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# Maternal vitamin levels in pregnancies affected by congenital malformations other than neural tube defects

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## Abstract

**Background—**Periconceptional use of folic acid prevents most neural tube defects (NTDs). Whether folic acid and/or multivitamins can prevent other congenital anomalies is not clear. This study tested whether maternal blood levels of folate and vitamin B12 in pregnancies affected by congenital malformations excluding NTDs are lower when compared to non-affected pregnancies.

**Methods—**We measured pregnancy red cell folate (RCF), vitamin B12, and homocysteine (tHcy) concentrations in blood samples taken at the first antenatal clinic in Dublin maternity hospitals in 1986–1990 when vitamin supplementation was rare. The cases were mothers who delivered a baby with a congenital malformation other than NTD identified by the Dublin EUROCAT Registry; controls were a systematic sample of mothers of offspring without congenital malformations from the same hospitals in the same time period.

**Results—**The median maternal levels of RCF and tHcy did not differ significantly between cases and controls for any of the congenital malformation groups examined (RCF: all malformations 275.9 ug/L v controls 271.2; p=0.77; tHcy: all malformations 7.5 umol/L v controls 7.6; p=0.57). In an unadjusted analysis vitamin B12 was significantly higher in case-mothers whose babies had cleft palate only (p=0.006), musculoskeletal malformations (p=0.034) and midline defects (p=0.039) but not after adjustment for multiple testing.

**Conclusions—**Our data suggest that low maternal folate and B12 levels or high tHcy levels in early pregnancy are not associated with all congenital malformations excluding NTDs. Fortification with folic acid or B12 may not have a beneficial effect in the prevention of these anomalies.

## Introduction

Fifty percent or more of neural tube defects (NTDs) can be prevented by an adequate periconceptional intake of folic acid. It has been suggested that folic acid and/or multivitamins, taken periconceptionally, have a role in the prevention of many other congenital anomalies but the results of these studies have not been consistent (Botto et al. 2000; Bower et al. 2006; Czeizel 1998; Shaw et al. 2000). Botto et al. (2004) conducted a comprehensive review of the preventive effect of multivitamin supplementation for all birth defects combined and specific congenital anomalies and concluded that periconceptional

multivitamin use reduces the overall occurrence of birth defects in addition to the established effect on NTDs. Food fortification with folic acid to prevent NTDs has made it possible to examine the effect of folic acid on rates of birth defects other than NTDs. The effect has been unclear with some studies showing a decrease in the rates of one or more defects while others have not confirmed these findings (Canfield et al. 2005; Castilla et al. 2003)

If vitamins have a role in prevention it is reasonable to expect lower maternal blood levels of these vitamins in pregnancies affected by congenital malformations other than NTDs. It has proved very difficult to conduct the necessary studies to test this hypothesis because blood samples can rarely be obtained from pregnant women before they begin taking prenatal vitamins. In Ireland in the 1980s few women used supplements routinely, there was no campaign to encourage women to take folic acid before becoming pregnant and fortified foods were rare (Molloy et al. 2009). We collected blood samples from pregnant women during this period. These samples present a rare opportunity to investigate a link between maternal vitamin status and congenital malformations other than NTDs by actually measuring maternal vitamin levels during the pregnancy of interest. In this study the maternal antenatal blood samples were examined to test if there was an association between low folate/vitamin B12 or high homocysteine levels and congenital defects other than NTDs.

## Materials and Methods

### Study Subjects

Approximately 90% of births to residents of the greater Dublin area occurred in the three main Dublin maternity hospitals during the study period. Between March 1986 and March 1990, blood samples were collected from 56,049 women attending their first antenatal visit at these hospitals as part of our investigation into causes and prevention of NTDs. Information on demographic details for the population in the present study was not collected due to resource constraints. However based on a previous nested case control study in this population, the median age of the cohort was 27 years and median gestational age at first visit was 15 weeks (Kirke et al. 1993).

