Prenatal omega-6:omega-3 ratio and attention deficit and hyperactivity disorder symptoms

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Abbreviations: AA: arachidonic acid (C20:4n-6); ADHD: attention deficit and hyperactivity disorder; CI: confidence interval; CPRS-R:S: Revised Conners' Parent Rating Scale Short Form; DHA: docosahexaenoic acid (C22:6n-3); DSM-IV: Diagnostic Manual of Mental Disorders, 4th edition; EPA: eicosapentaenoic acid (C20:5n-3); FFQ: Food Frequency Questionnaire; INMA: Infancia y Medio Ambiente [Environment and Childhood]; IPW: inverse probability weighting; IQ: intelligence quotient; IRR: incidence rate ratio; LCPUFA: long-chain polyunsaturated fatty acids; n-6: omega-6; n-3: omega-3; OR: Odds Ratio; SCL-90-R: Symptom Checklist-90-R; SD: standard deviation; WAIS-III: Wechsler Adult Intelligence, 3rd edition.

Abstract

Objective

To evaluate whether higher omega-6:omega-3 (n-6:n-3) long-chain polyunsaturated fatty acid (LCPUFA) ratio in cord plasma is associated with more child Attention Deficit and Hyperactivity Disorder (ADHD) symptoms at 4 and 7 years old.

Study design

This study was based on a population-based birth cohort in Spain. N-6 arachidonic acid, and n-3 eicosapentaenoic and docosahexaenoic acid concentrations were measured in cord plasma. At 4 years old, ADHD symptoms were reported by teachers through the ADHD-DSM-IV checklist (n=580). At 7 years old, ADHD symptoms were reported by parents through the Conners' Rating Scale-Revised (short form) (n=642). ADHD variable was treated as continuous (score) and as dichotomous (symptom diagnostic criteria). Child and family general characteristics were prospectively collected through questionnaires. We applied pooled zero-inflated negative binomial and logistic regressions adjusted for covariates.

Results

Higher n-6:n-3 LCPUFA ratio in cord plasma was associated with higher ADHD index (Incidence Rate Ratio [IRR]=1.13; 95% Confidence Interval [CI]=1.03, 1.23) at 7 years old. The association was not observed at 4 years old (IRR=1.04; 95%CI=0.92, 1.18). No associations were found using ADHD symptom diagnostic criteria.

Conclusions

High prenatal n-6:n-3 LCPUFA ratio preceded the appearance of subclinical ADHD symptoms during mid-childhood. Our findings suggest that maternal diet during pregnancy may modulate the risk to develop long-term ADHD symptoms in the offspring.

Introduction

Omega-3 (n-3) and omega-6 (n-6) long-chain polyunsaturated fatty acids (LCPUFA) are among the main components of cell membranes (1). The main source of these LCPUFA in humans is diet (2), which is estimated to contain a n-6:n-3 ratio of 15-20:1 in western countries (3). A balanced intake of both series of omega is particularly important, as n-3 and n-6 compete for incorporation into cell membranes (4) and have opposing physiological functions. N-6 promotes systemic pro-inflammatory states, while n-3 promotes anti-inflammatory states (3).

N-3 and n-6 LCPUFA comprise approximately 15 to 30% of the brain's dry weight (5). Three of these LCPUFA play a crucial role in the function and architecture of the central nervous system (6–8), particularly during the later stages of gestation and early postnatal life (9): the n-3 docosahexaenoic (DHA) and eicosapentaenoic (EPA) acids, and the n-6 arachidonic acid (AA). During these periods, the main source of n-3 and n-6 LCPUFA are through placental transfer of these substances and breastfeeding (9). An inadequate maternal nutritional pattern during this period could be related to child Attention Deficit and Hyperactivity Disorder (ADHD), a neurodevelopmental disorder that is estimated to affect 5.3% of the children globally (10). Children with ADHD symptoms have higher n-6:n-3 ratio compared to children without symptoms which could be due to dietary patterns, altered gut microbiota or abnormal LCPUFA metabolism (7,11–13). Whether high prenatal n-6:n-3 LCPUFA ratio precedes the appearance of ADHD symptoms during childhood, which may suggest a role in the development of the symptoms, remains unknown.

Following developmental origins of health and disease (DOHAD) theory (14), our study aimed to analyze the association between n-6(AA):n-3(DHA+EPA) LCPUFA ratio

concentration in cord plasma, a proxy of fetal LCPUFA availability during late gestation (15), and child ADHD symptoms at 4 and 7 years of age. We hypothesized that higher n-6:n-3 ratios were associated with more child ADHD symptoms.

Methods

Subjects

This study was based on a Spanish population-based birth cohort, including four Spanish regions: Asturias (n=494), Gipuzkoa (Basque Country) (n=638), Sabadell (Catalonia) (n=657), and Valencia (n=855). Between November 2003 and January 2008, pregnant women who visited the public health centers for their first trimester ultrasound examination were invited to participate in the project if they fulfilled the following inclusion criteria: age 16 years or older, singleton pregnancy, no use of assisted reproductive techniques, intention to deliver at the reference hospital, and ability to speak and understand Spanish or a local language. A baseline survey was performed at enrollment (approximately 12 weeks of pregnancy), and follow-up surveys were performed at 20 and 32 weeks of pregnancy, at birth, and when children were 6 months, and 1, 2, 4 or 5, and 7 years old. LCPUFA were assayed in 953 cord plasma samples based on availability. In total, 580 and 642 children in the 4-year-old and 7-year-old assessment periods, respectively, had data both on LCPUFA and ADHD and were included in the present analyses (Figure 1). All parents signed the informed consent form approved by the Clinical Research Ethical Committees of the Asturias, Donostia (Gipuzkoa), La Fe (Valencia) Hospitals, and the Medical Assistance Municipal Institute (Barcelona).

LCPUFA levels

Whole blood samples were collected by using venipuncture of cord vessels before the placenta was delivered. Samples were processed, separated into aliquots of 1 mL, and then frozen to −80°C until the time of analysis. In a subsample of cord plasma, LCPUFA methyl esters were prepared and extracted following the method developed and validated by Moltó-Puigmartí et al. (16). LCPUFA were then separated and quantified by using fast-gas chromatography with flame ionization detection. The measurement of LCPUFA in plasma reflects the dietary availability of these components during the last 2-3 days (17). The relative amount of each LCPUFA quantified was expressed as the percentage of the total FAs. The n-6:n-3 LCPUFA ratio was calculated by dividing AA percentage by the sum of the DHA and EPA percentages (3,18). Higher ratio reflects higher imbalance between both series of omega.

