood[1], a finding confirmed in two later studies in China.[2] Since then, many studies focused on the association of *individual* foods and nutrients with child psychopathology. However, the possible effects of the prenatal diet *composition* received less attention. By means of dietary pattern analysis[3], we derived three patterns: 1) a 'Mediterranean' diet (high intake of vegetables, fish & shellfish, vegetable oil, fruit, and eggs, and relatively low intake of processed meat, 2) a 'Traditionally Dutch' diet (high intake of fresh and processed meat, potatoes and margarines, and a very low intake of soy and diet products), and 3) a 'Confectionary diet' (high intake of cakes, sugar & confectionary products, tea, cereals, fruit and dairy products). Similar to the study by Jacka et al.[4], we found that children of mothers with a low adherence to a 'healthy' Mediterranean diet or a high adherence to a 'less healthy' Traditionally Dutch diet during pregnancy were at increased risk of behavioural problems throughout early childhood.

These results are also in line with studies of effects of dietary pattern on neural tube defects[5] and foetal growth[6], which in turn are related to child neurodevelopment. Therefore, the beneficial effect of more healthy dietary patterns on child behaviour may be mediated by foetal growth.[7] In our sample however, we did not find evidence for an association between foetal growth and child problem behaviour[8] and inclusion of birth weight in the model did not materially change the association between maternal prenatal diet and child behaviour. Nevertheless, we cannot exclude the possibility that other characteristics of foetal growth, such as disproportionate growth resulting from placental insufficiency, may explain the association.[9]

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A second possible mechanism relates to the neurodevelopmental origin of psychiatric disorders.[10] For example, increased prenatal oxidative stress may mediate effects of an imbalanced maternal diet on offspring neurodevelopment. Oxidative stress represents an imbalance in the body, characterised by an excessive production of free radicals and inadequate antioxidant defence mechanisms. A diet high in saturated fat, such as the Traditionally Dutch diet, increases the level of oxidative stress. Antioxidants, mainly found in vegetables and fruits as in the Mediterranean diet, decrease the level of oxidative stress. Maternal micronutrient imbalance has been found to reduce antioxidant enzymes in rat brain at birth.[11] In addition, maternal fatty acid status during foetal development has been hypothesised to affect child problem behaviour because of the important direct role in brain development of omega-3 (n-3) and omega-6 (n-6) fatty acids. Mothers with a high adherence to a Mediterranean-type diet may have a better n-3:n-6 fatty acid ratio than mothers who highly adhere to the Traditionally Dutch pattern, which mainly provides them with n-6 fatty acids. Indeed, we found these fatty acids, measured in maternal blood during pregnancy, to be associated with child emotional and behavioural problems. As a third potential mechanism, our findings may also be the result of confounding, which I will discuss in more detail later.

Influences of maternal folate status on offspring brain and behaviour

Emerging evidence has shown that maternal folic acid supplementation during pregnancy is associated with reduced risk of several neurodevelopmental disorders in offspring, including language delays[12], problem behaviour[13] and autism spectrum disorders (ASD).[14,15] However, the underlying mechanism remains unclear. To further support a potential relationship between folic acid supplement use and neurodevelopmental disorders, nutritional biomarkers and biological intermediates should be examined. Therefore, we aimed to study whether maternal folic acid supplementation and folate concentrations during pregnancy are related to offspring head growth, to general emotional and behavioural problems, and particularly to autistic traits.

Folic acid supplementation was associated with larger prenatal head size at 20 weeks of gestation (chapter 3.1), and with decreased risk of child emotional problems (chapter 3.2) and autistic traits (chapter 3.3). Higher maternal folate concentration in early pregnancy was related to a slightly faster foetal head *growth* from the second to the third trimester of pregnancy, resulting in a larger head size at 30 weeks of gestation (chapter 3.1). Low folate was also found to affect child emotional problems, which could partly explain the association of maternal folic acid supplementation with child emotional problems (chapter 3.2). Folate was, however, not related to child autistic traits (chapter 3.3).

It is tempting to speculate about a mechanistic pathway from prenatal folic acid supplementation through maternal blood folate status via prenatal head growth to child emotional problems. We must be cautious with our conclusions though, since

earlier analyses in our study sample did not show evidence for an association between foetal head growth and child emotional problems.[8] Moreover, the associations of prenatal folic acid use with child emotional problems and autistic traits could not be (entirely) explained by folate status. Folic acid use is a marker of good health literacy, and is thus associated with many factors that reduce the background risk of problem behaviour in the offspring. For example, folic acid use differs between mothers from different national origins.[16] In our studies, inadequate use is reported by 16% of the Dutch mothers and by 40% of the non-Dutch mothers. Child problem behaviour is also associated with national origin, with more (reported) problems in children from non-Dutch origin.[17] Emotional problems were more frequent in children of non-Dutch mothers (up to 37% of the children of inadequate users of folic acid supplements) than in children of Dutch mothers (roughly 14% of children of adequate and inadequate users of folic acid supplements). When we stratified our analyses of folic acid supplement use with child emotional problems or autistic traits by national origin, the reported associations were indeed only found within non-Dutch mothers. Apart from these effect-modifying effects of national origin, interpretation of the associations is further complicated by confounding factors related to national origin, folic acid use and reporting of child problem behaviour, such as home environment.[18] Therefore, we cannot eliminate the possibility that the observed association of prenatal folic acid use with child psychopathology in non-Dutch women is explained by such other, unmeasured factors.

Maternal fatty acids and child emotional and behavioural development

In chapter 4, we studied maternal prenatal fatty acid status in relation to child problem behaviour. The n-3 and n-6 polyunsaturated fatty acids (PUFAs) are important for brain development, as they affect numerous processes, including membrane fluidity, neurotransmission, and gene expression.[19] Whereas both types of PUFAs are important for brain development, they exert different and sometimes even opposite effects.[20] The prenatal phase is a time of both rapid neurodevelopment and increased foetal demand of PUFAs[21], for which the foetus depends on maternal supply.[22] Therefore, maternal PUFAs during foetal development have been hypothesised to affect child problem behaviour. Research in this field is however scarce and results are conflicting.[23-26] Moreover, most studies solely relied on maternal report of child problem behaviour, whereas multiple informants can provide information from different perspectives and in different contexts.[27] In addition, during the last century, the Western diet has provided a lower n-3 and higher n-6 intake than in previous generations, diminishing the n-3:n-6 ratio[20], which has been suggested to contribute to the possibly increased emergence of ASD over the past decades.[28] Little is known about the role of maternal PUFAs in the development of child ASD.[15,29,30] But perhaps even more important, nutritional biomarkers were not incorporated in earlier research to further support a potential relationship

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between maternal fatty acid intake and child ASD. Our studies aimed to gain insight in the associations of maternal blood PUFA concentrations and the n-3:n-6 ratio with child emotional and behavioural problems, and more specifically, with child autistic traits.

Higher maternal DHA (docosahexaenoic acid, n-3) levels and n-3:n-6 ratio were associated with fewer offspring emotional problems. Higher maternal AA (arachidonic acid, n-6) levels were associated with more child behavioural problems. Our results showed some discrepancies when different informants of child problem behaviour were used, which I will discuss later in more detail.

Furthermore, lower maternal n-3:n-6 ratio during pregnancy was associated with more autistic traits in the offspring. The effect of this presumably less healthy balance in PUFAs was mainly driven by the effects of higher n-6 concentrations and, more specifically, by higher maternal linoleic acid (n-6) levels. We did not find prenatal fish intake, a major source of n-3 PUFAs, to be associated with autistic traits in the offspring. This is in line with previous studies[30] and, against the background of our results from the biomarker analyses, may indicate that focus should not only be on increasing dietary n-3 intake, but also on lowering the intake of food products with high n-6 content.

