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Reduced vitamin B12 binding by transcobalamin II increases the risk of neural tube defects

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Summary

Periconceptional folic acid supplementation reduces the risk of neural tube defects (NTD). Homocysteine levels are elevated in mothers of NTD children, which may be due to decreased cellular vitamin B12 levels, as vitamin B12 is a cofactor for the methylation of homocysteine. Transcobalamin II (TC II) transports vitamin B12 to the tissues. To examine whether altered plasma transcobalamin levels are a risk factor for NTD, we determined the apo and holo form of TC II and haptocorrin (TCI+TCIII), vitamin B12 and homocysteine concentrations in the plasma of 46 mothers with NTD children, and in 73 female controls. Holo-tc II levels and holo-tc II percentages (holo-tc II/total tc II) in the first quartile of the control distribution were related to a three-fold (OR 2.9, 95%CI 0.9–9.2) and five-fold (OR 5.0, 95%CI 1.3–19.3) risk, respectively, for having a child with NTD, when compared with the last quartile. Homocysteine levels were significantly higher among individuals with low holo-tc II, low total vitamin B12 concentrations and low holo-tc II percentages. These low holo-tc II percentages are probably caused by reduced affinity of TC II for vitamin B12, which may be explained by genetic variation in the TC II gene. Vitamin B12 supplementation might therefore be warranted, in addition to folate, in the prevention of NTD.

Introduction

Neural tube defects (NTD) arise due to failure of closure of the neural tube, and are one of the most common congenital malformations. Periconceptional intake of folic acid reduces the occurrence and recurrence risk of neural tube defects (NTD).[1,2] The mechanism of this protective effect is still unknown. Steegers *et al*.[3] were the first to show that elevated homocysteine concentrations are present in mothers of NTD children. Later, we identified the first genetic risk factor for NTD, i.e. the C677T mutation in the methylenetetrahydrofolate reductase (MTHFR) gene, which causes elevated homocysteine concentrations.[4] This C677T mutation in the MTHFR gene can, however, only partly explain the protective effect of folate, and the high homocysteine levels present among mothers of NTD children. Therefore, other functional variants in genes involved in homocysteine metabolism may be present.[5]

Vitamin B12 (cobalamin) is a cofactor in the folate-dependent homocysteine metabolism. Methionine synthase requires vitamin B12 to transfer the methylgroup of 5-methyltetrahydrofolate to homocysteine. The products formed are methionine and tetrahydrofolate. Intracellular vitamin B12 deficiency may therefore result in increased plasma homocysteine concentrations. Low vitamin B12 concentrations in the cell could be the result of a low vitamin B12 intake, but could also be due to a disturbance in the absorption, transport or cellular uptake of this vitamin. Upon entering the stomach, vitamin B12 is bound to

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haptocorrin (HC) and transferred to the duodenum, where intrinsic factor (IF), arriving from the stomach, binds vitamin B12.[6] The IF-vitamin B12 complex is absorbed via the IF-B12 receptor, and vitamin B12 is subsequently bound to transcobalamin II (TC II) and released into the circulation.[7] TC II facilitates the transport of vitamin B12 in blood to various tissues. Only 20% of the vitamin B12 in plasma is bound to TC II, the remaining 80% being bound to HC, also referred to as TC I and TC III. So far the role of HC in plasma is unclear, it may function as storage for vitamin B12.[8] Holo-tc II is the proportion of TC II that contains vitamin B12, and apo-tc II is the proportion of TC II which does not contain vitamin B12. A similar nomenclature holds for holo- and apo-hc. The total amount of vitamin B12 in plasma is the sum of holo-tc II and holo-hc. Since TC II transports the vitamin B12 to the cell, low plasma holo-tc II concentrations may be the earliest indicator of a negative vitamin B12 balance[9] and may be a better indicator of vitamin B12 status than plasma vitamin B12 concentrations (holo-tc II + holo-hc).

