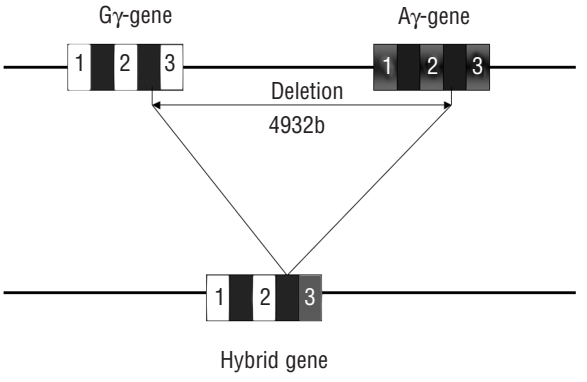


**Figure 2.** Schematic representation of the deletional γ-thalassemia in the β-globin locus; sequence co-ordinates: Gγ gene: 5232678-5230978, Ag 5227754-5226050.

by standard PCR (Figure 1D) through the amplification of an abnormal fragment 4.8 kb shorter than expected. The junction fragment was sequenced (Applied Biosystems 3130XL) and revealed the presence of a hybrid gene identical to Gγ-gene before the polymorphic TG repeat in IVS2[4] and identical to Aγ-gene after this repeat. As no abnormal sequence is created by the rearrangement, we assume that the breakpoint lies in this polymorphic sequence. The size of the deletion corresponds exactly to the size of DNA which separates the γ-genes (Figure 2). The hybrid gene produces an Aγ-globin chain under the control of the Gγ-gene promoter i.e. at a level normally seen for Gγ, explaining why the deletion is not symptomatic even in the homozygous condition. Such a deletion-fusion is also a common event occurring in α-globin locus: indeed, like the fetal γ-globin genes, α-globin genes present highly homologous flanking sequences which represent a potential target area for genetic rearrangements.[5] The resulting deletions, called type 2 α-thalassemia deletions, are very frequent in Asians, Africans and Mediterraneans and cause no clinical consequences.

Although we cannot confirm the presence of the deletion on the paternal chromosome which carries the βS allele, we hypothesize that the deletion is located in cis of the βS allele. Removing parts of the fetal coding region, it has led to premature interactions between LCR and the βS gene promoter, resulting in a high level of expression of the βS gene before birth. Thus, at birth, the proportion of HbS was higher than that of HbA, evocating SCD. In the weeks following birth, the switch on normal βA allele was processed, leading to the rise in the level of HbA, whereas the HbS level remained stable, and to the restoration of a typical heterozygous profile.

In conclusion, we show that a γ-thalassemia associated with a βS allele represents a pitfall in the neonatal screening for SCD.

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## Long term biweekly 1 mg oral vitamin $B_{12}$ ensures normal hematological parameters, but does not correct all other markers of vitamin $B_{12}$ deficiency. A study in patients with inherited vitamin $B_{12}$ deficiency

Parenteral vitamin $B_{12}$ is considered the treatment of choice for vitamin $B_{12}$ deficiency, but also treatment with 1-2 mg daily oral vitamin $B_{12}$ is recommended.[1,2] Recently, alternative regimes have been proposed, such as an oral dose of 1 mg vitamin $B_{12}$ daily for ten days, weekly for four weeks, and monthly or biweekly for life.[3,4] One short-term study assessed this regime employing hematologic markers and total plasma cobalamins.[4] It is unclear to what extent the patients included had a total or only a partial lack of active absorption of vitamin $B_{12}$.

In the present study, we examined the long-term efficacy of biweekly oral treatment with 1 mg vitamin $B_{12}$ by studying hematologic parameters, cobalamins, methylmalonic acid (MMA), total homocysteine (tHcy), and cobalamin saturated transcobalamin (holo-TC) in patients with congenital vitamin $B_{12}$ deficiency treated for at least five years, patients not following this treatment for at least one year, their biological parents, and healthy controls.

