Holotranscobalamin and Total Transcobalamin in Human Plasma: Determination, Determinants, and Reference Values in Healthy Adults

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Background: We developed microbiological assays (MBAs) to identify determinants and to establish reference values for cobalamin bound to transcobalamin [holotranscobalamin (holoTC)] and total TC in plasma. Methods: We captured holoTC with magnetic beads with TC antibodies and used a conventional MBA for cobalamin measurements. Total TC was determined as holoTC after TC was saturated with cyanocobalamin. The new assays were compared with published methods. Determinants and reference values were determined in 500 blood donors, ages 18–69 years. Results: Determination of cobalamin, holoTC, and TC by MBA required <150 μL. HoloTC and TC by MBA correlated with holoTC by RIA (r = 0.95) and TC by ELISA (r = 0.79), respectively. Between-day CVs for holoTC and total TC were 4%–9%. Women had lower holoTC than men, but only at age ≤45 years. In multivariate regression analyses, holoTC was positively associated with age (in women only), creatinine (in men only), and plasma concentrations of total TC, folate, and cysteine, but inversely correlated with homocysteine and methylmalonic acid. For all study participants, total TC was associated with holoTC and number of TCN2 766C alleles; in female participants only, total TC was also associated with age, homocysteine, and cysteine. Reference values were 670–1270 pmol/L for TC and 42–157 pmol/L for holoTC, but they differed according to age and sex. Conclusions: Our MBAs for TC and holoTC required low plasma volume and performed acceptably compared with other methods. Determinants of holoTC and TC differed between men and women and according to age. Separate reference intervals for holoTC should be considered in younger women.

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Cobalamin (vitamin B12) is essential for 1-carbon metabolism and cell division. The clinical consequences of cobalamin deficiency include megaloblastic anemia and neurologic disease. Deficiency also leads to increased total homocysteine (tHcy)5 concentrations, which have been associated with serious conditions such as cardiovascular disease, birth defects, pregnancy complications, osteoporosis, neuropsychiatric disorders, and dementia (1, 2).

Holotranscobalamin (holoTC) is the portion of cobalamin that is bound to the transport protein transcobalamin (TC), which facilitates cellular uptake of cobalamin (3). Less than 30% of the cobalamin in plasma circulates as holoTC. The remaining proportion is bound to haptocorrins (4, 5), the function of which is not known. Recent studies have suggested that holoTC is a better early marker for changes in cobalamin status than cobalamin concentration (4, 6) and is more strongly associated with conditions related to impaired cobalamin function (7–10). The relationship of total TC to cobalamin status is not as well understood, but together with holoTC, total TC measurements can be used to determine TC saturation, which may be better than holoTC alone for detecting changes in cobalamin status (4, 6). Furthermore, measurement of total TC concentrations may be useful for diagnosis of inherited TC deficiency. A recent study showed

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5 Nonstandard abbreviations: tHcy, total homocysteine; holoTC, holotranscobalamin; TC, transcobalamin; TCN2, transcobalamin 2 gene; MBA, microbiological assay; and MMA, methylmalonic acid.
that the plasma total TC concentration is related to long-term memory, independent of holoTC concentrations (11).

Measurements of total TC and holoTC were difficult to obtain (12) until cloning of the transcobalamin 2 gene (TCN2) led to new measurement methods (13–15). The commercial RIA for holoTC (13), however, requires 400–800 μL of plasma, an amount that makes this assay unsuitable for studies with limited available sample volume. We changed this assay into a microbiological assay (MBA) that uses less plasma volume and further modified it for use in determining total TC. Using the new assays, we searched for determinants and determined reference values for plasma holoTC and total TC in healthy adults.