Kirke *et al*. found an association between decreased maternal plasma vitamin B12 and an increased risk for having a child with NTD.[10] In addition, Steen *et al*.[11] and Steegers *et al*.[12] observed decreased vitamin B12 levels in the amniotic fluid of mothers with a NTD child. These findings point to a possible role for vitamin B12 in the aetiology of NTD. Magnus *et al*.[13] showed that apo-tc I and apo-tc II in midtrimester amniotic fluid of mothers who had previously had a child with NTD were significantly elevated when compared to control levels. Furthermore, Gardiki-Kouidou and Seller[14] found significantly elevated apo-tc II and apo-hc (apo-tc I and -III) levels and significantly decreased vitamin B12 levels in amniotic fluids of mothers carrying a child with NTD, or who had had a child with NTD. They concluded that this might indicate a genetic defect in the transport of vitamin B12, resulting in a disturbed vitamin B12 metabolism.

In the present study, we examined a possible association between the apo- and holo-forms of TC II and HC levels in plasma and the risk for having a child with NTD, and whether changes in these concentrations had an affect on homocysteine metabolism. We therefore determined the apo- and holo-forms of TC II and HC, total vitamin B12 (holo-tc II + holo-hc) and homocysteine concentrations in plasma of mothers with a NTD child and in female controls. In addition, we analysed whether known polymorphisms in the TC II gene, i.e. the Pro259Arg and the Gln234Arg mutations,[15] were associated with disturbed TC II function.

Methods

Study population

Mothers of patients with non-syndromic NTD were recruited by participation from the Pediatric Department of the University Hospital Nijmegen and were all Dutch Caucasians. The controls were healthy female volunteers with no history of NTD in their family and were also Dutch Caucasians. The study population consisted of 46 mothers with a NTD child (mean age 38 ± 4 years) and 73 controls (mean age 35 ± 8 years). The protocol was approved by the local ethics committee and written informed consent was obtained.

Laboratory methods

Holo-tc II and holo-hc levels were determined in EDTA plasma according to the method described by Benhayoun *et al*.[16] Plasma vitamin B12 (holo-tc II + holo-hc) was determined using the Dualcount Solid Phase Boil Radioassay (Diagnostic Products). After determination of plasma vitamin B12 concentration, TC II was removed with Heparin-Conjugated-Sepharose, and the remaining holo-hc was also determined by the Dualcount Solid Phase Biol Radioassay. Holo-tc II levels were calculated as the difference between holo-hc levels and plasma vitamin B12 levels.

Plasma apo-tc II and apo-hc levels were determined according to the method described by Nielsen *et al*.[17] Briefly, serum samples were incubated with excess ^{57}Co-labelled cyanocobalamin. Subsequently, each sample was loaded on a Sephacryl column (HRS300 Hiload 16/60, Pharmacia), with an automatic fraction collector, and 170 fractions were collected. Radioactivity was counted in each fraction by a gamma scintillation spectrometer. By integration of the resulting elution peaks, the levels of apo-tc II and apo-hc were calculated by using the specific radioactivity of Co-Cbl. The lower detection limit was 1 pmol/l and the run-to-run precision of apo-tc II after 20 runs was 9.6% (mean 216 pmol/l) and 10.7% for apo-hc (mean 529 pmol/l).

Homocysteine concentrations were determined in EDTA plasma by HPLC with fluorescent detection.[18] Erythrocyte vitamin B12 and plasma folate levels were determined by using the Dualcount Solid Phase Boil Radioassay (Diagnostic Products).

The prevalence of the TC II Pro259Arg and the TCII Gln234Arg polymorphism was determined by PCR on genomic DNA, using restriction enzyme digestion with *Scr*FI for the Pro259Arg, and *MSP*I for the Gln234Arg variant, followed by agarose gel

electrophoresis. No DNA was available of four mothers with NTD children.

The MTHFR C677T mutation was determined as described by Frosst *et al*.[19]

Statistical analyses

p values for differences in characteristics between mothers of NTD children and controls were calculated using linear regression analysis, and were adjusted for age. Odds ratios (OR) with 95%CIs for having a child with NTD were calculated by logistic regression analysis, and were also adjusted for age. Tests for linear trends in the observed distribution of these ORs used logistic regression analysis.

Homocysteine levels showed a non-normal distribution, and logarithmically-transformed homocysteine concentrations were used in all calculations. Mean homocysteine values were expressed as geometric means. *p* values for differences in homocysteine levels between TC percentiles were calculated using linear regression, and were corrected for age, plasma folate, the MTHFR C677T genotype and plasma HC concentrations where necessary. For correlations, Pearson's correlation coefficient was used. All calculations were performed by using the SPSS-software package 9.0; statistical significance was set at $p < 0.05$.