The findings in chapter 4.1 and 4.2 both suggest that low maternal n-3 and high n-6 PUFA levels, resulting in an unbalanced n-3:n-6 ratio, put the unborn child at risk of psychopathology in later life. This is in line with earlier studies regarding PUFA status and other health related outcomes in adults, such as cardiovascular disease, cancer, and inflammatory and auto-immune diseases. It has been hypothesised that high intake of n-6 PUFAs and a low n-3:n-6 ratio promote the underlying pathogenesis in these disorders.[31] Our results indicate a biological pathway between maternal PUFA intake, in particular the n-3:n-6 ratio, and the development of emotional and behavioural problems and autistic traits in the offspring, but the methodological limitations must be kept in mind.

METHODOLOGICAL CONSIDERATIONS

The strengths and limitations of the studies described in chapter 2–4 have been discussed in the specific chapters. In the following paragraph, I will discuss general methodological considerations with regard to studies of nutritional epidemiology and child psychopathology. These considerations will be related to the nature of determinants and their assessment, the use of multiple informants in assessment of child problem behaviour, and residual confounding.

Bias in dietary intake

Throughout this thesis, different determinants of maternal nutrition were used to gain insight in the association between maternal prenatal nutrition and offspring emotional

and behavioural problems. In chapter 2, an FFQ was used in order to measure average dietary intake in early pregnancy. Because it is relatively inexpensive and easy to administer, the FFQ is the most common method used in epidemiological studies to evaluate an individual's usual intake of type and amount of foods over a defined period of time. As such, it is a retrospective method of estimating nutritional intake. This retrospective nature, however, along with self-report, increases the likelihood of information bias due to measurement error in the collected exposure data. Subject-specific error, which can affect estimated effect sizes, increase uncertainty and decrease study power, is of major concern for analyses and interpretation of results.[32]

Subject specific error may result from over- or under-reporting of food consumption. A study by Lutomski et al.[33] showed that about 22% of women were under-reporters of total energy intake, and about 17% were over-reporters. In pregnant women, these numbers were respectively 33% and 13%.[34] In both studies, subjects with overweight were more likely to be under-reporters, whereas subjects of low socioeconomic status were more likely to be over-reporters. This measurement error, or 'misclassification', can be either differential or non-differential.[35] Differential exposure measurement error is error that is related to the outcome of interest. It occurs for example when mothers of subjects with problem behaviour (the outcome) recall their diet differently than mothers of subjects without problem behaviour. It is often assumed that exposure measurement error in nutritional epidemiological studies is non-differential, because diet is usually reported long before the manifestation of 'disease'.[35] However, although in our study mothers reported their diet before their child was even born, both diet and the reporting of diet are related to many prognostic factors of (reporting of) child psychopathology, such as socioeconomic status. This is suggestive of a differential measurement error in our study, leading to a biased observed estimate of the association between maternal diet and child problem behaviour. The degree of this bias, and whether our associations are an underestimation or overestimation of the 'true' effect, however, cannot be predicted without knowledge of parameters of true dietary intake.[36]

Another limitation of the FFQ used in our studies is that it is only suitable for assessment in Dutch women, as it was designed to capture Dutch dietary habits. Nevertheless, in one of our studies (chapter 4.2) we used fish intake data assessed by the FFQ in all participating mothers, regardless of being of Dutch national origin. Inclusion of non-Dutch mothers did not change the conclusions with regard to the reported associations. Associations of maternal fish intake with child autistic traits were, however, less strong in non-Dutch mothers as compared with those in Dutch mothers (unpublished data). Either the association is indeed less strong in other ethnicities (effect modification by national origin), or information bias has been introduced by inclusion of fish consumption data from women of non-Dutch origin. Considering the fact that mothers of non-

Dutch origin generally have a higher BMI and yet report lower energy intake than Dutch mothers within the Generation R study sample, under-reporting may have occurred in this group, leading to a biased effect estimate.

Bias in supplement use

In chapter 3, we assessed the associations of self-reported folic acid supplement use during pregnancy with offspring head growth and psychopathology. I previously described the possibility that confounding factors related to national origin, folic acid use and (reporting of) child problem behaviour, distort the association of prenatal folic acid supplementation with child emotional problems. Similar to the assessment of dietary intake, however, self-report of supplement use is prone to information bias, for example social desirability bias. Reporting of inadequate use of folic acid supplements may reflect the 'true' situation, being the result of actual unawareness - which is higher among non-Dutch than Dutch women[37] - whereas the reporting of adequate supplement use may be biased. I can only speculate whether the lack of evidence for an association between adequate folic acid supplement use and offspring emotional problems in Dutch mothers may be an underestimation of the effect or reflects the true situation.

Bias in biomarkers

In order to provide support for a biological pathway underlying the associations of maternal folic acid supplement use with child psychopathology, we subsequently assessed the relation between folate concentrations measured in maternal blood plasma and child brain and behaviour. It must be noted, that nutritional biomarkers such as folate, but also fatty acid concentrations used in chapter 4, do not directly reflect supplement use or dietary intake. Blood concentrations of nutrients reflect both dietary intake from supplements and (fortified) foods, and biological processes. For example, the bioavailability of folic acid, i.e. the proportion of the vitamin that is physiologically absorbed and utilised in the organism, depends on many factors, such as the chemical form of folate, the chemical environment of the intestinal tract and factors that affect folate's metabolic utilisation after absorption.[38]

   Our biomarker assessments of folate and fatty acids were performed in blood plasma, which is generally assumed to provide a short-term indication of nutrient status over the past few days.[39,40] Assessment in red blood cells may have given insight in the long-term status over the past month, although Hodson et al. recently showed that erythrocyte and plasma fatty acids reflect intake of saturated and n-6 fatty acids over a similar period of time.[41] Assessments in erythrocytes are usually more expensive and time consuming and therefore more difficult to conduct in large cohort studies like ours. In addition, folate concentrations, for example, are less reliably measured in red blood cells than in plasma.[42] Nevertheless, one single measure of folate and fatty acid concentration,

although indicative, is not a reliable reflection of a mother's long-term nutrient status because fluctuations in concentrations occur due to variation in dietary intake. Multiple measures over a longer period of time may provide a better estimate of nutrient status.

As described above, different determinants of the exposure in this thesis, i.e. nutrition during pregnancy, all face methodological limitations that increase the potential for bias. Indeed, the studies in this thesis show some inconsistent findings with regard to the association between prenatal nutrition and child problem behaviour. Bias in the assessment of child problem behaviour, which I will discuss below, may further contribute to these inconsistencies.

Bias in assessment of child problem behaviour

In professional child mental health care, it is common practice to gather as much information on a child's social, emotional and behavioural functioning as possible. To gain insight in a child's core characteristics from different perspectives and in different contexts, clinicians consult multiple informants, such as parents and teachers, and, from the age of 11 years onwards, the child itself.

In most scientific research studies on nutrition and child psychopathology, however, only one informant, usually the mother, is used. Although the mother is generally the first caregiver, the chance of information bias, particularly shared method variance bias, is introduced by including only mother reports of dietary intake and child problem behaviour.[43] Potential solutions are the use of nutritional data that do not rely on self-report, such as biomarker data, or to include reports of child problem behaviour from other informants.