**Materials and Methods**

**Materials, Methods, and Instrumentation**

Cyanocobalamin and mineral oil were from Sigma, colistin sulfate was provided by Spodefell Ltd., and Difco B12 assay medium was from BD UK, Ltd. Other reagents used in the extraction buffer and growth media were from Sigma. Magnetic beads (micspheres with immobilized monoclonal antibody specific for human TC), holoTC calibrator samples, and control samples were kindly donated by Axis-Shield plc. A stock of *Lactobacillus leichmannii* (NCIB 12519, ATCC 43787) was provided by Professor John M. Scott, Departments of Biochemistry and Clinical Medicine, Trinity College, Dublin, Ireland. The magnetic separator was from Aggene. Screw-cap tubes (1.5-mL conical and 2-mL ribbed and skirted) were from Starlab. Microtiter plates (flat-bottomed 96-well microtest plates; well volume, 360 μL) were from Sarstedt, and adhesive sealing film for the plates was from Fisher Scientific. The table autoclave (12 L CertoClav) was from Radleys. The plate reader, with a 590 nm multispan filter, was from Thermo Electron Corporation.

**MBA for Cobalamin, HoloTC, and Total TC**

Plasma cobalamin was measured by MBA using a colistin sulfate–resistant strain of *L. leichmannii* (16, 17). For holoTC, the major change, compared with the published HoloTC RIA [Axis Shield ASA (13)], was that the RIA has been replaced with the MBA developed for cobalamin determination. Total TC was measured as holoTC after the binding sides had been saturated with cyanocobalamin. The MBAs for holoTC, total TC, and cobalamin are described in detail in Fig. 1. Separation of holoTC (and total TC) from the remaining cobalamin was achieved by use of the magnetic beads from Axis Shield ASA. The bead solution, as supplied by the company, is diluted 20-fold with phosphate-buffered saline before use. The extraction buffer contains 20.7 mmol/L acetic acid, 8.3 mmol/L NaOH, and 0.45 mmol/L sodium cyanide, pH 4.5. The growth medium with the *L. leichmannii* was prepared as follows: 6.2 g of Difco B12 assay medium and 150 μL of Tween 80 were mixed with 100 mL of sterile H2O, heated, and boiled for 3 min. After the mixture cooled, colistin sulfate (11 mg) and manganese sulfate (15 mg) were added. Immediately before use, we added 200 μL of the bacterial culture grown to an absorbance of 0.8 at 590 nm (17). We assessed bacterial growth by measuring absorbance at 590 nm with a plate reader. Cobalamin concentrations were calculated with a calibration curve derived from samples with known concentrations of holoTC or cobalamin.

**Calibrator samples for holoTC and total TC measurements**

HoloTC calibrators (Axis-Shield) were prepared gravimetrically from a stock solution of recombinant holoTC saturated with cobalamin. The purity of the recombinant holoTC was determined by sodium dodecyl sulfate electrophoresis and spectroscopically from the ratio of the protein component and the cobalamin component. The concentration of holoTC in the stock solution was determined (a) from the absorbance at 362 nm, based on a molar absorptivity of 30 000 cm⁻¹ (18), and (b) by determination in a vitamin B12 assay. The holoTC calibrator set routinely included the following concentrations: 0, 10, 20, 40, 80, and 160 pmol/L. We included 2 additional concentrations: 30 pmol/L was prepared with a starting volume of 75 μL of 40 pmol/L calibrator, and 200 pmol/L with 125 μL of 160 pmol/L calibrator. The holoTC calibrator set was also used for total TC; these were analyzed as the holoTC calibrators, i.e., without saturation with cyanocobalamin (Fig. 1). Because we used one-tenth the plasma volume for total TC compared with holoTC, the results were multiplied by 10.

