Effect of Vitamin B\textsubscript{12} Treatment on Haptocorrin

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Background: Haptocorrin (HC) carries the major part of circulating cobalamin, but whether HC is altered on treatment with vitamin B\textsubscript{12} remains unknown.

Methods: Our study included 3 populations: a population of vegan men (n = 174; vegan population), of whom 63 were treated daily with 5 mg of oral vitamin B\textsubscript{12} for 3 months; a group of patients with a previous methylmalonic acid (MMA) concentration >0.4 \textmu mol/L. (n = 140; population with suspected deficiency), of which 69 were treated with weekly vitamin B\textsubscript{12} injections (1 mg) for 4 weeks; and a subgroup of participants in a vitamin B\textsubscript{12} intervention study (n = 88; nondeficient population), of whom 45 were treated daily with 0.4 mg of oral vitamin B\textsubscript{12} for 3 months. Total HC and holoHC were measured by ELISA. Cobalamin was measured by an intrinsic factor (IF)-based assay. Samples were collected at baseline and 3 months after start of treatment.

Results: Compared with baseline results for the 3 study populations, total HC and holoHC increased 30 pmol/L for every 100 pmol/L increase in cobalamin. After treatment with vitamin B\textsubscript{12}, holoHC (P <0.0001) and total HC (P <0.0001) increased significantly in the vegan population. Only holoHC increased in the population with suspected deficiency (P <0.0001), whereas no alteration was observed in the nondeficient population.

Conclusions: The HC concentration is decreased in severely cobalamin-deficient individuals and increases on treatment. The concentration of cobalamin also relates significantly to the HC concentration in nondeficient individuals.

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Three proteins—intrinsic factor (IF),\textsuperscript{7} transcobalamin (TC), and haptocorrin (HC)—are involved in the uptake and transport of cobalamin. Cobalamin bound to IF is transported from the intestine to the circulation, where it attaches to 2 binding proteins, TC and HC. Holotranscobalamin (holoTC) and holohaptocorrin (holoHC) denote the binding proteins with cobalamin attached, whereas their apoforms refer to the fraction unsaturated with cobalamin. Unlike holoTC, holoHC does not seem to deliver cobalamin to most cells. The majority of circulating HC exists as holoHC (~80%) (1), and holoHC represents the bulk (~75%) of cobalamin in plasma. In spite of the considerable fraction of plasma cobalamin carried by HC, the role of HC in vitamin B\textsubscript{12} metabolism and transport is still unclear (2, 3).

In contrast to the 2 other binding proteins, IF and TC, HC is characterized by its ability to bind both cobalamin and other corrinoids, the so-called vitamin B\textsubscript{12} analogs (2). It has been suggested that ~30% of plasma cobalamin consists of analogs unable to serve as coenzymes (4).

A strong association between total HC and cobalamin in plasma has been shown previously (5, 6). This result could support the previously published view that the HC genotype determines the HC concentration, and thereby the concentration of circulating cobalamin (7). Another explanation is that the HC concentration is influenced by the vitamin B\textsubscript{12} status of the individual.

Previously, HC was quantified by use of techniques based on immunoadsorption separation and subsequent direct or indirect measurement of apoHC and holoHC, the sum of the 2 representing total HC (8, 9). In the 1970s and 1980s, two RIAs for measurement of HC were developed, enabling direct quantification of HC (5, 10, 11).

\textsuperscript{7} Nonstandard abbreviations: IF, intrinsic factor; TC, transcobalamin; HC, haptocorrin; holoTC, holotranscobalamin; holoHC, holohaptocorrin; MMA, methylmalonic acid; tHcy, total homocysteine; and GC-MS, gas chromatography–mass spectrometry.
this study, an ELISA method was used for quantification of HC. The assay includes a deglycosylation step, which we found to be required for reliable measurement of this protein (6). HoloHC is measured by the same HC ELISA after precipitation of the unsaturated cobalamin-binding proteins with vitamin B_{12}-coated beads (12). Our holoHC method measures HC with cobalamin or vitamin B_{12} analogs attached.

We used those new methods to further study the relationship between HC and vitamin B_{12} status. We investigated the HC concentrations in a control population and pre- and post-vitamin B_{12} treatment in 3 populations: vitamin B_{12}-deficient vegan men, patients with suspected vitamin B_{12} deficiency, and a group of individuals with normal vitamin B_{12} status.