Subsequently any of these pregnancies that resulted in the birth of a baby with a congenital anomaly was ascertained through EUROCAT, a European network of population-based registries for the epidemiologic surveillance of congenital anomalies. The current study uses a nested case-control design where mothers of babies with a congenital malformation were the cases; 1695 mothers were reported by the Dublin EUROCAT Registry as giving birth to a baby with a congenital malformation other than NTD in the study hospitals during June 1986 – October 1990. Babies born with NTDs were not included as congenital anomalies as they had been reported previously (Kirke et al. 1993). Mothers with a history of NTD affected pregnancies were also excluded. The control group of 1502 births was a systematic sample (one in 45) of all births born without congenital malformations in the same hospitals in the same time period as the cases. In total this amounted to 3197 births but 210 mothers had ante-natal visits outside the blood collection period and were thus excluded. From the stored samples there was a retrieval rate of 54.5% which resulted in 853 cases and 776 controls. Further exclusions were 123 cases and 80 controls for whom there was insufficient blood for analysis of the biochemical parameters and a further 141 cases were excluded for a number of reasons (55 Down’s syndrome who had been previously reported (O’Leary et al. 2002), 39 metabolic disorders, and 47 cases who had minor anomalies that were subsequently excluded from the EUROCAT register or where there was insufficient diagnostic information). This resulted in 589 cases and 696 controls. Informed consent and ethical approval were obtained for the samples collected.

## Laboratory Methods

Bloods were collected into K EDTA. An aliquot in 1% ascorbic acid for red cell folate (RCF), a plasma sample and a whole blood sample were stored at −20°C for each participant. Details of collection and storage have been described previously (Kirke et al. 1993; Molloy et al. 2009). Plasma vitamin B12 (B12) and RCF were measured by microbiological methods as previously described (Kirke et al. 1993). Plasma total homocysteine (tHcy) concentrations were measured by HPLC with fluorescence detection (Mills et al. 1995; Ubbink et al. 1991). Analyses were completed between 6–10 years from sample collection, with each group analyzed as a batch in a continuous run of assays. Cases and controls were randomly mixed in every assay and operators were not aware of the status of any sample. Inter- and intra-assay coefficients of variation (CVs) were within 10.4%, 12% and 5.9% for folate, B12 and tHcy respectively. An ongoing quality control system within the laboratory ensured long term performance of the assays within established limits over the timescale of analysis of the three groups. Plasma folate was not included because of the reported instability of this compound after long term storage at −20°C.

## Congenital Anomaly Assessment

EUROCAT defines the term congenital anomaly as referring to structural defects (congenital malformations, deformations, disruptions and dysplasia), chromosomal abnormalities, inborn errors of metabolism and hereditary diseases (EUROCAT Working Group 1991). We limited our investigation to subjects with structural abnormalities. EUROCAT data come from multiple sources which include birth notifications, the Hospital Inpatient Enquiry System, death certificates, pathology reports and long term illness records. Information is collected by EUROCAT on length of gestation, type of birth, birth weight, outcome, malformations present and data on frequency of antenatal diagnosis and results of karyotyping and postmortem examinations. Cases are normally ascertained within 24 months of the date of birth, but cases diagnosed up to five years after birth are included. The sub-groups of diagnostic classifications were based on the EUROCAT sub-groups for example “all congenital heart defects” are based on EUROCAT Q20–25 and “all orofacial clefts” are based on EUROCAT Q35–37 (EUROCAT). In addition, some malformations were grouped into categories used in previous studies so that we could evaluate hypotheses generated by these studies. For example midline closure defects are thought by some to be developmentally related (Czeizel 1981; Khoury et al. 1989; Opitz 1982) and included in this group are facial clefts, tracheoesophageal fistula/oesophageal atresia, imperforate anus, gastrointestinal atresia, exomphalos, hypospadias, diaphragmatic hernia and conotruncal heart defects (including persistent truncus arteriosus, transposition of the great vessels, tetralogy of Fallot and double outlet right ventricle). A syndrome was defined as a recognisable pattern of anomalies which are known or thought to be causally related (Opitz 1994) and in this study syndromes were classified according to the EUROCAT classification (EUROCAT), but modified to exclude the VATER diagnostic association.

## Data Analysis

We looked for relationships between RCF, plasma B12 and tHcy and all congenital malformations plus various categories of congenital malformations. RCF and B12 were examined to determine if pregnancies affected by congenital malformations (other than NTDs) were associated with low maternal levels of these metabolites. RCF was selected because it is a better measure of long term folate status than serum folate as well as being more stable. tHcy was studied because it is a stable indicator of sub-optimal folate or B12 status and because raised tHcy levels might be toxic to the developing fetus (Mills et al. 1995; van der Put et al. 2001). Analysis was performed using the SPSS (version 16) statistical package. Because the biochemical determinants were not normally distributed, medians were compared and the Wilcoxon Rank Sum test was used to test significant

differences with a p value of <0.05 accepted as statistically significant. The proportions of cases and controls below the 25$^{th}$ percentile and above the 75$^{th}$ percentile for each of the biochemical parameters under investigation were compared using Pearson's chi-squared test.