ADHD characterization

At 4 year of age, teachers reported the ADHD symptoms of the participants by using the ADHD-DSM-IV (Diagnostic Manual of Mental Disorders, 4th ed) form list (19), which consists of 18 symptom-items categorized under two symptom subscales: the inattention subscale (nine symptoms) and the hyperactivity-impulsivity subscale (nine symptoms). Each ADHD symptom-item was rated on a four-point scale (0=never or rarely, 1=sometimes, 2=often, or 3=very often), so the total ADHD symptoms score ranges between 0 and 54, and the inattention and the hyperactivity-impulsivity subscales scores range from 0 to 27. We recorded the options 0 and 1 as 0 (symptom absent), and ratings of 2 and 3 as 1 (symptom present) (20). We generated a dichotomous variable of ADHD symptom diagnostic criteria, using 6 symptoms as cut off point. The ADHD-DSM-IV form list showed good internal consistency, with Cronbach Alpha coefficients of 0.90 for the total symptoms score, 0.88 for the inattention subscale, and 0.87 for the hyperactivity-impulsivity subscale.

At 7 years of age, parents reported the ADHD symptoms by using the Revised Conners' Parent Rating Scale Short Form (CPRS-R:S) (21), which consists of 27 items summarized on four subscales: ADHD index (twelve items), oppositional (six items), cognitive problems/inattention (six items), and hyperactivity (six items). Each item was rated on a four-point scale (0=never or rarely, 1=sometimes, 2=often, or 3=very often). Therefore, the ADHD index score ranges between 0 and 36, and the other subscales range between 0 and 18. We generated a dichotomous variable of ADHD symptom diagnostic criteria, using T score of 66 as cut off point (21). The CPRS-R:S showed good internal consistency, with Cronbach Alpha coefficients of 0.91 for the ADHD index, 0.85 for the oppositional subscale, 0.89 for the cognitive problems/inattention subscale, and 0.82 for the hyperactivity subscale.

Higher scores in ADHD-DSM-IV and CPRS-R:S indicates more ADHD symptoms.

Covariates

We used face-to-face questionnaires to collect data on maternal education (primary or lower, secondary and university), occupation, maternal age, and parity during the first trimester of pregnancy (zero, one and two or more). Data on maternal tobacco use was collected at the third trimester of pregnancy. Child's date of birth, gender, and birth weight were obtained from clinical records. Gestational age at delivery was based on the self-reported date of last menstrual period and it was corrected using crown-rump length measurement from an early ultrasound. We collected data on breastfeeding at 6 months and 1.5 years through a questionnaire. We calculated child's age based on birth date and ADHD assessment date. We used the Food Frequency Questionnaire (FFQ) (22) to collect information on maternal diet between weeks 12 and 32 of pregnancy and on child diet at 4 years old. We calculated the relative Mediterranean Diet Score, an

indicator of adherence to the Mediterranean diet, for the mothers based on the consumption of vegetables, fruits and nuts, cereals, legumes, fish, olive oil, meat, and dairy products (23). Maternal mental health was measured through the Global Severity Index of the Symptom Checklist-90-R (SCL-90-R) (24) and their verbal IQ proxy was tested through the Similarities subtest of the Wechsler Adult Intelligence, 3rd edition (WAIS-III) (25) when children were 4 years old.

Statistical analyses

We evaluated differences in n-6:n-3 ratio and ADHD symptom scores by socio-demographic characteristics using Kruskal-Wallis test. The continuous variables of child age, maternal mental health and maternal IQ proxy were split in categories for these tests. We used the median for splitting child age and tertiles for maternal variables. We evaluated sample differences between complete cases and incomplete cases and between cohort regions using Fisher's exact test for categorical variables and Kruskal-Wallis test for continuous variables.

We evaluated crude and adjusted associations between n-6:n-3 ratio and ADHD symptoms at 4 (n=580), and at 7 years old (n=642). To avoid underestimation of the outcome due to missing values in scale items, we multiplied the obtained raw score by the total number of items on the scale, divided by the total number of items that had responses, and then rounded to the nearest whole number (21). As ADHD symptoms occur as a continuum in the general population (26), this variable was primarily treated as continuous (score) and as dichotomous (symptom diagnostic criteria) to follow a more clinical approach. For the continuous outcomes, we applied zero-inflated negative binomial regression models, previously shown to improve the statistical modelling of ADHD studies (27). This method combines and simultaneously estimates two separate

regression models. The excess of zeros is modelled under a logistic distribution, and the count data with the standard negative binomial distribution. For the dichotomous outcome, we performed logistic regression models. We adjusted the models for maternal education, sex, age, and region. The confounders were selected a priori based on previous studies (28,29) and after building a Directed Acyclic Graph (DAG) (Figure 2; online). We also tested the heterogeneity of the associations between regions using the I^2 statistic, as well as the interaction terms between n-6:n-3 ratio and sex in the association with ADHD symptoms.

We applied inverse probability weighting (IPW) to control the potential selection bias induced by restricting the analysis to complete cases (30). Only individuals with observed data were analyzed but we used weights to rebalance the set of complete cases so that it is representative of the whole sample. We predicted the probability of being a complete case using logistic regression to generate the inverse probability weights (Tables 1 and 2; online). Breastfeeding, maternal social class, prematurity and smoking during pregnancy contained missing values (<2%) that were singly imputed as the variable median value.

We performed some sensitivity analyses. First, we ran the model without including IPW. Second, we additionally adjusted the models for maternal mental health and IQ proxy, maternal and child fish intake (together and separately), and maternal adherence to Mediterranean diet. Although these variables were not considered confounders and the sample size was reduced up to 16%, we tested whether the inclusion of these variables in the models modified the effect estimates of the main analyses. Third, we repeated the models for each specific LCPUFA (AA, EPA and DHA).

We used R (3.0.2; R Foundation for Statistical Computing) and Stata 12.1 (Stata Corporation, College Station, Texas) to perform the statistical analyses.

Results

Participants had a mean age of 4.85 (SD=0.72) and 7.35 (SD=0.63) years old at each of the assessment periods. The sample was equally distributed by sex (48% of the participants were females). The mean of maternal age at delivery was 32 years old (SD=4), 45% of the mothers were manual workers and 40% had secondary education levels. Three percent of the participants were born preterm and the breastfeeding duration mean was of 27 weeks (SD=22).