Materials and Methods

STUDY POPULATIONS

This study included (a) a control population (6); (b) a population of vegan men (13), of whom a subgroup was allocated to receive oral vitamin B_{12} treatment (vegan population); (c) a population of individuals with suspected vitamin B_{12} deficiency, of whom a subgroup received intramuscular injections of vitamin B_{12} (population with suspected deficiency) (14); and (d) a population of vitamin B_{12}-replete patients, of whom a subgroup was allocated to receive oral vitamin B_{12} treatment (nondeficient population) (15, 16).

CONTROL POPULATION

Blood samples (EDTA plasma) from a population of healthy persons [women <50 years of age (n = 36); women >50 years of age (n = 35); men <50 years of age (n = 37), and men >50 years of age (n = 40)] were used to establish a reference interval for holoHC and HC saturation. The overall reference interval for total HC in this population was reported previously by Mørkbak et al. (6).

The vegan population consisted of 174 vegan men recruited from the Oxford cohort of the European Prospective Investigation into Cancer and Nutrition and from the London Vegan Society. Exclusion criteria for participation in the study were a history of major gastrointestinal disease, presence of parietal cell or IF antibodies, liver disease, diabetes mellitus, vitamin B_{12} injections, or medication for psychiatric disorders (13). A subset of the vegans with cobalamin concentrations <148 pmol/L (measured with the Bayer Immuno I assay) participated in a randomized double-blind controlled intervention study (n = 77). Of these men, those with a cobalamin concentration <89 pmol/L were automatically allocated to vitamin B_{12} treatment (n = 42) and individuals with cobalamin concentrations between 89 and 148 pmol/L were randomized to vitamin B_{12} treatment (n = 21) or placebo (n = 14). In total, 63 individuals were treated with a high oral dose of vitamin B_{12} in the form of cyanocobalamin (5 mg/day) for 3 months (B_{12}-treated vegan population).

The population with suspected deficiency was recruited from 937 individuals with suspected vitamin B_{12} deficiency [based on methylmalonic acid (MMA) concentration ≥0.28 μmol/L within the past 4 years] (14). Persons with MMA <0.40 or >2.00 μmol/L, plasma thyroid-stimulating hormone ≥4.1 mIU/L, plasma creatinine >120 μmol/L (females) or >133 μmol/L (males), unable to cooperate or currently participating in other clinical studies, or receiving vitamin B_{12} injections were excluded. A total of 140 patients with plasma MMA between 0.40 and 2.00 μmol/L [based on a sample collected 2–12 weeks (median, 4 weeks) before baseline] were randomized to receive weekly intramuscular injections containing cyanocobalamin (1 mg; n = 69; B_{12}-treated population with suspected deficiency) or placebo (1 mL of isotonic sodium chloride; n = 71) weekly for 4 weeks (14). After 3 months, 137 of the 140 individuals were reexamined.

The nondeficient population was a part of the Western Norway B-vitamin Intervention Trial, a prospective randomized double-blind study on the effect of homocysteine-lowering therapy in patients with coronary artery disease. Exclusion criteria were participation in other studies, malignant disease, alcohol abuse, mental illness, or unwillingness to do long-term follow-up (15, 16). In the Western Norway B-vitamin Intervention Trial, we categorized 88 persons with sufficient volumes of stored plasma in 2 groups: one group (n = 45; B_{12}-treated nondeficient population) received daily oral treatment with cyanocobalamin (0.4 mg) and folic acid (0.8 mg) either with (n = 22) or without (n = 23) vitamin B_{6} (40 mg); and the other group (n = 43) received vitamin B_{6} (40 mg; n = 20) or placebo (n = 23). Forty-two of the 45 persons chosen for vitamin B_{12} supplementation were revisited after 3 months.

For the purposes of the present study of HC, vitamin B_{12}, total homocysteine (tHcy), and MMA concentrations, we used samples collected at baseline and after treatment in the above 3 studies. To estimate the intraindividual variations in HC concentrations, we used samples collected at baseline and 3, 14, 28, and 84 days after the start of the study from the nondeficient “placebo” subgroup (n = 23). Written informed consent was obtained from all patients, and the study protocols were approved by the regional ethics committees.