The use of multiple informants, as e.g. in chapter 4.1, introduces difficulties in interpretation when leading to inconsistent findings. Similar discrepancies in results using maternal and teacher report have been found before.[26,44] As a consequence, strong statements about the associations can only be made when results are consistent between informants. Therefore, not only findings from studies using one informant, but also findings from our study should be interpreted with caution. Agreement on child problem behaviour between parents and teachers is quite low in general[27], which in part results from actual behaviour differences in the child across settings and site differences in ability to elicit behaviour being assessed.[45] In our study, although we used instruments from the family of the Achenbach System of Empirically Based Assessment, we were unable to use Child Behavior Checklists (CBCL) and Teacher Report Forms (TRF) of the same age range due to study design, which may have contributed to this discrepancy in agreement too.

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Residual confounding

Throughout the studies described in this thesis, a rather large decline in effect estimates was observed after adjustment for factors such as maternal psychopathology, national origin, educational level, and family income. This phenomenon is called confounding, which occurs when an extraneous factor (such as national origin) distorts the effect of the exposure (in this case maternal nutrition) on the outcome (child psychopathology).[46] This was not only the case in associations where the determinant was more prone to information bias, such as maternal dietary intake and folic acid supplement use in chapter 2 and 3, but also when we used nutritional biomarkers (chapter 3 and 4). Whereas residual confounding, i.e. confounding by factors that were not measured in the study, can never be ruled out in epidemiological studies, the fact that both the determinant and the outcome in our studies are strongly related to many epiphenomenae of socioeconomic status makes the issue of residual confounding of specific concern.

In particular, I expect other nutrients that are associated with neurodevelopment and that simultaneously affect folate or fatty acid status, such as iron[47,48], to be potential confounders. Also, maternal psychopathology was one of the main confounders in the studies described in this thesis. Possibly, our assessment of maternal psychiatric symptoms did not fully capture all aspects of psychopathology that may affect offspring problem behaviour. Next, although we accounted for many factors such as educational level, income and child daycare attendance, other measures of family functioning such as home environment and parenting style might further explain the reported associations. In addition, the postnatal diet of the child may be an unmeasured, confounding factor in the association between maternal prenatal diet and child psychopathology. As the brain continues to develop rapidly in the first two years of life, it has been suggested that early life nutrition plays an important role in the development of emotional and behavioural problems, and many studies have indeed reported on associations of child nutrition with child problem behaviour.[49,50] I acknowledge the influence of the postnatal diet on child psychopathology and the importance of its incorporation in studies of maternal nutrition and child problem behaviour. However, regarding the fact that the brain most prominently develops during the prenatal phase, I consider it less likely that the postnatal diet would largely explain the associations of maternal prenatal nutrition with offspring psychopathology. We were not able to properly adjust for child dietary intake or folate/fatty acid concentrations. Nevertheless, we did not find any effects of breastfeeding, postnatal n-3 and n-6 intake or consumption of snacks and sugar containing beverages on the associations under investigation. In addition, other studies that were able to take into account child diet or nutritional status have shown effects of maternal nutrition on child psychopathology independent of child nutrition.[4,23,25] Moreover, animal studies have shown, for example, that prenatal and not postnatal DHA deficiency was associated with increased depression and anxiety later in life[51], and that

detrimental changes in offspring anxiety after prenatal exposure to a n-3 PUFA deficient diet could not be reversed by postnatal supplementation with n-3 PUFAs.[52]

CLINICAL AND SCIENTIFIC IMPLICATIONS

Although the results from the studies presented in this thesis alone do not provide a basis to make strong statements and further research is certainly warranted, they provide suggestions for both primary and secondary prevention strategies.

Currently, pregnant women usually receive nutritional advice upon their first visit to the midwife, usually around eight weeks of pregnancy. They are asked about their use of folic acid supplements and receive some dietary information mainly focused at foods that should be avoided due to possible toxic effects for the developing foetus. The results in chapter 2 suggest that nutritional advice should not (only) be aimed at individual foods, but should encompass the diet as a whole. Considering the limited amount of time during a visit, midwives could offer pregnant women written information about specific foods to avoid and on the type of diet recommended (e.g. via magazines or leaflets). Also, adherence to recommendations is usually better when people are motivated. Whereas the importance of good nutrition for physical development of the foetus is generally accepted, there is much less awareness of the potential influence of maternal nutrition on foetal brain and subsequent child mental development. In addition, with regard to the use of folic acid supplements, the current recommendation is to start four weeks before conception and to continue until eight weeks after conception.[53] There is a need to perform randomised controlled trials to confirm our results presented in chapter 3, which suggest that the use of folic acid supplements should perhaps be continued throughout pregnancy in order to lower the risk of development of autistic traits in children.

To date, there is no official advice on fatty acid intake for pregnant women. The general population is advised to consume two portions of fish per week, of which at least one should be fatty fish.[54] Our results in chapter 4 suggest that pregnant women should receive advice, not only with regard to n-3, but also n-6 fatty acid intake. Current literature emphasises the importance of the third trimester of pregnancy for the influence of fatty acids[21], but based on our findings the advice should perhaps be aimed at earlier stages of pregnancy, or even, similar to folic acid supplementation, at the preconceptional period.

Possibly, the best window of opportunity for nutritional prevention strategies is indeed the preconceptional period. There are emerging observations that parental exposures before conception, including maternal nutrition, affect offspring phenotype.[55] For example, mice embryos exposed to preconception zinc-deficiency generated foetuses

prone to neural tube defects[56], a periconceptional low protein diet in mice was associated with offspring abnormal anxiety-related behaviour[57], and murine oocyte quality was found to be improved by a preconception diet high in n-3 fatty acids, whereas a diet high in n-6 fatty acids resulted in very poor quality.[58]

Finally, women at high risk, e.g. women with (severe) psychopathology, could be screened for folate deficiency and imbalanced n-3:n-6 ratio early in pregnancy. Similar to the current standard assessment of thyroid stimulating hormone in pregnant psychiatric patients, folate and fatty acid status could be determined by biomarker assessment. If deficiencies are found, dietary changes or supplementation could be advised. However, I will argue that we need more randomised trials before we implement such policies.

In order to further support these guidelines for clinical practice, future research should focus on the timing of the potential prenatal effects of folate and fatty acid status on child psychopathology by including multiple assessments of biomarkers throughout pregnancy. By combining these biomarker assessments with trials of continuation of folic acid supplementation, the effects of folic acid supplementation on offspring problem behaviour can further be examined. For example, pregnant women could be randomised into four different arms of a trial (e.g. no continuation of folic acid supplementation after 10 weeks of pregnancy, continuation until 20 weeks of pregnancy, continuation until 30 weeks of pregnancy, and continuation throughout pregnancy). Long-term follow-up in the offspring is needed to show potentially beneficial effects of folic acid supplement continuation after embryogenesis. Such a trial will be expensive and time-consuming, but the effective control for confounding by randomisation is the central advantage over observational studies as ours. In a similar way, recommendations about optimal prenatal fatty acid status throughout pregnancy can be attained when biomarker assessments are incorporated in trials with various dosages of n-3 and n-6 fatty acid supplementation.

In addition, our results with regard to child autistic traits are indicative of associations between maternal nutrient status during pregnancy and child neurodevelopmental disorders. In this respect, further research into other neurodevelopmental disorders later in life, such as psychotic or severe mood disorders, is of interest.

To gain further insight in the mechanisms underlying the relation between periconceptional/prenatal nutrition and child psychopathology, research into the epigenetic effects of nutrition on foetal and postnatal brain development and subsequent child behaviour should be considered. Folate, for example, is involved in DNA methylation, which has been shown to be a candidate mechanism by which in utero conditions affect later health.[59] Recent studies additionally provide evidence for various other epigenetic effects of periconceptional nutrition that program the developmental trajectory of the embryo and the foetus.[55] Prenatal 3D ultrasounds of the foetal brain might be used to

improve measurement accuracy in brain development as compared with current 2D images. Postnatally, alternative imaging measures, such as brain MRI, can be used to measure not only brain structures, but also brain connectivity.