The concentrations of LCPUFA in cord plasma and the ADHD scores at both assessment periods are described in Table 3.

Table 4 presents the median values of the n-6:n-3 LCPUFA ratio concentrations and the ADHD scores according to socio-demographic characteristics. The ADHD symptom reports were associated with child's age and sex, maternal education, mental health and IQ. The n-6:n-3 ratio was associated with child's age and maternal education. Tables 5 and 6 (online) show that the main variables were differently distributed across the Spanish cohort regions included in this study.

Table 7 describes weighted crude and adjusted associations between n-6:n-3 ratio and ADHD symptoms, treated as dichotomous and continuous variable, at 4 and 7 years old. We observed that n-6:n-3 LCPUFA ratio in cord plasma did not predict the risk of presenting ADHD symptom diagnostic symptom criteria at 4 years old (OR=1.11; 95%CI=0.73, 1.71). Similarly, no association was observed with the continuous ADHD symptoms score (IRR=1.04; 95%CI=0.92, 1.18), without differences between subscales. We did not observe substantial heterogeneity among cohorts (I^2=5.7%, *P*=0.365), nor

interaction by child sex (*P* for interaction=0.124). At 7 years old, we did not obtain a statistically significant association using the outcome variable as dichotomous (OR=1.40; 95%CI=0.98, 2.00), however, we found that the ADHD index increased a 13% per each n-6:n-3 LCPUFA ratio unit (IRR=1.13; 95%CI=1.03, 1.23). The subscales that showed statistically significant associations were the cognitive problems/inattention (IRR=1.12; 95%CI=1.01, 1.25) and the hyperactivity subscales (IRR=1.13; 95%CI=1.02, 1.25). There was a significant heterogeneity between cohort regions in some subscale outcomes, specifically in the oppositional and the hyperactivity subscales, causing almost 60% of the total variability in the estimates. In those cases, the associations were strong in Asturias, while they were weak in the other regions (data not shown). The interaction term between n-6:n-3 ratio and sex in the association with ADHD index was not significant (*P* for interaction=0.418).

The results were similar but weaker when IPW was not applied in the models (Table 8; online). The inclusion of maternal mental health and IQ proxy scores, maternal and child fish intake (together and separately), and maternal Mediterranean diet in the final weighted models did not change the results meaningfully (Table 9; online).

In further analyses using each LCPUFA component separately as exposure in weighted and adjusted models, we observed that DHA was negatively associated with ADHD symptoms at 7 years old, EPA was positively associated with ADHD symptoms at 4 years old, and AA was not associated with ADHD symptoms (Table 10; online).

Discussion

In this study, we observed an association between n-6:n-3 ratio (AA/EPA+DHA) concentration in cord plasma and subclinical ADHD symptom scores during childhood. We found that ADHD symptom scores increased about 13% per each n-6:n-3 ratio unit

at 7 years old, although this association was not observed at 4 years old. In separated association analyses, DHA seemed to present a major role in this association. The longitudinal approach of this study allowed us to demonstrate that higher prenatal n-6:n-3 LCPUFA ratio preceded subclinical ADHD symptoms during childhood.

The present findings are consistent with previous studies that have linked the prenatal and perinatal n-6:n-3 ratio intake or in maternal fluids with early neurodevelopment (31–34). N-6:n-3 ratio intake during pregnancy showed negative associations with language, psychomotor, cognitive and social development at early ages (31,32). Higher ratio measured in maternal serum during pregnancy was related to slower psychomotor development at 9 months (33). N-6:n-3 ratio in breast milk and in plasma phospholipids at 44 weeks of gestational age was negatively associated with mental and motor development in premature infants (34).

Both series of omega have important physiological functions in the brain, n-3 modulates the synthesis, transport and release of neurotransmitters (8), and n-6 is involved in signal activation and reception (2). When n-6 ingestion is higher than n-3 LCPUFA, the first one replaces the last in the neuron membrane, altering its function and promoting a pro-inflammatory state (4). According to our finding, high prenatal n-6:n-3 ratio concentration in cord plasma is associated with ADHD symptoms during childhood and this association may be driven by low DHA concentrations. The high ratio during development could be related to the appearance of ADHD symptoms through early fetal programming. The nutrient supply during early stages of life programmes the structure and the function of the organs, which has an impact on individual's health (35). The brain is particularly vulnerable to misprogramming due to its long period of development (36,37). These alterations could lead to neurodevelopmental disorders, such as ADHD, since monoaminergic systems have been found to be affected by

LCPUFA status (38). It has been demonstrated in rodents that decreased concentrations of DHA during development alter neurobiological pathways, which has long-term negative consequences on behaviour (39). A recent study in humans showed an early association of LCPUFA-related genotypes with cognitive performance at school age after correction for current DHA blood concentrations, demonstrating the programming effect of these nutrients on the brain development (40). Nevertheless, the use of LCPUFA supplements during pregnancy for preventing behavioral problems is not supported by clinical trials (41–44). Interestingly, we also observed a positive association between EPA and ADHD symptoms at 4 years old. Although we are not aware of any explanation for this unexpected finding, a similar result was reported in a German birth cohort study. The authors of this study observed that higher EPA concentrations in cord blood serum were associated with more conduct problems at 10 years old (45).

In the present study, we assessed ADHD symptoms during two different periods, at 4 and 7 years old, but we only observed associations with n-6:n-3 ratio in the second period. This finding could be explained by the measurement error at early ages, since the ADHD symptoms reported could be originated by a delay in neurodevelopment within normality (46). Indeed, ADHD symptom phenotype is usually detected at school age (10). Furthermore, the different instruments and informants used at 4 and 7 years old could also explain the observed differences, since agreement between parents and teachers regarding ADHD symptoms is usually relatively poor (47). Our main findings remained significant after adjusting the models for key confounders selected by DAG models, and for other variables, identified in the literature, that could modify the associations as well. Such associations were not explained by maternal mental health, IQ proxy, and other LCPUFA-related nutrients obtained from diet during pregnancy,

neither by child diet. This is probably explained by the fact that the LCPUFA ratio in cord plasma is a valid measurement of the LCPUFA internal concentration and the one that the fetus gets at the end of the pregnancy period. Furthermore, the LCPUFA ratio associations observed here were partly driven by Asturias, where the fish consumption, both in mothers during pregnancy and in children, was higher than in the rest of the cohort regions. These differential dietary habits, combined with the relatively low prevalence of ADHD symptoms, could explain the heterogeneity detected in the associations between cohort regions.

