Vitamin B_{12} and the Risk of Neural Tube Defects in a Folic-Acid-Fortified Population

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Background: Low maternal vitamin B_{12} status may be a risk factor for neural tube defects (NTDs). Prior studies used relatively insensitive measures of B_{12}, did not adjust for folate levels, and were conducted in countries without folic acid food fortification. In Canada, flour has been fortified with folic acid since mid-1997.

Methods: We completed a population-based case–control study in Ontario. We measured serum holotranscobalamin (holoTC), a sensitive indicator of B_{12} status, at 15 to 20 weeks' gestation. There were 89 women with an NTD and 422 unaffected pregnant controls. A low serum holoTC was defined as less than 55.3 pmol/L, the bottom quartile value in the controls.

Results: The geometric mean serum holoTC levels were 67.8 pmol/L in cases and 81.2 pmol/L in controls. There was a trend of increasing risk with lower levels of holoTC, reaching an adjusted odds ratio of 2.9 (95% confidence interval = 1.2–6.9) when comparing the lowest versus highest quartile.

Conclusions: There was almost a tripling in the risk for NTD in the presence of low maternal B_{12} status, measured by holoTC. The benefits of adding synthetic B_{12} to current recommendations for periconceptional folic acid tablet supplements or folic-acid-fortified foods need to be considered. It remains to be determined what fraction of NTD cases in a universally folate-fortified environment might be prevented by higher periconceptional intake of B_{12}.

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Neural tube defects (NTD), manifesting as anencephaly or spina bifida, are to some degree preventable developmental anomalies. The risk of NTD can be reduced nearly 50% with periconceptional maternal exposure to folic acid tablet supplements[1] or fortified flour, as we have seen in Canada.[2] Despite these remarkable accomplishments, about 6 to 12 in every 10,000 fetuses in Canada have NTD.[2,3]

A systematic overview of previously published data suggested that a deficiency of vitamin B_{12} (B_{12}, cobalamin) might also be associated with a higher risk of NTD.[4] However, these studies are characterized by relatively few participants, lack of adjustment for maternal folate status, variability in the time of specimen collection during pregnancy, and relatively inaccurate measurement of bioavailable maternal B_{12}.[4] As a consequence, there is ongoing debate about whether B_{12} should be added to folic acid tablet supplements or fortified foods.[5] We evaluated serum holotranscobalamin (holoTC)—the fraction of total circulating B_{12} bound to transcobalamin II. Since holoTC is the only form of B_{12} taken up by tissues, it should be a sensitive measure of maternal B_{12} status, as it may be related to NTD risk.[6] B_{12} also appears to be the leading nutritional determinant of hyperhomocysteinemia within folic acid-fortified populations.[7] We therefore studied whether this associated risk changed after the fortification of Canadian flour by folic acid.

METHODS

We performed a population-based case–control study.[2,8] Since 1993, under the universal Ontario Health Insurance Plan all pregnant women are offered standardized maternal serum screening at no financial cost. Maternal screening is made available at 15 to 20 weeks' gestation through a physician or midwife; about 60% of pregnant women are screened. Self-reported maternal date of birth, gravidity, ethnicity, weight at the time of screening, and pregestational diabetes mellitus were recorded on the screening requisition sheet.

Women with a positive screen are referred for counseling at one of 17 genetics centers in Ontario. Each center supplies follow-up data to the Ontario Maternal Serum Screening Database. Open NTD cases are detected antenatally by ultrasonography or fetal autopsy, or postnatally through data linkage of the mother's insurance plan number with that of her infant during the delivery hospitalization, through the Canadian Institute for Health Information Discharge Abstract Database.[2,8]

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Two large metropolitan genetics centers in Southern Ontario—the North York General Hospital (Toronto) and The Credit Valley Hospital (Mississauga)—handle about half of all serum specimens acquired at the time of screening and stored frozen. Blood samples were collected in a red top vacutainer or serum separator tube, and the serum transferred into a plastic transport tube, without a preservative, and then frozen at 70°C.

