

# Measurement of Transcobalamin by ELISA

EBBA NEXO,<sup>1\*</sup> ANNA-LISA CHRISTENSEN,<sup>1</sup> TORBEN E. PETERSEN,<sup>2</sup> and SERGEY N. FEDOSOV<sup>2</sup>

**Background:** Transcobalamin is essential for the cellular internalization of cobalamin. Methods to quantify the unsaturated protein are available, but few attempts have been made to develop methods to quantify the sum of unsaturated and cobalamin saturated transcobalamin.

**Methods:**  $\gamma$ -Globulins from two polyclonal rabbit antibodies against recombinant human transcobalamin were used as capture and detection antibodies, and recombinant human transcobalamin was used as calibrator in an ELISA design.

**Results:** The ELISA is specific for transcobalamin and has a detection limit of  $<1.6$  pmol/L. The imprecision (CV) is 4–6% for mean concentrations of 13–70 pmol/L. The central 95% interval for serum from healthy blood donors ( $n = 77$ ) was  $\sim 600$ –1500 pmol/L and showed limited variation with age and sex. No correlation was observed between the marker of acute phase reaction, C-reactive protein, and transcobalamin in plasma.

**Conclusions:** The ELISA measures total transcobalamin in serum and thus can be used for measurement of transcobalamin in patients treated with cobalamin.

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Cobalamin (vitamin B<sub>12</sub>) is an essential nutrient that is absorbed and distributed by three soluble proteins. Intrinsic factor is needed for its uptake in the intestine, and plasma transcobalamin ensures the internalization of the vitamin into all cells of the body. The third protein, plasma haptocorrin, carries most of the circulating cobalamin, but its function is unknown (1, 2).

The central role of transcobalamin in the metabolism of cobalamin has challenged researchers since its discovery in the 1960s (3). An important challenge is to diagnose children born with transcobalamin deficiency, a rare autosomal recessive condition in which the child presents with a broad spectrum of symptoms, including impaired

development, susceptibility to infections, and anemia (4, 5). Another challenge is to further explore the observation that decreased cobalamin-saturated transcobalamin is an early marker of cobalamin deficiency (6, 7).

Unsaturated transcobalamin can be measured by labeled cobalamin, but measurement of total transcobalamin and cobalamin-saturated transcobalamin is somewhat more complicated (8). Development of immunological methods for measurement of transcobalamin have been hampered by the limited availability of pure human transcobalamin and by the fact that this protein easily precipitates in solution. Recently, we expressed human transcobalamin in yeast and showed that the purified protein remains stable in solution (9).

In this report, we describe the development of an ELISA to quantify total transcobalamin in human serum or plasma.

## Materials and Methods

### TRANSCOBALAMIN, ANTIBODIES, AND CALIBRATORS

Human transcobalamin was cloned and purified as described previously (9), and used for preparation of calibrators and for production of rabbit antibodies. The antibodies were produced in three rabbits by Dako A/S, Denmark. The rabbits were each immunized with 300  $\mu$ g of human recombinant transcobalamin mixed with 50  $\mu$ L of 100 nmol/L NaCl four times with 4-week intervals. The  $\gamma$ -globulin fraction of serum obtained from each of the three rabbits (F5121, F5123, and F4957) was purified by chromatography as described previously (10). Calibrators were prepared from a stock solution of 4.34 g/L (94.3  $\mu$ mol/L) human recombinant transcobalamin saturated with hydroxocobalamin. This stock solution was diluted in polystyrene test tubes with assay buffer, composed of 0.1 mol/L sodium phosphate, pH 8.0, containing 1 g/L human albumin (Behringwerke) to obtain calibrators ranging from 1.6 to 100 pmol/L. The assay buffer was used as zero calibrator.

### ELISA

The ELISA was based on an immobilized antibody that captures the analyte and a biotinylated detection antibody that reacts with horseradish peroxidase-avidin and produces a color reaction that increases with increasing

<sup>1</sup> Department of Clinical Biochemistry, AKH, Aarhus University Hospital, Norrebrogade 44, DK-8000 Aarhus C, Denmark.