**Calibrator samples for cobalamin determination.** A 50 μg/L solution of cyanocobalamin of was first prepared and the concentration checked by the absorbance at 362 nm, based on a molar absorptivity of 30 000 cm⁻¹. The solution was then diluted to 100 μg/L in water, divided into aliquots, and stored at –80 °C. Each aliquot was thawed only once. Starting with an initial dilution to 50 ng/L (36.9 pmol/L), we made the following calibrator samples: 0, 1.36, 2.04, 2.72, 3.39, 4.14, 5.44, 8.16, 10.89, 13.61, 19.06, and 24.50 pmol/L. Because calibrators were diluted less than plasma samples (Fig. 1), these calibrator samples corresponded to plasma concentrations of 0, 52, 77, 103, 129, 157, 206, 310, 413, 516, 723, and 930 pmol/L.

**Performance of MBA for holoTC and total TC**

**Samples for testing linearity and imprecision.** For holoTC, 2 pools of human plasma with no added cobalamin were used: Sample 1 (S1) contained a holoTC concentration close to the upper reference limit, and S5 contained a holoTC concentration below the lower reference limit. S1 and S5 were then mixed so that samples S2, S3, and S4 contained 50%, 25%, and 12.5% of S1 and 50%, 75%, and 87.5% of S5, respectively. For total TC, we used 3 pools of human plasma; in pool 1 (P1) the concentration was above the upper reference limit, in P2 within the reference
interval, and in P3 in the low end of the reference interval. To assess variation in the measurement of TC saturation, we used 20 samples from the reference population (see below) that differed in TC saturation, holoTC, and total TC concentration.

**Within- and between-day variation.** We assessed the within-day CV by measuring 10 parallels of samples S1 through S5 for cobalamin and for holoTC, and 10 parallels of pools P1 through P3 for total TC. We assessed the between-day CV by measuring the same samples and pools over a total of 8 days: Each sample (S1 through S5) was measured in duplicate for cobalamin and holoTC and each pool (P1 through P3) in duplicate for total TC. In addition, we assessed between-day variation for holoTC by analyzing the control samples for holoTC provided by Axis-Shield.

**Linearity.** We assessed holoTC linearity with samples S1 through S5, as described previously (19, 20). The “true values” of S1 and S5 were estimated as the overall means obtained from the between-day experiments. The “true concentrations” of S2, S3, and S4 were calculated from the fractional composition of S1 and S5 and then compared with the measured results.
Comparison of MBA assays with established methods. Using samples from the reference population (see below), we compared holoTC results obtained by MBA with those from a commercial holoTC assay (13). In the commercial assay, the magnetic beads for capturing TC are the same, but cobalamins are determined by RIA. Total TC measurement by MBA was compared with total TC determination by ELISA (14). In the ELISA, γ-globulins from 2 polyclonal rabbit antibodies against recombinant human TC were used as capture and detection antibodies, and recombinant human TC was used as a calibrator.

Matrix effects. Using 30 samples with variable holoTC (range, 28–227 pmol/L) and total TC concentrations (range, 477–2088 pmol/L), we searched for matrix effects by measuring cobalamin, holoTC, and total TC in serum and in EDTA-, heparin-, and citrate plasma.

REFERENCE POPULATION
The reference population included 500 healthy, nonfasting blood donors, ages 18–69 years. Blood samples were collected between 0730 and 1130 in the morning, at the end of each blood donation. Blood was collected into tubes without additives (for serum) and into tubes containing EDTA, heparin, or citrate as anticoagulant. Tubes with anticoagulant were placed on ice, and serum tubes were kept at room temperature. The tubes were centrifuged within 2 h, and aliquots of plasma and serum were collected and stored at −80 °C. From each participant, serum and EDTA whole blood were sent to the clinical chemistry laboratory at Haukeland University Hospital for measurement of serum creatinine and hematologic variables with Technicon Chem 1® (Bayer) and CELL-DYN® 4000 (Abbott) platforms. In addition to cobalamin, holoTC, and total TC measurements, serum folate was determined by MBA (21, 22), and tHcy, total cysteine, methionine, and methylmalonic acid (MMA) were measured with a modified gas chromatography–mass spectrometry method based on ethylchloroformate derivatization (23). The TCN2 766C>G polymorphism was determined with universal energy transfer–labeled primers (Amplifluor™; Chemicon International) combined with allele-specific amplification in a real-time PCR method (24).