Cases were all women who had a pregnancy affected by a myelomeningocele or anencephaly, and whose screening database record corresponded to one of the 2 genetics centers. For each case, 5 maternal controls with a healthy pregnancy were randomly selected from these 2 centers within a plus-or-minus 12 months of the case specimen. Serum HoloTC was determined using a radioimmunoassay, according to the manufacturer's protocol (Axis-Shield, Ireland).[9] Analytic sensitivity is about 5 pmol/L, with a between-assay imprecision of 8.5% at 32 pmol/L and 7.6% at 94 pmol/L. Serum folate was analyzed using the Bayer Centaur immunoassay analyzer method (Bayer Diagnostics Division, Toronto, ON). The distributions of the holoTC and folate values were positively skewed, so we log-transformed these measures and used inverse transformations to generate geometric means and standard deviations.

Statistical Analysis

We compared the year of screening for cases and controls using a χ^2 test for trend. The mean serum folate and mean holoTC concentrations among cases and controls were compared using an unpaired *t* test. An abnormally low holoTC was defined, a priori, as a concentration less than or equal to the bottom quartile value in the control group. For the main analysis, we calculated a crude odds ratio (OR) and 95% confidence interval (CI). We used logistic regression to estimate the adjusted OR, with the highest quartile concentration of holoTC as the referent. We adjusted for maternal age (1-year increments), gravidity, race (white versus nonwhite), weight, low-income status, presence of pregestational diabetes mellitus and serum folate concentration (as a continuous variable) at the time of screening. Low-income status was based on data from the 2001 Canadian Census.[10] This method uses the first 3 digits of each woman's 6-digit postal code to designate a geographical region, and then determines the prevalence of households in that same region who spend 55% or more of their income on food, shelter, and clothing.[10] A secondary analysis, limited to the period after March 1, 1997, when folic acid food fortification was begun,[11] used the same multivariate model as above. Finally, we estimated a population attributable risk percent of NTD in relation to low holoTC, using the adjusted OR.[12,13]

All variables were included in the model a priori. The research protocol was approved by the Ministry of Health and Longterm Care in Ontario, as well as the Research Ethics Boards of Sunnybrook Health Sciences Centre, St. Michael's Hospital and The North York General Hospital, with participant identifiers removed from the data set prior to analysis.

RESULTS

There were 317 cases of NTD in Ontario during the period of study. Of these, 89 cases originated from the North York General and Credit Valley Hospitals. Of the 434 unaffected pregnant controls selected from these 2 centers, 12 were excluded due to an insufficient volume of serum, leaving 422 controls (Table 1). About half (n = 248) of all participants were enrolled after fortification of flour had begun. Of 89 NTD cases, 67 (75%) were diagnosed antenatally. No difference was observed between cases and controls in the year of specimen collection (χ^2 test for trend: $P = 0.99$) (Table 1). The mean concentration of serum folate did not differ appreciably between cases and controls across the entire study period (13.3 versus 13.9 nmol/L; $P = 0.07$).

The overall geometric mean serum holoTC level was substantially lower among cases (67.8 pmol/L) than controls (81.2 pmol/L), a mean of difference of 13.4 pmol/L (95% CI = 13.0–13.8) (Table 1). Comparing the lowest with the highest quartile of maternal holoTC concentration, the crude OR for NTD was 2.0 (95% CI = 1.1–3.9), increasing to 2.9 (1.2–6.9) in the adjusted model (Table 2). The corresponding population-attributable risk for NTD in relation to low holoTC was 34%.

TABLE 1. Characteristics of Selected Women in Ontario With (Cases) and Without (Controls) a Pregnancy Affected by a Neural Tube Defect (NTD), 1993–2004

Maternal Characteristic	Cases (n = 89)	Controls (n = 422)
Age (yrs); mean ± SD	28.6 ± 4.8	29.8 ± 5.1
Gravidity; median	2.0	2.0
Weight (kg); mean ± SD	68.6 ± 15.5	67.3 ± 13.6
Prepregnancy diabetes mellitus; no. (%)	3 (3)	5 (1)
Nonwhite ethnicity; no. (%)	23 (29)	60 (14)
Postfortification of flour; no. (%)	44 (49)	204 (48)
Percent low income status; mean ± SD	13.0 ± 6.7	13.2 ± 7.5
Year of enrollment; no. (%)		
1993	5 (6)	23 (5)
1994	24 (27)	120 (28)
1995	16 (18)	75 (18)
1996–1997*	7 (8)	29 (7)
1998	12 (14)	59 (14)
1999	6 (7)	26 (6)
2000	5 (6)	25 (6)
2001	3 (3)	21 (5)
2002	6 (7)	20 (5)
2003–2004*	5 (6)	24 (6)
Serum folate (nmol/L); geometric mean ± SD	13.3 ± 3.0	13.9 ± 2.8
Serum holotranscobalamin concentration (pmol/L)		
Geometric mean ± SD	67.8 ± 2.0	81.2 ± 1.8
Lowest quartile	—	55.3