<sup>2</sup> Protein Chemistry Laboratory, Department of Molecular and Structural Biology, University of Aarhus, DK-8000 Aarhus, Denmark.

\*Author for correspondence. Fax 45-8949-3060; e-mail ene@post9.tele.dk.

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concentration of the analyte. The ELISA was established in three steps: (a) the  $\gamma$ -globulin fraction from each of the three rabbits was tested in a checkerboard design to select the optimal combination of capture and detection antibodies; (b) the optimal dilution of the detection antibody was established; and (c) the characteristics of the assay were studied.

Coating of the ELISA plates and preparation of the biotinylated antibody were performed essentially as described previously for epidermal growth factor (11). Briefly, MaxiSorp F-96 immunoplates (Life Technologies) were coated by adding 0.8  $\mu$ g of anti-human transcobalamin IgG (F5123, F4957, or F5121) in 100  $\mu$ L of 15 mmol/L sodium carbonate, 35 mmol/L sodium bicarbonate, pH 9.6, to each well. The plates were incubated for 20 h at 4 °C before the wells were emptied and 200  $\mu$ L of 1 mol/L ethanolamine, pH 8–9, was added to each well. The plates were incubated for 20 h at 4 °C and subsequently stored at –20 °C.

For evaluation as detection antibody, the same three anti-human transcobalamin IgGs were biotinylated. Briefly, 1 mL of IgG (1 g/L) that had been dialyzed overnight against 0.1 mol/L sodium bicarbonate, pH 8.3, was mixed gently for 4 h at room temperature in the dark with 50  $\mu$ L of 4.4 mmol/L biotinamidocaproate *N*-hydroxysuccinimide ester (Sigma) dissolved in dimethyl sulfoxide (Merck). Subsequently, 50  $\mu$ L of 0.1 mol/L L-lysine (Fluka) was added, and after 15 min, 50  $\mu$ g of rabbit  $\gamma$ -globulin (Calbiochem) was added. The sample was dialyzed for 72 h against 10 mmol/L sodium phosphate buffer containing 145 mmol/L NaCl; 1 g/L sodium azide was added to the buffer during the last 24 h. The biotinylated IgG was stored at –20 °C, and before use it was diluted in assay buffer.

For each assay, plates were washed three times with 10 mmol/L sodium phosphate buffer, pH 7.4, containing 145 mmol/L NaCl, 1 g/L bovine albumin (Sigma), and 1 g/L Tween 20 (washing buffer). Calibrator, control, or sample (100  $\mu$ L) was then added in duplicate and incubated for 30 min. After the plates were washed three times, 100  $\mu$ L of biotinylated antibody was added, and the plates were incubated for 30 min. After the plates were washed three times, 100  $\mu$ L of horseradish peroxidase-avidin (Dakopatt) diluted 1:2000 in 10 mmol/L sodium phosphate, pH 7.4, 400 mmol/L NaCl, 0.2 g/L lysozyme (Sigma) was added to each well, and the plates were again incubated for 30 min, followed by three washes. The color reaction was developed by the addition of 100  $\mu$ L of TMB Microwell Substrate System (Kirkegaard and Perry Laboratories) to each well and incubation at room temperature for ~10 min. The reaction was stopped by the addition of 100  $\mu$ L of 1 mol/L phosphoric acid to each well. The color developed was measured photometrically at 450 nm and corrected for the absorbance at 620 nm. In the final step of the analysis, 1 nmol/L cyanocobalamin (Sigma) was added to the assay buffer to ensure that all transcobalamin was saturated with cobalamin before analysis. A

serum pool diluted 1:20 (to ~50 pmol/L transcobalamin) was used as a daily control.

#### INTERFERING SUBSTANCES

Human haptocorrin and human intrinsic factor (12) were prepared at a concentration of 100 nmol/L in assay buffer to test possible cross-reactivity.

Human serum was fractionated using a SMART system (Pharmacia) with a Superdex 200 HR10/30 column and 0.1 mol/L Tris, pH 8.0, 1 mol/L NaCl, 0.2 g/L sodium azide, 0.5 g/L human albumin. Fractions of 400  $\mu$ L were collected and analyzed for transcobalamin.

