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Maternal Vitamin B₁₂ Status and Risk of Neural Tube Defects in a Population With High Neural Tube Defect Prevalence and No Folic Acid Fortification

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What’s Known on This Subject

- Folic acid can prevent many, but not all, NTDs. Vitamin B₁₂ interacts closely with folate metabolism and may play a role in NTD prevention. Some studies have found low vitamin B₁₂ status in mothers of NTD-affected children.

What This Study Adds

- This study confirms that low maternal vitamin B₁₂ status is an independent risk factor for having an NTD-affected pregnancy and is the first to address the public health question of what B₁₂ level might be protective for women entering pregnancy.

ABSTRACT

OBJECTIVE. Folic acid fortification has reduced neural tube defect prevalence by 50% to 70%. It is unlikely that fortification levels will be increased to reduce neural tube defect prevalence further. Therefore, it is important to identify other modifiable risk factors. Vitamin B₁₂ is metabolically related to folate; moreover, previous studies have found low B₁₂ status in mothers of children affected by neural tube defect. Our objective was to quantify the effect of low B₁₂ status on neural tube defect risk in a high-prevalence, unfortified population.

METHODS. We assessed pregnancy vitamin B₁₂ status concentrations in blood samples taken at an average of 15 weeks’ gestation from 3 independent nested case-control groups of Irish women within population-based cohorts, at a time when vitamin supplementation or food fortification was rare. Group 1 blood samples were from 95 women during a neural tube defect–affected pregnancy and 265 control subjects. Group 2 included blood samples from 107 women who had a previous neural tube defect birth but whose current pregnancy was not affected and 414 control subjects. Group 3 samples were from 76 women during an affected pregnancy and 222 control subjects.

RESULTS. Mothers of children affected by neural tube defect had significantly lower B₁₂ status. In all 3 groups those in the lowest B₁₂ quartiles, compared with the highest, had between two and threefold higher adjusted odds ratios for being the mother of a child affected by neural tube defect. Pregnancy blood B₁₂ concentrations of <250 ng/L were associated with the highest risks.

CONCLUSIONS. Deficient or inadequate maternal vitamin B₁₂ status is associated with a significantly increased risk for neural tube defects. We suggest that women have vitamin B₁₂ levels of >300 ng/L (221 pmol/L) before becoming pregnant. Improving B₁₂ status beyond this level may afford a further reduction in risk, but this is uncertain. Pediatrics 2009;123:917–923

FOLIC ACID CAN prevent up to three fourths of neural tube defects (NTDs).¹⁻³ Folic acid fortification of grain products in the United States was initially reported to reduce the incidence of NTDs by 19%,¹ but this was probably an underestimate.³ Recent studies have shown reductions between 35% and 78% since mandatory fortification programs were introduced.⁶⁻¹⁰ There is debate on whether all folic acid-preventable NTDs are being prevented¹¹⁻¹³ and whether the observed range of effectiveness can be explained by underlying ethnic differences in susceptibility¹⁰ or differences in completeness of case ascertainment.¹ The argument is that insufficient folic acid has been added, and there have been calls to increase the level of fortification in the United States.¹¹⁻¹³ Nevertheless, it is generally agreed that not all NTDs are preventable by folic acid. Therefore, to further reduce NTDs, other modifiable risk factors must be found.

Maternal obesity has been identified as 1 modifiable risk factor.¹⁴⁻¹⁶ Vitamin B₁₂ (B₁₂) status might be another,
given the close metabolic association between B12 and folate and the importance of B12 status as a determinant of plasma homocysteine. A link between low maternal serum B12 level and anencephaly was suggested as far back as 1980. Several studies found differences in maternal B12 status (measured by serum total B12 or by holotranscobalamin) both during and after an NTD-affected pregnancy (AP). Lower amniotic fluid B12 or lower B12 binding capacity was also reported in NTD-APs. The 2 largest positive studies, conducted during the introduction of folic acid fortification in the United States and postfortification in Canada, found a tripling of risk between the lowest and highest quintiles of serum B12 or quartile of holotranscobalamin.