Patients (n=16) were unable to actively absorb vitamin $B_{12}$ because of defects in the cubilin or amnionless receptors (n=12) or inherited lack of intrinsic factor (IF) (n=4). Genotyping was available for 11 patients.[5-7] Lack of vitamin $B_{12}$ absorption was recently confirmed in all 16 patients.[7] After the initial diagnosis, all 16 patients except

**Table 1A.** Data on patients with congenital vitamin $B_{12}$ deficiency treated or not with oral vitamin $B_{12}$, their parents and healthy individuals (controls). Median and range is indicated, Table 1A.

| *Subjects Group n (females)* | *Age (years)* | *Hb[3] (g/dL)* | *MCV (fL)* | *Cobalamins (pmol/L)* | *Holo-TC (pmol/L)* | *MMA (μmol/L)* | *tHcy (μmol/L)* |
|---|---|---|---|---|---|---|---|
| Controls 44 (22) | 25[a] (9-58) | 13.7 (8.4-16.4) | 86 (63-91) | 204[a] (124-467) | 35[a] (17-91) | 0.22[a] (0.12-0.79) | 10[a] (6-27) |
| Parents 19 (9) | 41[b] (30-57) | 14.4 (9.9-15.9) | 87 (70-92) | 214[a] (108-297) | 33[a] (14-68) | 0.28[a] (0.10-0.71) | 12[a] (7-18) |
| Patients treatment 4 (1) | 12[c] (8-23) | 12.8 (11-16) | 86 (82-105) | 96[b] (92-122) | 7[c] (5-7) | 2.06[b] (1.68-2.99) | 24[b] (18-30) |
| Patients treatment 12 (7) | 17[c] (9-32) | 13.5 (10.9-16.4) | 87 (80-92) | 161[a] (113-384) | 16[b] (6-47) | 0.34[a] (0.14-1.44) | 12[a] (6-28) |
| Reference intervals | – | 11.5-17.5[1)] | 77-102[1)] | 125-455[2)] | 21-88[2)] | <0.51[2)] | <22[2)] |

*Age of participants, concentrations of holo-TC, cobalamins, tHcy, and MMA, differed significantly between the four groups (All p-values <0.001, one-way analysis of variance, after log transformation). In each column a different superscript letters (a, b or c) indicates that corresponding values are significantly different from each other, p<0.01. Tukey's method was used for the pair-wise comparisons.*

**Table 1B.** Data for each of the included patients.

| *Patients #* | *$B_{12}$ treatment* | *Sex* | *Mutated Gene[3)]* | *Siblings[4]* | *Age (years)* | *Hb[5] (g/dL)* | *MCV (fL)* | *Cobalamins (pmol/L)* | *Holo-TC (pmol/L)* | *MMA (μmol/L)* | *tHcy (μmol/L)* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | *Data obtained for present study (data obtained at the time of diagnosis)* | | | | | | |
| 1 | + | F | AMN | – | 32 (3)[6] | 12.5 (8.4) | 80.3 (89) | 221 | 47 | 0.14 | 6 |
| 5 | + | F | CUBN | – | 13 (1) | 11.5 (4.7) | 82.6 (102) | 135 | 15 | 0.39 | 13 |
| 6 | + | F | GIF | – | 10 (1) | 14.1 (6.4) | 82 (94) | 122 | 29 | 0.23 | 9 |
| 7 | + | F | AMN | – | 18 (3) | 11.6 (7) | 91.7 (120) | 384 | 30 | 0.38 | 19 |
| 8 | + | M | na | B | 15 (1.5)[6] | 15.8 (4.2) | 89.6 (90) | 184 | 18 | 0.54 | 13 |
| 9 | + | M | ma | B | 17 (3)[6] | 14.6 (7.2) | 86.9 (86) | 135 | 17 | 0.61 | 12 |
| 10 | + | M | CUBN | – | 17 (1)[6] | 15.8 (7.2) | 85.3 (87) | 167 | 16 | 0.26 | 16 |
| 12 | + | M | na | C | 9 (0.5) | 14 (8.4) | 87 (108) | 113 | 13 | 0.83 | 13 |
| 13 | + | F | AMN | D | 18 (1) | 13 (5.6) | 89.8 (100) | 187 | 22 | 0.17 | 11 |
| 14 | + | F | AMN | D | 17 (1.5)[7] | 12.7 (5.0) | 86.4 (-) | 201 | 15 | 0.14 | 9 |
| 15 | + | F | na | – | 15 (1.5) | 10.9 (4.8) | 86.6 (102) | 149 | 6 | 0.30 | 10 |
| 16 | + | M | AMN | – | 17 (4) | 16.4 (7.9) | 91.9 (124) | 155 | 12 | 1.43 | 28 |
| 3 | – | M | GIF | A | 9 (1.5) | 12 (4.1) | 82 (96) | 92 | 5 | 2.98 | 30 |
| 4 | – | F | GIF | A | 8 (1) | 11 (7.5) | 104.9 (93) | 95 | 7 | 2.11 | 24 |
| 11 | – | M | na | C | 15 (3.5) | 13.5 (4.5) | 86 (118) | 96 | 7 | 2.02 | 24 |
| 17 | – | M | GIF | – | 23 (4) | 16 (5.3) | 86.2 (110) | 122 | 6 | 1.68 | 18 |
| Reference intervals | | | | | | 11.5-17.5[1] | 77-102[1] | 125-455[2] | 21-88[2] | <0.51[2)] | <22[2] |