Blood for preparation of plasma (EDTA) and serum was obtained from 20 healthy blood donors to examine whether the two matrixes can be used interchangeably.

#### LINEARITY AND IMPRECISION

Linearity and imprecision were tested essentially as described previously (13).

A pool of human plasma diluted 1:20 with assay buffer (S1) was prepared and diluted to contain 80% (S2), 60% (S3) 40% (S4), and 20% (S5) S1. Based on the ELISA measurements, we assigned a transcobalamin concentration of 40 pmol/L to S3, and the concentrations in the other pools were calculated from this value. Aliquots of each S sample were frozen at –20 °C until analyzed. The samples were analyzed in duplicate in 27 independent analyses over a period of 2 months, using three different batches of coated microtiter plates.

#### CENTRAL 95% INTERVAL FOR HEALTHY BLOOD DONORS

Blood for preparation of serum was obtained from 77 healthy blood donors [37 women and 40 men; age groups, <50 years (n = 39) and  $\geq$ 50 years (n = 38)].

#### TOTAL TRANSCOBALAMIN COMPARED WITH UNSATURATED TRANSCOBALAMIN

The samples used to establish the central 95% interval were analyzed for unsaturated transcobalamin and haptocorrin as described previously (14). Briefly, 250 fmol (25  $\mu$ L) of  $^{57}$ Co-labeled cyanocobalamin (Amersham-Pharmacia Biotech), adjusted to a specific activity of 1.5 pmol/ $\mu$ Ci by the addition of cyanocobalamin was incubated with 100  $\mu$ L of serum and 200  $\mu$ L of assay buffer for 15 min. Free and protein-bound cobalamin were separated by the addition of 500  $\mu$ L of hemoglobin-coated charcoal [2.5 g of charcoal (Difco) and 0.25 g of hemoglobin (Sigma) in 100 mL of 0.154 mol/L NaCl], incubation for 10 min, and centrifugation for 10 min at 2500g. The supernatant containing labeled transcobalamin and haptocorrin was removed and added to 100  $\mu$ L of silica gel suspension [40 g of FK383DS silica gel (Bie & Berntsen) dissolved in 1 L of 0.154 mol/L NaCl]. After centrifugation for 10 min at 2500g, the supernatant containing haptocorrin and the

precipitate containing transcobalamin were counted in a gamma counter (Wallac), and the concentration of the unsaturated binding proteins was calculated from the specific activity of the labeled cyanocobalamin. The method has been characterized previously (15).

#### TRANSCOBALAMIN AS AN ACUTE PHASE REACTANT

Plasma samples ( $n = 59$ ) with C-reactive protein concentrations up to 3660 nmol/L, as determined by a routine immunological method according to the manufacturer (Integra; Roche Diagnostics), were obtained from the routine laboratory and analyzed for transcobalamin.

#### STATISTICAL ANALYSES

The total and within-day variances were calculated using nested analysis of variance. Regression analysis was used to analyze linearity, the relationship between unsaturated and total transcobalamin, and the relationship between results for C-reactive protein and transcobalamin. An unpaired *t*-test (Mann-Whitney) was used to analyze the central 95% interval as a function of age and sex, and a paired *t*-test was used to compare results for plasma and serum transcobalamin. The GraphPad Prism program (GraphPad Software) was used for data analyses.

## Results

#### ESTABLISHMENT OF THE ELISA

We used an assay format with a capture antibody immobilized on microtiter plates and a biotinylated detection antibody visualized via an avidin-coupled enzymatic reaction.

Three polyclonal rabbit anti-recombinant human transcobalamin antibodies were tested as both capture and detection antibodies according to a previously described method (16). All three antibodies performed equally well as capture antibodies, and two of the three antibodies performed comparably as detection antibodies (F4957 and F5123; Fig. 1A). Our choice was to develop the ELISA using F5123 as capture and F4957 as detection antibody. Although we used a standard amount of 0.8  $\mu\text{g}$  of IgG per well as capture antibody, the detection antibody was titrated to optimize the analytical range of the assay and the incubation time needed to develop the color reaction (Fig. 1B). Our choice was to use 0.09  $\mu\text{g}$ /well of the detection antibody, which allowed for a color development of  $\sim 10$  min.