In a previous study, undertaken primarily to examine the association of folate status with risk of NTD, we found that low maternal B12 status during an AP was associated with risk, independent of folate status. To gain further insight into the role of B12 in the prevention of NTDs, we present results on 2 additional groups, using pregnancy blood samples from over the same years, which predate the era of widespread food fortification and when medical advice was to avoid unnecessary prophylactic supplements during early pregnancy, including vitamin supplements. For comparison, we also included hitherto unreported risk analysis from our previous study, using B12 data only from women in the 1986–1990 biobank described above. Laboratory B12 results were missing for 5 case subjects and 25 control subjects. We excluded 65 women because they were taking vitamins, mainly as participants in the Irish Trial for the prevention of NTD. A further 9 women had insufficient sample for analysis. We had blood samples for 1 woman during both an AP and an NAP pregnancy. We included her AP blood in group 3 below and excluded her NAP sample. Control subjects were obtained for each case by selecting 4 to 5 women who attended the antenatal clinic in the same hospital on the same day. Hospital charts for these women were scrutinized for maternal B12, serum folate, and RCF were measured by microbiologic methods, as described previously. All of the vitamin analyses were completed between 3 and 9 years from the sample collection, with each group analyzed as a batch in a continuous run of assays. Case and control samples were randomly mixed in every assay, and operators were not aware of the sample status. Interassay and intra-assay coefficients of variation were within 10.4% and 12.0% for folate and B12, respectively. An ongoing laboratory quality-control system ensured long-term performance of the assays within established limits over the time scale of analysis.

Groups 2 and 3
Between 1986 and 1990, research blood samples were collected from 56,049 women at their first antenatal visit in the 3 major Dublin maternity hospitals. This represents ~70% of women who delivered in these hospitals during the period. Additional details have been published elsewhere. An aliquot in 1% ascorbic acid for red cell folate (RCF) and a plasma sample were stored at −20°C for each participant.

Group 2 includes blood samples within the above biobank that were collected from women with a history of NTD-APs but who had an NAP between 1986 and 1990. From the EUROCAT birth defects registry and hospital records we ascertained that there were 303 such women during the time period in the 3 hospitals. Of these, 187 women had given research blood samples. We excluded 65 women because they were taking vitamins, mainly as participants in the Irish Trial for the prevention of NTD. A further 9 women had insufficient sample for analysis. We had blood samples for 1 woman during both an AP and an NAP pregnancy. We included her AP blood in group 3 below and excluded her NAP sample. Control subjects were obtained for each case by selecting 4 to 5 women who attended the antenatal clinic in the same hospital on the same day. Hospital charts for these women were scrutinized for vitamin supplementation and other demographic details. From this, samples from 439 nonsupplemented women were eligible for analysis. Laboratory B12 results were missing for 5 case subjects and 25 control subjects, leaving a final data set of 107 case subjects and 414 control subjects.

Group 3 represents a previously unpublished analysis from our earlier study on maternal folate and B12 in NTD-APs. These blood samples were also obtained from the 1986–1990 biobank described above. For the current analysis, all of the known vitamin supplement users were removed, leaving data from 76 case mothers and 222 control mothers.
isons were conducted using Wilcoxon 2-sample tests. Logistic regression models were used to test whether decreasing levels of B12 were a significant risk for NTD in each of the groups. B12 was entered as a continuous variable or as quartiles of the control population. Adjustments were made for year of sampling and for folate status. Analysis of risk by maternal B12 status was conducted using cutoffs for B12 that represented deficient (0–149 ng/L), borderline-deficient (150–199 ng/L), adequate-good (300–399 ng/L) and good (>400 ng/L) status. Based on NTD prevalence data in the Dublin maternity hospitals, an average NTD rate of 2.9 per 1000 births was used for group 1, collected between 1984 and 1986, and 1.9 per 1000 births for groups 2 and 3, collected between 1986 and 1990. Confidence limits for risk estimates were calculated by assigning each control a representative weight (based on assumed overall risk) and assuming no sampling variability in the population and a Poisson distribution for the number of cases. Comparison of risk for the B12 status categories within each study group was based on an analysis of maximum likelihood odds ratio (OR) estimates, using the highest B12 category as the reference. All of the analyses were done using SAS 9 (SAS Institute, Cary, NC). Significant effects were those with 2-tailed P values <.05.