The study was unlinked and anonymous, but each participant completed a simple questionnaire through which we obtained data on sex, age, time of blood collection, time of last meal, time of last big meal (i.e., dinner), use of vitamin supplements, and whether the person was a smoker, ex-smoker, or never-smoker. The study was approved by the regional ethics committee, and the participants gave informed consent.

STATISTICAL ANALYSES
Markedly skewed variables (cobalamin, holoTC, total TC, TC saturation, tHcy, MMA, folate) were logarithmically transformed before statistical analysis. For comparison between groups, the Student t-test for independent samples, ANOVA, or the χ² test was used. When significant differences among the means were observed, a post hoc test with Bonferroni correction was performed to identify significantly different group means. Matrix effects were tested by paired-sample t-test. Comparison of methods was performed by Spearman correlations and by Bland–Altman plots (25).

Results
O b j e c t i v e s
Optimization and performance of the holoTC and total TC assays
Initial experiments showed that the MBA could be used instead of RIA for cobalamin measurements and that adequate sensitivity and precision were achieved with 100 µL of plasma. In samples with very low holoTC concentrations, sensitivity and precision could be increased when a higher plasma volume was used. In the total TC assay, incubation of the sample in the presence of a cyanocobalamin concentration ~10 times the mean total TC concentration ensured maximum saturation without demanding repeated washing steps to remove excess cyanocobalamin. The optimized procedures for cobalamin, holoTC, total TC, and their calibrators are described in Fig. 1.

Performance. We assessed linearity for the holoTC assay with 5 samples with interrelated concentrations (see Table S1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol52/issue1/). The measured holoTC values were close to the expected values, indicating that the assay was linear from below to above the reference interval.

The between-day CVs were 4.3%–9.3% for holoTC and 6.8%–7.2% for total TC. Between-day CVs were 1%–2% lower when we used duplicate rather than singular measurements (see Table S1 in the online Data Supplement). We assessed long-term imprecision for holoTC with the control samples, which were measured in duplicate in each run. Over a 15-month period, 2 technicians performed 75 different runs, with 3 batches of control samples. The between-day CVs were 3.8%–5.7% for the high control samples and 4.9%–7.3% for the low control samples.

Imprecision of TC saturation was difficult to assess because we usually measured total TC and holoTC on different days. We compared the variation between duplicate measurements of total TC, holoTC, and TC saturation with 20 samples, all measured on the same day with the same calibration curve. The mean CVs were 1.6%
which dilutes the blood by effect is attributable partly to the use of liquid citrate, EDTA for holoTC, 86% for total TC). This apparent matrix lower concentrations than other matrixes (81% relative to total TC in EDTA plasma, heparin plasma, and serum.

Comparison between methods. Using all samples from the reference population, we found that holoTC by MBA strongly correlated \((r = 0.95\), Spearman correlation) with holoTC by RIA (13), but the values were consistently \(\sim 20\%\) higher by MBA than by RIA, as confirmed by a Bland–Altman plot (see Fig. S1 in the online Data Supplement). Total TC by MBA had a correlation coefficient of 0.79 with total TC by ELISA (14), and the values were \(\sim 6\%\) higher than those obtained by ELISA. A Bland–Altman plot revealed that the difference between the 2 methods was small at low total TC concentrations but increased significantly at higher TC concentrations (see Fig. S1 in the online Data Supplement). Furthermore, the difference in total TC concentrations between methods depended on the TCN2 776 C>G genotype; it was greatest for the GG genotype (MBA 11.5% above ELISA), intermediate for the CG genotype (6.4%), and least for the CC genotype (3.6%; data not shown).