The range of transcobalamin concentrations in our ELISA was 1.6–100 pmol/L (Fig. 2A). The signal obtained for the lowest calibrator was 1.7–3.4 times the signal

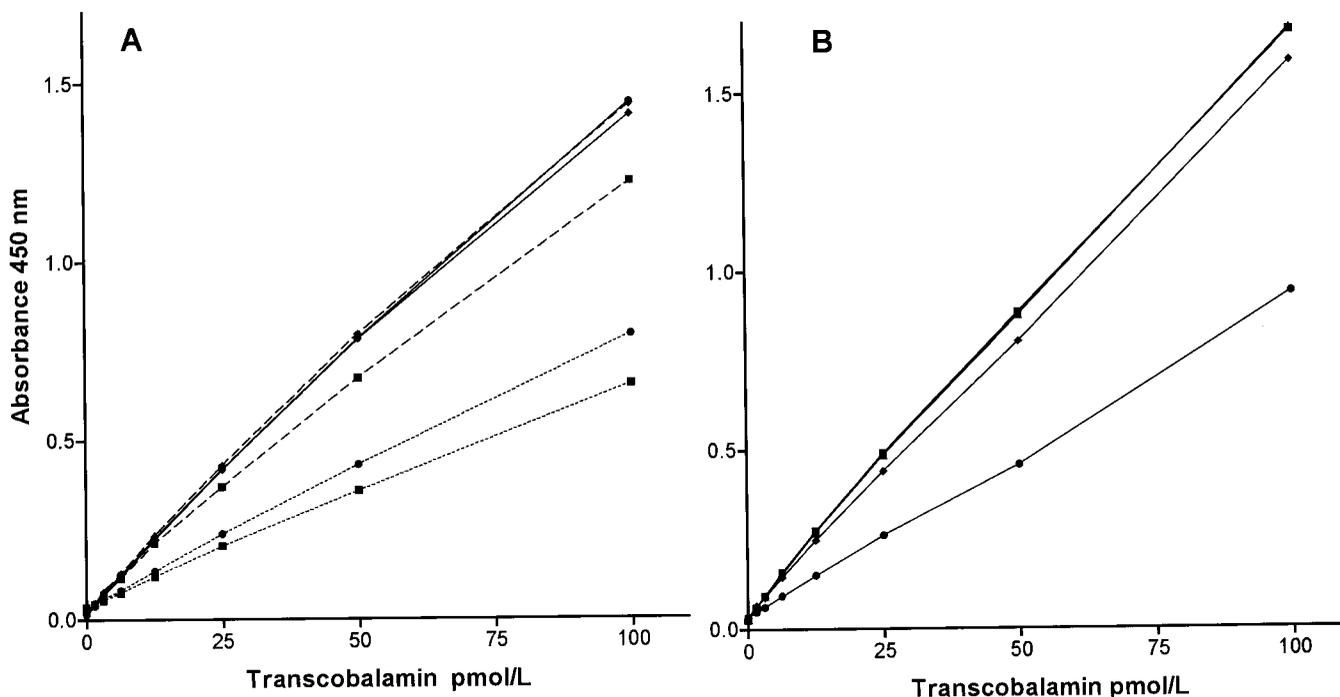


Fig. 1. Evaluation of antibodies for an ELISA for transcobalamin.

(A), three polyclonal rabbit antibodies were used as capture and detection antibodies in an ELISA design using avidin-coupled enzyme for measurement of the detection antibody. Calibration curves for the six combinations using different antibodies for capture and detection are shown. Capture antibodies: F4957 (■), F5121 (◆), and F5123 (●). Detection antibodies: F4957 (—), F5121 (···), and F5123 (---). Three combinations, F5123/F4957, F5121/F4957, and F5123/F5121 (capture/detection antibody), showed almost identical results. The antibody combination F5123 (capture antibody) and F4957 (detection antibody) was selected for development of the ELISA. (B), titration of the detection antibody F4957. A decrease in the amount of detection antibody to  $<0.09$   $\mu\text{g}$ /well increased the time needed for color development and reduced the final signal obtained. An amount of 0.09  $\mu\text{g}$ /well was chosen for further studies. Reaction conditions: ■, detection antibody, 0.11  $\mu\text{g}$ /well, 5-min color development; ▲, detection antibody, 0.09  $\mu\text{g}$ /well, 7-min color development; ◆, detection antibody, 0.05  $\mu\text{g}$ /well, 15-min color development; ●, detection antibody, 0.02  $\mu\text{g}$ /well, 20-min color development.