SAMPLING AND ANALYTICAL METHODS

EDTA-plasma or serum (vegan population) was collected at baseline and after −3 months of treatment with vitamin B_{12} or placebo in the 3 populations. From the nondeficient “placebo” subgroup (n = 23), additional samples were collected after 3, 14, and 28 days. Plasma or serum was separated from the blood cells within 2 h after sample collection and stored below −70 °C until used.

Total HC was measured by an in-house sandwich ELISA with an imprecision (CV) of ~5% (6). holoHC was measured by the HC ELISA after removal of the apoHC...
with vitamin B₁₂-coated beads as described previously for measurement of holoTC (12). The CV was ~10%. HC saturation was calculated as holoHC/total HC. The HC-derived markers were analyzed on thawed samples kept below ~70 °C for 4–6 years. Total HC is stable during prolonged storage as judged from repeat analysis of a control sample run over the course of 1.5 years and showing a CV of ~8% (n = 58) with no systematic drift.

Serum or plasma cobalamin was measured on the Advia Centaur (Bayer A/S) with an imprecision <10% (13–16). Measurement of serum cobalamin before randomization in the vegan population was performed with the Immuno I analyzer (Bayer A/S).

MMA was measured by gas chromatography–mass spectrometry (GC-MS; vegan and suspected deficiency populations) (17,18) and a modified GC-MS method involving ethyl chloroformate derivatization (19) (nondeficient population) with an imprecision <8% [Refs. (13, 14) and personal communication from H. Refsum]. tHcy in the vegan population was measured by GC-MS (17), in the population with suspected deficiency by an immunologic method on the IMx (Abbott), and in the nondeficient population by a modified GC-MS method involving ethyl chloroformate derivatization (19), with an imprecision ≤5% (13, 14, 20).

**Statistical Analysis**

Associations were assessed by Spearman ranks correlation coefficients. Statistical analysis was performed with the Wilcoxon matched-pairs signed-rank test. All statistical analyses were performed with GraphPad Prism (Ver. 4.00 for Windows; GraphPad Software).

**Results**

**Reference Intervals and Intraindividual Variation for Total HC, HoloHC, and HC Saturation**

The 95% reference intervals for holoHC and HC saturation were 190–590 pmol/L and 0.6–1 based on analysis of the control population. Because the absolute variations in the reference intervals between men and women and between the 2 age groups were small for total HC, holoHC, and HC saturation, we used a common reference interval for both sexes and all age groups (see Table S1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol52/issue6/).

The intraindividual variation was calculated based on results obtained in the nondeficient “placebo” subgroup (n = 23), based on blood samples collected at baseline and after 3, 14, 28, and 84 days. The calculated median (range) intraindividual variation was 6 (2–14)% for total HC, 6 (2–17)% for holoHC and 4 (1–7)% for HC saturation.

The pre- (n = 177) and posttreatment (n = 171) interindividual variation was calculated based on baseline samples and 3-month samples obtained in the 3 subgroups allocated to vitamin B₁₂ treatment. The pretreatment (posttreatment) interindividual variation was 30% (30%), 31% (32%), and 16% (8%) for total HC, holoHC, and HC saturation, respectively.

**HoloHC and Total HC Compared with Other Biochemical Markers of Vitamin B₁₂ Deficiency**

To compare the relationship between HC, cobalamin, and the metabolic markers of vitamin B₁₂ deficiency, tHcy and MMA, we used baseline results obtained for all 3 study populations (see Table S2 in the online Data Supplement), i.e., a total of 402 persons. Both total HC (r = 0.4; P <0.0001) and holoHC (r = 0.4; P <0.0001) were significantly associated with cobalamin (Fig. 1). Regression analysis of the relationship between cobalamin and total HC (holoHC) gave an intercept for the regression line of 300 pmol/L (250 pmol/L) and a slope indicating an increase in total HC (holoHC) of 30 pmol/L (30 pmol/L) per 100 pmol/L increase in cobalamin (Fig. 1).

![Fig. 1. Baseline cobalamin vs baseline total HC (top) and holoHC (bottom) in the 3 study populations (n = 402).](image-url)
Table 1. Treatment regimens and age, sex, and pre- and posttreatment cobalamin, tHcy, MMA, total HC, and holoHC concentrations for the 3 subgroups selected for vitamin B₁₂ treatment.

