

Nitrate in drinking water and risk of birth defects: Findings from a cohort study of over one million births in Denmark

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Summary

Background A few studies have reported an increased risk of birth defects (BD) with maternal exposure to nitrate in drinking water. We examined this association in a large cohort study with well-characterized exposure.

Methods Danish singletons liveborn to Danish-born parents from 1991–2013 were identified using civil and patient registries (n=1,018,914). Exposure to nitrate was estimated using a spatial model based on national data linked with individual addresses. Odds ratios (OR) and 95% confidence intervals (95% CI) were estimated using logistic regression.

Findings In total, 33,182 cases of BD were identified. Nitrate concentrations were generally well below US and EU standards. We observed an exposure-response relationship ($p=0.004$) between nitrate during pregnancy and eye BD, and increased risk in the highest exposure group (≥ 25 mg/L nitrate) (OR: 1.29; 95% CI: 1.00, 1.66). An interaction was observed between maternal age and continuous nitrate exposure for nervous system BD ($p<0.001$) indicating an increased risk among mothers <25 years-of-age (OR for 10 mg/L (OR₁₀): 1.20; 95% CI: 1.06, 1.35). An interaction ($p<0.01$) with maternal age and continuous nitrate exposure was also observed for ear, face, and neck BD indicating an increased risk among babies born to mothers <25 years-of-age (OR₁₀: 1.35; 95% CI: 1.11, 1.66). There was evidence of an inverse exposure-response relationship for any, digestive system, female genital, and urinary BD.

Interpretation Our study is the first to report an association between nitrate and eye BD and BD of the ear, face, and neck. It also provides support to prior reports of increased risk of nervous system BD.

Funding This study was supported by a grant from the United States National Institute of Environmental Health Sciences (R01 ES027823-01A1).

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Keywords: Nitrate; Drinking Water; Birth Defects; Congenital Malformations

Introduction

Nitrate is one of the most common contaminants in drinking water worldwide.¹ Contamination results primarily from animal manure and the use of nitrogen fertilizers. It is of particular concern to communities that have intensive animal farming and extensive use of

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The Lancet Regional Health - Europe 2021;14: 100286

Published online 12 January 2022

<https://doi.org/10.1016/j.lanepe.2021.100286>

Research in context

Evidence before this study

Experimental studies with mice, rats, and hamsters exposed to *N*-nitroso compounds, which are formed endogenously after ingestion of nitrate, have demonstrated an increase in central nervous system birth defects. Eight epidemiologic studies have examined whether an association exists between nitrate in drinking water and the risk of birth defects in humans. Of these, six case-control and one cohort study have reported an increased risk of nervous system birth defects. All studies were identified through PubMed using “birth defects,” “congenital malformations,” and “nitrate exposure” as search terms and through checking the papers cited in previous relevant publication.

Added value of this study

Most of the prior epidemiological studies identified relied on smaller study populations, crude surrogate measures of nitrate exposure, a case-control study design, and were designed to only examine one birth defect (e.g. nervous system birth defects). Our study used a cohort design to assess a wide range of birth defects. We also utilized high quality individual-level estimates of exposure to nitrate in drinking water for over 1 million births in Denmark, making this the most comprehensive and largest study to date. Our study provides evidence of an increased risk of birth defects of the eye from prenatal exposure to nitrate in drinking water. We also noted an increased risk of nervous system and ear, face, and neck birth defects, but only among children of mothers who were under 25 years old.

Implications of all the available evidence

An association between prenatal exposure to nitrate and birth defects of the eye has not been previously studied, and additional studies are thus warranted. Our effect estimates were unchanged when we restricted analyses to mothers whose drinking water met current European Union and United States nitrate standards. This, along with evidence from prior studies, suggests that the current standards may not be sufficient to prevent birth defects and other adverse birth outcomes from prenatal exposure to nitrate in drinking water.

nitrogen fertilizers, including the United States (US) and Denmark. Excess nitrogen on agricultural fields may leach as water-soluble nitrate into groundwater depending on local soil and hydrogeological conditions. In 2012, approximately 2.5% of the Danish population that received drinking water from public supplies, while 37% of the population that received water from private wells had elevated concentrations of nitrate (≥ 25 mg/L).²

Birth defects (BD) are a leading cause of infant mortality and may be associated with substantial morbidity

and long-term disability. *N*-nitroso compounds, which are formed endogenously after the ingestion of nitrate and nitrite, have been found to increase central nervous system (CNS) BD in mice,³ hamsters,⁴ and rats.⁵ Nitrate has also been shown to be a disruptor of gonadal steroidogenesis and thyroid function in humans.⁶

Several epidemiologic studies have reported an increased risk of BD from exposure to nitrate in drinking water.^{7–14} The evidence is particularly strong for CNS BD and subcategories of CNS (*i.e.*, neural tube defects), which has been reported in six case-control^{7,8,10–12,14} and one cohort study.¹³ An association with nitrate in drinking water has also been reported in some studies of limb deficiencies,^{11,13} cleft lip or palate,¹¹ and BD of the heart.^{9,11,13}

Common limitations of several of these previous studies relate to their use of ecologic measures of nitrate exposure and small sample size. Only one of the prior studies was a cohort study.¹³ Several of the case-control studies were designed to only examine the association with CNS BD,^{8,10,14} and one only examined heart defects.⁹ Thus these studies could not examine the risk for other major BD.

The objective of our study was to fill this data gap by assessing the potential association between nitrate in drinking water and the risk of specific BD in a large, population-based cohort study with well-characterized estimates of individual-level exposure to nitrate in drinking water during pregnancy.

Methods

Using the Danish Civil Registration System¹⁵ we identified all singletons liveborn in Denmark between 1991–2013 to Danish-born parents where the mother resided in Denmark during her entire pregnancy. The study began in 1991 because this was the first year in Denmark when nearly complete information was available on nitrate concentrations in public water systems. Children of non-Danish born parents were excluded to minimize the potential for confounding by diet (*e.g.*, nitrite and antioxidants), adherence to prenatal care, and other possible life-style factors. Primary analyses excluded children of women who resided in a house with private well use during their pregnancy or who had incomplete covariate or nitrate data.

Birth defects that were diagnosed until two years-of-age were identified using the Danish National Patient Registry.¹⁷ We linked individual-level data from the Danish Medical Birth Registry¹⁶ the Danish Civil Registration System with the BD identified in the Danish National Patient Registry using the unique personal Danish identification numbers (CPR). Data from the Danish Medical Birth Registry was only available for births up to 2015. Since we included birth defects that occurred up to age 2 we only included births up to 2013 in order to allow each child to have two years of follow-

up. BD were identified using the ICD-8 revision for births from 1991–1993 and the ICD-10 revision for births thereafter. BD were grouped for analysis using the 11 major BD defects categories defined in the EURO-CAT guide 1.4¹⁸ for ICD-10, and on a modified version of a translation to ICD-8 published by Cohen *et al.*¹⁹ A category for any BD was created in which cases with multiple birth defects were counted only once. The codes used to classify the BD are presented in Supplemental Table 1.