RESULTS

Table 1 gives available characteristics of the 3 groups. Median gestation was 15 weeks. Group 2 case mothers tended to be older than control subjects and had more pregnancies (P < .001), which is consistent with these case subjects having a previous pregnancy history. In all of the groups, the median B12 concentration was between 13% and 19% lower in case subjects (P < .002; Table 2). RCF was significantly lower in group 3 case subjects (238 vs 315 ng/mL; P = .0001) and marginally lower in group 2 case subjects (median: 255 vs 291 ng/mL; P = .079). RCF concentrations were not available for group 1, but the serum folate was not different between case and control subjects (2.85 vs 3.3 g/L; P = .42). There was a weak correlation between B12 and RCF in group 3 samples (r = 0.16; P = .006; N = 298) but little correlation between the B12 and RCF in group 2 (r = 0.07; P = .11; N = 514). There was little correlation between serum/plasma folate and B12 in any of the 3 groups (r = 0.09, 0.07, and 0.10 for groups 1, 2, and 3, respectively). The median B12 of control subjects in group 1, sampled between 1984 and 1986, was some

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**TABLE 1** Characteristics of Pregnant Case and Control Women in the 3 Study Groups

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Case Subjects</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median age (IQR), y</td>
<td>27.0 (22–32)</td>
<td>32.1 (29–36)*</td>
<td>26.5 (24–33)</td>
</tr>
<tr>
<td>Median weeks’ gestation (IQR)</td>
<td>16.0 (12–21)</td>
<td>15.3 (11–21)</td>
<td>14.4 (12–20)</td>
</tr>
<tr>
<td>Median No. of pregnancies (range)</td>
<td>1 (0–9)</td>
<td>4 (1–11)*</td>
<td>0 (0–14)</td>
</tr>
<tr>
<td>NTD type, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spina bifida only</td>
<td>47 (49.5)</td>
<td>47 (43.9)</td>
<td>36 (47.4)</td>
</tr>
<tr>
<td>Anencephaly only</td>
<td>27 (28.4)</td>
<td>35 (32.7)</td>
<td>24 (31.6)</td>
</tr>
<tr>
<td>Spina bifida + anencephaly</td>
<td>6 (6.3)</td>
<td>16 (15.0)</td>
<td>9 (11.8)</td>
</tr>
<tr>
<td>Encephalocele only</td>
<td>9 (9.5)</td>
<td>7 (6.5)</td>
<td>3 (3.9)</td>
</tr>
<tr>
<td>Other</td>
<td>6 (6.3)</td>
<td>2 (1.8)</td>
<td>4 (5.3)</td>
</tr>
<tr>
<td><strong>Control Subjects</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median age (IQR), y</td>
<td>28.0 (24–32)</td>
<td>27.9 (24–32)*</td>
<td>28.0 (24–33)</td>
</tr>
<tr>
<td>Median weeks’ gestation (IQR)</td>
<td>15.0 (11–20)</td>
<td>14.5 (12–19)</td>
<td>15.1 (12–20)</td>
</tr>
<tr>
<td>Median No. of pregnancies (range)</td>
<td>1 (0–8)</td>
<td>1 (0–10)*</td>
<td>1 (0–13)</td>
</tr>
<tr>
<td>NTD type, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spina bifida only</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Anencephaly only</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Spina bifida + anencephaly</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Encephalocele only</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Other</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td><strong>Sample year, n (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1983</td>
<td>16 (16.8)</td>
<td>37 (14.0)</td>
<td>—</td>
</tr>
<tr>
<td>1984</td>
<td>49 (51.6)</td>
<td>122 (46.0)</td>
<td>—</td>
</tr>
<tr>
<td>1985</td>
<td>28 (29.5)</td>
<td>103 (38.9)</td>
<td>—</td>
</tr>
<tr>
<td>1986</td>
<td>2 (2.1)</td>
<td>3 (1.1)</td>
<td>36 (33.6)</td>
</tr>
<tr>
<td>1987</td>
<td>—</td>
<td>—</td>
<td>28 (26.2)</td>
</tr>
<tr>
<td>1988</td>
<td>—</td>
<td>—</td>
<td>31 (29.0)</td>
</tr>
<tr>
<td>1989 and 1990</td>
<td>—</td>
<td>—</td>
<td>12 (11.2)</td>
</tr>
</tbody>
</table>