<table>
<thead>
<tr>
<th>Subgroups selected for vitamin B₁₂ treatment</th>
<th>Vegan&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Suspected deficiency&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Nondeficient</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Active ingredient</strong></td>
<td>Cyanocobalamin</td>
<td>Cyanocobalamin</td>
<td>Cyanocobalamin</td>
</tr>
<tr>
<td>Dosage</td>
<td>5 mg/day</td>
<td>1 mg/week for 4 weeks</td>
<td>0.4 mg/day</td>
</tr>
<tr>
<td>Administration route</td>
<td>Oral</td>
<td>Intramuscular</td>
<td>Oral</td>
</tr>
<tr>
<td>Median (range) age, years</td>
<td>44 (22–78)</td>
<td>74 (20–92)</td>
<td>61 (38–79)</td>
</tr>
<tr>
<td>Sex, % women</td>
<td>0</td>
<td>72</td>
<td>20</td>
</tr>
<tr>
<td><strong>Analytes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. in group</td>
<td>Reference interval</td>
<td>Pretreatment</td>
<td>Posttreatment</td>
</tr>
<tr>
<td>Cobalamin, pmol/L</td>
<td>200–600&lt;sup&gt;d&lt;/sup&gt;</td>
<td>97 (32–286)</td>
<td>1016 (142–2896)&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>tHcy, µmol/L</td>
<td>4.5–11.9&lt;sup&gt;e&lt;/sup&gt;</td>
<td>18.3 (8.4–183)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>8.0 (4.9–59.9)&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>MMA, µmol/L</td>
<td>0.08–0.28&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.7 (0.1–4.3)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.1 (0.1–0.3)&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total HC, pmol/L</td>
<td>240–680</td>
<td>330 (118–616)</td>
<td>377 (143–684)&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>HoloHC, pmol/L</td>
<td>190–590</td>
<td>286 (101–626)</td>
<td>365 (135–700)&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Randomization was based on cobalamin results obtained with the Bayer Immuno I analyzer. Cobalamin results shown were obtained with the Advia Centaur method (see Materials and Methods).

<sup>b</sup> Randomization was based on MMA results obtained on a sample collected 2–12 weeks (median, 4 weeks) before baseline. Baseline MMA results are shown.

<sup>c</sup> Pre- and posttreatment concentrations are given as the median (range).

<sup>d</sup> Reference intervals were obtained from Refs. (17, 24). The reference intervals for tHcy are as follows: for age <30 years, 4.6–8.1 µmol/L for both males and females; for age 30–59 years, 4.5–7.9 µmol/L for females and 6.3–11.2 µmol/L for males; for age >60 years, 5.8–11.9 µmol/L for both males and females.

<sup>e</sup> Significant difference between the pre- and posttreatment concentrations (Wilcoxon matched-pairs signed-rank test, \( P < 0.0001 \)).

<sup>f</sup> Only 62 persons in this group because sample was not available for 1 person for both tHcy and MMA.

<sup>g</sup> The posttreatment decrease in tHcy for the vitamin B₁₂-treated nondeficient subgroup may have been induced in part by the concomitant administration of folate and/or vitamin B₆ (see Materials and Methods).
HoloHC and HC saturation were associated with both tHcy \( [r = -0.14 \ (P = 0.014; \ n = 401)] \) and MMA \( [r = -0.17 \ (P = 0.0015; \ n = 401)] \) and MMA \( [r = -0.22 \ (P = 0.0002; \ n = 401)] \). These associations were lower than those observed for cobalamin vs tHcy \( [r = -0.27 \ (P < 0.0001; \ n = 401)] \) and MMA \( [r = -0.32 \ (P < 0.0001; \ n = 401)] \). We observed no association between total HC and the 2 metabolic markers of vitamin B\(_{12}\) deficiency.