*Few cases and controls were recorded, and are therefore combined over the two-year period.

TABLE 2. Risk of an Open Neural Tube Defect in Relation to Low Holotranscobalamin Concentrations

Serum holoTC Quartile (pmol/L)	Cases		Controls		Crude Odds Ratio	(95% CI)	Adjusted Odds Ratio*	(95% CI)
	No.	%	No.	%				
≤55.3	35	39	106	25	2.0	(1.1–3.9)	2.9	(1.2–6.9)
>55.3–84.0	19	21	105	25	1.1	(0.55–2.3)	2.0	(0.75–5.1)
>84.0–121.0	18	20	106	25	1.0	(0.51–2.1)	1.1	(0.40–2.9)
>121.0†	17	19	105	25	1.0		1.0	

*Adjusted for maternal age, gravidity, weight, ethnicity, low-income status, presence of pregestational diabetes mellitus and serum folate concentration.
†Reference category.

In the period before fortification, the mean ± SD serum folate concentrations were 8.8 ± 3.5 nmol/L among the cases and 10.3 ± 3. nmol/L in the controls. After fortification, serum folate concentrations rose to 20.9 ± 2.0 nmol/L for cases and 19.4 ± 2.2 nmol/L for controls. Similarly, the levels of holoTC were 58.5 ± 1.9 pmol/L in cases and 72.7 ± 1.8 pmol/L among the controls and after fortification 78.8 ± 2.0 pmol/L in cases and 91.3 ± 1.8 pmol/L in controls. Restricting the analyses to the time after food fortification with folic acid, the association of low holoTC with risk of NTD remained strong (adjusted OR = 3.2; CI = 0.94–11.0).

DISCUSSION

Using a sensitive and specific measure of bioavailable cobalamin, we observed nearly a tripling in the risk of NTD in the presence of low maternal B_{12} status. In a Canadian setting of moderate folic acid tablet supplement use[14] and universal folic acid flour fortification,[2] these data suggest that about 34% of NTD may be due to low B_{12}.

Our study has some limitations. The collection of maternal specimens about 15 weeks after conception would be expected to dilute any true relationship between B_{12} insufficiency and NTD risk.[15] We do not know how many cases or controls were taking folic acid or B_{12}-containing tablet supplements periconceptionally (ie, before and after conception), nor could we assess a "contamination" effect on measured holoTC concentration from B_{12} supplement use started after conception. We adjusted for serum folate concentration at the time of screening—which differed little between cases and controls—as well as maternal age and socioeconomic status, each predictors of early access to antenatal care and periconceptional vitamin supplement use.[14] There were more nonwhite women among cases than controls, and we adjusted for nonwhite ethnicity. This heterogeneous group of women (mainly Asian and black) may differ from whites in terms of dietary intake and metabolism of B_{12}.[16]

Surveys suggest that the majority of women in Ontario take a periconceptional tablet supplement containing folic

TABLE 3. Published Case–Control Studies Evaluating the Risk of Neural Tube Defects in Association With Indicators of Maternal B_{12} Status