## Results

The study included blood samples for 589 cases and 696 controls. Table 1 shows the median maternal levels of RCF, B12 and tHcy in the cases and controls at the first antenatal visit for each malformation group. For the category, all congenital malformations, there were no significant differences between cases and controls in RCF, B12 or tHcy. When individual defects or diagnostic subgroups were examined, RCF and tHcy levels did not differ significantly between cases and controls. However, vitamin B12 was significantly higher in case mothers for the subgroups, orofacial clefts (p=0.03) which on sub-analysis was confined to cleft palate only (p=0.006), all musculoskeletal defects (malformations and deformations) (p=0.03) and musculoskeletal malformations (p=0.03) with the subgroup talipes being just outside the level of significance (p=0.056).

In Table 2 various malformations are grouped together to reflect embryologically related defects and syndromes. There was only one significant outcome for these categories: when midline defects were examined the maternal B12 level was significantly higher in cases than controls (p=0.039).

We compared the proportions of cases and controls in the upper and lower quartiles (determined by the quartile level in the controls) for RCF, B12 and tHcy. There was no significant difference in the proportion of cases and controls in the quartile categories for any of the three metabolites.

## Discussion

In this study, pregnant women's levels of RCF, vitamin B12 and tHcy were measured generally in early second trimester pregnancy. This was in a time period when exposure to vitamin supplementation or fortification was rare. Mothers of children with birth defects did not have lower RCF or B12 levels or higher tHcy levels than mothers of unaffected children. Significantly higher B12 concentrations were found in mothers of cases with cleft palate only, all musculoskeletal defects and musculoskeletal malformations. Significantly higher B12 concentrations were also found in the combined group of midline defects. These findings may be due to chance given the number of comparisons that were made. They are of interest because low B12 has been shown to be an independent risk factor for NTDs (Molloy et al. 2009). Our results strongly suggest that low B12 is unlikely to be an important risk factor for defects other than NTDs. This is particularly noteworthy in the case of oral clefts where folate has been suggested to play a role because it suggests that the effect of folate, if it is a factor, is not mediated by the methylation pathway in which folate and B12 interact.

Two main studies examined the role of multivitamins containing folic acid in reducing the risk of all birth defects excluding NTDs and both suggested a significant reduction with prenatal vitamin supplementation (Botto et al. 2004; Czeizel 1998). Our findings do not support this.

Many studies have investigated a role for folic acid on its own or as part of a multivitamin preparation in the possible prevention of individual congenital malformations other than NTDs but results have been inconsistent (Botto et al. 2000; Bower et al. 2006; Canfield et al. 2005; Czeizel 1998; Hayes et al. 1996; Shaw et al. 2000; Shaw et al. 1995a; Shaw et al.

1995b). Among the studies that examined vitamin supplement use and the prevention of individual defects at least four showed a significant reduction in orofacial clefts in vitamin supplement users; but all examined multivitamin supplements that included folic acid in doses ranging from 0.2 mg to 10 mg.(Czeizel et al. 1996; Itikala et al. 2001; Shaw et al. 1995a; Tolarova and Harris 1995). Johnson and Little (2008) conducted a systematic review and meta-analysis to assemble evidence on the role of folate in the aetiology of orofacial clefts. This review of 22 studies included some of the studies listed above. They concluded that there is no strong evidence for an association between oral clefts and folic acid alone but that multivitamins may protect against oral clefts. Furthermore, in contrast to the effect on NTD prevalence, the fortification programme in Canada between 1998 and 2000 had no effect on the rate of orofacial clefts (Ray et al. 2003).