Results

An overview of the median (range) age and of important biochemical variables of mothers with NTD children and controls are summarized in Table 1. Age was significantly higher in the mothers of NTD children compared with the controls; therefore, all other *p* values were adjusted for age. Other parameters were not significantly different between mothers of NTD children and controls. To examine whether altered transcobalamin concentrations increased the risk of having a child with NTD, we calculated the odds ratios (OR) for the different quartiles of transcobalamins on the basis of the control distribution. We calculated the OR for each quartile by using the first (0–25th percentile) or the last (75–100th percentile) quartile as the reference category. Figure 1 shows the ORs for NTD, with 95%CIs, for holo-tc II, apo-tc II, the percentage holo-tc II (holo-tc II/total tc II), which is an indication for the proportion vitamin B12 bound to TC II, and plasma vitamin B12 (holo-tc II + holo-hc). A holo-tc II level in the first quartile of the distribution was associated with a nearly three-fold increased risk for having a child with NTD (OR 2.9, 95%CI 0.9–9.2). A holo-tc II percentage in the first quartile was associated with a significant five-fold increase in risk for having a child with NTD (OR 5.0, 95%CI 1.3–19.3). The OR for having a child with NTD associated with apo-tc II levels in the last quartile was 2.8 (95%CI 0.9–8.6). To examine a possible trend in ORs for NTD, a linear trend test was performed on the consecutive 10th percentiles of the different transcobalamin concentrations. The ORs calculated for holo-tc II concentrations and holo-tc II percentages gave a significant *p* value for trend ($p = 0.04$ and $p = 0.03$), indicating that a decrease of holo-tc II or holo-tcII percentage is related to an increase in risk for having a child with NTD.

To investigate the relationship between the different TC and HC levels and homocysteine concentrations, we combined the mothers of the NTD children and the controls to increase statistical power. We calculated geometric mean homocysteine concentrations in each quartile of the different TC concentrations (Figure 2). All *p* values were adjusted for age, plasma folate, the MTHFR C677T genotype and, when necessary, for HC. For holo-tc II, mean homocysteine levels differed significantly between the first and third ($p = 0.025$), first and last ($p = 0.003$) and the second and last quartiles ($p = 0.005$). For the holo-tc II percentage, mean homocysteine levels were significantly different between the first and third ($p = 0.0001$), first and

Table 1 Characteristics of mothers of children with NTD, and controls

	Mothers ($n = 46$)	Controls ($n = 73$)	*p*
Age (years)	38.0 (29–48)	34.0 (20–55)	0.03*
Holo-tc II (pmol/l)	41.0 (1–372)	50.0 (0.5–186)	0.27
Apo-tc II (pmol/l)	607.5 (294–908)	557.0 (344–946)	0.24
Holo-hc (pmol/l)	161.0 (37–628)	168.0 (52–434)	0.58
Apo-hc (pmol/l)	56.5 (26–190)	49.0 (19–332)	0.71
Plasma vitamin B12 (pmol/l)	220.0 (70–1000)	220.0 (89–600)	0.40
Erythrocyte vitamin B12 (pmol/l)	35.0 (20–180)	39.0 (20–150)	0.76
Homocysteine (μmol/l)	12.4 (6.3–27.1)	12.2 (7.3–19.8)	0.16

All values are medians (range). *p* values are adjusted for age. *Significant difference ($p < 0.05$).