CONCLUSION

In a multi-causal phenomenon such as development of the brain and behaviour, large effects of nutrition are not expected. Indeed, the effect sizes presented throughout the studies in this thesis are quite small. Nevertheless, each small effect contributes to development and should therefore be considered as an opportunity to improve pre-natal conditions in such a way that the foetus can optimally develop. In contrast to, for example, genetic traits and socioeconomic status, prenatal nutrition is a modifiable risk factor for the development of psychopathology in early childhood.

The studies presented in this thesis also demonstrate the crucial issue of residual con-founding in epidemiological studies of maternal nutrition and child problem behaviour, and stress the importance of inclusion of many well-measured environmental, social and psychological factors. These should not be restricted to the prenatal phase, as the brain continues to develop rapidly throughout the first two years of life. Because a better understanding of any modifiable risk factor is important, I advocate combining research on prenatal and early postnatal nutrition to further gain insight in the aetiology of child psychopathology, in order to decrease the burden that emotional and behavioural problems place on children, their families and society.

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Appendices

SUMMARY

Various childhood emotional and behavioural disorders are considered to be neurode-velopmental in origin. Their development is assumed to be a multi-causal phenomenon, influenced by genetic and environmental factors and their interaction. As the brain develops rapidly during gestation, the prenatal phase may be an important opportunity for the prevention of mental health problems later in life. One of the environmental factors with potential for intervention that have been found to contribute to foetal brain development is *maternal nutrition during pregnancy*. Support for a relationship between prenatal nutrition and offspring problem behaviour has primarily been pro-vided by animal studies through prenatal deficiency and enrichment studies. In humans, some promising results have been found in studies on maternal supplementation and nutritional intake during pregnancy. Folic acid supplementation during pregnancy has been associated with fewer child emotional and behavioural problems, better social competence, and lower risk of autism spectrum disorder (ASD; impairments in social communication and restricted repetitive behaviours) in young children. Also, increased maternal dietary intake of omega-6 fatty acids has been associated with reduced risk of ASD in the offspring. Studies linking actual prenatal nutrient status – as measured in blood – to child psychopathology, however, show less consistent findings.

This thesis aims to extend existing knowledge on the relation between maternal nutri-tion during pregnancy and offspring emotional and behavioural development. To this end, we explored whether the maternal prenatal diet as a whole, as well as maternal blood concentrations of specific nutrients (the B-vitamin folate and omega-3 (n-3) and -6 (n-6) fatty acids) affect early childhood risk of general emotional and behavioural problems, and autistic traits more specifically, within the general population. The stud-ies in this thesis were conducted within the Generation R Study, a population-based cohort from early foetal life until young adulthood in Rotterdam, the Netherlands.

In **chapter 2**, we study the effect of maternal dietary patterns on child emotional and behavioural problems during early childhood. Three patterns were derived from reported food intake during the first trimester of pregnancy: 1) a 'Mediterranean' diet (high intake of vegetables, fish & shellfish, vegetable oil, fruit, and eggs, and relatively low intake of processed meat), 2) a 'Traditionally Dutch' diet (high intake of fresh and processed meat, potatoes and margarines, and a very low intake of soy and diet products), and 3) a 'Confectionary diet' (high intake of cakes, sugar & confectionary products, tea, cereals, fruit and dairy products). We found that children of mothers with a low adherence to a 'healthy' Mediterranean diet or a high adherence to a 'less healthy' Traditionally Dutch diet during pregnancy were at increased risk of behavioural problems throughout early childhood. These results suggest that the development of child behavioural problems is affected by the overall composition of maternal diet during gestation.

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In **chapter 3**, we examine whether maternal folic acid supplementation (a synthetic form of folate) and folate concentrations during pregnancy are related to offspring head growth, general emotional and behavioural problems, and autistic traits in particular. Folic acid supplementation was associated with larger prenatal head *size* at 20 weeks of gestation (**chapter 3.1**), and with decreased risk of child emotional problems (**chapter 3.2**) and autistic traits (**chapter 3.3**). Higher maternal folate concentration in early pregnancy was related to a slightly faster foetal head *growth* from the second to the third trimester of pregnancy, resulting in a larger head size at 30 weeks of gestation (**chapter 3.1**). Low folate was also found to affect child emotional problems, which partly explained the association of maternal folic acid supplementation with child emotional problems (**chapter 3.2**). Folate was, however, not related to child autistic traits (**chapter 3.3**). These results provide support for a biological pathway underlying the associations of maternal folic acid supplement use with general child psychopathology, but not autistic traits, through higher maternal folate concentrations.

In **chapter 4**, we describe associations of maternal prenatal polyunsaturated fatty acid (PUFA) status with child problem behaviour, and more specifically with child autistic traits. Higher maternal DHA (docosahexaenoic acid, n-3) levels and a higher n-3:n-6 ratio was associated with fewer offspring emotional problems. Higher maternal AA (arachidonic acid, n-6) levels were associated with more child behavioural problems. Our results showed some discrepancies if different informants (parents versus teachers) of child problem behaviour were used and should therefore be interpreted with caution (**chapter 4.1**). Furthermore, lower maternal n-3:n-6 ratio during pregnancy was associated with more autistic traits in the offspring (**chapter 4.2**). The effect of this presumably less healthy balance in PUFAs was largely accounted for by the effects of higher n-6 instead of lower n-3 status. Likewise, we did not find prenatal fish intake - a major source of n-3 PUFAs - to be associated with autistic traits in the offspring. Overall, the results suggest that low maternal n-3 and high n-6 PUFA levels, resulting in an unbalanced n-3:n-6 ratio, put the unborn child at risk of psychopathology during childhood.

In **chapter 5**, the main findings of the studies in this thesis are summarised and major methodological issues with regard to studies of nutritional epidemiology and child psychopathology are discussed. The chapter is concluded with some implications for clinical practice and suggestions for future studies.

SAMENVATTING

Van verschillende gedrags- en emotionele stoornissen bij jonge kinderen wordt verondersteld dat hun oorsprong in de ontwikkeling van de hersenen ligt. Het ontstaan van deze stoornissen wordt beschouwd als een multi-causaal fenomeen; het wordt beïnvloed door omgevings- en genetische factoren alsmede de interactie tussen deze. Omdat de hersenen zeer snel ontwikkelen tijdens de zwangerschap, is de prenatale fase mogelijk een belangrijke periode om het ontstaan van latere problemen in de geestelijke gezondheid te voorkomen. Een omgevingsfactor die beïnvloed kan worden, en waarvan is aangetoond dat zij bijdraagt aan de foetale hersenontwikkeling, is *voeding tijdens de zwangerschap*. Bewijs voor een verband tussen prenatale voeding en probleemgedrag in het nageslacht is voornamelijk geleverd door studies naar voedingstekorten en dieetverrijking bij dieren. Bij onderzoek met mensen zijn veelbelovende resultaten gevonden op het gebied van voedingssuppletie en -inname tijdens de zwangerschap. Zo werd het gebruik van foliumzuursupplementen tijdens de zwangerschap in verband gebracht met een vermindering van gedrags- en emotionele problemen, betere sociale competenties en een verminderd risico op autismespectrumstoornis (ASS; 1) tekortkomingen in sociale communicatie en interactie en 2) beperkt en repetitief gedrag) bij jonge kinderen. Ook werd een verhoogde inname van omega-6 vetzuren tijdens de zwangerschap gerelateerd aan een kleiner risico op ASS bij jonge kinderen. Studies die aan de hand van bloedbepalingen de daadwerkelijke voedingsstatus van zwangere vrouwen in verband trachten te brengen met kinderpsychopathologie tonen echter minder consistente bevindingen.