In this study, prenatal n-6:n-3 ratio did not influence the risk of presenting ADHD symptom diagnostic criteria during childhood. However, we found significant associations with the continuous score of ADHD symptoms. Despite the relatively low and clinically irrelevant effect estimates obtained, these are important at the population level. If the whole population is exposed to high n-6:n-3 ratio, the distribution for ADHD symptom scores would likely move to the right, and the prevalence of extreme values would increase substantially, which may have a negative impact on the community's health costs and productivity (48).

This study presents some limitations. The measurement of LCPUFA in cord plasma did not reflect long-term dietary exposure to these components, as adipose tissue or red blood cells. The missing data due to the lack of cord plasma samples and follow up lost could have biased the results and led to low external validity. This limitation was minimized by applying Inverse Probability Weighting (IPW) in all the analyses. Moreover, we measured ADHD symptoms of children indirectly, and the informants and instruments were different at each assessment period. This could reduce the comparability of results between both age periods, limiting our ability of disentangling age from both instrument and informant influence on ADHD symptoms score.

This study encompasses several strengths. We used a large sample size from a population-based birth cohort in which different Spanish regions were represented, which guarantees a relative degree of generalization of the results to the general population. The longitudinal design of this study allowed us to discard the reverse causation that can affect cross-sectional studies. We collected the key LCPUFA for brain development, AA, DHA, and EPA, from cord plasma, instead of using a single determination collected from maternal biological samples or intake estimations. The use of a dimensional approach for the ADHD variables allowed us to study subtle subclinical dysfunction and increase statistical power. Finally, the models were adjusted for the main confounders identified in the literature after following DAG models and other additional important variables that could influence the tested associations.

Conclusion

High prenatal n-6:n-3 ratio was associated with subclinical ADHD symptoms during mid-childhood. Our finding suggests that maternal diet during pregnancy may modulate the risk to develop long-term ADHD symptoms in the offspring. Future research should measure ADHD symptoms using the same rating scales across assessment periods to further understand the trajectory patterns of this complex outcome. Longer follow-ups are warranted to explore the stability of this long-term association until adolescence periods; and randomized trials are needed to explore the potential nutritional sources of such LCPUFA ratio to improve nutritional guidelines during pregnancy.

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Figure 1. Flowchart of the study population

ADHD: Attention Deficit and Hyperactivity Disorder; ADHD-DSM-IV: Diagnostic Manual of Mental Disorders, 4th edition; CPRS-R:S: Revised Conners' Parent Rating Scale Short Form; LCPUFA: long-chain polyunsaturated fatty acids

Figure 2. Directed Acyclic Graph

ADHD: Attention Deficit and Hyperactivity Disorder; IQ: Intelligence Quotient; LCPUFA: long-chain polyunsaturated fatty acids

Table 1. Socio-demographic characteristics of the study participants[a] and non-participants at 4-year-old period

	Complete cases[a] (n=580)	Incomplete cases (n=2064)	P[b]
Child age in years, mean (SD)	4.85 (0.72)	4.94 (0.68)	<0.001
Child sex, % females	48.28	48.57	0.469
Region, %			<0.001
Asturias	13.79	20.06	
Gipuzkoa	18.45	25.73	
Sabadell	43.79	19.53	
Valencia	23.97	34.69	
Birth weight in grams, mean (SD)	3296.39 (439.10)	3240.50 (494.71)	0.022
Gestational age in weeks, mean (SD)	39.73 (1.39)	39.54 (1.80)	0.114
Preterm, %	2.97	5.19	0.015
Any breastfeeding in weeks, mean (SD)	27.29 (22.32)	23.80 (21.77)	<0.001
Maternal age, mean (SD)	31.74 (3.81)	31.27 (4.51)	0.017
Maternal occupation, %			<0.001
I/II managers/technicians	22.97	20.87	
III non-manual	31.26	24.12	
IV/V manual	45.77	55.01	
Maternal education level, %			0.013
Primary	20.69	26.27	
Secondary	42.24	41.22	
University	37.07	32.51	
Parity, %			0.662
0	55.96	56.32	
1	36.27	36.96	
2+	7.77	6.72	
Smoking at week 32 of pregnancy (cigarettes/day), mean (SD)	0.77 (2.38)	1.19 (3.21)	0.021
Maternal mental health, mean (SD)[c]	50.36 (10.37)	49.76 (9.71)	0.472

SD=Standard Deviation. [a]Number of children with cord blood LCPUFA, ADHD-DSM-IV, and potential confounders available. [b]Kruskal-Wallis test for continuous variables and Fisher's exact test for categorical variables. [c]Global Severity Index of the Symptom Checklist-90-R (SCL-90R), T score.

Table 2; online only

Table 2. Socio-demographic characteristics of the study participants[a] and non-participants at 7-year-old period

	Complete cases[a] (n=642)	Incomplete cases (n=2002)	P[b]
Child age in years, mean (SD)	7.35 (0.63)	7.66 (0.60)	<0.001
Child sex, % females	48.91	48.36	0.423
Region, %			<0.001
Asturias	11.37	21.03	
Gipuzkoa	22.12	24.78	
Sabadell	42.37	19.23	
Valencia	24.14	34.97	
Birth weight in grams, mean (SD)	3291.82 (423.34)	3240.21 (501.27)	0.032
Gestational age in weeks, mean (SD)	39.74 (1.39)	39.53 (1.81)	0.084
Preterm, %	2.52	5.41	0.001
Any breastfeeding in weeks, mean (SD)	27.24 (21.73)	23.69 (21.96)	<0.001
Maternal age, mean (SD)	31.63 (3.89)	31.29 (4.52)	0.104
Maternal occupation, %			<0.001
I/II managers/technicians	24.49	20.31	
III non-manual	30.58	24.12	
IV/V manual	44.93	55.57	
Maternal education level, %			0.003
Primary	21.03	26.34	
Secondary	40.65	41.70	
University	38.32	31.96	
Parity, %			0.551
0	56.01	56.32	
1	37.91	36.44	
2+	6.08	7.24	
Smoking at week 32 of pregnancy (cigarettes/day), mean (SD)	0.73 (2.36)	1.22 (3.24)	0.002
Maternal mental health, mean (SD)[c]	49.97 (10.04)	49.95 (9.88)	0.858

SD=Standard Deviation. [a]Number of children with cord blood LCPUFA, Conners' Rating Scale-Revised, and potential confounders available. [b]Kruskal-Wallis test for continuous variables and Fisher's exact test for categorical variables. [c]Global Severity Index of the Symptom Checklist-90-R (SCL-90R), T score.