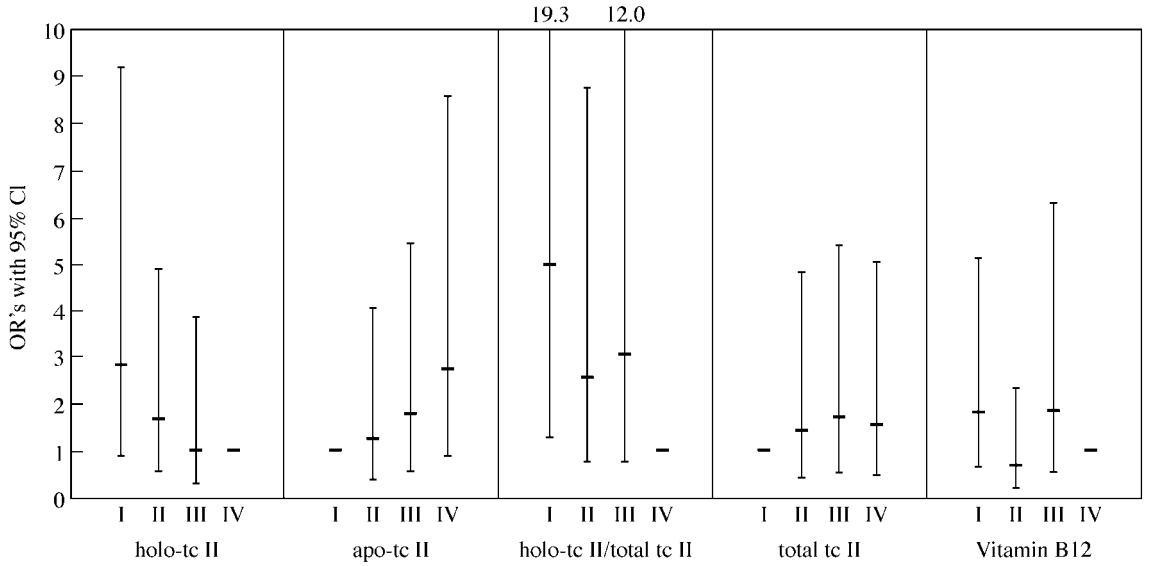

Figure 1. Odds ratios (OR) for having a child with NTD, for the different quartiles of plasma holo-tc II, apo-tc II, holo-tc II/total tc II, total tc II and plasma vitamin B12, compared to their respective reference quartiles. ORs are shown as thick horizontal bars, 95%CIs as thin vertical lines. Quartile I, 0–25th percentile; II, 25–50th percentile; III, 50–75th percentile; IV, 75–100th percentile.

last ($p = 0.016$), second and third ($p = 0.001$) and the second and last quartiles ($p = 0.036$). For vitamin B12, mean homocysteine levels were significantly different between the first and third ($p = 0.004$) and the first and last ($p = 0.013$) quartiles of total vitamin B12. Another functional effect of low holo-tc II concentrations, in addition to the high homocysteine concentration, is the vitamin B12 concentration in the cell. Therefore we calculated the mean erythrocyte vitamin B12 concentration per quartile of holo-tc II (Figure 3). The mean erythrocyte vitamin B12 concentration was significantly different when comparing the first with the third quartile ($p = 0.03$) and the first with the last quartile ($p = 0.006$).

To investigate whether the low amount of vitamin B12 bound to TC II, the low holo-tc II percentage, (holo-tc II/total tc II) is caused by a deficiency of vitamin B12 or by disturbed binding of vitamin B12 to TC II, we calculated mean plasma vitamin B12 concentrations with 95%CIs per quartile of holo-tc II (Figure 4). We also plotted the vitamin B12 concentrations against the holo-tc II concentrations below vs. above the 50th percentile of the control distribution (Figure 5). Holo-tc II concentrations above the 50th percentile showed a positive correlation ($r = 0.838$, $p = 0.00001$) with plasma vitamin B12, whereas holo-tc II concentrations below the 50th percentile showed no correlation ($r = -0.063$, $p = 0.60$) between holo-tc II and vitamin B12.

The frequency of the Pro259Arg polymorphism did not vary significantly between mothers of NTD children (14%) and controls (21%) (Table 2). The Gln234Arg polymorphism was not observed in the study population. For mean holo-tc II and homocysteine concentrations per genotype, we combined the mothers with the controls. Holo-tc II levels differed significantly between the three Pro259Arg genotypes with, respectively, mean holo-tc II concentrations of 34.8 ($\pm$24.9) pmol/l for the 259-Arg homozygotes, 48.8 ($\pm$33.2) pmol/l for the heterozygotes and 61.8 ($\pm$35.6) pmol/l for the 259-Pro homozygotes. Homocysteine levels did not differ significantly between the genotypes; mean homocysteine concentrations were 13.5 $\pm$ 3.3 μmol/l for the 259-Arg homozygotes, 12.9 $\pm$ 3.6 μmol/l for the heterozygotes and 12.0 $\pm$ 2.9 μmol/l for the 259-Pro homozygotes.