Het doel van dit proefschrift is de bestaande kennis over de relatie tussen maternale voeding tijdens de zwangerschap en de ontwikkeling van gedrags- en emotionele problemen bij jonge kinderen uit te breiden. Daartoe werd bij een grote groep mensen uit de algemene bevolking onderzocht of het dieetpatroon en de bloedconcentraties van specifieke voedingsstoffen (de B-vitamine folaat en omega-3 (n-3) en -6 (n-6) vetzuren) tijdens de zwangerschap gerelateerd zijn aan het latere risico op algemene gedrags- en emotionele problemen, en meer specifiek autistische symptomen, tijdens de vroege kindertijd. De studies in dit proefschrift werden uitgevoerd binnen de Generation R Studie, een grootschalig cohortonderzoek vanaf het vroege foetale leven tot in de jongvolwassenheid in Rotterdam.

In **hoofdstuk 2** bestuderen we het effect van dieetpatronen tijdens de zwangerschap op het ontstaan van gedrags- en emotionele problemen bij jonge kinderen. Uit de gerapporteerde voedingsinname tijdens het eerste trimester van de zwangerschap werden drie dieetpatronen afgeleid: 1) een 'Mediterraan' dieet (hoge inname van groenten, vis & schaaldieren, plantaardige olie, fruit en eieren, en een relatief lage consumptie van verwerkt vlees), 2) een 'Traditioneel Hollands' dieet (hoge inname van vers en verwerkt

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vlees, aardappelen en margarines, en een zeer lage consumptie van soja en dieetpro-
ducten), en 3) een 'Banket' dieet (hoge inname van cake, suiker en banketproducten,
thee, granen, fruit en zuivelproducten). Kinderen van moeders die laag scoorden op het
'gezonde' Mediterrane dieet of die hoog scoorden op het 'minder gezonde' Traditioneel
Hollandse dieet hadden een verhoogd risico op gedragsproblemen tijdens de kindertijd.
Deze bevindingen suggereren dat de gedragsontwikkeling van jonge kinderen wordt
beïnvloed door de samenstelling van hun moeders dieet tijdens de zwangerschap.

In **hoofdstuk 3** onderzoeken we of folaatconcentraties en het gebruik van foliumzuur-
supplementen (een synthetische vorm van folaat) tijdens de zwangerschap gerelateerd
zijn aan hoofdgroei, algemene gedrags- en emotionele problemen, en meer specifiek
autistische symptomen bij kinderen. Foliumzuursuppletie was geassocieerd met een
grotere foetale *hoofdomtrek* op 20 weken zwangerschap (**hoofdstuk 3.1**) en met een
verminderd risico op emotionele problemen (**hoofdstuk 3.2**) en autistische symptomen
(**hoofdstuk 3.3**) bij jonge kinderen. Een hogere folaatconcentratie bij de moeders in
het eerste trimester van de zwangerschap was gerelateerd aan een enigszins snellere
hoofdgroei tussen het tweede en derde trimester van de zwangerschap, hetgeen resul-
teerde in een grotere hoofomtrek van de foetus bij 30 weken zwangerschap (**hoofdstuk
3.1**). Een lage hoeveelheid folaat bleek ook van invloed op emotionele problemen
van kinderen, wat deels het verband tussen prenatale foliumzuursuppletie en emoti-
onele problemen in de kindertijd verklaarde (**hoofdstuk 3.2**). Folaat was echter niet
gerelateerd aan autistische symptomen bij kinderen (**hoofdstuk 3.3**). Deze resultaten
ondersteunen de hypothese dat een biologisch mechanisme ten grondslag ligt aan
het verband tussen foliumzuursuppletie tijdens de zwangerschap en latere algemene
kinderpsychopathologie (maar niet autistische symptomen).

In **hoofdstuk 4** beschrijven we verbanden tussen de status van meervoudig onverza-
digde vetzuren (PUFAs) bij zwangere vrouwen en probleemgedrag, en meer specifiek
autistische symptomen, bij kinderen. We vonden dat hogere niveaus van docosa-
hexaeenzuur (DHA, n-3) en een grotere verhouding tussen n-3 en n-6 vetzuren (hogere
n-3:n-6 ratio) tijdens de zwangerschap geassocieerd waren met minder emotionele
problemen bij kinderen. Hogere niveaus van arachidonzuur (AA, n-6) waren gerelateerd
aan meer gedragsproblemen bij jonge kinderen. Omdat we discrepanties vonden in
resultaten wanneer probleemgedrag door verschillende informanten (ouders versus
leraren) werd gerapporteerd, dienen deze bevindingen met zorg te worden geïnterpre-
teerd (**hoofdstuk 4.1**). Voorts bleek een lage n-3:n-6 ratio tijdens de zwangerschap ook
gerelateerd te zijn aan meer autistische symptomen bij kinderen (**hoofdstuk 4.2**). Het
effect van deze ogenschijnlijk minder gezonde balans in PUFAs kon met name worden
toegeschreven aan een hogere n-6 in plaats van aan een lagere n-3 status. Evenzo
vonden we geen verband tussen prenatale inname van vis - een belangrijke bron van
n-3 PUFAs - en autistische symptomen bij kinderen. Over het geheel suggereren de

resultaten dat lage n-3 en hoge n-6 niveaus tijdens de zwangerschap, resulterend in een ongebalanceerde n-3:n-6 ratio, het ongeboren kind blootstellen aan een verhoogd risico op psychopathologie in de kindertijd.

In **hoofdstuk 5** wordt een samenvatting van de voornaamste bevindingen van dit proefschrift gegeven en worden belangrijke methodologische aspecten met betrekking tot studies op het raakvlak van voedingsepidemiologie en kinderpsychopathologie besproken. Het hoofdstuk wordt afgesloten met praktische implicaties en suggesties voor toekomstig onderzoek.

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Maartje Basten, Hanan El Marroun, Akhgar Ghassabian, Vincent WV Jaddoe, Jolien Rijlaarsdam, Jolien Steenweg-de Graaff, Alette N Walstra

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ABOUT THE AUTHOR

Jolien Steenweg-de Graaff was born in Geldrop, the Netherlands, on November 9th 1980. She grew up in Ter Aar and passed secondary school at the Groene Hart Lyceum in Alphen aan den Rijn in 1998. In the same year, she started her study Technology, Policy and Management at Delft Technical University. During this study, she became an active member of the student volleyball society "D.S.V.V. Punch", where she was chairwoman of the board (one year) and she participated in several committees. In 2004, after a 6 month internship at the European Aeronautic Defence and Space Company in Munich, Germany, she obtained her Master's degree in Systems Engineering. She then worked as a test engineer at Sogeti Netherlands B.V. for 2.5 years, during which she started her study Paedagogy at Leiden University in 2007. After obtaining the Master's degree in Child and Family Studies in 2009, she started a PhD project at the Generation R Study Group and the department of Child and Adolescent Psychiatry/psychology at the Erasmus Medical Centre, Rotterdam, under supervision of prof.dr. Frank C. Verhulst, prof. dr. Henning Tiemeier and Dr. Sabine J. Roza. The results of this research are presented in this thesis. During her PhD program, she completed the Master of Science program in Epidemiology at the Netherlands Institute for Health Sciences in 2012. From February 2015 onwards, Jolien works as a postdoctoral researcher in ErasmusAGE, Erasmus Medical Centre, Rotterdam. She is involved in research that aims to identify nutritional and lifestyle factors that are associated with maternal and child health.