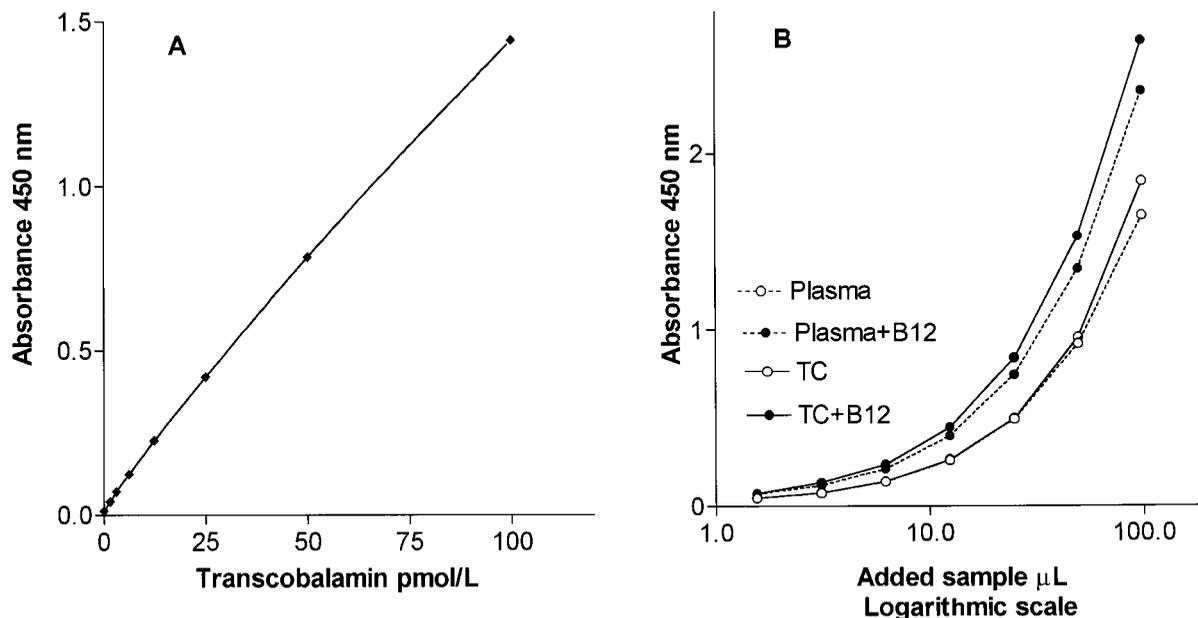


Fig. 2. Measurement of transcobalamin by ELISA.

An ELISA was developed that uses two rabbit antibodies against recombinant human transcobalamin as capture (F5123) and detection (F4957) antibodies and recombinant human transcobalamin as calibrator. (A), typical calibration curve covering transcobalamin concentrations of 1.6–100 pmol/L. (B), serial dilutions of a calibrator (TC) containing 0.1 pmol/L and a plasma sample prediluted 1:20 assayed in the presence (filled symbols) and absence (open symbols) of 1 mol/L added cyanocobalamin (B12). The x axis indicates μL of sample present in the dilution.

obtained for the zero calibrator (mean ± SD, 2.5 ± 0.3; n = 27). On the basis of these results, we judged the detection limit of the assay to be 1.6 pmol/L or lower. Because serum contains transcobalamin in a concentration of ~1000 pmol/L, serum and plasma samples were diluted 1:20 before analysis.

A dilution curve for the recombinant transcobalamin and for plasma is shown in Fig. 2B. Transcobalamin saturated with cobalamin showed a higher absorbency in the ELISA than did unsaturated transcobalamin. Because of that we chose to add cobalamin to both calibrators and samples before analysis. The similarity between the curves for plasma and recombinant transcobalamin supports that the antibodies recognize endogenous transcobalamin and recombinant transcobalamin equally well.