*The table shows values obtained at the time of this study and at the time of diagnosis (in brackets). [1)]The interval of reference differs with sex and age. The values cover both sexes and the age group from 6-49 yrs. as indicated by the laboratory performing the analysis. [2)]The interval of reference covers 95% of the values obtained for the controls included in this study. [3)]A genetic diagnosis was available for 11 patients, as described in Tanner et al.[5,6] and in Bor et al.[7] Five patients (na, not available) are still under evaluation, but they are believed to have Imerslund-Grasbeck syndrome. AMN, amnionless, GIF, gastric IF; CUBN, cubilin. [4)]Patients who are siblings are indicated by the same letter. [5]Hemoglobin in g/dL can be converted to mmol/L by multiplying with 0.621. [6]Patients who received blood transfusion and [7]patient who started treatment with vitamin B12 prior to diagnosis at Hacettepe University.*

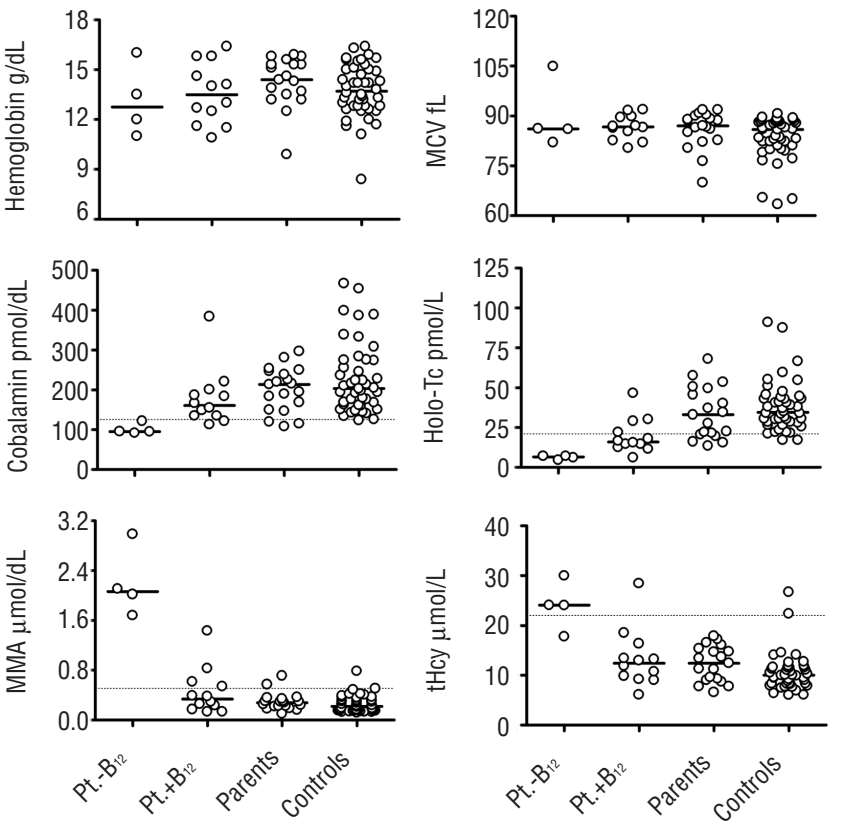


**Figure 1.** Biochemical indices in patients with congenital vitamin $B_{12}$ deficiency and in control groups. The median values for hematologic and vitamin $B_{12}$ indices are presented in 1) patients with congenital vitamin $B_{12}$ deficiency not treated biweekly with 1 mg oral vitamin $B_{12}$ for at least one year (n=4, Pt. -B12), 2) such patients treated biweekly with 1 mg oral vitamin B12 for at least five years (n=12, Pt. + $B_{12}$), 3) the obligate heterozygous biological parents (n=19), and 4) healthy controls (n=44). The dotted vertical lines represent upper or lower values of the reference interval calculated from values obtained for the control group. MCV; middle cell volume, MMA: methylmalonic acid, tHcy: total homocysteine, Holo-TC: cobalamin saturated transcobalamin.

2 (patients # 4,16) received 100 µg vitramin $B_{12}$ intramuscularly daily during the first week, every other day during the second week, twice a week until the hemoglobin had normalized, and every month thereafter. This treatment regime was changed to biweekly 1 mg oral treatment in 1996. Patients #4,16, diagnosed in 1997, received only oral treatment as indicated above after a loading dose of 1 mg oral vitamin $B_{12}$ daily for ten days and 0.5 mg oral vitamin $B_{12}$ daily for an additional ten days.