Jolien is married to Rob Steenweg and together they have two daughters, Eva (September 2012) and Sanne (October 2014).

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PUBLICATIONS AND MANUSCRIPTS

1. **Steenweg-de Graaff J**, Tiemeier H, Basten MG, Rijlaarsdam J, Demmelmair H, Koletzko B, Hofman A, Jaddoe VW, Verhulst FC, Roza SJ. Maternal LC-PUFA status during pregnancy and child problem behavior: the Generation R Study. *Pediatr Res. 2015;77(3):489-97*.

2. **Steenweg-de Graaff J**, Ghassabian A, Jaddoe VW, Tiemeier H, Roza SJ. Folate concentrations during pregnancy and autistic traits in the offspring. The Generation R Study. *Eur J Public Health. 2014 [Epub ahead of print]*.

3. Ghassabian A, **Steenweg-de Graaff J**, Peeters RP, Ross HA, Jaddoe VW, Hofman A, Verhulst FC, White T, Tiemeier H. Maternal urinary iodine concentration in pregnancy and children's cognition: results from a population-based birth cohort in an iodine-sufficient area. *BMJ Open. 2014;4(6):e005520*.

4. Micali N, dos-Santos-Silva I, De Stavola B, **Steenweg-de Graaff J**, Jaddoe VW, Hofman A, Verhulst FC, Steegers E, Tiemeier H. Fertility treatment, twin births, and unplanned pregnancies in women with eating disorders: findings from a population-based birth cohort. *BJOG. 2014;121(4):408-16*.

5. **Steenweg-de Graaff J**, Tiemeier H, Steegers-Theunissen RP, Hofman A, Jaddoe VW, Verhulst FC, Roza SJ. Maternal dietary patterns during pregnancy and child internalising and externalising problems. The Generation R Study. *Clin Nutr. 2014;33(1):115-21*.

6. Ringoot AP, Jansen PW, **Steenweg-de Graaff J**, Measelle JR, van der Ende J, Raat H, Jaddoe VW, Hofman A, Verhulst FC, Tiemeier H. Young children's self-reported emotional, behavioral, and peer problems: the Berkeley Puppet Interview. *Psychol Assess. 2013;25(4):1273-85*.

7. Micali N, De Stavola B, dos-Santos-Silva I, **Steenweg-de Graaff J**, Jansen PW, Jaddoe VW, Hofman A, Verhulst FC, Steegers EA, Tiemeier H. Perinatal outcomes and gestational weight gain in women with eating disorders: a population-based cohort study. *BJOG. 2012;119(12):1493-502*.

8. **Steenweg-de Graaff J**, Roza SJ, Steegers EA, Jaddoe VW, Hofman A, Verhulst FC, Tiemeier H. Maternal folate status in early pregnancy and child emotional and behavioral problems: the Generation R Study. *Am J Clin Nutr. 2012;95(6):1413-21*.

9. Vidakovic AJ, Gishti O, **Steenweg-de Graaff J**, Hofman A, Tiemeier H, Jaddoe VW, Gaillard R. Maternal fatty acid levels during pregnancy and childhood blood pressure. The Generation R Study. *Revision.*

10. Bautista Niño PK, Tielemans MJ, Schalekamp-Timmermans S, **Steenweg-de Graaff J**, Hofman A, Tiemeier H, Jaddoe VW, Steegers EA, Felix JF, Franco OH. Maternal fish consumption, fatty acid levels and angiogenic factors: The Generation R Study. *Revision.*

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11. Rucci E, den Dekker HT, de Jongste JC, **Steenweg-de Graaff J**, Gaillard R, Pasmans S, Hofman A, Tiemeier H, Jaddoe VW, Duijts L. Maternal fatty acid levels during pregnancy and risks of childhood eczema and asthma. The Generation R Study. *Revision.*

12. Ars C*, Nijs IM*, El Marroun H, Muetzel R, Schmidt M, **Steenweg-de Graaff J**, van der Lugt A, Jaddoe VW, Hofman A, Steegers EA, Verhulst FC, Tiemeier H, White T. Prenatal maternal folate insufficiency is associated with reduced brain volume in school-aged offspring: The Generation R Study. *Revision.*
    * These authors contributed equally.

13. **Steenweg-de Graaff J**, Roza SJ, Walstra AN, Marroun H, Steegers EA, Jaddoe VW, Hofman A, Verhulst FC, Tiemeier H, White T. Associations of maternal folic acid supplementation and folate concentrations during pregnancy with foetal and child head growth. The Generation R Study. *Submitted for publication.*

14. **Steenweg-de Graaff J**, Tiemeier H, Ghassabian A, Rijlaarsdam J, Hofman A, Jaddoe VW, Verhulst FC, Roza SJ. Maternal fatty acid status during pregnancy and child autistic traits. The Generation R Study. *Submitted for publication.*

15. van Mil NH, Tiemeier H, **Steenweg-de Graaff J**, Verhulst FC, Hofman A, Jaddoe VW, Steegers EA, Steegers-Theunissen RP. Maternal mid-pregnancy trans fatty acids and vascular related pregnancy complications in mother and infant. *Submitted for publication.*

16. van Mil NH, Tiemeier H, **Steenweg-de Graaff J**, Koletzko B, Demmelmair H, Verhulst FC, Hofman A, Jaddoe VW, Steegers EA, Steegers-Theunissen RP. Maternal plasma n-3:n-6 fatty acids during pregnancy and associations with features of fetal growth and birth outcomes. *Submitted for publication.*

PhD PORTFOLIO

Name PhD student:	Jolien Steenweg-de Graaff
Erasmus MC department:	Child & Adolescent Psychiatry/psychology
Research school:	Netherlands Institute for Health Sciences
PhD period:	May 2009 – June 2014
Promotors:	Prof.dr. F.C. Verhulst, Prof.dr. H. Tiemeier
Copromotor:	Dr. S.J. Roza

	Year	Workload (ECTS)
1. PhD training		
Master's degree Health Sciences, specialisation Epidemiology, NIHES, Erasmus University Rotterdam, the Netherlands	2009-2012	
General courses		
Principles of Research in Medicine and Epidemiology		0.7
Principles of Epidemiologic Data-analysis		0.7
History of Epidemiologic Ideas		0.7
Conceptual Foundation of Epidemiologic Study Design		0.7
Cohort Studies		0.7
Case-control Studies		0.7
Introduction to Public Health		0.7
Methods of Public Health Research		0.7
Primary and Secondary Prevention Research		0.7
Health Economics		0.7
Principles of Genetic Epidemiology		0.7
Genome Wide Association Analysis		1.4
Core courses		
Study Design		4.3
Classical Methods for Data-analysis		5.7
Modern Statistical Methods		4.3
Methodological Topics in Epidemiologic Research		1.4
Public Health Research Methods		5.7
In-depth courses		
International Course in Nutritional Epidemiology, Imperial College London, UK		2.9
Missing Values in Clinical Research		0.7

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Courses for the Quantitative Researcher		1.4
Maternal and Child Health		0.9

Seminars, workshops and symposia

Generation R Research meetings, Erasmus MC	2009-2014	1.0
Symposium 40 years Epidemiology, Erasmus MC	2009	0.3
PhD day, Erasmus MC	2009	0.3
Nutrimenthe Project Meeting, Tarragona, Spain. *Oral presentation*	2009	1.4
Unilever Nutrition Symposium, Essential fats for future health, Vlaardingen, the Netherlands	2010	0.3
Nutrimenthe Workshop: training Neuropsychological Test Administration, York, UK	2010	0.6
Nutrimenthe Project Meeting, Munich, Germany. *Oral presentation*	2010	1.0
Nutrimenthe Working group: Neuropsychological Test Administration, Warsaw, Poland	2011	0.6
EUCCONET International workshop 'Nutrition resources in longitudinal studies; what can we learn from each other?' Bristol, UK: *2 oral presentations*	2011	2.0
Generation R Research Meeting. *Oral presentation*	2012	0.6
Nutrimenthe Project Meeting, Rotterdam, the Netherlands. *Organisation*	2012	1.0