Table 3

Table 3. Description of the LCPUFA concentrations in cord plasma and the ADHD variables at 4-year-old and 7-year-old assessment periods of the final sample

		Minimum	25th percentile	50th percentile	75th percentile	Maximum
Cord plasma (n=715)	**% AA among total fatty acids**	0.32	12.74	14.33	15.94	25.02
	% DHA among total fatty acids	1.68	3.89	4.71	5.95	11.42
	% EPA among total fatty acids	0	0.13	0.19	0.30	2.09
	n-6:n-3 ratio[a]	0.07	2.33	2.86	3.47	6.28
4-year-old period (n=580)	**ADHD symptom score[b]**	0	1	4	10	52
	Inattention	0	0	2	5	25
	Hyperactivity-impulsivity	0	0	2	5	27
7-year-old period (n=642)	**ADHD index[c]**	0	3	6	11	36
	Oppositional	0	1	3	5	18
	Cognitive problems/inattention	0	1	2	5	18
	Hyperactivity	0	1	3	6	18

AA: arachidonic acid; ADHD: Attention Deficit and Hyperactivity Disorder; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; LCPUFA: long-chain polyunsaturated fatty acids. [a]AA:(DHA+EPA); [b]ADHD-DSM-IV (Diagnostic Manual of Mental Disorders, 4th edition) symptom score based on 18 items (teachers); [c]Conners' Rating Scale-Revised (short form) ADHD index based on 12 items (parents).

Table 4

Table 4. Median n-6:n-3 ratio in cord plasma and ADHD symptom scores at 4- and 7-year-old periods by socio-demographic characteristics

	n-6:n-3 ratio in cord plasma (N=715)	*P*[a]	ADHD[b] 4-year-old (N=580)	*P*[a]	ADHD[c] 7-year-old (N=642)	*P*[a]
Child age (4-year-old)		0.844		0.029		
<4.5	2.83		4			
≥4.5	2.90		5			
Child age (7-year-old)		<0.001				1.000
<7.5	3.13				6	
≥7.5	2.71				6	
Child sex		0.269		<0.001		<0.001
Females	2.93		3		5	
Males	2.83		6		8	
Maternal mental health[d]		0.360		0.458		<0.001
1st tertile (35.5-44.2)	2.95		4		4.5	
2nd tertile (44.3-52.5)	2.83		5		6	
3rd tertile (52.6-96.6)	2.95		5		9	
Maternal IQ[e]		0.466		0.184		0.046
1st tertile (0-9.0)	2.95		4		8	
2nd tertile (9.1-11.2)	2.84		5		6	
3rd tertile (11.3-18.6)	2.89		4		6	
Maternal education		<0.001		0.178		0.001
Primary	3.03		5		8	
Secondary	3.00		5		7	
University	2.66		4		5	

ADHD: Attention Deficit and Hyperactivity Disorder; IQ: intelligence quotient. [a]Kruskal-Wallis test. [b]ADHD-DSM-IV (Diagnostic Manual of Mental Disorders, 4th edition) symptom score (teachers). [c]Conners' Rating Scale-Revised (short form) (parents). [d]Global Severity Index the Symptom Checklist-90-R (SCL-90R), T score. [e]Similarities subtest of the Wechsler Adult Intelligence, 3rd edition (WAIS III), (mean=10, SD=3).

Table 5; online only

Table 5. Characteristics of the study participants according to cohort region at 4-year-old period

	Asturias (n=80)	Gipuzkoa (n=107)	Sabadell (n=254)	Valencia (n=139)	P[a]
Child age in years, mean (SD)	4.77 (0.39)	4.40 (0.21)	4.44 (0.25)	5.99 (0.40)	<0.001
Child sex, % females	43.75	49.53	51.18	44.60	0.507
% AA among total fatty acids, mean (SD)	13.26 (1.80)	17.58 (2.29)	13.69 (1.76)	13.51 (2.14)	<0.001
% EPA among total fatty acids, mean (SD)	0.30 (0.15)	0.33 (0.27)	0.19 (0.12)	0.22 (0.18)	<0.001
% DHA among total fatty acids, mean (SD)	4.85 (1.18)	6.90 (1.65)	4.42 (1.16)	4.60 (1.39)	<0.001
n-6:n-3 ratio, mean (SD)	2.73 (0.76)	2.58 (0.77)	3.15 (0.81)	3.01 (0.95)	<0.001
ADHD symptom score, mean (SD)[b]					
All	9.90 (9.94)	5.22 (7.04)	6.70 (7.84)	7.63 (9.07)	0.001
Males	11.93 (10.56)	6.67 (8.47)	8.23 (9.05)	9.22 (9.82)	0.014
Females	7.29 (8.51)	3.75 (4.86)	5.25 (6.16)	5.66 (7.66)	0.182
ADHD diagnostic criteria, %[c]					
All	16.25	4.67	4.72	17.00	0.001
Males	22.22	7.41	7.26	18.18	0.014
Females	8.57	1.89	2.31	4.84	0.276
Maternal mental health, mean (SD)[d]	50.15 (11.42)	50.35 (9.32)	50.29 (10.51)	50.61 (10.05)	0.833
Maternal IQ, mean (SD)[e]	9.59 (3.09)	9.60 (2.97)	10.62 (2.89)	10.10 (3.11)	0.013
Maternal education, %					<0.001
Primary	11.25	13.08	22.05	29.50	
Secondary	40.00	37.38	43.70	44.60	
University	48.75	49.53	34.25	25.90	
Maternal fish intake, mean (SD)[f]	83.64 (52.52)	79.94 (34.13)	64.11 (33.67)	65.36 (37.22)	<0.001
Child fish intake, mean (SD)[g]	39.96 (20.37)	34.71 (14.49)	36.78 (17.22)	31.09 (16.90)	0.001
Maternal Mediterranean diet, mean (SD)[h]	8.08 (2.36)	9.25 (2.62)	7.84 (2.40)	7.32 (2.35)	<0.001

SD=Standard Deviation; AA= arachidonic acid; DHA=docosahexaenoic acid; EPA=eicosapentaenoic acid; ADHD=Attention Deficit and Hyperactivity Disorder; IQ=intelligence quotient. [a]Kruskal-Wallis test for continuous variables and Fisher's exact test for categorical variables. [b]ADHD-DSM-IV (Diagnostic Manual of Mental

Disorders, 4th edition) symptom score (teachers). [c]Six or more symptoms. [d]Global Severity Index of the Symptom Checklist-90-R (SCL-90R), T score. [e]Similarities subtest of the Wechsler Adult Intelligence, 3rd edition (WAIS III), (mean=10, SD=3). [f]Food Frequency Questionnaire (FFQ), grams per day, between weeks 12 and 32 of pregnancy. [g]Food Frequency Questionnaire (FFQ), grams per day, at 4 years old. [h]Relative Mediterranean Diet Score, constructed with FFQ data considering the consumption of vegetables, fruits and nuts, cereals, legumes, fish, olive oil, meat, and dairy products.