Since prior studies have reported an association with neural tube defects, analyses were conducted for subcategories of nervous system BD. *Post-hoc* subcategory analyses were also conducted for BD of the eye, since a strong association was observed between nitrate and this category.

Exposure assessment

Groundwater is the source of water for nearly all public water systems in Denmark. The methodology for the estimation of household levels of nitrate in drinking water has been thoroughly described in previous publications.^{2,20} In brief, we developed a spatial model linking drinking water quality measurements from the national monitoring database, *Jupiter*, at the waterworks-level with the locations of all Danish residential addresses. We have refined our spatial model by taking into further account historical changes in the water supply areas.

In total, we calculated annual average nitrate concentrations from 130,944 drinking water samples in 3,907 public waterworks taken between 1991–2013. Measurements of nitrate in water were taken at the exit of the waterworks, in the distribution system, and at the consumer taps which have all been found to be highly correlated.²¹ Approximately 11% of water supply areas had a nitrate sample from more than one water works in a given year. In these cases, we computed average concentrations, weighted by the annual drinking water production volume of each waterworks. Although nitrate levels have been declining over time in Denmark, yearly averages are adequate since no seasonal variation of nitrate concentrations in Danish public supplies has been observed and levels seldom change over a year.²⁰

We imputed annual nitrate concentrations for years with missing information (19% of all water supply area/year dyads) using linear interpolation between two sampled years. We only imputed missing exposure when a nitrate sample was available within three years. At the tails, we used the closest observation moving forward in time for the earliest year and moving backward in time for the latest year.

Longitudinal residential address histories for each mother and child pair were obtained from the Danish Civil Registration System.¹⁵ Addresses were linked to the calendar year-specific average exposure estimates for their water system. We computed time-weighted average exposure for each mother for 90 days prior to

conception, during pregnancy, and for each pregnancy trimester.

We excluded births to mothers who lived in households that had private wells anytime during their pregnancy from the main analysis because monitoring of private wells for nitrate is much less complete (~50% of wells) and private wells have generally poorer water quality than public supplies.²

Covariates

Covariates in our main models were chosen *a priori* based on their known or suspected association with BD and their potential for an association with nitrate exposure. Information on birth weight (continuous), birth order (1st, 2nd, ≥3rd), maternal and paternal age (continuous), maternal smoking during pregnancy (yes/no), and pre-pregnancy maternal height and weight (continuous) were obtained from the Danish Medical Birth Registry.¹⁶ Maternal height and weight were used to calculate pre-pregnancy body mass index (BMI). Information on parental employment (employed, unemployed, not in the workforce), parental education (primary, high school, or higher), and parental income (continuous), which was normalized for inflation to 2013 using the Consumer Price Index, was obtained from the Integrated Database for Longitudinal Labour Market Research.²² Income, education, and employment data from two years prior to birth were used.

Statistical analysis

The association between the covariates with any BD and nitrate exposure was examined using contingency tables and tested using a Pearson chi-square test. Logistic regression models were used to examine the association between nitrate in maternal drinking water and BD. Models were fit using generalized estimating equations and robust standard errors to account for the clustering of children born to the same mother. Unadjusted models and models that controlled for year of birth, birth order, birth weight, sex, parental age, and maternal smoking, education, income, and employment status were fitted. The continuous covariates (birth weight, parental age, and income) were modeled using restricted cubic splines with four knots placed at 0.05, 0.35, 0.65, and 0.95 quantiles of the nitrate exposure distribution. The statistical significance of the exposure-response relationships was determined by fitting the continuous measures of exposure and computing the Wald statistic and associated *p*-value. Finally, for ease of interpretation, marginal standardization²³ was used to derive absolute risks from models with significant exposure-response relationships.

Nitrate concentrations were modeled as both categorical and continuous variables. In the main analyses, the exposure was averaged over the pregnancy considering changes over time, and in maternal addresses. Four

categories of exposure were defined *a priori* based on the distribution of exposure in the population and their usefulness for policy considerations. The highest category ≥ 25 mg/L nitrate is half of the current EU standard. The lowest category, < 2 mg/L nitrate, was used as the referent.

For each major outcome category, statistical interactions between nitrate and each of the covariates were examined in models that adjusted for all of the covariates. The significance of the interactions was assessed using a likelihood ratio test comparing the model with the interaction term to a model with just the covariates and nitrate exposure variables. *A priori* we decided to only report interactions that were below $p < 0.01$ due to the large number of interactions examined. All tests of statistical significance were two-sided.

Sensitivity analyses were performed to assess whether our results changed when we: 1) modeled pre-conception and trimester-specific nitrate exposure rather than the pregnancy average; 2) included children born to mothers who lived in homes with private wells during their pregnancy; 3) included additional adjustment for paternal employment, education, and income; 4) included maternal BMI in the model, which was only available from 2003 onward; 5) compared results for births before and after 2004 when Denmark initiated a BD screening program; and 6) dropped individuals who had average nitrate exposure levels greater than the EU standard of 50 mg/L nitrate to evaluate whether or not the standard is adequate to prevent an increased risk of BD.

All statistical analyses were conducted using Stata Version 16.1.

Ethical considerations

In keeping with Danish legislation, the Danish Data Protection Agency, the Danish Health Data Authority, and Statistics Denmark approved this study. This study has also been approved by the University of Illinois at Chicago's Institutional Review Board. Due to Danish legislation and the use of de-identified data, informed consent was not needed for this study.

Role of the funding source

This work was supported by funding that was provided by the National Institutes for Health/National Institute for Environmental Health Sciences grant (R01 ES027823-01A1). The funders had no role in the design, conduct, or interpretation of the study findings, and in the decision to submit this paper for publication.

Results

A total of 1,138,562 live-born singletons who met the study entrance criteria of having been born in Denmark between 1991–2013 to Danish-born parents and a mother who resided in Denmark during her pregnancy

were identified. We excluded children of women who resided in a house with private well use during their pregnancy ($n=55,282$, 5%), had incomplete covariate data ($n=50,580$, 4%), or incomplete nitrate data ($n=13,786$, 1%) resulting in an analytic data set of 1,018,914 births (Figure 1). In total, 33,182 BD diagnosed within the first two years of life were identified.