(Inter)national conferences

The Power of Programming: Developmental Origins of Health and Disease, Munich, Germany	2010	1.0
WCRF International Conference on Nutrition, Physical Activity & Cancer Prevention: Current Challenges, New Horizons, Londen, UK	2010	0.3
Developmental Origins of Health and Disease (DOHaD), Rotterdam, the Netherlands. *Poster presentation*	2012	1.0
20th International Congress of Nutrition, Granada, Spain. *Oral presentation*	2013	1.4
Nutrimenthe International Conference, Granada, Spain. *Oral presentation Young Scientists' Forum Competition* (2nd price)	2013	1.4
150 years Sophia Children's Hospital: Children of the future, Rotterdam, the Netherlands. *Oral presentation*	2013	1.0

2. Teaching activities

Training students (Nutrimenthe Neuropsychological Test Battery)	2011	1.0
Supervising theses:		
BSc. student Lisette de Jong. '*Maternal folate concentration in early pregnancy and psychomotor development in 1-year old children*'	2011	3.0
Medical student Annemiek van der Zee. '*Maternal fatty acid concentrations in early pregnancy and child emotional and behavioral problems*'	2012	3.0
MSc. student Sanne Cnossen. '*Maternal folate concentration in early pregnancy and child cognitive development*'	2013	3.0

Tutoring:

Diederik Tichler & Jeske Boeding. Short Review. *'Do prenatal maternal micronutrient concentrations have an effect on the offspring's mental development? A systematic review'*	2012	0.5
Didactic skills course, Onderwijscentrum, Erasmus MC	2014	1.0

Note: 1 ECTS (European Credit Transfer System) is equal to a workload of 28 hours.

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DANKWOORD

Een dankwoord wordt geschreven om aan betrokkenen te laten weten dat hun hulp en steun erg gewaardeerd zijn. Het zou echter toch veel mooier zijn als we met onze dankbetuigingen niet wachten tot het schrijven van ons dankwoord, maar we tijdens de daadwerkelijke uitvoer van het werk onze dank en waardering al laten blijken! Helaas ben ook ik in dit laatste tekortgeschoten, vandaar dat ik de mogelijkheid van het schrijven van een dankwoord met beide handen aangrijp!

Graag wil ik alle deelnemers aan het Generation R onderzoek, jong en oud, bedanken voor hun inzet door de jaren heen: dankzij jullie deelname kunnen mijn collega's en ik onderzoek doen met als ultieme doel de gezondheid van komende generaties te verbeteren!

Ook wil ik laten weten hoe zeer ik het getroffen heb met mijn ijzersterke begeleiders-trio van de Kinder- en Jeugdpsychiatrie. Professor Tiemeier, beste Henning, dat ik 6 jaar geleden bij jou op gesprek kwam, was eigenlijk helemaal niet de bedoeling. Ik zou immers "nooit gaan promoveren"… Jouw enthousiasme voor onderzoek en je eerlijke woorden ("Ik leid niet op tot specialisten, maar tot generalisten.") haalden me echter over. En je hebt woord gehouden: je bleek een echte leraar te zijn, die zijn studenten algemene kennis van de wetenschapsuitoefening bijbrengt, ruimte geeft voor het maken van fouten, maar ook helpt die weer teboven te komen. Dank je wel voor je sterke begeleiding! Daarnaast heb ik dankzij jou ook nog een onvergetelijke, niet-wetenschappelijke ervaring opgedaan. Doordat wij dermate in gesprek waren, misten we de gate-wijziging t.b.v. een Nutrimenthe-reis en ben ik voor het eerst in mijn leven (en hopelijk ook meteen voor het laatst!) op Schiphol omgeroepen: 'Mr Tiemeier and Miss de Graaff, you are delaying the flight…'.

Professor Verhulst, beste Frank, ik ben erg onder de indruk van al jouw werk in de kinder- en jeugdpsychiatrie. Je hebt een geweldige (onderzoeks)afdeling opgezet. Dank je wel voor je vakinhoudelijke input tijdens onze projectoverleggen en bij het schrijven van de artikelen. Jouw klinische ervaring hielp mij om de bevindingen van onze studies te relativeren en in perspectief te plaatsen, iets wat men uit enthousiasme en naïviteit gemakkelijk uit het oog kan verliezen.

Doctor Roza, lieve Sabine, de rol van copromotor was mij aan het begin van mijn promotietraject niet geheel duidelijk. Gedurende het project kreeg ik echter in de gaten dat jij één van de hoofdrollen vervulde! Je bracht duidelijkheid, stuurde bij waar nodig en had vertrouwen in me. Naast het feit dat je zowel wetenschappelijk als vakinhoudelijk een wandelende encyclopedie voor mij was, was je ook op persoonlijk vlak altijd betrokken en hebben we veel kunnen lachen, zeker ook als één van ons (weer eens) zwanger

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was ;-) (dankzij jouw reactie op mijn anekdote over de door Rob meegebrachte 'vette' vis had ik al snel een 11^e stelling! ☺). Ik zou hier nog veel meer over kunnen schrijven, maar ik vat mijn dank en waardering het beste samen als ik zeg: "Jouw begeleiding wens ik iedere promovendus toe; ik hoop dat ik eenzelfde rol kan/mag vervullen in mijn verdere werk!"

Geachte Professor Franco, beste Oscar, dank voor je bereidheid zitting te nemen in de leescommissie en het secretarisschap op je te nemen. Geachte Professor van Baar, hartelijk dank voor het beoordelen van mijn manuscript, alsmede voor uw aanwezigheid tijdens mijn verdediging. Dear Professor Dye, thank you very much for your willingness to participate in my dissertation committee and for having reviewed my thesis. Geachte Professor Steegers-Theunissen en Professor Brouwer, dank u wel voor uw aanwezigheid tijdens mijn verdediging.

Graag wil ik ook mijn dank betuigen aan alle co-auteurs; het is erg prettig om in een team wetenschap te kunnen beoefenen; te kunnen ontwerpen, analyseren, schrijven, her-analyseren, herschrijven en perfectioneren! Professor Jaddoe, beste Vincent, dank je voor je scherpe blik op al mijn artikelen.

Een belangrijke voorwaarde voor plezier hebben in mijn werk, is het omringd worden door leuke collega's. Gelukkig zijn er daar bij Generation R een heleboel van! ☺ Ik heb het voorrecht gehad om te mogen werken in een grote groep gezellige en tevens behulpzame collega's, van wie ik er graag een aantal bij naam wil noemen.

Beste Focus-dames, ik heb genoten van mijn tijd op het Focuscentrum. In het bijzonder dank ik Yvonne en de dames van 'het Gedragsblok', Anneke en Ineke, voor de prettige samenwerking en gezellige gesprekken! Beste Patricia, Laureen, Rose en Erica, dank jullie wel voor alle broodnodige secretariële ondersteuning; jullie kennis van de organisatie is onmisbaar! Beste Alwin, dank je wel voor je altijd snelle hulp op IT-vlak. Beste Claudia, dank je voor je hulp bij het laten bepalen van de vetzuurconcentraties! Ook dank ik jou en Marjolein voor alle snel aangeleverde datasets en jullie hulp bij het opschonen.