Table 6; online only

Table 6. Characteristics of the study participants according to cohort region at 7-year-old period

	Asturias (n=73)	Gipuzkoa (n=142)	Sabadell (n=272)	Valencia (n=155)	P[a]
Child age in years, mean (SD)	8.24 (0.33)	7.76 (0.13)	6.73 (0.37)	7.64 (0.19)	<0.001
Child sex, % females	42.47	49.30	51.47	47.10	0.547
% AA among total fatty acids, mean (SD)	13.34 (1.94)	17.67 (2.21)	13.67 (1.87)	13.54 (2.08)	<0.001
% EPA among total fatty acids, mean (SD)	0.29 (0.12)	0.32 (0.27)	0.19 (0.13)	0.23 (0.18)	<0.001
% DHA among total fatty acids, mean (SD)	4.82 (1.18)	6.77 (1.68)	4.44 (1.14)	4.68 (1.44)	<0.001
n-6:n-3 ratio, mean (SD)	2.76 (0.74)	2.65 (0.75)	3.13 (0.81)	2.96 (0.91)	<0.001
ADHD symptom score, mean (SD)[b]					
All	7.40 (6.64)	7.28 (7.46)	7.96 (7.06)	9.43 (7.12)	0.003
Males	7.95 (6.42)	8.51 (8.68)	9.50 (7.51)	11.47 (7.51)	0.003
Females	6.65 (6.96)	6.01 (5.74)	6.51 (6.29)	7.14 (5.90)	0.482
ADHD diagnostic criteria, %[c]					
All	5.48	9.15	8.82	10.32	0.692
Males	4.76	11.11	9.09	10.98	0.670
Females	6.45	7.14	8.57	9.59	0.933
Maternal mental health, mean (SD)[d]	49.40 (10.77)	49.24 (9.01)	50.33 (10.55)	50.01 (9.37)	0.721
Maternal IQ, mean (SD)[e]	9.64 (3.23)	9.85 (2.86)	10.69 (2.94)	10.16 (3.29)	0.019
Maternal education, %					<0.001
Primary	13.70	10.56	22.06	32.26	
Secondary	35.62	35.21	44.49	41.29	
University	50.68	54.23	33.46	26.45	
Maternal fish intake, mean (SD)[f]	80.53 (51.17)	78.75 (29.85)	66.61 (40.16)	65.03 (40.43)	<0.001
Child fish intake, mean (SD)[g]	39.54 (20.81)	34.57 (14.62)	37.48 (17.30)	31.68 (17.14)	0.001
Maternal Mediterranean diet, mean (SD)[h]	7.93 (2.35)	9.24 (2.64)	7.83 (2.33)	7.30 (2.27)	<0.001

SD=Standard Deviation; AA= arachidonic acid; DHA=docosahexaenoic acid; EPA=eicosapentaenoic acid; ADHD=Attention Deficit and Hyperactivity Disorder; IQ=intelligence quotient. [a]Kruskal-Wallis test for continuous variables and Fisher's exact test for categorical variables. [b]Conners' Rating Scale-Revised (short form) (parents).

[c] T score >66. [d]Global Severity Index of the Symptom Checklist-90-R (SCL-90R), T score. [e]Similarities subtest of the Wechsler Adult Intelligence, 3rd edition (WAIS III), (mean=10, SD=3). [f]Food Frequency Questionnaire (FFQ), grams per day, between weeks 12 and 32 of pregnancy. [g]Food Frequency Questionnaire (FFQ), grams per day, at 4 years old. [h]Relative Mediterranean Diet Score, constructed with FFQ data considering the consumption of vegetables, fruits and nuts, cereals, legumes, fish, olive oil, meat, and dairy products.

Table 7

Table 7. Crude and adjusted associations[a] between n-6:n-3 ratio in cord plasma and ADHD symptoms

	Crude			Adjusted[b]			Between-regions
Subscale (range)	**Estimate**	**95% CI**		**Estimate**	**95% CI**		**heterogeneity (I^2)[c]**
4-year-old period[d]							
ADHD diagnostic criteria (6 or more symptoms)	1.13	0.77	1.64	1.11	0.73	1.71	0%
ADHD (0-52)	1.09	0.95	1.25	1.04	0.92	1.18	5.70%
Inattention (0-27)	1.09	0.96	1.25	1.05	0.93	1.18	0%
Hyperactivity-impulsivity (0-27)	1.10	0.93	1.30	1.04	0.89	1.22	0%
7-year-old period[e]							
ADHD diagnostic criteria (T score >66)	1.52	1.10	2.11	1.40	0.98	2.00	35.20%
ADHD (0-36)	1.15	1.06	1.25	1.13	1.03	1.23	23.40%
Oppositional (0-18)	1.04	0.95	1.15	1.04	0.95	1.15	56.50%*
Cognitive problems/inattention (0-18)	1.16	1.05	1.28	1.12	1.01	1.25	0%
Hyperactivity (0-18)	1.16	1.04	1.28	1.13	1.02	1.25	59.20%*

ADHD: Attention Deficit and Hyperactivity Disorder; CI: Confidence Interval. [a]Incidence Rate Ratio estimated by zero-inflated negative binomial regression models for the continuous outcomes and Odds Ratio estimated by logistic regression models for the dichotomous outcome. We applied IPW (Inverse Probability Weighting) in all models. [b]Models were adjusted for maternal education, child sex, child age and region. [c]Percentage of the total variability in the estimates that is attributable to heterogeneity between regions. [d]ADHD-DSM-IV (Diagnostic Manual of Mental Disorders, 4th edition) symptom score (teachers). [d]Conners' Rating Scale-Revised (short form) (parents). * test for heterogeneity with p-value<0.10.