Discussion

We found that loss of affinity of TC II for vitamin B12 is relatively common and is related to an increased risk for NTD. Low holo-tc II levels were associated with a three-fold increased risk (OR 2.9, 95%CI 0.9–9.2) and reduced holo-tc II percentages, which indicate the proportion of vitamin B12 bound to the total amount of TC II protein, increased the risk five-fold (OR 5.0, 95% CI 1.3–19.3). The significant linear trend analyses confirmed the relation between holo-tc II and holo-tc II percentage and the risk of having a child with NTD.

There may be three causes for a low holo-tc II percentage. Firstly, total TC II concentrations may be increased, for example, due to increased

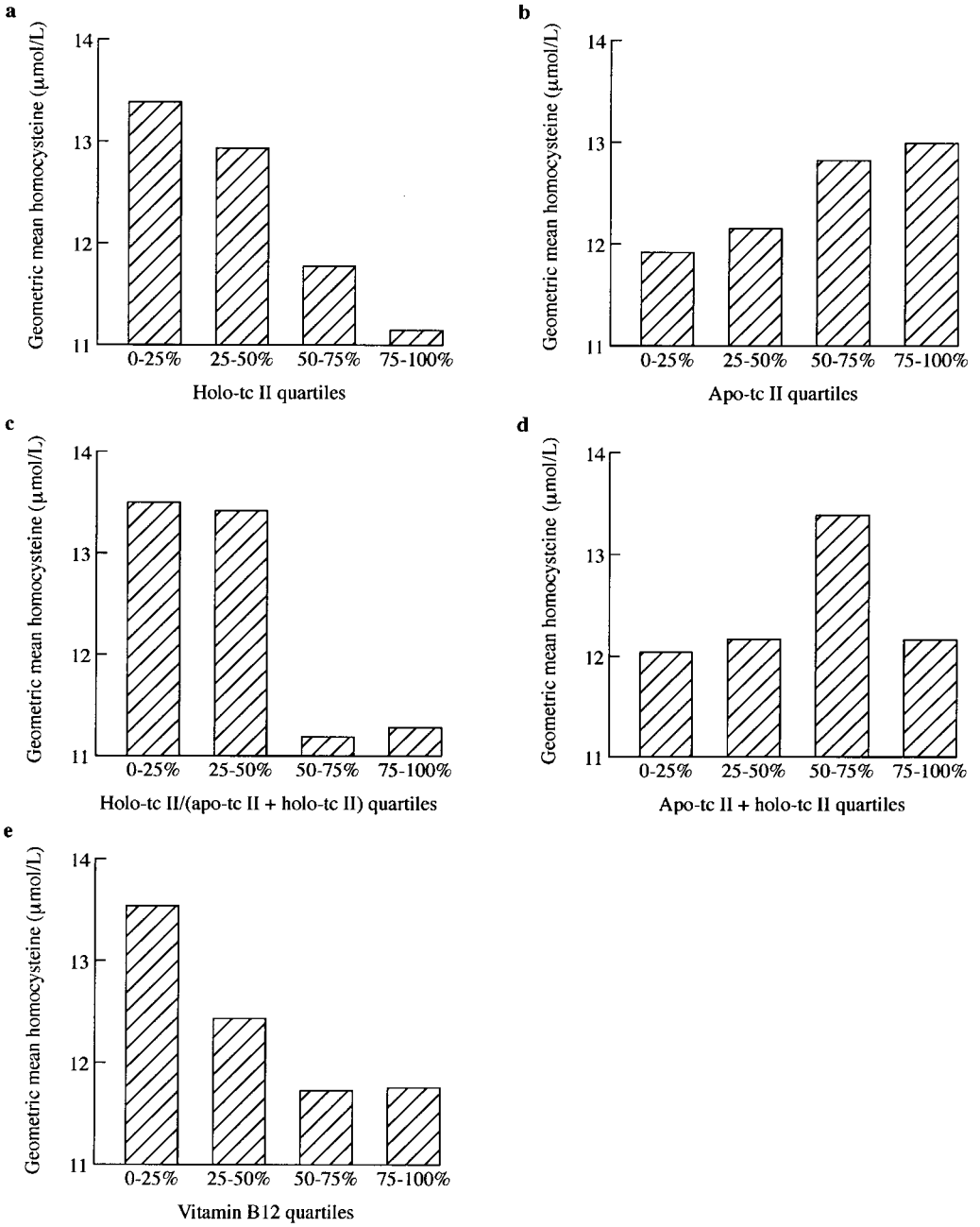

Figure 2. Relationship between quartiles of holo-tc II (**a**), apo-tc II (**b**), holo-tc II/total tc II (**c**), total tc II (**d**), total vitamin B12 (**e**) and mean homocysteine (hcy) concentration in mothers of a NTD child and controls together. **a.** Mean hcy levels differ significantly between the first and third ($p = 0.025$), the first and last ($p = 0.003$) and between the second and last quartile ($p = 0.005$) of holo-tc II. **b.** There are no significant differences in mean hcy between quartiles of apo-tc II. **c.** For holo-tc II/total tc II mean hcy levels are significantly different between the first and third ($p = 0.0001$), first and last ($p = 0.016$), second and third ($p = 0.001$) and the second and last quartiles ($p = 0.03$). **d.** There are no significant differences in mean hcy between quartiles of total tc II. **e.** Mean hcy levels are significantly different between the first and third ($p = 0.004$) and the first and last ($p = 0.013$) quartiles of total vitamin B12. Quartiles are based on the distribution in controls. All p values are corrected for age, plasma folate, HC levels and the MTHFR C677T mutation. p values for plasma vitamin B12 are not corrected for HC levels.