INTERFERING SUBSTANCES

No cross-reactivity was observed for the two other cobalamin-transporting proteins, intrinsic factor and haptocorrin. Concentrations of 100 nmol/L in the assayed samples, corresponding to a plasma concentration of 2000 nmol/L (>1000-fold higher than the usual concentration in plasma), were below the detection limit of the assay.

To ensure that the ELISA assay recognized only transcobalamin, we analyzed serum separated by gel filtration. Only one peak corresponding to the size of transcobalamin was observed (data not shown).

A comparison of the results obtained for 20 serum samples showed significantly higher values (P < 0.001) for plasma than for serum. Plasma values were on average 6% higher than the values obtained for serum. We did not explore this difference further, but chose to establish the central 95% interval for serum to match the material most often used for determination of total cobalamin.

**Table 1. Total and intraassay imprecision (CV) for measurement of transcobalamin by ELISA.<sup>a</sup>**

Assigned value, pmol/L	Measured mean, pmol/L	Estimates (runs), n	Total CV, %	Intraassay CV, %
66.7	73	53 (27)	4.0	2.3
53.3	55	55 (27)	4.4	2.5
40.0	39	52 (25)	6.2	2.4
26.7	26	54 (27)	5.8	2.6
13.3	13.7	54 (27)	4.1	2.9

<sup>a</sup> A pool of human plasma diluted 1:20 with assay buffer (S1) was prepared and diluted to contain 80%, 60% (S3), 40%, and 20% S1. The transcobalamin concentration in S3 was assigned to be 40 pmol/L, and the concentrations in the other pools were calculated from this value. The samples were analyzed in duplicate in 27 independent assays over a period of 2 months, using three different batches of coated microtiter plates.

**Table 2. Serum total transcobalamin in blood donors.**

Age	Range, pmol/L (n)		
	Males	Females	All
<50 years	560–1550 (18)	610–1400 (21)	
≥50 years	680–1400 (19)	560–1300 (19)	
All			560–1550 (77)

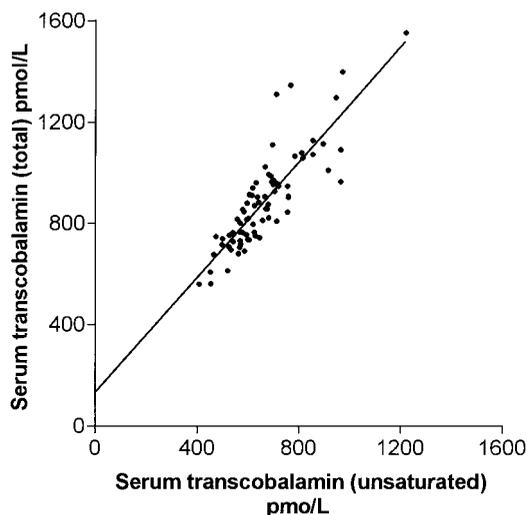


Fig. 3. Unsaturated cobalamin binding capacity for transcobalamin compared with total transcobalamin in serum samples from 77 blood donors.

Total transcobalamin was measured by ELISA, and unsaturated transcobalamin was measured based on the ability of the protein to bind labeled cobalamin.

#### LINEARITY AND IMPRECISION

Linearity and imprecision were tested by analyzing five diluted plasma samples (S1–S5) with interrelated concentrations (13.3–66.7 pmol/L) two times per assay in a total of 27 assays over a period of 2 months. Because there is no reference method, the exact concentrations were not known, but the relative concentrations in the samples were calculated based on the assumption that S3 had a concentration of 40 pmol/L. The results showed minor deviations from linearity in the range tested, but regression analyses ( $x$  = the assigned value;  $y$  = the measured value) showed a correlation coefficient of 0.997, and the intercept ( $-2.8 \pm 2.2$ ) did not deviate significantly from zero.

The total imprecision (CV) was 4.0–6.2%, and the intraassay imprecision was 2.3–2.9% (Table 1). Surprisingly, the assay performed equally well at low and high concentrations of the analyte, most likely because the lowest concentration tested (13 pmol/L) was substantially above the detection limit of the assay (1.6 pmol/L).