IQR indicates interquartile range, denoting the 25th to 75th percentile values; —, no data.

* P < .001.

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**TABLE 2** Serum B12 Concentrations During Pregnancy in Mothers With a History of Pregnancies Affected by NTD (Cases) and Nonaffected Mothers (Controls) Matched for Group

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group 1 (AP)</th>
<th>Group 2 (NAP)</th>
<th>Group 3 (AP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case B12, median (IQR), ng/L</td>
<td>210 (162–252)</td>
<td>270 (208–360)</td>
<td>244 (208–330)</td>
</tr>
<tr>
<td>No.</td>
<td>95</td>
<td>107</td>
<td>76</td>
</tr>
<tr>
<td>Control B12, median (IQR), ng/L</td>
<td>242 (190–297)</td>
<td>314 (253–404)</td>
<td>300 (237–366)</td>
</tr>
<tr>
<td>No.</td>
<td>265</td>
<td>414</td>
<td>222</td>
</tr>
</tbody>
</table>

Wilcoxon test, P = .0003 .0004 .0018

IQR indicates interquartile range, denoting the 25th to 75th percentile values; AP, case samples were taken during an AP; NAP, case samples were taken during an NAP from women who previously had an NTD-AP.

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20% lower than groups 2 and 3, sampled between 1986 and 1990 ($P < .0001$). When the median B12 was plotted by year, there was weak general trend toward increased status over the period of the study (data not shown). We noted a 4% difference ($P = .023$) in median B12 among control subjects in groups 2 and 3, both of which were sampled from the same population of 56,049 blood samples. This was probably because of differences in the year of analysis and minor fluctuations in assay performance but was well within the interassay coefficients of variation of our laboratory.

To determine the independent contributions of B12 and RCF to risk of NTD, we used logistic regression analysis with B12 and RCF (groups 2 and 3) as continuous variables. There were highly significant associations (Table 3), such that each unit increase in B12 concentration provided an 0.3% reduction in risk, independent of RCF. RCF was a significant factor only in group 3.

To explore whether the associations with risk were confined to particular sectors of the B12 distribution, we categorized the data by quartile of B12 concentration among control subjects. Logistic regression analysis, using the highest B12 quartile as the reference group and adjusting for RCF and year of sampling (Table 4), showed that risk of NTD was significantly increased only in the lowest quartile for groups 2 and 3 but extended to the second quartile for group 1 (adjusted for serum folate). The upper cutoffs for the second quartile in group 1 (242 ng/L) and the lowest quartile in group 2 (252 ng/L) and group 3 (237 ng/L) were remarkably similar. In all 3 of the groups, those with B12 concentrations of <250 ng/L had a 2.5- to 3-fold higher risk of being the mother of an NTD-affected child, after adjusting for folate. Adjusting for gestation did not substantially change the magnitude of the effects (data not shown).