**Alteration in HC-derived markers after treatment with vitamin B\(_{12}\)**

In each of the 3 study populations, a proportion of the persons were treated with vitamin B\(_{12}\). The characteristics of the individuals before treatment and 3 months after start of vitamin B\(_{12}\) intervention are summarized in Table 1. After treatment, both holoHC (median, 365 pmol/L vs 286 pmol/L at baseline; \( P < 0.0001 \)) and total HC (377 pmol/L vs 330 pmol/L at baseline; \( P < 0.0001 \)) increased significantly in the B\(_{12}\)-treated vegan population (Table 1). In the vitamin B\(_{12}\)-treated subgroup of the population with suspected deficiency, only holoHC increased significantly (from 336 to 377 pmol/L; \( P < 0.0001 \); Table 1). No alteration in holoHC or total HC was observed in the subgroup of the nondeficient population treated with vitamin B\(_{12}\). The changes as a function of the pretreatment cobalamin concentration divided into tertiles in the 3 vitamin B\(_{12}\)-treated populations studied are shown in Fig. 2. As is clear in Fig. 2, those with the lowest pretreatment cobalamin concentration (the B\(_{12}\)-treated vegan population) show the highest increase in the HC-related markers. However, as can also be seen in Fig. 2, a relationship existed between HC and cobalamin that remained in individuals for whom vitamin B\(_{12}\) treatment did not lead to an increase in HC (cobalamins and total HC are significantly associated in the subgroup of the nondeficient population treated with vitamin B\(_{12}\) \( r = 0.62; \ P < 0.0001; \ n = 45 \)). No changes in total HC and holoHC were observed after treatment in the placebo groups (data not shown).

To further explore the relationship between HC and vitamin B\(_{12}\) status, we performed additional analysis on the combined group of individuals treated with vitamin B\(_{12}\) (\( n = 170 \)). We first examined changes in total and holoHC after vitamin B\(_{12}\) treatment in 3 groups classified based on pretreatment laboratory results as deficient (cobalamin \( <200 \) pmol/L and MMA \( >0.4 \) mmol/L; \( n = 53 \)), nondeficient (cobalamin \( \geq 200 \) pmol/L and MMA \( <0.28 \) mmol/L; \( n = 39 \)), or “conflicting” (cobalamin \( \geq 200 \) pmol/L and MMA \( \geq 0.28 \) mmol/L or cobalamin \( <200 \) pmol/L and MMA \( \leq 0.40 \) mmol/L; \( n = 78 \)). As shown in Fig. 3, we observed significant increases in holoHC (P

![Fig. 2. Tertiles of pretreatment cobalamin vs pretreatment (○) and posttreatment (●) holoHC and total HC (mean (SD; error bars)).
Cobalamin values on the x axes are the mean (range). (Left), vitamin B\(_{12}\)-treated vegan population (\( n = 63 \)); (middle), vitamin B\(_{12}\)-treated population with suspected deficiency (\( n = 66 \)); (right), vitamin B\(_{12}\)-treated nondeficient population (\( n = 42 \)). * significant difference between pre- and posttreatment concentrations (Wilcoxon matched-pairs signed-rank test, \( P \leq 0.009 \)).](image-url)
individuals with cobalamin significant difference between pre- and posttreatment concentrations (Wilcoxon/H11021 cobalamin MMA and cobalamin (cobalamin/H11350 cobalamin/H11022 treatment.

B12 status in 3 populations before and after vitamin B12 examination and describe a relationship between HC and vitamin H11005 metabolism marker MMA after treatment with vitamin B12. We observed posttreatment increases in holoHC and total HC concentrations in vitamin B12-treated groups, classified according to pretreatment laboratory results. We also observed smaller, but highly significant, increases in holoHC (P <0.0001) and total HC (P <0.0001) in the deficient group. We also observed smaller, but highly significant, increases in conflicting results for cobalamin and MMA. No increase was seen in the nondeficient group. We next examined changes in HC in relation to alterations in the metabolic marker MMA after treatment with vitamin B12. We observed posttreatment increases in holoHC and total HC in the 2 tertiles with the highest decrease in MMA (P <0.01), whereas we observed no change in the tertile with the lowest posttreatment decrease in MMA (Table 2).

Discussion

We report reference intervals for holoHC and HC saturation and describe a relationship between HC and vitamin B12 status in 3 populations before and after vitamin B12 treatment.

We used a method for estimating holoHC that measures the protein moiety of HC not removed with magnetic beads coated with vitamin B12. Our reference interval compares well with previously published values obtained by methods using immunoprecipitation of HC and subsequent measurement of cobalamin or vitamin B12 analogs bound to HC by assays that used HC as binding protein (8, 9).

The intraindividual variation of total HC (6%) and holoHC (6%) in our study was low compared with previously reported results for cobalamin (9%) and holoTC (16%) (12), which most likely reflects that holoHC is a more stable marker over time than is holoTC, as judged from its half-life of more than a week compared with the half-life of TC, which is in the magnitude of hours (2).