#### TOTAL TRANSCOBALAMIN COMPARED WITH UNSATURATED TRANSCOBALAMIN

The ELISA measures total transcobalamin. To date, most studies concerning transcobalamin have been performed with assays that quantify only cobalamin-unsaturated transcobalamin. We compared the results obtained for total and unsaturated transcobalamin in serum samples (Fig. 3). Total transcobalamin was measured by the ELISA, and unsaturated transcobalamin was measured from the ability of the protein to bind labeled cobalamin. As expected, higher values were obtained for total transcobalamin than for unsaturated transcobalamin, and the mean difference was 220 pmol/L. A correlation was

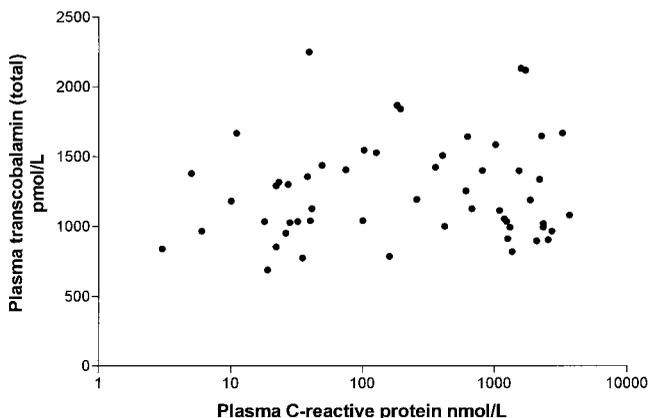


Fig. 4. Comparison of plasma C-reactive protein and total transcobalamin.

Plasma samples from 59 patients with plasma C-reactive protein concentrations  $\leq 3660$  nmol/L were analyzed. The reference interval for plasma C-reactive protein is  $<40$  nmol/L. Note that the x axis is given in a logarithmic scale.

observed between the concentration of unsaturated and total transcobalamin ( $r = 0.85$ ;  $P < 0.001$ ). The regression line showed a slope of  $1.13 \pm 0.08$ , and an intercept of  $135 \pm 54$  pmol/L (mean  $\pm$  SD).

#### SERUM TOTAL TRANSCOBALAMIN IN BLOOD DONORS

The central 95% interval for total transcobalamin in serum from healthy blood donors is given in Table 2. We found no statistical difference between men and women and no age-related difference in men. The values obtained for women below 50 years were significantly ( $P < 0.01$ ) lower than values obtained for women 50 years or older. Until this observation is substantiated, we suggest a common central 95% interval of  $\sim 600$ – $1500$  pmol/L.

#### TRANSCOBALAMIN IS NOT AN ACUTE PHASE REACTANT

Transcobalamin has been considered an acute phase reactant (1, 2). To examine the relationship between acute phase reaction and total transcobalamin, we analyzed plasma samples from 59 patients with various concentrations of C-reactive protein (reference interval,  $<40$  nmol/L). No correlation between transcobalamin and C-reactive protein was observed for plasma C-reactive protein concentrations up to 3660 nmol/L (Fig. 4).

#### Discussion

We have developed a specific and reproducible ELISA for quantification of the cobalamin-binding protein transcobalamin in serum samples. The concentration of transcobalamin ( $\sim 600$ – $1500$  pmol/L) in samples from healthy blood donors is comparable to values obtained by previously developed radioimmunoassays (17, 18).

All circulating cobalamin is attached to either transcobalamin or haptocorrin, and the two proteins also account for the unsaturated cobalamin binding capacity present in plasma (1). Thus, measurement of plasma cobalamins is at the same time a measure for the sum of haptocorrin and

transcobalamin saturated with cobalamin. Likewise, the cobalamin-binding capacity measures the sum of unsaturated transcobalamin and haptocorrin (1, 2).

Over the years, there has been increasing interest in methods that allow the two proteins to be examined independently. Studies of transcobalamin are important because this protein is essential for the cellular uptake of cobalamin (1, 2). Haptocorrin is also of some interest because concentrations of this protein of unknown function are increased in several malignancies, such as chronic myeloid leukemia (1, 19). It is important that methods developed to study one of the proteins show no cross-reactivity with the other protein. Our ELISA for transcobalamin showed no cross-reactivity with haptocorrin in concentrations >1000-fold higher than the concentrations usually observed in plasma.