All covariates were significantly ($p < 0.001$) associated with both case status (Table 1) and pregnancy average nitrate exposure (Supplemental Table 2), except for sex ($p=0.05$). Pregnancy average nitrate exposure was also significantly associated with any BD. Non-cases had a slightly larger percentage (3.8%) in the highest exposure group (≥ 25 mg/L nitrate) than cases (3.5%). The distribution of the nitrate exposure was highly right skewed. The mean exposure in the highest exposure group (> 25 mg/L) was 34.8 mg/L (SD=8.5). Approximately 0.2% ($n=2,026$) of the cohort had drinking water exposures that exceeded the EU standard of 50 mg/L nitrate (Figure 2).

Findings from unadjusted and adjusted models (Table 2) were similar in magnitude and direction and we therefore focused on adjusted model results. The risk of having any BD decreased with nitrate exposure in both the categorical and continuous models ($p=0.02$). Evidence of an inverse exposure-response relationship was also observed for most of the specific BD categories and was particularly strong for digestive system ($p=0.03$), female genital ($p=0.002$), and urinary ($p=0.001$) BD. BD of the eye was the only category that demonstrated a significant positive exposure-response ($p=0.004$) with nitrate exposure. The odds ratio (OR) for eye BD was 1.29 (95% confidence interval (95% CI): 1.00, 1.66) for the highest exposure group (≥ 25 mg/L nitrate) compared to the lowest exposure group (< 2 mg/L nitrate) and the OR per 10 mg/L nitrate (OR₁₀) was 1.09 (95% CI: 1.03, 1.16).

There was no evidence of an association between prenatal nitrate exposure and any of the subcategories of nervous system BD (Table 3). An association was observed between prenatal nitrate exposure and congenital cataracts in the second to highest exposure category (5–25 mg/L nitrate) (OR: 1.41, 95% CI: 1.05, 1.90). Evidence of an exposure-response relationship ($p=0.0005$) was observed for the other eye BD (Table 4).

Strong evidence of an interaction with maternal age was observed for nervous system BD ($p < 0.001$). The risk of nervous system BD increased with exposure for mothers < 25 years-of-age (OR₁₀: 1.20, 95% CI: 1.06, 1.35) and decreased with exposure in the other age groups. We also observed evidence ($p < 0.01$) of an interaction between maternal age and nitrate exposure for ear, face, and neck BD. The pattern of the interaction was similar with an increased risk for mothers < 25 years-of-age (OR₁₀: 1.35, 95% CI: 1.11, 1.66; $p=0.003$) and no increased risk in the other age categories. There was no evidence of an interaction between maternal age and nitrate for other BD or with other covariates (Table 5).

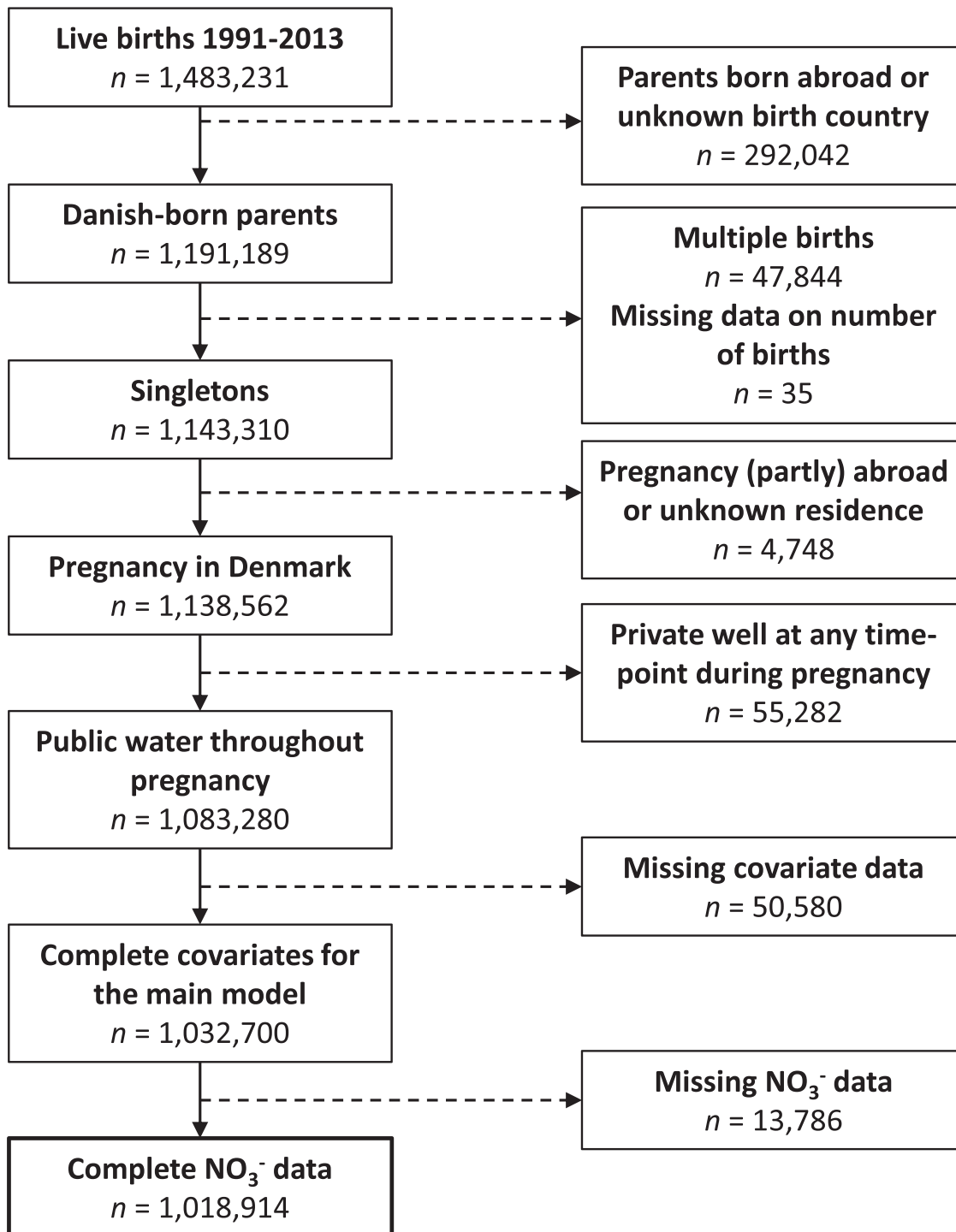


Figure 1. Flowchart of the birth cohort enumeration.

The predicted absolute risks from marginal standardized models for eye neoplasms are illustrated in [Figure 3](#). Results for children of mothers < 25 years old for nervous system and ear, face and neck BD are illustrated in [Figures 4](#) and [5](#).