Matrix effects. In some methods, holoTC and total TC concentrations are higher in EDTA plasma than in serum (13, 14). With our MBA, concentrations of holoTC and total TC in EDTA plasma, heparin plasma, and serum were similar, whereas citrate plasma had significantly lower concentrations than other matrices (81% relative to EDTA for holoTC, 86% for total TC). This apparent matrix effect is attributable partly to the use of liquid citrate, which dilutes the blood by \(\sim 10\%\).

Variables associated with holoTC and total TC

We examined blood samples from 500 healthy blood donors for physiologic and biochemical variables associated with holoTC and total TC. The characteristics of the total population and for men and women separately are listed in Table S2 in the online Data Supplement. Median age was 44 years; 49% of the donors were women, 21.7% were smokers, and 92.5% were nonfasting at the time of blood collection. Multi- or B-vitamin supplements were used by 15.6%. Cobalamin, holoTC, and TC saturation were significantly lower in women than in men \((P < 0.05)\); we therefore routinely performed separate analyses for men and women.

The Spearman correlations between cobalamin, holoTC, and total TC and the various physiologic and biochemical variables are shown in Table 1. The correlations for TC saturation were very similar to those for holoTC, and usually slightly weaker (data not shown). Results obtained by MBA were used, but with few exceptions (listed below), we found very similar results for holoTC by RIA and total TC by ELISA.

### Table 1. Correlations in 254 men and 244 women, 18–69 years of age.\(^a\)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cobalamin</th>
<th>HoloTC</th>
<th>Total TC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Men</td>
<td>Women</td>
<td>Men</td>
</tr>
<tr>
<td>HoloTC</td>
<td>(0.65^a)</td>
<td>(0.61^a)</td>
<td></td>
</tr>
<tr>
<td>Total TC</td>
<td>(-0.08)</td>
<td>(0.04)</td>
<td>(0.14^a)</td>
</tr>
<tr>
<td>TC saturation</td>
<td>(0.65^a)</td>
<td>(0.58^a)</td>
<td>(0.85^a)</td>
</tr>
<tr>
<td>Age</td>
<td>(-0.08)</td>
<td>(0.23^a)</td>
<td></td>
</tr>
<tr>
<td>Creatinine</td>
<td>(0.00)</td>
<td>(-0.02)</td>
<td>(0.13^a)</td>
</tr>
<tr>
<td>Plasma folate</td>
<td>(0.09)</td>
<td>(0.25^a)</td>
<td>(0.22^a)</td>
</tr>
<tr>
<td>Total cysteine</td>
<td>(0.13^a)</td>
<td>(0.12)</td>
<td>(0.16^a)</td>
</tr>
<tr>
<td>IHcy</td>
<td>(-0.18^a)</td>
<td>(-0.21^a)</td>
<td>(-0.28^a)</td>
</tr>
<tr>
<td>MMA</td>
<td>(-0.07)</td>
<td>(-0.16^a)</td>
<td>(-0.18^a)</td>
</tr>
<tr>
<td>TCN2 776 C&gt;G</td>
<td>(0.07)</td>
<td>(0.03)</td>
<td>(-0.05)</td>
</tr>
</tbody>
</table>

\(^a\) Spearman rank correlations. Significant correlations are indicated in bold.

**Interrrelationships between cobalamin, holoTC, and total TC.** As expected, the interrelationships between cobalamin, holoTC, and TC saturation were strong (Table 1). In our data set, \(\sim 27\%\) of plasma cobalamin was bound to TC (holoTC), and \(\sim 9\%\) of TC existed as holoTC (TC saturation; see Table S2 in the online Data Supplement). These relationships changed, however, according to the cobalamin concentration: In the top 5th percentile of the cobalamin distribution (median cobalamin, 534 pmol/L), \(\sim 22\%\) existed as holoTC (median holoTC, 119 pmol/L), whereas in the bottom 5th percentile of the cobalamin distribution (median cobalamin, 157 pmol/L), \(\sim 37\%\) was in the form of holoTC (median holoTC, 57 pmol/L). The corresponding numbers for TC saturation were \(\sim 6\%\) and \(\sim 14\%\), respectively.