expression of the TC II gene; secondly, vitamin B12 levels may be deficient, leading to low plasma vitamin B12 and low holo-tc II concentrations; and thirdly, the binding of vitamin B12 to TC II can be disturbed, which will result in low holo-tc II levels in the presence of normal vitamin B12 levels. The data presented in Table 1 and Figure 2 demonstrate no differences between the mothers of NTD children and the controls in the total TC II concentrations, indicating that the observed low

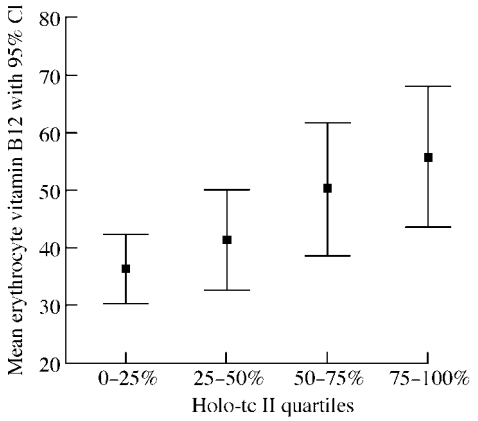

Figure 3. Mean erythrocyte vitamin B12 concentrations with 95%CI per quartile of holo-tc II.

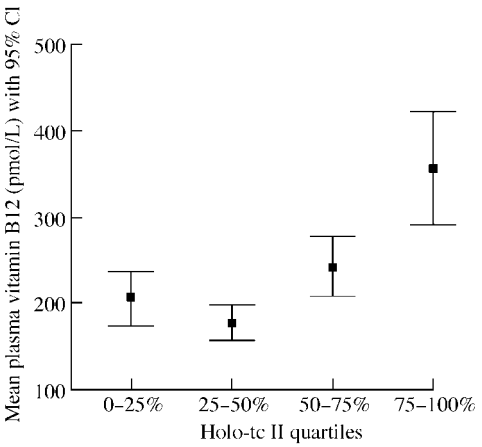

Figure 4. Mean plasma vitamin B12 concentrations with 95%CI per quartile of holo-tc II.

holo-tc II percentages are not due to increased transcription and translation of TC II. Figure 4 shows that low holo-tc II levels are not due to low plasma vitamin B12, but rather are the result of reduced binding of vitamin B12 to TC II. The latter is made clearer in Figure 5, which demonstrates a lack of correlation between holo-tc II and vitamin B12 concentrations when the holo-tc II concentration is below the 50th percentile. Obviously TC II in these individuals has lost its normal affinity for vitamin B12. The disturbed binding of vitamin B12 to TC II could be caused by genetic variation in the TC II gene, resulting in an altered binding site of vitamin B12 or a different folding of the TC II protein that reduces the binding of vitamin B12 to TCII, which would both result in lower holo-tc II concentrations. Although the Pro259Arg polymorphism significantly decreases holo-tc II concentrations, the frequencies of this mutation did not explain the higher risk for NTD from low holo-tc II concentrations and low holo-tc II percentages. Moreover, no significant differences in homocysteine levels were observed between the different genotypes of this polymorphism. Therefore it is unlikely that the Pro259Arg polymorphism in the TC II gene strongly influences the risk for NTD.