Sensitivity analyses

The results were generally unchanged when pre-conception (Supplemental Table 3) or trimester-specific (Supplemental Table 4) estimates of exposure were used rather than the pregnancy average. This is largely

Characteristic	Non-Cases		Cases	
	n	%	n	%
Birth Year				
1991–1995	227 037	23.0	6 542	19.7
1996–2000	220 757	22.4	6 817	20.5
2001–2005	215 859	21.9	7 362	22.2
2006–2010	211 092	21.4	7 896	23.8
2010–2013	110 987	11.3	4 565	13.8
Birth Order				
First	435 906	44.2	15 426	46.5
Second	380 611	38.6	11 951	36.0
≥Third	169 215	17.2	5 805	17.5
Maternal Age (years)				
<25	134 895	13.7	4 844	14.6
25 to <30	361 475	36.7	11 559	34.8
30 to <35	340 976	34.6	11 220	33.8
≥35	148 386	15.1	5 559	16.8
Maternal Education				
Elementary	229 475	23.3	8 627	26.0
High School	464 586	47.1	15 111	45.5
Higher Education	291 671	29.6	9 444	28.5
Maternal Income (Kroner/year)				
<175 000	239 007	24.2	8 506	25.6
175 000 to <250 000	266 041	27.0	8 761	26.4
250 000 to <300 000	195 984	19.9	6 376	19.2
≥300 000	284 700	28.9	9 539	28.7
Maternal Employment				
Employed	801 661	81.3	26 364	79.5
Unemployed	60 620	6.1	2 080	6.3
Not in Workforce	123 451	12.5	4 738	14.3
Paternal Age (years)				
<25	67 255	6.8	2 455	7.4
25 to <30	271 851	27.6	8 870	26.7
30 to <35	363 912	36.9	11 951	36.0
≥35	282 714	28.7	9 906	29.9
Sex				
Male	503 519	51.1	19 831	59.8
Female	482 213	48.9	13 351	40.2
Maternal Smoking				
Non-smoker	769 506	78.1	25 633	77.2
Smoker	216 226	21.9	7 549	22.8
Mean Nitrate During Pregnancy (mg/L)				
<2	510 195	51.8	17 264	52.0
2 to <5	310 986	31.5	10 653	32.1
5 to <25	126 645	12.8	4 092	12.3
≥25	37 906	3.8	1 173	3.5

Table 1: Study population characteristics by any birth defect case status.

explained by the fact the preconception and trimester specific nitrate exposure estimates were highly correlated ($R^2=0.96, 0.99$) with the pregnancy average. The exposure-response relationship for eye BD was somewhat stronger for third trimester ($OR_{10}: 1.10, 95\%$

CI: 1.04, 1.17), than for the first ($OR_{10}: 1.07, 95\%$ CI: 1.01, 1.14) or second trimester ($OR_{10}: 1.09, 95\%$ CI: 1.03, 1.16). The results were largely unchanged when we included children whose mothers lived in homes with private wells sometime during their pregnancy (Supplemental Table 5) or when paternal socioeconomic status variables (income, education, and employment) were included (Supplemental Table 6). The addition of BMI to the model, which was only available from 2003 onward, resulted in a slightly stronger exposure-response relationship for eye BD ($OR_{10}: 1.14, 95\%$ CI: 1.04, 1.25) (Supplemental Table 7) than in the main analysis ($OR_{10}: 1.09, 95\%$ CI: 1.03, 1.16) (Table 2).

Stratifying the analysis by pre- and post-2004, which is the year that ultrasound screening during gestational week 18–20 was widely implemented in Denmark, revealed stronger evidence of an exposure-response relationship for eye BD in the 2004–2015 time period ($OR_{10}: 1.14, 95\%$ CI: 1.03, 1.25; $p=0.01$) than in 1991–2003 ($OR_{10}: 1.07, 95\%$ CI: 0.98, 1.15; $p=0.12$) (Supplemental Table 8). Finally, our findings were unchanged when children of mothers who had average nitrate exposure drinking water concentrations greater than the EU standard of 50 mg/L nitrate were excluded from the analysis (Supplemental Table 9).

Discussion

We observed strong evidence of an exposure-response relationship ($p=0.004$) between nitrate and BD of the eye ($OR_{10}: 1.09, 95\%$ CI: 1.03, 1.16) in our study. Ours is the first study to report findings of an association between nitrate exposure from residential tap water and BD of the eye. The register-based retrospective cohort design and large sample size of our study permitted us to examine eye and other BD that were not examined in prior studies. An association between the use of nitrosatable drugs during pregnancy and the risk of eye BD was reported in a study by Olshan and Faustman.²⁴ Nitrosatable drugs may react with exogenous and endogenous nitrate and nitrite to form *N*-nitroso compounds, which are known to be teratogenic in experimental animals.^{3–5} Nitrate has also been found to be highly concentrated in eye tissues where it plays an important role in nitrous oxide regulation.²⁵

Prior epidemiologic studies have most consistently reported an association between nitrate in drinking water and the risk of nervous system BD, particularly neural tube defects or spina bifida.^{7,8,10,11,13,14} *N*-nitroso compounds, which are formed endogenously have been found to increase central nervous system BD in several animal species as well.^{3–5}

Overall, we did not observe an association between nervous system BD and nitrate in our study. However, we did observe strong evidence ($p=0.005$) of an interaction between maternal age and nitrate exposure for

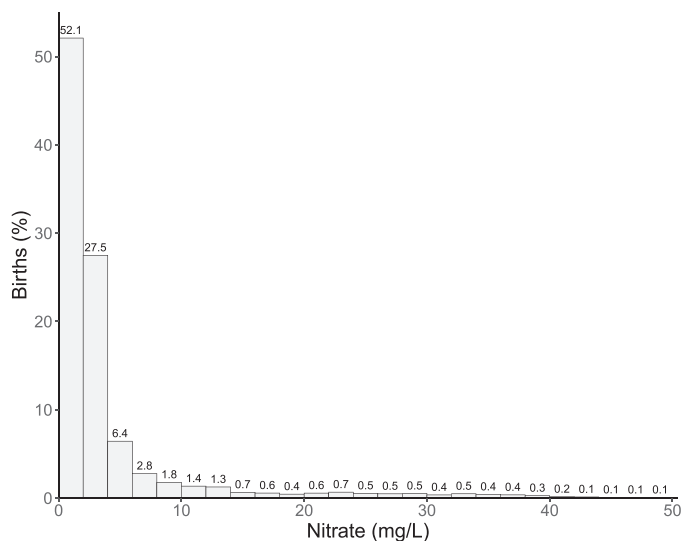


Figure 2. Histogram of the distribution of average pregnancy nitrate exposure.