We then divided the B12 levels into 5 nutritionally relevant groups (deficient, borderline deficient, etc) and calculated relative risks for each group, based on the known NTD prevalence rates at the time of sampling. Point estimates with SEs are shown on Table 5. These data show clear trends of risk reduction across the 5 categories in all of the groups, with those in the lowest category (pregnancy B12 concentrations of <150 ng/L) having −5-times higher risk compared with those with pregnancy B12 levels of >400 ng/L. The effects are significant below a concentration of 200 ng/L in groups 1 and 2. Effects were not significant for group 3, but smaller numbers are likely to have been a factor. Figure 1 shows trends across the B12 distributions. These suggest that some further reduction in risk may be afforded by having a B12 status ≥320 to 350 ng/L, but there is no statistically significant effect.

**DISCUSSION**

We have shown, in 3 separate groups, that low B12 status is an independent maternal risk factor for having an NTD-AP. Moreover, our study is the first to examine the risk by concentration of B12. Our data indicate that...
women with pregnancy B12 concentrations of <200 ng/L are at 3 times greater risk than those with levels of >400 ng/L. These results agree remarkably with effects observed by others.22–26 Our analysis indicates that the majority of risk is confined to B12 levels below ~250 ng/L. (Tables 4 and 5), although the trend line in Fig 1 suggests that further risk reduction might be achieved by having a B12 status >320 to 350 ng/L. Considering that these women were sampled at an average of 15 weeks’ gestation and, by that time, there is a natural physiologic drop of ~20% to 25% in serum B12 from the prepregnancy level.40,41 our data indicate that women should aim to enter pregnancy with serum B12 concentrations of >300 ng/L (221 pmol/L) and that levels above 400 ng/L (295 pmol/L) might be desirable, although we found no statistically significant benefit.

It is uncertain whether further reduction in NTDs can be achieved in the United States by increasing the level of grain fortification with folic acid. Moreover, recent reports on possible adverse effects of high folic acid consumption make such a strategy unlikely.42–44 The addition of B12 in conjunction with folic acid has been proposed but mainly to protect individuals with low B12 status.12,13,45,46 There is little information on the proportion of women who enter pregnancy with B12 levels of <300 ng/L, although a recent National Health and Nutrition Examination Survey report found a mean serum B12 of just over 400 ng/L (300 pmol/L) in women between 20 and 39 years old.47 Our study suggests that the addition of B12 to fortified grains may be a useful and acceptable way to further reduce the prevalence of NTD, but more studies are needed to establish the safety of fortifying with B12 and the dose of B12 that might be required to reach an effective level of protection.

Previous case-control reports revealed lower B12 status in women with a history of NTD-APs who were not pregnant at the time of study.21,23–27 Our study is unusual in that we observed lower B12 concentrations during an NAP in such mothers (our group 2 case subjects), There are 2 possible explanations for this finding. One is that B12 is merely marking low folate status. However, we found little interaction between B12 and folate in any of our groups. This is not surprising, because in a nonfortified, nonsupplemented population, vitamin status is determined by dietary sources of folate and B12, and the 2 are quite different. The second, more likely explanation, is that long-term low B12 status may act in synergy with low folate status to precipitate an NTD-AP. B12 status, along with genetic differences, may help to explain the low NTD rates seen in some ethnic groups and may also help in understanding why low maternal folate status alone usually does not result in NTD-APs. For example, blacks, who have both lower NTD rates and lower folate levels than other ethnic groups in the United States, have significantly higher B12 status, and this high status is also seen during pregnancy.50 Our case mothers were all of Irish descent (ie, white) and lived in a region of traditionally high NTD prevalence, suggesting a moderately high genetic predisposition. The importance of B12 as a synergistic factor is also supported by our previous observation28 that women in the bottom quartile of both plasma folate and B12 had >5 times higher ORs of a birth affected by NTD than those in the highest quartile of both vitamins. Women in the bottom quartile of plasma folate but the highest B12 quartile had less than half that risk.