Magnus *et al.*[13] found elevated mean apo-tc II levels in the amniotic fluid of mothers with a previous NTD pregnancy when compared to controls. They concluded that this could be due to a defect in the gene or in its regulation of the expression, or to differences in intake of vitamin B12. Gardiki-Kouidou and Seller[14] also found significantly elevated median apo-tc II levels and reduced

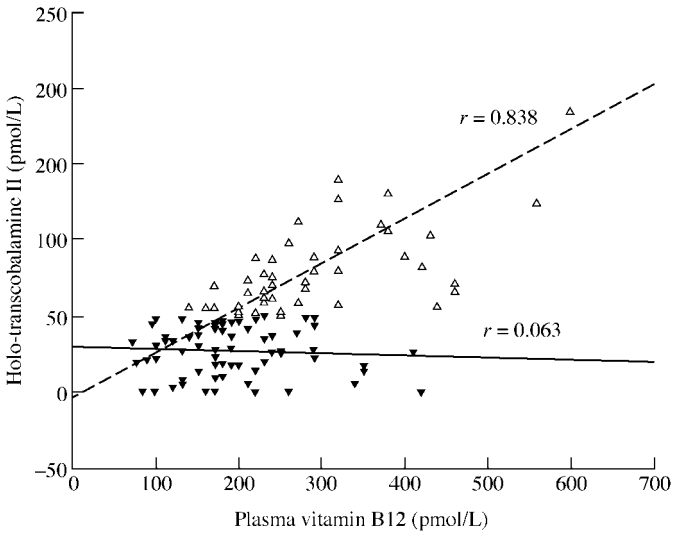

Figure 5. Relationship between plasma vitamin B12 and plasma holo-tc II concentrations, the straight line indicates the relation for holo-tc II concentrations below the 50th percentile of holo-tc II and the dashed line the relationship above the 50th percentile. Individual values above the 50th percentile are represented by grey triangles and individual values below the 50th percentile are represented by black triangles. The holo-tc II level of the 50th percentile was 50.0 pmol/l, based on the control distribution.

Table 2 Genotype distribution of the TC II Pro259Arg polymorphism in mothers of NTD children and in controls

	Arg-259 homozygous	Pro259Arg heterozygous	Pro-259 homozygous
Mothers ($n = 42$)	6 (14%)	25 (60%)	11 (26%)
Controls ($n = 73$)	15 (21%)	36 (49%)	22 (30%)

vitamin B12 levels in amniotic fluid. They also concluded that this could be due to a genetic defect in the production or transport of transcobalamins. We did not find a significant difference in apo-tc II concentration between mothers of NTD children and controls. A possible explanation for this may be that we used plasma from non-pregnant women instead of amniotic fluid. However, our results are in agreement with the elevated apo-tc II levels reported by Magnus *et al.*[13] and Gardiki-Kouidou and Seller,[14] because a reduced binding of vitamin B12 to TC II results in lower holo-tc II levels and, as a consequence, in higher apo-tc II levels.

Vitamin B12 is required in the remethylation of homocysteine to methionine. Therefore, low vitamin B12 levels in the cell could lead to increased plasma homocysteine concentration. The functional effect of low holo-tc II concentrations and low holo-tc II percentages is demonstrated by significantly higher homocysteine levels in these groups. Another functional effect of low holo-tc II levels resulting in reduced delivery of vitamin B12 to the cell, is shown by the clear relation between holo-tc II and vitamin B12 concentrations in the cell, in this case the erythrocyte (Figure 3).

Low plasma holo-tc II concentrations and low holo-tc II percentages are relatively common and increase the risk of NTD. Both findings are best explained by a disturbed binding of vitamin B12 to holo-tc II. Therefore, our future research will focus on mutations in the TC II gene that may explain the observed low holo-tc II concentrations, low holo-tc II percentages, increased homocysteine and decreased erythrocyte vitamin B12 concentrations. Supplementation of vitamin B12 will raise holo-tc II levels and holo-tc II percentages, and thereby lower homocysteine levels and increase cellular vitamin B12, which could result in a decreased risk for NTD. In addition to folic acid supplementation, vitamin B12 supplementation should be considered in the prevention of NTD in women planning a pregnancy.

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