There was a weak but significant positive association between holoTC and total TC, which remained significant in linear regression models after adjustment for age, sex, TCN2 genotypes, and the other variables listed in Table 1. This association was also present when we replaced the MBA values with holoTC by RIA and total TC by ELISA (\(r_s = 0.16\)).

Fasting state, smoking status, and B-vitamin supplements. Our samples were collected between 0730 and 1130 in the morning, and the group naturally separated into those who had a meal in the morning (sample obtained \(\leq 5\ h\) after a meal) and those who did not (\(> 8\ h\); fasting state). There were no differences in holoTC and total TC concentrations in fasting vs nonfasting individuals, and we found no correlation between time since last meal and holoTC or total TC concentrations. Neither smoking status nor use of B-vitamin or multivitamin supplements was associated with cobalamin, holoTC, or total TC.

Age, sex, and creatinine. Both cobalamin and holoTC concentrations were lower in women than in men, and they increased with age. Further analyses revealed that the age effect was limited to women, and the sex differences were
confined to those ≤45 years (see Fig. S2 and Table S3 in the online Data Supplement). In women ≤45 years of age, there was a complete shift of the holoTC distribution toward lower concentrations (see Fig. S3 in the online Data Supplement).

There was no significant sex difference in total TC concentrations, but total TC increased with age in women but not in men (see Fig. S2 and Table S3 in the online Data Supplement).

In these healthy blood donors, serum creatinine was not associated with total TC in either sex, and it was not associated (women) or was weakly associated (men) with holoTC. Further analyses showed that the positive association between creatinine and holoTC was confined to men >45 years of age (r_s = 0.25).

Plasma amino acids and MMA. HoloTC was inversely associated with tHcy and MMA concentrations. The association between holoTC and tHcy or MMA remained significant in multivariate regression analyses. The lower association between holoTC and tHcy or MMA remained associated with tHcy and MMA concentrations. The association between cysteine and holoTC (both sexes) and total TC remained significant, and for holoTC, it became stronger (women). In regression analyses, this association disappeared after age was included in the model. There was a significant association between cysteine and holoTC in men, but not in women (data not shown). In a regression analysis, the association disappeared after age was included in the model. There was a significant association between cysteine and holoTC (both sexes) and total TC (men). In regression analyses, this association remained significant, and for holoTC, it became stronger after adjustment for age, tHcy, creatinine, or the other variables listed in Table 1.

Plasma folate and hematologic variables. Cobalamin in women and holoTC in both sexes were associated with plasma folate (Table 1). This association remained significant after adjustment for age and creatinine concentration, but became nonsignificant after inclusion of tHcy in the model. In women, holoTC was weakly but significantly associated with hemoglobin concentrations (r_s = 0.20), hematocrit (r_s = 0.16), and mean corpuscular volume (r_s = 0.13), and total TC was associated with hemoglobin (r_s = 0.17) and hematocrit (r_s = 0.14). In linear regression analyses, neither holoTC nor total TC remained significantly associated with these variables after inclusion of age and total cysteine in the model.

TCN2 776C>G polymorphism. The allele frequency of the TCN2 C>G polymorphism was 39.8%, and 16% of the population had the GG genotype. Total TC concentrations decreased with the number of G alleles (geometric means, 986 vs 908 vs 847 pmol/L; P < 0.001, adjusted for age and sex). The genotype effect was more pronounced when total TC concentrations were measured by ELISA (geometric means, 957 vs 863 vs 764 pmol/L; P < 0.001, adjusted for age and sex). In regression analyses including age, sex, tHcy, cysteine, holoTC, and the TCN2 genotype, the genotype was by far the strongest determinant of total TC (partial R for MBA = −0.27, for ELISA = −0.43). There was no effect of the polymorphism on cobalamin or holoTC concentrations in the total group, but an analysis confined to participants >45 years of age showed a significant difference in holoTC concentrations according to genotype in women (geometric mean, 102 vs 86 vs 80 pmol/L; P = 0.041, adjusted for age) but not in men (P = 0.22).