In studies showing a risk reduction for congenital heart defects, urinary tract defects and limb deficiency defects, the periconceptional exposure was also with a multivitamin but, with the exception of the Hungarian randomized controlled trial which used 0.8 mg, the dose of folic acid was not reported (Botto et al. 2000; Czeizel 1998; Li et al. 1995; Werler et al. 1999; Yang et al. 1997). A recent study which looked at severe congenital heart defects in Canada pre and post fortification of grain with folic acid, found that there was a significant decrease in birth prevalence of both conotruncal and non-conotruncal defects post fortification (Ionescu-Ittu et al. 2009).

Contradictory findings on vitamin use with regard to the prevention of specific birth defects could occur for many reasons including differences in dose, constituents, frequency and timing of consumption of the vitamin supplementation. This is not an issue in the present study as we measured maternal blood levels in early pregnancy in the era before periconceptional vitamin supplementation was recommended. Our study shows that low maternal folate or B12 levels or elevated tHcy were not associated with the occurrence of congenital defects other than NTDs.

The strengths of this study are as follows: it is an unselected cohort relating to children born with congenital defects; our study samples were taken from a population at a time when women were not exposed to periconceptional vitamin supplementation; there was no termination of pregnancy and control subjects were matched by gestational, temporal and storage variables likely to affect the metabolite levels of cases and controls. Limitations of the study include: even in a large prospective study like this, sufficient numbers of cases were not available for all types of birth defects limiting our power to investigate some defects. We have a limited amount of data on the defects and no data were available on risk factors for birth defects including family history, maternal medical history and demographic information.

The link between low maternal folate levels and NTDs has been clearly established and our research group and others have also found NTD pregnancies to be associated with low maternal levels of B12 independent of folate status (Molloy et al. 2009). This is not surprising given the critical role B12 plays in folate metabolism. Folate and B12 status are important determinants of plasma homocysteine and raised total homocysteine levels in maternal blood and amniotic fluid are associated with an increased risk of NTD pregnancies (Mills et al. 1995; Steegers-Theunissen et al. 1995). Thus, it is important to determine whether B12 might play a role in the etiology of other malformations. Our data indicate that B12 levels were not lower in women whose conceptuses had other types of defects. In fact they were significantly higher for some defects. Even if we account for the non-independence of our defect groups in our statistical correction for multiple comparisons, our findings for B12 would no longer be significant. On the other hand, it is worth noting that all of the unadjusted positive findings occurred in the B12 analysis and all showed an increased

risk with higher B12. This was not an a priori hypothesis but the finding that higher B12 levels were associated with increased malformation rates is important and requires investigation. There is a possibility that the serum B12 concentration may be a surrogate marker for a diet that is rich in animal food products that also contain high concentrations of an unidentified risk factor. The higher serum B12 levels in cases could also be the result of genetic factors that impair transport of B12 into tissues. Thus further investigation is needed especially in light of suggestions that B12 be added to fortification programmes.

We found no studies that measured vitamin levels during pregnancies affected by malformations other than NTDs for comparison with our results. Our findings show that within the physiological range of folate and homocysteine there was no association between levels and malformation risk. This does not, however, preclude the possibility that raising folate levels to the pharmacologic range could prevent some malformations as the effect could well be dose-dependent. Overall this study suggests that fortification with folic acid or vitamin B12 may not have a major beneficial effect in the prevention of congenital malformations other than NTDs.

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**Table 1**

Maternal red cell folate (RCF), total plasma homocysteine (tHcy) and plasma vitamin B12 levels in pregnancies affected by selected birth defects, other than NTDs, categorised by anatomical system