[3] Cobalamin (cyanocobalamin) (1 mg ampoule, Deva) was mixed with water or fruit juice and was self-administered orally. Blood sampling was performed two weeks after the last oral intake of vitamin $B_{12}$ in 2003. Four of the patients (#3, 4, 11, 17) initially followed the biweekly oral treatment as described above but did not receive any treatment with vitamin $B_{12}$ for at least one year prior to the study. We included parents of the patients who were all expected to be heterozygous for the inherited vitamin $B_{12}$ deficiency (n=19), and healthy controls (n=44) with no clinical evidence for vitamin B12 deficiency. Inclusion criteria were no known disorders related to vitamin $B_{12}$ deficiency, no chronic systemic disease and no medical treatment, including vitamin tablets, within the previous week.

Laboratory data and other information on all patients and controls are indicated in Tables 1A and 1B. The Research Ethics Committee of Hacettepe University Hospital approved the study protocol.

Cobalamins was measured on the Advia Centaur Analyzer (CV<10%). Serum holo-TC was measured by an in-house ELISA (CV<10%).[8] MMA and tHcy were determined by gas chromatography–mass spectrometry after derivatization with methylchloroformate[9] (CV < 4%). Hematologic parameters were measured using a Beckman Coulter.

All patients with congenital vitamin $B_{12}$ malabsorption showed an increase in hemoglobin and most of them showed a decrease in MCV between the time of diagnosis and the time of the current study (Table 1B). At the time of the study there was no difference in hematologic parameters (Table 1A) between patients with congenital vitamin $B_{12}$ malabsorption and the control groups. The four untreated patients had low serum cobalamins, and holo-TC concentrations, elevated tHcy and MMA ($p$<0.001) as compared to values obtained for the controls, but, unexpectedly, normal hematologic parameters (Table 1A, Figure 1).