Birth Defect (number of cases)	Unadjusted			Adjusted ^a		
	OR	95%CI	p-value	OR	95%CI	p-value
Any (n=33 182)						
<2 mg/L	Ref.			Ref.		
2 to <5 mg/L	1.01	0.99 1.04	0.34	1.01	0.98 1.04	0.45
5 to <25 mg/L	0.95	0.92 0.99	0.001	0.99	0.95 1.02	0.40
≥25 mg/L	0.91	0.86 0.97	0.004	0.93	0.88 0.99	0.02
Continuous ^c	0.97	0.96 0.99	0.001	0.98	0.97 1.00	0.02
Abdominal Wall (n=445)						
<2 mg/L	Ref.			Ref.		
2 to <5 mg/L	1.01	0.82 1.25	0.92	0.92	0.74 1.14	0.44
5 to <25 mg/L	1.13	0.86 1.50	0.38	0.87	0.66 1.16	0.35
≥25 mg/L	1.21	0.76 1.90	0.42	1.01	0.64 1.59	0.97
Continuous ^c	1.08	0.96 1.21	0.20	1.00	0.88 1.13	0.98
Digestive System (n=2 139)						
<2 mg/L	Ref.			Ref.		
2 to <5 mg/L	0.98	0.88 1.10	0.78	0.96	0.87 1.06	0.44
5 to <25 mg/L	0.93	0.80 1.08	0.34	1.02	0.89 1.16	0.81
≥25 mg/L	0.75	0.56 1.00	0.05	0.70	0.54 0.92	0.01
Continuous ^c	0.90	0.85 0.97	0.003	0.93	0.87 0.99	0.03
Ear, Face and Neck (n=448)						
<2 mg/L	Ref.			Ref.		
2 to <5 mg/L	1.22	0.99 1.50	0.06	0.96	0.87 1.06	0.44
5 to <25 mg/L	1.04	0.77 1.39	0.81	1.02	0.89 1.16	0.81
≥25 mg/L	1.26	0.80 2.00	0.32	0.70	0.54 0.92	0.001
Continuous ^c	1.02	0.90 1.15	0.78	1.03	0.91 1.17	0.60
Eye (n=1 402)						
<2 mg/L	Ref.			Ref.		
2 to <5 mg/L	1.22	0.99 1.50	0.06	1.02	0.91 1.16	0.69
5 to <25 mg/L	1.04	0.77 1.39	0.81	1.23	1.05 1.44	0.01
≥25 mg/L	1.26	0.80 2.00	0.32	1.29	1.00 1.66	0.05
Continuous ^c	1.08	1.01 1.15	0.02	1.09	1.03 1.16	0.004
Male Genital (n=3 103)						
<2 mg/L	Ref.			Ref.		
2 to <5 mg/L	1.10	1.04 1.18	0.002	1.09	1.02 1.16	0.01
5 to <25 mg/L	1.02	0.93 1.12	0.64	0.99	0.91 1.09	0.87
≥25 mg/L	0.88	0.75 1.03	0.11	0.86	0.74 1.02	0.08
Continuous ^b	0.97	0.93 1.01	0.12	0.96	0.92 1.00	0.06

Table 2 (Continued)

Birth Defect (number of cases)	Unadjusted				Adjusted ^a			
	OR	95%CI		p-value	OR	95%CI		p-value
Female Genital (n=203)^c								
<2 mg/L	Ref.				Ref.			
2 to <5 mg/L	1.16	0.90	0.98	0.01	1.12	1.06	1.20	0.0002
5 to <25 mg/L	1.11	0.92	1.04	0.47	0.99	0.91	1.08	0.83
≥25 mg/L	NA ^c	NA ^c	NA ^c	NA ^c	NA ^c	NA ^c	NA ^c	NA ^c
Continuous ^b	0.97	0.98	1.03	0.87	0.94	0.90	0.98	0.002
Heart (n=9 752)								
<2 mg/L	Ref.				Ref.			
2 to <5 mg/L	0.94	0.90	0.98	0.01	0.94	0.90	0.99	0.01
5 to <25 mg/L	0.98	0.92	1.04	0.47	1.01	0.95	1.08	0.77
≥25 mg/L	1.02	0.92	1.13	0.68	1.04	0.94	1.15	0.45
Continuous ^c	1.00	0.98	1.03	0.87	1.01	0.99	1.04	0.37
Limbs (n=5 528)								
<2 mg/L	Ref.				Ref.			
2 to <5 mg/L	0.94	0.90	0.98	0.01	1.02	0.96	1.08	0.54
5 to <25 mg/L	0.98	0.92	1.04	0.47	0.97	0.89	1.06	0.52
≥25 mg/L	1.02	0.92	1.13	0.68	0.91	0.78	1.06	0.21
Continuous ^c	1.00	0.96	1.03	0.83	0.99	0.95	1.03	0.64
Nervous System (n=1 702)								
<2 mg/L	Ref.				Ref.			
2 to <5 mg/L	0.98	0.88	1.10	0.78	0.98	0.88	1.10	0.76
5 to <25 mg/L	0.93	0.80	1.08	0.34	0.93	0.80	1.09	0.37
≥25 mg/L	0.75	0.56	1.00	0.05	0.75	0.57	1.00	0.05
Continuous ^c	0.94	0.87	1.01	0.09	0.94	0.87	1.01	0.10
Oro-facial Clefts (n=2154)								
<2 mg/L	Ref.				Ref.			
2 to <5 mg/L	1.07	0.97	1.17	0.18	1.07	0.97	1.17	0.18
5 to <25 mg/L	1.00	0.88	1.14	0.99	0.97	0.85	1.11	0.68
≥25 mg/L	0.97	0.77	1.23	0.82	0.95	0.76	1.20	0.69
Continuous ^b	0.99	0.93	1.05	0.71	0.98	0.92	1.04	0.52
Respiratory (n=1287)								
<2 mg/L	Ref.				Ref.			
2 to <5 mg/L	0.97	0.90	1.05	0.46	1.22	1.08	1.38	0.001
5 to <25 mg/L	0.85	0.76	0.95	0.004	1.15	0.97	1.37	0.11
≥25 mg/L	0.66	0.53	0.81	0.0001	0.85	0.61	1.18	0.34
Continuous ^b	0.94	0.87	1.02	0.12	0.95	0.88	1.03	0.22
Urinary (n=3055)								
<2 mg/L	Ref.				Ref.			
2 to <5 mg/L	0.97	0.90	1.05	0.46	0.96	0.89	1.04	0.35
5 to <25 mg/L	0.85	0.76	0.95	0.004	0.88	0.79	0.98	0.02
≥25 mg/L	0.66	0.53	0.81	0.0001	0.67	0.54	0.83	0.0002
Continuous ^b	0.89	0.84	0.94	<0.0001	0.90	0.85	0.95	0.0001
Other (n=4244)								
<2 mg/L	Ref.				Ref.			
2 to <5 mg/L	1.07	1.01	1.13	0.02	0.92	0.74	1.14	0.44
5 to <25 mg/L	0.91	0.84	0.98	0.02	0.87	0.66	1.16	0.35
≥25 mg/L	0.92	0.81	1.05	0.21	1.01	0.64	1.59	0.97
Continuous ^b	0.96	0.93	0.99	0.02	0.99	0.95	1.02	0.40

Table 2: Results for the major birth defect categories from crude and adjusted models using categorical or continuous average estimates of prenatal exposure to nitrate.