Table 8. Adjusted associations[a] between n-6:n-3 ratio in cord plasma and ADHD symptoms without applying IPW

Subscale (range)	Estimate	95% CI	
4-year-old period[b]			
ADHD diagnostic criteria (6 or more symptoms)	1.13	0.78	1.66
ADHD (0-52)	1.05	0.94	1.17
Inattention (0-27)	1.04	0.92	1.17
Hyperactivity-impulsivity (0-27)	1.06	0.93	1.22
7-year-old period[c]			
ADHD diagnostic criteria (T score >66)	1.27	0.91	1.76
ADHD (0-36)	1.09	1.00	1.18
Oppositional (0-18)	1.02	0.94	1.12
Cognitive problems/inattention (0-18)	1.09	0.99	1.21
Hyperactivity (0-18)	1.09	0.99	1.19

ADHD: Attention Deficit and Hyperactivity Disorder; CI: Confidence Interval; IPW: Inverse Probability Weighting. [a]Incidence Rate Ratio estimated by zero-inflated negative binomial regression models for the continuous outcomes and Odds Ratio estimated by logistic regression models for the dichotomous outcome. Models were adjusted for maternal education, child sex, child age and region. [b]ADHD-DSM-IV (Diagnostic Manual of Mental Disorders, 4th edition) symptom score (teachers). [c]Conners' Rating Scale-Revised (short form) (parents).

Table 9; online only

Table 9. Associations[a] between n-6:n-3 ratio in cord plasma and ADHD symptoms adjusted for additional variables

Subscale (range)	Maternal mental health and IQ (estimate, 95% CI)[b]			Maternal fish intake (estimate, 95% CI)[c]			Child fish intake (estimate, 95% CI)[d]			Maternal and child fish intake (estimate, 95% CI)			Maternal Mediterranean diet (estimate, 95% CI)[e]		
4-year-old period[f]															
ADHD diagnostic criteria (6 or more symptoms)	1.12	0.71	1.76	1.15	0.74	1.79	1.23	0.80	1.89	1.27	0.81	2.00	1.12	0.72	1.74
ADHD (0-52)	1.08	0.94	1.24	1.08	0.95	1.23	1.09	0.97	1.23	1.11	0.98	1.27	1.07	0.94	1.21
Inattention (0-27)	1.12	0.99	1.26	1.08	0.96	1.22	1.06	0.94	1.21	1.09	0.96	1.24	1.07	0.95	1.21
Hyperactivity-impulsivity (0-27)	1.06	0.89	1.25	1.09	0.92	1.28	1.12	0.96	1.30	1.14	0.98	1.34	1.07	0.91	1.26
7-year-old period[g]															
ADHD diagnostic criteria (T score >66)	1.14	0.78	1.65	1.40	0.97	2.02	1.37	0.96	1.97	1.35	0.93	1.97	1.42	0.98	2.04
ADHD (0-36)	1.09	1.00	1.20	1.11	1.02	1.21	1.13	1.03	1.24	1.12	1.02	1.23	1.12	1.02	1.23
Oppositional (0-18)	1.00	0.91	1.11	1.04	0.95	1.15	1.06	0.96	1.18	1.07	0.96	1.18	1.04	0.95	1.14
Cognitive problems/inattention (0-18)	1.09	0.98	1.22	1.12	1.01	1.24	1.12	1.01	1.26	1.12	1.01	1.25	1.13	1.02	1.25
Hyperactivity (0-18)	1.08	0.97	1.20	1.12	1.01	1.24	1.13	1.01	1.26	1.13	1.01	1.26	1.12	1.01	1.24

ADHD: Attention Deficit and Hyperactivity Disorder; CI: Confidence Interval. [a]Incidence Rate Ratio estimated by zero-inflated negative binomial regression models for the continuous outcomes and Odds Ratio estimated by logistic regression models for the dichotomous outcome. We applied IPW (Inverse Probability Weighting) in all models. Models were also adjusted for maternal education, child sex, child age and region. [b]Global Severity Index of the Symptom Checklist-90-R (SCL-90R), T score, and Similarities subtest of the Wechsler Adult Intelligence, 3rd edition (WAIS III), mean=10, SD=3, at the 4-year-old wave. [c]Food Frequency Questionnaire (FFQ), grams per day, between weeks 12 and 32 of pregnancy. [d]Food Frequency Questionnaire (FFQ), grams per day, at 4 years old. [e]Relative Mediterranean Diet Score, constructed with FFQ data considering the consumption of vegetables, fruits and nuts, cereals, legumes, fish, olive oil, meat, and dairy products. [f]ADHD-DSM-IV (Diagnostic Manual of Mental Disorders, 4th edition) symptom score (teachers). [g]Conners' Rating Scale-Revised (short form) (parents).

Table 10; online only

Table 10. Adjusted associations[a] between each LCPUFA (AA, DHA, and EPA) in cord plasma and ADHD symptoms

	AA, C20:4n-6			DHA, C22:6n-3			EPA, C20:5n-3		
Subscale (range)	**Estimate**	**95% CI**		**Estimate**	**95% CI**		**Estimate**	**95% CI**	
***4-year-old period*[c]**									
ADHD diagnostic criteria (6 or more symptoms)	0.97	0.81	1.16	0.92	0.68	1.24	5.72	1.92	17.03
ADHD (0-52)	0.99	0.94	1.04	0.96	0.89	1.04	1.59	1.06	2.38
Inattention (0-27)	0.98	0.93	1.03	0.94	0.88	1.02	1.25	0.83	1.88
Hyperactivity-impulsivity (0-27)	1.00	0.94	1.06	0.95	0.86	1.05	1.75	1.04	2.96
***7-year-old period*[d]**									
ADHD diagnostic criteria (T score >66)	1.04	0.91	1.20	0.75	0.57	0.99	2.88	0.78	10.66
ADHD (0-36)	1.02	0.98	1.05	0.93	0.89	0.98	1.31	0.98	1.77
Oppositional (0-18)	1.01	0.98	1.05	0.99	0.93	1.05	1.37	1.00	1.88
Cognitive problems/inattention (0-18)	1.01	0.96	1.05	0.92	0.87	0.98	1.30	0.89	1.92
Hyperactivity (0-18)	1.03	0.99	1.08	0.95	0.89	1.01	1.25	0.89	1.75

AA: arachidonic acid; ADHD: Attention Deficit and Hyperactivity Disorder; CI: Confidence Interval; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; IRR: Incidence Rate Ratio. [a]Incidence Rate Ratio estimated by zero-inflated negative binomial regression models for the continuous outcomes and Odds Ratio estimated by logistic regression models for the dichotomous outcome. We applied IPW (Inverse Probability Weighting) in all models. Models were adjusted for maternal education, child sex, child age and region. [c]ADHD-DSM-IV (Diagnostic Manual of Mental Disorders, 4th edition) symptom score (teachers). [d]Conners' Rating Scale-Revised (short form) (parents).