Rather tedious methods have been developed to isolate transcobalamin before analyzing the cobalamin binding capacity or to analyze the amount of cobalamin attached to the protein (8). These studies indicate that the major part of transcobalamin circulates in an unsaturated form, a finding confirmed in our study. We found that total and unsaturated transcobalamin in serum correlate strongly to each other. The amount of cobalamin-saturated transcobalamin was calculated by subtracting the result for unsaturated transcobalamin from the result for total transcobalamin. The mean value, 220 pmol/L, is somewhat higher than previous estimates obtained by direct analysis of cobalamin attached to transcobalamin (6, 7, 20). However, our estimate is rather uncertain because it is derived by subtracting two large values obtained by two different methodologies.

To date, analysis of transcobalamin has been performed mainly according to the unsaturated binding capacity of the protein (1, 8). From such studies it has been concluded that transcobalamin circulates at increased concentrations in some patients with autoimmune diseases, in patients with autoantibodies against transcobalamin, and in patients with an acute phase reaction (21–23). To examine the relationship between acute phase reaction and total plasma transcobalamin, we analyzed samples from patients covering a wide range of values for C-reactive protein. We did not find any correlation between the two components. Interestingly, three patients with intermediate C-reactive protein concentrations had plasma transcobalamin concentrations exceeding 2000 pmol/L. We speculate that conditions exist that simultaneously increase the concentrations of C-reactive protein and transcobalamin. One possibility is conditions in which the macrophage system is activated, such as histiocytosis (24).

Patients with decreased concentrations of unsaturated transcobalamin have also been reported. Most of these patients had received recent treatment with cobalamin injections; therefore, the concentration of unsaturated transcobalamin does not reflect the concentration of total transcobalamin. In rare cases, however, the result may

reflect an inborn defect in transcobalamin and thereby an urgent need to treat the patient with large doses of cobalamin to circumvent the transcobalamin-mediated internalization of cobalamin (4, 5). Although transcobalamin deficiency has been described in fewer than 50 cases (4, 5), the number of infants suspected to suffer from the condition is considerably higher because the presenting symptoms may be nonspecific. Determination of total transcobalamin in connection with diagnosis of possible transcobalamin deficiency has several advantages compared with the currently used analyses of unsaturated binding capacity. Most importantly, the assay measures total transcobalamin. In addition, the analysis requires as little as 50  $\mu$ L of serum, and the low detection limit of the analyses makes it possible to measure subnormal values with high precision.

The low detection limit of our new assay also makes it of interest for researchers within the field. It is well known that cobalamin plays an important role for normal neurological function (1), and it is also known that transcobalamin is present in the cerebrospinal fluid (25). However, more refined studies linking the concentration of transcobalamin in cerebrospinal fluid to neurological diseases have been hampered because of the sensitivity and sample requirements of current assays.

Recently, it has been realized that transcobalamin is filtered in the kidney and reabsorbed via the multifunctional receptor megalin in the proximal tubules (26). As yet we do not know the implications concerning the metabolism of cobalamin for patients with kidney disease, but our assay will make it possible to analyze whether transcobalamin is excreted into the urine in these patients.

Today, measurement of total plasma cobalamin is widely used in connection with diagnosis of cobalamin deficiency (27). As mentioned, plasma cobalamin is the sum of cobalamin attached to transcobalamin and haptocorrin, but only the minor part of plasma cobalamin attached to transcobalamin is directly available to the cells. Measurement of the cobalamin bound to transcobalamin has therefore been suggested as a sensitive marker for cobalamin deficiency (6, 7), but currently no method suitable for routine use has been developed. Our ELISA may also be of interest in this connection, if a method that separates unsaturated and saturated transcobalamin can be developed.

In conclusion, we have developed an assay for quantification of minute amounts of transcobalamin. The assay measures total transcobalamin and may well be superior to current assays that measure only the unsaturated part of transcobalamin.

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