Note: Abbreviations: OR=odds ratio; 95% CI=95% confidence interval; Ref.=referent group.

^a Models adjusted for birth weight, birth order, birth year, sex, maternal and paternal age, and maternal smoking, income, education and employment status.

^b Results from the continuous model for an increase in exposure of 10 mg/L.

^c To preserve confidentiality and because models were based on less than five cases results are not available.

Birth Defect (number of cases)	Nitrate (mg/L)	OR	95%CI		p-value
Neural Tube (n=424)	<2 mg/L	Ref.			
	2 to <5 mg/L	0.89	0.72	1.10	0.29
	5 to <25 mg/L	0.72	0.52	1.00	0.05
	≥25 mg/L	0.88	0.53	1.46	0.62
	Continuous ^b	0.95	0.82	1.10	0.48
Anencephaly (n=12)^c	<2 mg/L	Ref.			
	2 to <5 mg/L	NA ^c	NA ^c	NA ^c	NA ^c
	5 to <25 mg/L	NA ^c	NA ^c	NA ^c	NA ^c
	≥25 mg/L	NA ^c	NA ^c	NA ^c	NA ^c
	Continuous ^b	1.19	0.70	2.01	0.52
Encephalocele (n=62)	<2 mg/L	Ref.			
	2 to <5 mg/L	0.77	0.43	1.37	0.37
	5 to <25 mg/L	0.49	0.19	1.24	0.13
	≥25 mg/L	NA ^c	NA ^c	NA ^c	NA ^c
	Continuous ^b	0.92	0.60	1.41	0.70
Hydrocephalus (n=501)	<2 mg/L	Ref.			
	2 to <5 mg/L	1.06	0.87	1.29	0.55
	5 to <25 mg/L	1.08	0.83	1.40	0.59
	≥25 mg/L	0.63	0.35	1.12	0.12
	Continuous ^b	0.92	0.81	1.04	0.18
Microcephalus (n=395)	<2 mg/L	Ref.			
	2 to <5 mg/L	1.00	0.80	1.26	0.97
	5 to <25 mg/L	0.87	0.62	1.20	0.39
	≥25 mg/L	0.69	0.38	1.26	0.22
	Continuous ^b	0.88	0.75	1.03	0.10
Spina Bifida (n=356)	<2 mg/L	Ref.			
	2 to <5 mg/L	0.90	0.71	1.13	0.36
	5 to <25 mg/L	0.78	0.55	1.09	0.15
	≥25 mg/L	0.80	0.44	1.42	0.44
	Continuous ^b	0.94	0.80	1.09	0.41
Other Nervous System (n=513)	<2 mg/L	Ref.			
	2 to <5 mg/L	0.93	0.76	1.13	0.47
	5 to <25 mg/L	1.06	0.81	1.39	0.66
	≥25 mg/L	0.72	0.42	1.23	0.23
	Continuous ^b	0.97	0.84	1.12	0.67

Table 3: Results for the subcategories of nervous system birth defects from adjusted models^a using categorical or continuous estimates of prenatal exposure to nitrate in household drinking water.

Note: Abbreviations: OR=odds ratio; 95% CI= 95% confidence interval; Ref.=referent group.

^a Models adjusted for birth weight, birth order, birth year, sex, maternal and paternal age, and maternal smoking, income, education and employment status.

^b Results from the continuous model for an increase in exposure of 10 mg/L.

^c To preserve confidentiality and because models were based on less than five cases results are not available.

nervous system BD, which revealed a strong exposure-response relationship for women <25 years-of-age. We also observed weaker evidence ($p < 0.01$) of an interaction between nitrate and ear, face and neck BD among children of mothers <25 years-of-age. An association between nitrate and BD of the ear, face, and neck BD has not been examined in prior studies.

It is unclear why there was an interaction with maternal age for these specific BD sites. One possibility is that women <25 years-of-age are more likely to have unplanned pregnancies than older women and thus less likely to take folate supplements during

preconception and early pregnancy. Neural tube malformations are believed to occur early in pregnancy when the neural tube is formed and before women may know they are pregnant.²⁶ Folate supplementation is well recognized to reduce neural tube defects and knowledge of the importance of folate supplementation during pregnancy has been reported to be low in Danish women <30 years-of-age.²⁷ Young women may also be more likely to reside in the larger cities as they may be students and thereby be co-exposed to ambient air pollution and other urban stressors. They may also be more likely to smoke and have more other unhealthy

Birth Defect (number of cases)	Nitrate	OR	95%CI		p-value
Anophthalmos (n=17)^b	<2 mg/L	NA ^c	NA ^c	NA ^c	NA ^c
	2 to <5 mg/L	NA ^c	NA ^c	NA ^c	NA ^c
	5 to <25 mg/L	NA ^c	NA ^c	NA ^c	NA ^c
	≥25 mg/L	NA ^c	NA ^c	NA ^c	NA ^c
	Continuous ^b	0.60	0.24	1.49	0.27
Anophthalmos/Microphthalmos (n=135)	<2 mg/L	Ref.			
	2 to <5 mg/L	1.18	0.81	1.73	0.39
	5 to <25 mg/L	1.06	0.62	1.80	0.84
	≥25 mg/L	1.23	0.53	2.83	0.63
	Continuous ^b	1.03	0.84	1.27	0.76
Congenital Cataracts (n=362)	<2 mg/L	Ref.			
	2 to <5 mg/L	1.03	0.81	1.31	0.83
	5 to <25 mg/L	1.41	1.05	1.90	0.02
	≥25 mg/L	1.01	0.57	1.78	0.97
	Continuous ^b	1.03	0.91	1.16	0.65
Microphthalmos (n=127)	<2 mg/L	Ref.			
	2 to <5 mg/L	1.20	0.81	1.78	0.37
	5 to <25 mg/L	1.15	0.67	1.96	0.62
	≥25 mg/L	1.33	0.58	3.07	0.50
	Continuous ^b	1.07	0.88	1.30	0.52
Other Eye (n=930)	<2 mg/L	Ref.			
	2 to <5 mg/L	0.99	0.86	1.15	0.93
	5 to <25 mg/L	1.19	0.98	1.45	0.08
	≥25 mg/L	1.43	1.06	1.92	0.02
	Continuous ^b	1.13	1.06	1.21	0.0005

Table 4: Results for the subcategories of eye BD from adjusted^a models using categorical or continuous estimates of prenatal exposure to nitrate.