REFERENCE VALUES IN HEALTHY BLOOD DONORS

The reference values in the total population and according to age and sex are listed in Table 2. An obvious difference between the groups was that reference values for cobalamin, holoTC, and TC saturation were lower for younger women than for the rest of the population.

We compared the reference values for holoTC (13, 14) obtained by MBA with those obtained by the other methods and found that the 5th to 95th percentile intervals were 15%–20% lower (36–129 pmol/L) for holoTC by RIA and 1%–6% lower (666–1189 pmol/L) for total TC by ELISA.

Discussion

Using an MBA to detect the cobalamin moiety, we measured cobalamin, holoTC, and total TC with only ~150 µL of plasma. The performance of the MBA was comparable to other assays (5, 13–15). According to our data, a single measurement is sufficient for most purposes. Because holoTC is up-concentrated by use of magnetic beads with TC antibodies (13), the lower limit of detection and the measurement precision can be further improved by use of

| Table 2. Reference values in healthy blood donors.\(^a\) |
|-----------------|-----------------|
|                 | Total population | Men | Women |
|                 | (18–69 years)    | 18–45 years | 46–68 years | 19–45 years | 46–69 years |
| n               |                 | 135 | 115 | 142 | 95 |
| HoloTC, pmol/L   | 42–157         | 46–152 | 41–176 | 34–141 | 48–174 |
| Total TC, pmol/L | 670–1270       | 624–1288 | 650–1296 | 671–1224 | 699–1297 |
| TC saturation, % | 4.4–16.9       | 5.0–17.3 | 3.8–17.9 | 4.0–15.0 | 4.7–16.9 |

\(^a\) 5th–95th percentiles.
a larger plasma volume in samples with very low holoTC concentrations.

We found that holoTC determined by MBA correlated strongly with an established RIA (13), but values were ~20% higher by MBA. This observed difference is probably caused by slightly different holoTC concentrations in different calibrator batches. Another possible cause is the presence of cobalamin analogs. Intrinsic factor–based assays, such as holoTC by RIA, have low affinity for analogs (26). In contrast, L. leichmannii can use some, but not all, analogs for growth (27, 28). In the total TC and holoTC assays, however, we measured cobalamin bound to TC. Although not as specific as intrinsic factor, TC has low affinity for analogs (26). It is thus unlikely that the presence of analogs explains the systematic 20% difference in holoTC measurements between the MBA and RIA.

The mean total TC concentrations measured by MBA were similar to total TC concentrations by ELISA. The correlation coefficient was relatively low, however, possibly as a consequence of low between-person variability in total TC, because correlation between measurements is dependent on the variability between samples (29). Furthermore, the 2 methods have a different approach: MBA measures the cobalamin moiety (16), whereas ELISA measures the protein moiety (14). Interestingly, the difference in total TC concentrations was most pronounced for the TCN2 776GG genotype, a finding that suggests that one or both assays give a systematic error depending on genotype, a matter that needs further elucidation.

In line with previous data (4–6, 30), we observed that ~27% of plasma cobalamin existed as holoTC, whereas TC saturation was ~9%. However, our data show that these proportions change according to cobalamin status: at low concentrations, a higher proportion of cobalamin exists as holoTC, suggesting that holoTC is better maintained than haptocorrins. In contrast, TC saturation becomes lower. Thus, the typical finding in individuals with low cobalamin concentrations will be a relatively high holoTC/cobalamin ratio, whereas TC saturation will be low.