Beste Rob, het GenR-uitje in Delft was een succes! Lieve Akhgar, dank voor de prettige samenwerking en de gezellige tijd in Granada. Lieve Jessica, dank je dat ik met al mijn voedings- en statistiekvragen altijd bij je aan mocht kloppen. Onze avonturen in Londen en Bristol waren super! Beste Ank, Marina, Maartje B, Nina, Jolien, Laura, Maartje L, Rolieke, Fleur, Eszter, Nicole, Sandra, Andrea, Dafna, Ralf, Anne W en Selma, dank jullie voor alle gezelligheid en het vele meedenken met mijn onderzoek. Dear Ayesha and Olivera, the three of us pregnant at the same time: it was so much fun! ☺ Beste Anne V en Alette, dank jullie wel voor mijn leuke begintijd in Ae-005: jullie vertrokken veel te snel! Dear Irene and Fadila, my roommates in 'the new building': thank you for your support and the enthusiastic atmosphere in NA-2819; I am convinced the both of you

are excellent researchers and I wish you all the best! En lieve Ryan en Sabine, kamergenoten in Ae-005, met jullie heb ik zo veel kunnen delen, van persoonlijke life-events tot het 1^e geaccepteerde paper. Jullie maakten dat ons kantoor voor mij aanvoelde als een huiskamer; dank jullie wel daarvoor!

Lieve vrienden en familie, van jullie kwam de onmisbare steun van buiten het werk!

Waarde kringgenoten, bedankt voor jullie luisterend oor en voorbeden door de jaren heen!

Beste volleybal-teamgenoten en -trainers, dank jullie wel voor jullie interesse in mijn onderzoek tijdens de afgelopen jaren, maar bovenal voor de ontspanning die jullie konden bieden: op het veld dacht ik even aan niets anders dan volleybal! ☺

Lieve 'Vriendjes', Stefan, Lize, Lotte, Jorrit, Paul, Willemijn, Didi, Thomas, Sabine, Reinoud, Geert-Jan, Bart: dank jullie wel voor de vele vragen naar mijn onderzoek als ook de afleiding die jullie boden aan de hand van skireisjes, bezoekjes aan Bebop, BBQ's, WK-wedstrijden kijken, en natuurlijk ook gewoon een hoop (beach)volleybal!

Lieve Astrid, al vanaf groep 4 zijn we vriendinnen, en ook al zijn we ieder een andere kant op gegaan, onze vriendschap heeft stand gehouden, iets waar ik ontzettend blij mee ben. Dank je voor je begrip als ik het weer eens te druk had om af te spreken; die tijd is nu voorbij!

Lieve Lize en Stefan, van elkaars studie- en volleybalvrienden tot dorpsgenoten, ceremoniemeesters en getuigen: wat ben ik blij met jullie vriendschap en het feit dat onze levens zich zo met elkaar verweven hebben! Lize, tijdens de laatste loodjes van mijn promotieonderzoek waren we ook nog samen zwanger: wat leuk om dat te kunnen delen en nog dichter naar elkaar toe te groeien.

Lieve paranimfen, Lize en Sabine, dank jullie wel dat jullie tijdens mijn verdediging naast me staan; jullie hebben – de één vanuit privésfeer, de ander vanuit werksfeer – beide de hoogte- en dieptepunten van mijn promotieonderzoek meebeleefd en hebben mij ontzettend veel steun gegeven. Daarnaast is het regelen van de hele promotiedag natuurlijk appeltje-eitje met twee van zulke organisatietalenten ;-).

Lieve Hennie en Leo, wat fijn dat jullie mijn schoonouders zijn! Jullie hebben mij in jullie gezin opgenomen als ware ik een eigen dochter; dat gevoel alleen al is goud waard. Dank jullie wel, daarnaast, voor het vele oppassen waardoor ik extra tijd had om aan mijn proefschrift te werken. En Leo, jij tevens enorm bedankt voor het doornemen van enkele hoofdstukken van dit proefschrift.

Lieve Marjan, Arjen, Suzan en Mark, dank jullie wel voor jullie steun en begrip als ik het weer eens druk had en voor alle gezelligheid die jullie steeds boden (en zeker nog vaak zullen bieden!); wat hebben we toch een geluk met elkaar en met al onze kindjes!

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Lieve Marc en Esther, dank jullie wel voor jullie voortdurende bewondering voor mijn werk. Weet echter, dat ik jullie nog veel meer bewonder! De manier waarop jullie in liefde en geduld jullie kinderen opvoeden, getuigt van doorzettingsvermogen waar geen promovendus aan kan tippen. Esther, jouw rake blogs over het leven met een zorgintensief kind ontroeren mij telkens weer. Ik weet dat mijn onderzoek aan jullie situatie niets kan veranderen, maar ik leef in de hoop dat het op een manier zal bijdragen aan het verder inzichtelijk maken van het ontstaan van ontwikkelingsstoornissen.

Lieve mam/moeke en pap/daddo, jullie hebben me van jongs af aan gestimuleerd om mezelf te ontwikkelen en moeilijke dingen niet uit de weg te gaan. Die insteek en jullie liefde in ons gezin hebben mij mede gevormd tot wie ik nu ben. Dank jullie wel daarvoor! En heel praktisch gezien: dank jullie wel voor al het oppassen en de huishoudelijke klusjes waarmee jullie ons geholpen hebben, met name tijdens mijn zwangerschap van Sanne en de laatste maanden voor de verdediging. Ik weet dat jullie het met liefde gedaan hebben, maar ik besef ook dat dit niet voor iedereen zo vanzelfsprekend is!

Lieve Eva en Sanne, 'meidjes van de mama', jullie komst heeft mijn leven zo verrijkt! Ook al was (en is) het niet altijd even makkelijk voor me om zorg met werk te combineren, een glimlach van jou, Sanne, of samen zingen met jou, Eva, maakt dat ik me weer realiseer hoe gelukkig en gezegend ik ben; daar kan geen promotiestress iets aan afdoen!

Lieve Rob, nog tijdens onze studietijd heb ik in jou de man gevonden met wie ik oud wil worden; het is zo fijn om iemand naast je te hebben die dezelfde interesses heeft en hetzelfde denkt over de dingen die je belangrijk vindt! Tegelijkertijd zijn we ook verschillend en beschik jij over eigenschappen die heel goed van pas zijn gekomen tijdens mijn promotieonderzoek. Dank je wel voor je onmetelijke geduld, voor je '> 2 SD' analytisch vermogen (waar menigmaal zelfs midden in de nacht beroep op werd gedaan als ik weer eens lag te tobben over analyses), voor je relativeringsvermogen ("Uiteindelijk komt alles goed." / 'Why? Because Rob says so!' ☺), voor het feit dat we overal grappen over (moeten) kunnen maken, voor... alles wat je in mij ziet, waarvan ik zelf niet altijd overtuigd ben dat het er is. Maar bovenal: dank je wel voor jouw liefde; ik prijs mezelf ultiem gelukkig met het feit dat wij bij elkaar zijn en ik kijk uit naar de rest van ons leven samen!

Met mijn dank aan al deze mensen dank ik God, die altijd bij mij is.

Prenatal **nutrition** and early childhood **behaviour**

This thesis focuses on the relation between maternal nutrition during pregnancy and offspring emotional and behavioural development within the general population. The studies described in this thesis explore whether the maternal prenatal diet as a whole, as well as maternal blood concentrations of specific nutrients (folate and omega-3 and -6 fatty acids) affect early childhood risk of general emotional and behavioural problems, and autistic traits more specifically. The research was conducted within the Generation R Study, a population-based cohort from early foetal life until young adulthood in Rotterdam, the Netherlands.