Note: Abbreviations: OR=odds ratio; 95%CI=95% confidence interval; Ref.=referent group.

^a Models adjusted for birth weight, birth order, birth year, sex, maternal and paternal age, and maternal smoking, income, education and employment status.

^b Results from the continuous model for an increase in exposure of 10 mg/L.

^c To preserve confidentiality and because models were based on less than five cases results are not available.

Birth Defect (number of cases)	Maternal age (years)	OR ₁₀	95%CI		p-value	p for interaction
Any (n=33182)	<25	1.00	0.96	1.03	0.83	0.71
	25 to <30	0.97	0.95	1.00	0.04	
	30 to <35	0.98	0.95	1.01	0.13	
	≥35	0.99	0.95	1.04	0.77	
Abdominal (n=445)	<25	1.01	0.83	1.25	0.89	0.33
	25 to <30	0.90	0.73	1.13	0.37	
	30 to <35	0.98	0.76	1.26	0.88	
	≥35	1.30	0.98	1.72	0.07	
Digestive System (n=2139)	<25	0.90	0.77	1.07	0.24	0.09
	25 to <30	0.92	0.83	1.02	0.13	
	30 to <35	0.86	0.76	0.98	0.02	
	≥35	1.09	0.96	1.25	0.19	
Ear, Face and Neck (n=448)	<25	1.35	1.11	1.66	0.003	0.0095
	25 to <30	1.08	0.90	1.30	0.39	
	30 to <35	0.84	0.64	1.12	0.23	
	≥35	0.65	0.36	1.17	0.16	

Table 5 (Continued)

Birth Defect (number of cases)	Maternal age (years)	OR ₁₀	95%CI		p-value	p for interaction
Eye (n=1402)	<25	1.13	0.97	1.31	0.13	0.57
	25 to <30	1.06	0.95	1.18	0.29	
	30 to <35	1.15	1.04	1.26	0.005	
	≥35	1.02	0.84	1.22	0.86	
Genital (Female; n=203)	<25	0.90	0.49	1.65	0.73	0.06
	25 to <30	0.60	0.35	1.04	0.07	
	30 to <35	0.97	0.67	1.39	0.86	
	≥35	0.94	0.01	1.12	0.06	
Genital (Male; n=3103)	<25	1.02	0.90	1.14	0.80	0.82
	25 to <30	0.96	0.89	1.04	0.35	
	30 to <35	1.01	0.93	1.10	0.80	
	≥35	0.98	0.85	1.12	0.74	
Heart (n=9752)	<25	0.99	0.93	1.06	0.80	0.61
	25 to <30	1.00	0.95	1.04	0.89	
	30 to <35	1.03	0.98	1.08	0.26	
	≥35	1.04	0.97	1.11	0.26	
Limb (n=5528)	<25	0.95	0.87	1.05	0.33	0.58
	25 to <30	1.02	0.97	1.08	0.46	
	30 to <35	0.98	0.91	1.04	0.47	
	≥35	0.98	0.89	1.08	0.73	
Nervous System (n=1702)	<25	1.20	1.06	1.35	0.003	0.0005
	25 to <30	0.88	0.78	0.99	0.03	
	30 to <35	0.89	0.78	1.03	0.11	
	≥35	0.76	0.59	0.98	0.03	
Oral (n=2154)	<25	0.96	0.83	1.11	0.58	0.74
	25 to <30	1.02	0.93	1.12	0.65	
	30 to <35	0.95	0.86	1.06	0.40	
	≥35	0.95	0.80	1.12	0.52	
Respiratory (n=1287)	<25	0.97	0.80	1.17	0.72	0.91
	25 to <30	0.93	0.81	1.06	0.32	
	30 to <35	0.94	0.82	1.09	0.43	
	≥35	1.02	0.84	1.23	0.87	
Urinary (n=3055)	<25	0.83	0.71	0.98	0.03	0.52
	25 to <30	0.92	0.84	1.01	0.07	
	30 to <35	0.86	0.77	0.95	0.004	
	≥35	0.94	0.81	1.08	0.35	
Other (n=4244)	<25	0.99	0.89	1.11	0.92	0.21
	25 to <30	0.92	0.85	1.00	0.04	
	30 to <35	1.02	0.95	1.09	0.58	
	≥35	1.03	0.93	1.14	0.63	

Table 5: Results from adjusted continuous models^{a, b} that include an interaction term between maternal age and nitrate exposure.

Note: Abbreviations: OR=odds ratio; 95%CI=95% confidence interval; Ref.=referent group.

^a Models adjusted for birth weight, birth order, birth year, sex, maternal and paternal age, and maternal smoking, income, education and employment status.

^b Results from the continuous model for an increase in exposure of 10 mg/L.

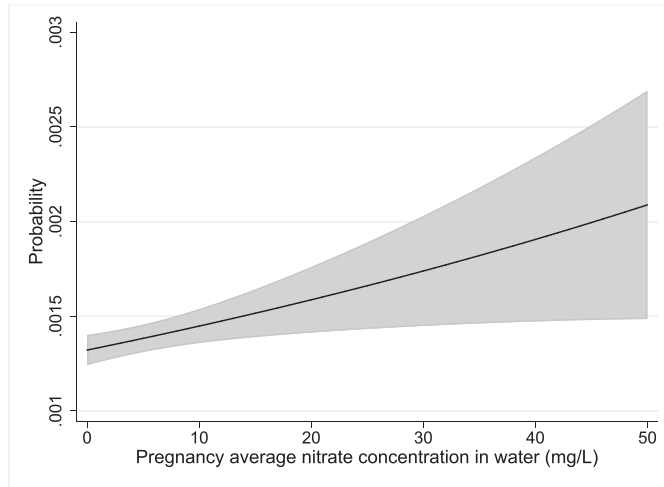


Figure 3. Marginally standardized plot of the predicted probability of eye birth defects as a function of nitrate exposure.

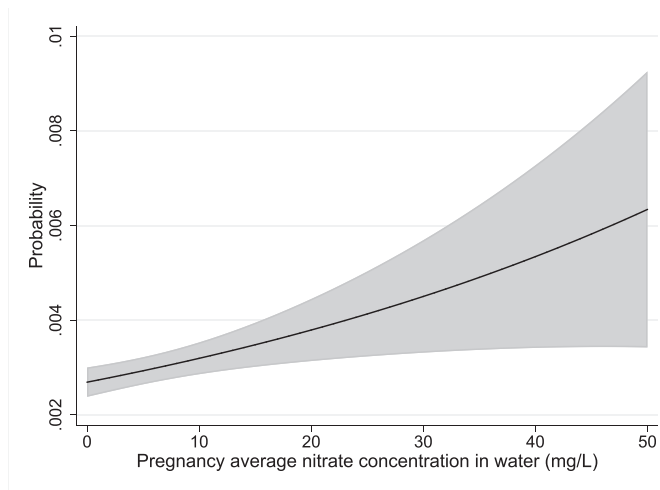


Figure 4. Marginally standardized plot of the probability of nervous system BD as a function of nitrate exposure for children with mothers age < 25.