It is not known how folate and B12 might interact to

### TABLE 5: Risk of NTD per 1000 Births According to Maternal B12 Status

<table>
<thead>
<tr>
<th>B12 Category, ng/L</th>
<th>Group 1 (AP)a</th>
<th>Group 2 (NAP)b</th>
<th>Group 3 (AP)c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Risk (CI)</td>
<td>P</td>
<td>Risk (CI)</td>
<td>P</td>
</tr>
<tr>
<td>0–149</td>
<td>5.1 (3.1–8.0)</td>
<td>.014</td>
<td>8.9 (4.5–15.9)</td>
</tr>
<tr>
<td>150–199</td>
<td>3.6 (2.2–5.5)</td>
<td>.050</td>
<td>4.5 (2.4–7.5)</td>
</tr>
<tr>
<td>200–299</td>
<td>2.9 (2.1–3.9)</td>
<td>.082</td>
<td>1.9 (1.3–2.6)</td>
</tr>
<tr>
<td>300–399</td>
<td>1.6 (0.7–3.1)</td>
<td>.502</td>
<td>1.7 (1.2–2.5)</td>
</tr>
<tr>
<td>≥400</td>
<td>1.0 (0.2–2.8)</td>
<td>Ref</td>
<td>1.1 (0.6–1.7)</td>
</tr>
</tbody>
</table>

Data are the relative risk (with confidence limits) of being a case mother compared with being a control mother, given a B12 concentration within the designated category. CI indicates confidence limit; Ref, reference.

a Case samples were taken during an AP at a time when the underlying prevalence of NTD was 2.9 per 1000 births.

b Case samples were taken during an NAP at a time when the underlying prevalence of NTD was 1.9 per 1000 births.

c Case samples were taken during an AP at a time when the underlying prevalence of NTD was 1.9 per 1000 births.
affect neural tube formation, but several mechanisms are possible. As cofactor to the enzyme methionine synthase, B$_{12}$ influences both the incorporation of folates into the cellular pool and the flux of folate derived 1-carbon units destined for DNA synthesis or for methylation reactions. DNA synthesis is an essential feature of embryonic development, but other factors that trigger developmental changes, such as cell-signaling events that lead to differential gene expression, are partially controlled by methylation reactions. Impairment of either of these systems could be involved in folate or B$_{12}$-responsive NTDs.

Our study has several strengths. First, our groups were large enough to detect an average B$_{12}$ difference of 15%, enabling us to get a good estimate of the critical level of B$_{12}$ required to prevent NTDs. Several other studies that found no difference were limited to ≈60 case subjects$^{51}$ or were conducted in an area of very low NTD prevalence with high reported B$_{12}$ levels.$^{52}$ Our study samples were taken from a population of high NTD risk, at a time when women were not exposed to prenatal vitamins before the blood draw. Such populations are difficult to find nowadays, but they have the advantage that the observed concentrations are more likely to reflect the blood vitamin level at the time of neural tube closure, because they have not been confounded by early pregnancy intake of vitamin supplements. Samples from all of our groups were assayed using the same methodology, and our collection strategy ensured that case and control subjects were matched for gestational, temporal, and storage variables likely to affect the B$_{12}$ content. One limitation is the fact that we could not also control for maternal age and number of pregnancies. However, we had no evidence that these variables would affect maternal B$_{12}$ status. The study is also limited by sparse demographic data on participants and by the lack of RCF data for group 1, which, because of its greater stability, may have been more informative than serum folate.

CONCLUSIONS
We have confirmed that low B$_{12}$ status is an important maternal risk factor for having an NTD-AP. More importantly, our study is first to address the public health question of what B$_{12}$ concentrations might be protective, although our calculations are limited by extrapolation from pregnancy values to a nonpregnant condition. Our logistic regression analysis suggests that women who start pregnancy with serum B$_{12}$ concentrations of <300 ng/L (221 pmol/L) are at significantly higher risk for NTDs. Improving B$_{12}$ status beyond 300 ng/L might offer further risk reduction, but this is unclear.

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