Figure 1

Click here to download Figure: Fig1.docx

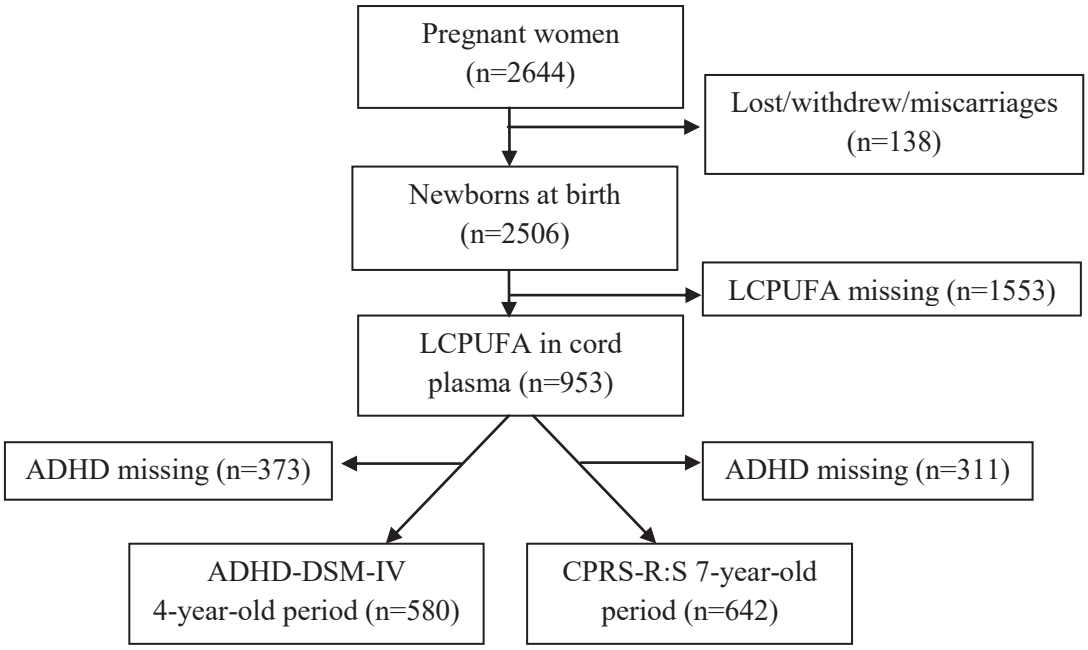

Figure 2; online only

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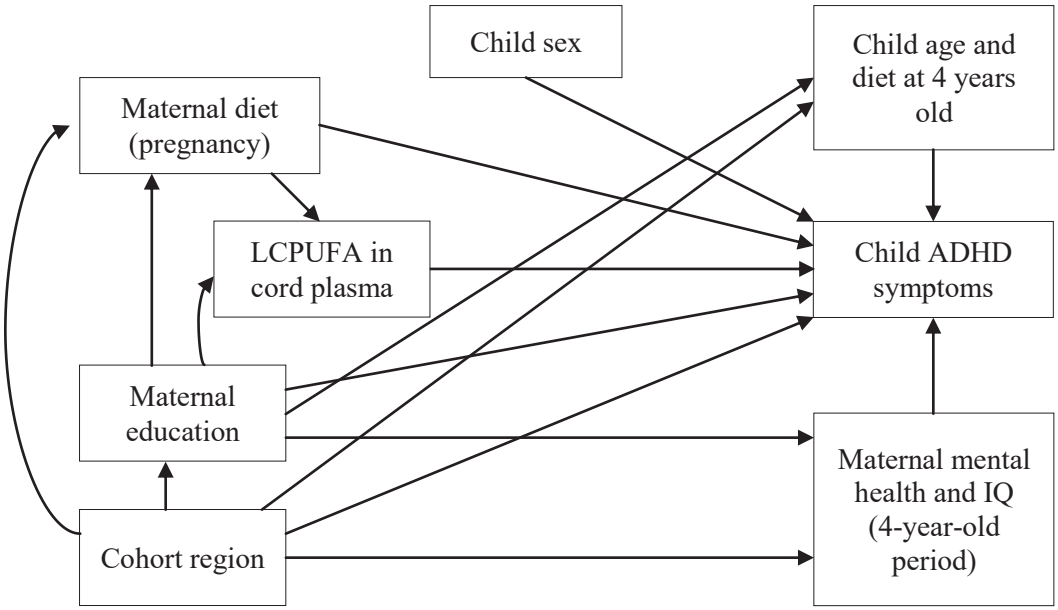

STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No	Recommendation	Page
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	Abstract (study design)
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	Abstract
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	Page 6
Objectives	3	State specific objectives, including any prespecified hypotheses	Page 6 (last paragraph)
Methods			
Study design	4	Present key elements of study design early in the paper	Page 7
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	Page 7
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants	Page 7
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case	
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	Pages 7-9
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	Pages 7-9
Bias	9	Describe any efforts to address potential sources of bias	Page 10
Study size	10	Explain how the study size was arrived at	Page 7, figure 1
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	Pages 10, 11 (statistical analyses)
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	Pages 10, 11 (statistical analyses)
		(*b*) Describe any methods used to examine subgroups and	Pages 10, 11

		interactions	(statistical analyses)
		(*c*) Explain how missing data were addressed	Pages 10, 11 (statistical analyses)
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy	Page 11 (IPW)
		(*e*) Describe any sensitivity analyses	Page 11
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	Page 7, figure 1
		(b) Give reasons for non-participation at each stage	Page 7, figure 1
		(c) Consider use of a flow diagram	Figure 1
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	Pages 7-9
		(b) Indicate number of participants with missing data for each variable of interest	Figure 1
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)	Page 7
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time	Table 3
		Case-control study—Report numbers in each exposure category, or summary measures of exposure	
		Cross-sectional study—Report numbers of outcome events or summary measures	
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	Table 7
		(*b*) Report category boundaries when continuous variables were categorized	Table 4
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	Page 11
Discussion			
Key results	18	Summarise key results with reference to study objectives	Pages 12, 13
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	Page 14
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	Pages 13-16

Generalisability	21	Discuss the generalisability (external validity) of the study results	Page 16
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	Title page

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.