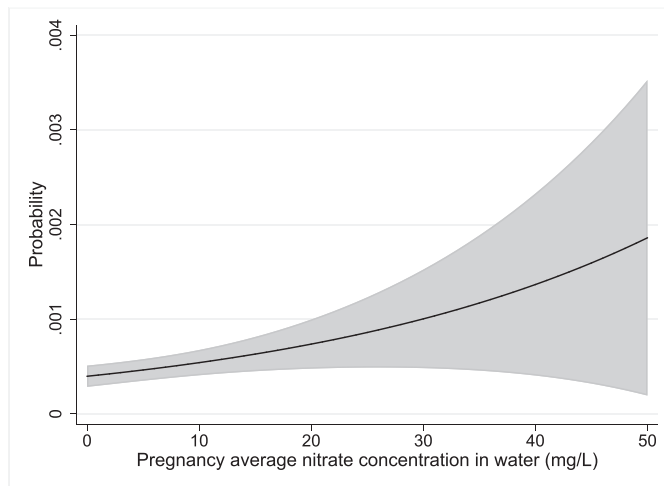


Figure 5. Marginally standardized plot of the probability of ear, face and neck BD as a function of nitrate exposure for children with mothers age < 25.

behaviors than women of older ages that have planned to become pregnant. Chance is also a possible explanation for these findings due to the many interactions tested in this study.

In addition to its size and cohort design, our study has several strengths over prior studies which mostly used surrogates of exposure such as private well use,⁸ ecologic measures of exposure,¹³ or exposures at birth.^{7,14} In contrast, our study used detailed estimates of nitrate exposure at the household level and address histories that considered maternal changes of residence. Only one prior study had information on bottled water use,¹³ which may be an important source of exposure misclassification in prior studies. We did not have information on bottled water use, but the use of bottled water in Denmark is low with only 20.5 L consumed per capita annually in 2014 and much less for earlier years.²⁸

Our study has a few limitations which future studies should address. We lacked information on dietary sources of nitrate and nitrite, intake of antioxidants that might modify the effect of nitrate exposure on the risk of birth defects, and on the use of nitrosatable drugs that may promote the formation of *N*-nitroso compounds.¹³ We also lacked information on the amount of water consumption at home and outside of the home. The effect of this misclassification of exposure is most likely to bias our findings towards the null assuming these are random errors that are non-differential with respect to BD. Although we did not have information on co-contaminants in drinking water, public water in Denmark is recognized to be high-quality and generally complies with legal guideline values.²⁹ The nitrate exposures in our study were quite low, which is both a limitation due to reduced statistical power, and a strength since we were able to examine risks at nitrate concentrations below the current standards.

Another limitation is our lack of access to BD in pregnancies that resulted in fetal loss. It is noteworthy that we observed a significant inverse exposure-response relationship for diagnosis of any BD ($p=0.02$) and for several of the specific BD categories. This seems to reflect a bias in our study, as nitrate is unlikely to prevent BD. A probable explanation for this is live birth bias, which may occur when the exposure causes fetal mortality from severe BD and thus prevents selection of these births into the study.³⁰ Use of nitrosatable prescription drugs has been reported to be associated with an increased risk of fetal loss³¹ and it is possible that nitrate may potentiate the effects of nitrosatable drugs. This bias might also explain the lack of evidence in our study for an increased risk of limb deficiencies and heart defects, which have been reported in some prior studies.^{13,14} It may also have negatively biased the results for other BD and particularly for anencephaly and other nervous system BD that have high fetal mortality.

Reporting of BD in Denmark is mandatory by law and we would expect compliance to be very high. Of course, the reporting may be more accurate and

complete for some anatomical sites than for others. For example, in a Danish study it was reported that only 4.5% (95% CI: 1.2-7.8) of major heart BD were detected at birth.³² In contrast, a European study of clubfoot reported that 100% of reported cases in Denmark were confirmed after review of the medical records.³³

In general, the nitrate concentrations in Danish groundwater have been decreasing since the 1980's.³⁴ This is due to implementation of national mitigation measures such as for the handling of animal manure, nitrogen quotas for specific crops, and growing catch crops after harvesting of the main crop. However, there is still a large regional and local variation in the content of nitrate in groundwater in Denmark mainly due to variation in animal density, nitrate leaching from fields, and vulnerability of the groundwater aquifers. The Danish drinking water nitrate trends are also affected by infrastructural changes as for example the location and abstraction depth of drinking water because waterworks are trying to avoid the upper nitrate containing groundwater.³⁵ Denmark is not yet meeting the demands of both national and EU legislations, and more locally targeted mitigation measures are being developed to further lower the nitrate content in groundwater.³⁶

Far higher levels of water contamination by nitrate may exist in other countries that are highly agricultural and do not use strict mitigation strategies. For example, concentrations of up to 1,500 mg/L were found in an agricultural area of India.³⁷ If causal, our findings raise serious concerns about the potential for an increased risk in BD in areas of the world with higher nitrate levels than Denmark.

In conclusion, we found strong evidence of an increased risk of BD of the eye. To our knowledge this association has not been examined in prior studies of nitrate contaminated drinking water. We also observed strong evidence of interaction by maternal age, indicating an increased risk of nervous system BD and ear, face, and neck BD among children whose mothers were <25 years-of-age at the time of birth. Our findings were unchanged when we restricted our analyses to children of mothers who had average exposures that were below the current EU standard of 50 mg/L nitrate, which is nearly equivalent to the US Environmental Protection Agency standard of 44 mg/L.¹ Our findings add to mounting evidence that the current nitrate in drinking water standards may not be sufficient to prevent an increased risk of BD and other adverse birth outcomes.

¹ The U.S. EPA's MCL for nitrate in drinking water is 10 mg/L measured as nitrogen ($\text{NO}_3^- \cdot \text{N}$). The EU standard is based on measurement of the whole nitrate ion (NO_3^-). Concentrations of $\text{NO}_3^- \cdot \text{N}$ can be converted to NO_3^- by multiplying by 4.42. Thus the U.S. MCL is 44.2 mg/L as NO_3^- , roughly equivalent to the EU standard of 50 mg/L.

Contributors

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Data availability statement

Due to privacy concerns, the data from this study are not available to outside researchers unless they have approval from the Danish Data Protection Agency.

Declaration of interests

The authors have no conflicts of interest to declare.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.lanepe.2021.100286.

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