As expected (5, 9, 30), holoTC concentrations were independently associated with tHcy and MMA, but the associations were relatively weak. This finding may be related to a generally good cobalamin status in the healthy blood donors. We assume that donor health status also explains the observation that none of the cobalamin variables was independently associated with the hematologic variables. More surprising is our observation that holoTC was independently associated with plasma folate and total cysteine. This relationship between holoTC and folate might indicate that individuals with good folate status also tend to have good cobalamin status. In relation to cysteine, one possible explanation could be that low holoTC is associated with high tHcy, which may displace cysteine from plasma (31). However, entering tHcy and possible confounders in the regression model strengthened the association. The clinical significance of these associations is uncertain.

Both holoTC and total TC are increased in renal failure (32, 33), and serum creatinine is an important determinant of holoTC concentrations in elderly persons and in individuals with impaired cobalamin status (9, 30). In our population, creatinine was associated with holoTC and TC concentrations only in men >45 years. Notably, creatinine is not a particularly good marker of renal function (34), and before old age, creatinine is actually positively associated with a healthy lifestyle (35). Furthermore, increased creatinine consistent with renal impairment was not observed in this population; only 1 of 500 individuals had creatinine above the upper limit of 120 μmol/L. Thus, our data suggest that, in healthy adults, creatinine within the reference interval is not a determinant of holoTC or total TC.

As reported previously (36, 37), we found that total TC concentrations decreased with the number of G alleles. In our data set, the TCN2 766C>G polymorphism was the strongest determinant of total TC concentrations, particularly when measured by ELISA. In contrast to published data (8, 38), the effect of the TCN2 polymorphism on cobalamin or holoTC concentrations was nonsignificant except in women >45 years. Overall, our data suggest that it is important to take this polymorphism into account when studying associations between total TC and clinical outcome.

Results of most other studies, based on smaller sample sizes or older study populations, suggested that there is no effect of age on holoTC (5, 15, 30) and that women have holoTC concentrations higher than (5) or similar to (15) those in men. These results contrast with our findings. Both cobalamin and holoTC concentrations were lower in women than in men, and they increased with age in women. Total TC increased moderately with age in women but not in men, confirming the findings of Nexo et al. (14), which suggested that that total TC in women <50 years might be lower than in older women.

In the reference population, the reference values in these blood donors were similar to those observed in another population of healthy blood donors (14, 15). In addition, we found that younger women had lower reference values for cobalamin, holoTC, and TC saturation than the rest of the population. This finding was related to a complete shift in cobalamin concentrations to the left, which was not associated with metabolic effects on tHcy or MMA. The observed effects in young women may be related to hormonal factors, because estrogen intake in men (39) and use of oral contraceptives in premenopausal women (40, 41) have been associated with lower cobalamin and holoTC concentrations. We lacked data on the use of hormonal replacement therapy or oral contraceptives; however, we observed the same low concentrations in women 35–45 years of age, a group that traditionally does not use oral contraceptives—in 40-year-old Norwegians, only 3.5% of the women used
combined hormonal contraceptives (42). Thus, it is unlikely that hormone use alone can explain the ~20% lower holoTC values in women ≤45 years.

Because of limited data collection, a shortcoming of our study, we do not have data on use of oral contraceptives or hormone replacement therapy, nor do we have any measurements of iron status, which is of central importance for evaluating associations to hematologic factors. Another potential weakness with this population was that we collected blood at the end of a routine blood donor session. Hemodilution develops gradually and can be expected to change hemoglobin values by ~5%, and less in those with a high hematocrit (43). We observed the same pattern in relation to age and sex when we adjusted for hematocrit or excluded individuals with low hematocrits.

In summary, our data show that age and sex are determinants of holoTC, total TC, and cobalamin concentrations in adults younger than 70 years, whereas serum creatinine is not. The major determinant of total TC concentration was the TCN2 776C>G genotype. Further investigations to elaborate accurate age- and sex-specific reference values are warranted.

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