First Author	No. NTD Cases	No. Non-NTD Controls	Serum/Plasma Analyte	Comparison of Abnormal vs. Normal Cut-Points	Odds Ratio	(95% CI)	Odds Ratio Adjusted for Maternal Folate Concentration?
Kirke[22]	81	247	B_{12} and folate	≤lower quartile vs. ≥upper quartile, both analytes	5.4	(1.2–25.2)	No
Molloy[23]	32	384	B_{12}	<185 pmol/L vs. ≥185 pmol/L	0.9	(0.4–1.9)	No
van der Put[24]	60	94	B_{12}	≤5th centile vs. >95 centile	3.9	(1.3–11.9)	No
Groenen[25]	44	83	B_{12}	<10th centile vs. ≥10th centile	3.5	(1.3–8.9)	No
Suarez[26]	157	186	B_{12}	≤lower quintile vs. ≥upper quintile	2.6	(1.2–5.4)	Yes
Wilson[27]	58	89	B_{12}	≤lower quartile vs. ≥second quartile	2.1	(0.9–5.2)	No
			B_{12} and MTRR	B_{12} as above, combined with MTRR homozygosity	4.8	(1.5–15.8)	No
Adams[28]	33	132	MMA	≥90th centile vs. <10th centile	13.3	(2.7–65.5)	Yes
Afman[19]	46	73	B_{12}	≤lower quartile vs. ≥upper quartile	1.8	(0.6–5.2)	No
			HoloTC	≤lower quartile vs. ≥upper quartile	2.9	(0.9–9.2)	No
Current	89	422	HoloTC	≤lower quartile vs. >upper quartile	2.9	(1.2–6.9)	Yes

MMA indicates methylmalonic acid.

acid,[14,17] in keeping with Canadian guidelines.[18] The small quantity of B_{12} contained in each prenatal multivitamin tablet in Canada (2.5 μg) is unlikely to explain the observed difference in holoTC levels (for example, if a greater number of controls than cases had initiated multivitamin tablets soon after conception).

The concentration of holoTC defining the bottom quartile among our pregnant controls (55 pmol/L) was comparable to the lower-quartile values described in other studies of pregnant women[19] and healthy adults.[9,20] A lower reference limit of 50 pmol/L is recommended for a diagnosis of B_{12} deficiency in young and middle-aged adults.[21] Together, this reinforces the robustness of the holoTC threshold that we used to define "abnormal," and the comparability of our controls to other study populations of young adults.

Though not a central focus of our study, we were surprised that the concentration of serum folate was only slightly lower among the cases than controls. This may have been due to the dilutional effect of pregnancy on folate levels, a similar prevalence of use of folic acid tablet supplements among cases and controls around the time of maternal screening, or perhaps the fact that nearly half of all study participants were screened after universal folic acid flour fortification. Other Canadian studies have found that serum folate levels have increased by more than 60% after the introduction of flour fortification, with 99% of women now replete.[11]

Previous studies have directly or indirectly assessed an association between low maternal B_{12} and NTD risk (Table 3). One small case–control study has observed an association of low holoTC with risk of NTD (crude OR = 2.9; 95% CI = 0.9–9.2).[19] Another study,[28] comprising 33 cases and 132 controls, found an adjusted OR for NTD of 13 (95% CI = 2.7–66) using another sensitive functional indicator of B_{12} deficiency (maternal serum methylmalonic acid concentration above the 90th centile).[29] The combination of a low B_{12} and a genetic polymorphism MTRR, which makes of methionine synthase reductase, (an enzyme that activates cobalamin-dependent methionine synthase), was associated with an OR for NTD of 4.8 (95% CI = 1.5–16).[27] In general, while there appears to be a consistent association between maternal B_{12} insufficiency and NTD risk, few studies have examined maternal B_{12} status using methods of sufficient sensitivity, or controlled for influential maternal risk factors such as folate status (Table 3).

Our findings suggest that as much as 34% of all NTDs in Canada may be attributable to low maternal B_{12} status, a conclusion which could have profound public health implications, if generalizable. However, only about 60% of pregnant women in Ontario have maternal screening, which limits the generalizability of our data. Women who do not access prenatal screening may differ from those who do,[30] with lower rates of pregnancy planning and periconceptional folic acid tablet supplement use.[14]

B_{12} and folic acid are the main nutritional determinants of homocysteine metabolism.[7] While folic acid supplementation may lower both plasma homocysteine and the risk of NTD, the impact of higher B_{12} intake on NTD risk is not known. Given our results and those of other studies, a randomized clinical trial of periconceptional B_{12} may be indicated. Adding B_{12} to folic acid in food fortification may help prevent NTD, while at the same time reducing concern about masking B_{12}-related neurologic disease, which can occur when fortifying with folic acid alone.[31,32]

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