In this study, we used an IF-based assay for measurement of cobalamin. IF has a low affinity for the B12 analogs, whereas HC binds cobalamin and the analogs equally well (2). Our assay for holoHC will detect HC saturated with cobalamin or B12 analogs. Thus, if plasma or serum contains sufficient amounts of cobalamin analogs, the holoHC concentration may exceed the total cobalamin concentration. In our study, we observed higher values for holoHC than for cobalamin in the vegan population and the population with suspected deficiency, suggesting that HC is partly saturated with analogs, at least in these 2 populations.

We observed a positive association between total HC and cobalamin both when analyzing the entire study cohort of 402 persons and when analyzing each of the 3 groups separately (Fig. 1). This observation is in accordance with previously reported results (5, 6) supporting the view that the HC genotype determines the concentration of HC and thereby the concentration of circulating cobalamin (7), but also the alternative explanation that the HC concentration is influenced by the vitamin B12 status of the individual.

We studied the changes in total and holoHC in 3 populations 3 months after treatment with vitamin B12 was initiated. One population, the population of vegan men, was generally highly vitamin B12 deficient before treatment, as judged from the biochemical markers (Table 1). Interestingly, this population had lower baseline concentrations of both total and holoHC, but after vitamin B12 treatment, the concentrations increased and were within the reference intervals. The results strongly suggest that the total HC concentration is regulated by vitamin B12 status. The implication of this result is quite important because it questions whether it is safe to assume that low cobalamin is caused simply by heterozygosity for a lack of HC (7).

Plasma was collected in the population with suspected deficiency and the nondeficient population, whereas serum was drawn from the vegan population. It has previously been reported that apoHC is artifically increased in serum presumably because of granulocytic release during the clotting process (21). This could cause an artificial overestimation of total HC in the vegan popula-

![Fig. 3. Mean (SD; error bars) pre- (○) and posttreatment (●) holoHC and total HC concentrations in vitamin B12-treated groups, classified according to pretreatment laboratory results.](image-url)
tion. However, because the concentrations of leukocytes and erythrocytes did not increase after treatment (data not shown), it is not likely that the increase in total HC could be explained by this artifact.

The second population undergoing vitamin $B_{12}$ treatment consisted of patients with subtle signs of vitamin $B_{12}$ deficiency. In this group, no change in total HC was observed, whereas the holoHC concentration increased after treatment with vitamin $B_{12}$. The results suggest that this population had a suboptimal concentration of holoHC before treatment, although no decrease in total HC was observed.

Interestingly, the population of vitamin $B_{12}$-nondeficient individuals showed no alteration in either holoHC or total HC, indicating that these compounds remain unchanged after treatment with pharmacologic doses of vitamin $B_{12}$ in nondeficient persons. This is in disagreement with previously published results on calculated holoHC (cobalamin – holoTC) in this population showing an increase in holoHC after treatment (15). At present, this disagreement cannot be explained.

An obvious question is whether differences in the 3 populations studied might be of importance for the conclusions drawn concerning the relationship between an increase in HC (holoHC and total HC) and vitamin $B_{12}$ treatment and status. Obviously, it is impossible to demonstrate whether differences in sex, age, clinical condition, and vitamin $B_{12}$ treatment (route and dose) among the 3 vitamin $B_{12}$-treated populations influenced the results obtained. Previously published results show that correction of vitamin $B_{12}$ status (indicated by lowering of MMA) depends on the dose of vitamin $B_{12}$, the route of administration, the duration of treatment, and age (22, 23). However, for the following reasons we do not believe this is the case. Despite the variation in treatment route and dose, all treated individuals were very likely to be $B_{12}$ replete 3 month after initiation of treatment (based on the decrease in the metabolic marker MMA and increase in cobalamin). Furthermore, results obtained by pooling of the data from the 3 populations treated with vitamin $B_{12}$ and analyzing the increases in holoHC and total HC as a function of the pretreatment vitamin $B_{12}$ status or the decrease in MMA strongly supported the observations made on the individual populations (Fig. 3 and Table 2).

In summary, our results indicate a complex relationship between HC and cobalamin. The HC concentration seems to be regulated by the vitamin $B_{12}$ status, but at the same time a correlation between HC and cobalamin existed in the cobalamin-replete and $B_{12}$-treated individuals.

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**References**