For the 12 treated patients, no markers except for holo-TC ($p$<0.001) showed a significant difference to those of the control subjects and their biological parents (Table 1A). However, values not within normal range as calculated from controls was observed for some of the 12 treated patients; MMA>0.51 µmol/L (n=4), tHcy>22 µmol/L (n=1), cobalamin <126 pmol/L (n=2) and holo-TC<21 pmol/L (n=8).

The main findings of the present study on patients with congenital vitamin $B_{12}$ deficiency are that biweekly oral treatment with 1 mg vitamin $B_{12}$ ensures normal hematologic parameters, but does not correct all other markers of vitamin $B_{12}$ deficiency. Thus, vitamin $B_{12}$ deficiency may exist in this population without hematologic signs, as previously demonstrated in other populations.[10]

Studies by Gangorase *et al.*[11] and subsequently by Altay and Cetin[3] showed that anemia due to inherited vitamin B12 deficiency could be corrected by oral vitamin $B_{12}$. In the latter study, biweekly 1 mg oral vitamin $B_{12}$ treatment

after a loading dose was sufficient for maintenance of normal hematologic parameters and cobalamin levels during a follow-up period of 12 months. Our study challenges the conclusion that biweekly 1 mg vitamin $B_{12}$ treatment is sufficient and questions hematologic parameters and plasma cobalamins as measures of inadequate vitamin $B_{12}$ status.

Elevated plasma concentrations of MMA and tHcy can be used as biochemical markers to aid in the diagnosis of vitamin $B_{12}$ deficiency and to monitor the response to cobalamin supplementation. In our study, more than 75% of the patients with congenital vitamin $B_{12}$ deficiency treated biweekly with 1 mg oral vitamin $B_{12}$ for more than five years had normal levels of MMA and tHcy, but only 33% had a normal holo-TC. This observation agrees with the view that holo-TC is an early marker of a suboptimal vitamin B12 homeostasis.[12]

In conclusion, biweekly treatment with 1 mg vitamin $B_{12}$ seems to normalize indices of vitamin $B_{12}$ status in some, but not all of the patients with congenital vitamin $B_{12}$ deficiency. We, therefore, believe that a more frequent intake of oral vitamin $B_{12}$ is required, but we question whether a daily dose of 1-2 mg vitamin $B_{12}$ is needed.

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*Key words: oral vitamin B12 treatment, holo-TC, cobalamin, inherited vitamin B12 deficiency*

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## Monitoring for cytomegalovirus and Epstein-Barr virus infection in chronic lymphocytic leukemia patients receiving i.v. fludarabine-cyclophosphamide combination and alemtuzumab as consolidation therapy

The combination of fludarabine and cyclophosphamide (FC) has become the standard of care in chronic lymphocytic leukemia (CLL) patients. Due to the well-recognized F-related immunosuppression,[1] a higher risk of opportunistic infections could be expected by adding another immunosuppressive agent. Randomized trials[2-4] do not report a significantly higher rate of infections in patients receiving FC combination versus F alone as first-line therapy, although they do not focus on reactivation of latent viral infections. However, the combination of FC and dexamethasone has been associated with Epstein-Barr virus (EBV) infection in heavily pre-treated patients with lymphoproliferative diseases[5] and with EBV-related histological transformation of CLL.[6] In addition, sporadic cases of cytomegalovirus (CMV) infection on F-based therapy have been reported.[7] The susceptibility to opportunistic infections related to defective cell-mediated immunity is even higher with alemtuzumab (AL),[8] with particular concern for CMV infection. We report results from our surveillance program for CMV and EBV infections in patients treated with FC and AL as consolidation of response.

Sixty-seven CLL patients ≤ 65 years old ( median age 54 yrs) received FC combination for progressive disease defined according to NCIWG criteria (as first-line therapy in 53 and second-line in 14). All patients had signed an informed consent before inclusion in treatment and monitoring programs. Median time to treatment was 36 months (range: 1-79 mos) for first-line patients, while median interval from previous therapy to FC administration was 15 months (range 5-32 mos). FC consisted of F