| Diagnostic Classification* | RCF | | | | tHcy | | | | B12 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Category | N† | Median (μg/L) | IQR | P value | N† | Median (μmol/L) | IQR | P value | N† | Median ng/L | IQR | P value |
| Controls | 568 | 271.2 | 148.5 | | 463 | 7.6 | 2.8 | | 471 | 326.0 | 167.0 | |
| All congenital malformations | 485 | 275.9 | 138.3 | 0.770 | 376 | 7.5 | 3.0 | 0.571 | 383 | 344.0 | 176.0 | 0.069 |
| All congenital heart defects | 83 | 256.0 | 152.5 | 0.473 | 66 | 7.5 | 3.3 | 0.973 | 67 | 354.0 | 152.0 | 0.078 |
| Conotruncal defects | 17 | 256.7 | 164.4 | 0.626 | 17 | 8.1 | 3.1 | 0.885 | 17 | 357.0 | 122.0 | 0.316 |
| VSDs | 34 | 269.6 | 177.7 | 0.667 | 25 | 7.5 | 4.5 | 0.877 | 26 | 331.5 | 144.0 | 0.578 |
| All orofacial clefts | 36 | 260.1 | 96.8 | 0.161 | 22 | 7.5 | 2.2 | 0.590 | 23 | 405.0 | 167.0 | 0.033 |
| Cleft palate | 18 | 260.1 | 107.7 | 0.305 | 9 | 7.4 | 4.1 | 0.793 | 10 | 470.0 | 143.0 | 0.006 |
| Cleft lip ± palate | 18 | 260.9 | 137.7 | 0.324 | 13 | 7.6 | 1.7 | 0.624 | 13 | 342.0 | 200.0 | 0.651 |
| Pyloric stenosis | 67 | 265.7 | 125.9 | 0.361 | 59 | 7.5 | 2.4 | 0.596 | 59 | 355.0 | 211.0 | 0.527 |
| Other digestive system defects | 34 | 306.2 | 124.8 | 0.615 | 21 | 7.7 | 4.1 | 0.415 | 21 | 298.0 | 90.0 | 0.243 |
| All urinary tract defects | 14 | 302.9 | 148.8 | 0.102 | 11 | 6.9 | 3.2 | 0.484 | 11 | 281.0 | 221.0 | 0.748 |
| All musculoskeletal defects | 173 | 280.0 | 149.1 | 0.621 | 135 | 7.3 | 3.2 | 0.209 | 139 | 353.0 | 163.0 | 0.032 |
| Malformations | 100 | 281.8 | 180.8 | 0.164 | 78 | 7.0 | 2.5 | 0.105 | 79 | 353.0 | 194.0 | 0.034 |
| Talipes | 53 | 264.3 | 130.8 | 0.938 | 43 | 6.9 | 3.3 | 0.232 | 43 | 392.0 | 203.0 | 0.056 |
| Poly/syndactyly | 21 | 333.3 | 156.4 | 0.092 | 11 | 6.9 | 2.0 | 0.237 | 12 | 386.5 | 191.0 | 0.153 |
| Deformations | 73 | 268.6 | 122.4 | 0.399 | 57 | 7.9 | 3.8 | 0.889 | 60 | 355.0 | 152.0 | 0.303 |
| Hypospadias | 21 | 287.2 | 145.1 | 0.720 | 13 | 7.6 | 2.8 | 0.968 | 14 | 314.5 | 251.0 | 0.962 |
| Other isolated congenital defects not included above | 27 | 225 | 182.8 | 0.173 | 27 | 8 | 3.3 | 0.250 | 27 | 359.0 | 169.0 | 0.287 |

*some diagnostic categories contain isolated and multiple defects

†variations in the numbers because the amount of blood available was not sufficient to perform all tests

**Table 2**

Maternal red cell folate (RCF), total plasma homocysteine (tHcy) and vitamin B12 levels in pregnancies affected by selected birth defects other than NTDs

| Diagnostic Classification | | RCF | | | | tHcy | | | | B12 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Category** | **N** | **Median (μg/L)** | **IQR** | **P value** | **N** | **Median (μmol/L)** | **IQR** | **P value** | **N** | **Median (ng/L)** | **IQR** | **P value** |
| Controls | 568 | 271.2 | 148.5 | | 463 | 7.6 | 2.8 | | 471 | 326.0 | 167 | |
| Midline defects* | 113 | 281.1 | 156.8 | 0.842 | 80 | 7.6 | 2.8 | 0.661 | 82 | 347.5 | 195 | 0.039 |
| Syndromes** | 15 | 283.3 | 188.6 | 0.678 | 11 | 7.0 | 2.1 | 0.513 | 11 | 245.0 | 157 | 0.064 |

* includes facial clefts, tracheoesophageal fistula/oesophageal atresia, imperforate anus, gastrointestinal atresia, exomphalos, hypospadias, diaphragmatic hernia and conotruncal heart defects

** includes 18 minus q deletion syndrome, Apert's syndrome, Cat eye syndrome, Noonans syndrome, Pena-Shokeir syndrome, Rubinstein-Taybi syndrome, Turners syndrome, William Beuren syndrome, Patau syndrome, Edwards syndrome, Di Deorge syndrome, Wiskott Aldrich syndrome and Alagille syndrome