Paper IV

ity from lot to lot in mobile phase or column packing material *(4)*. It appears that intermittent interferences from Hb C and S traits with some, but not all, ion-exchange GHb methods continues to be an issue.

In summary, some current GHb methods show clinically significant interferences with samples containing Hb C or S trait. These interferences are not necessarily consistent within method types, and with ion-exchange methods may vary over time with changes in column or reagent lots.

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Effects of Oral Contraceptives and Hormone Replacement Therapy on Markers of Cobalamin Status, *Bettina Riedel,[1*] Anne-Lise Bjørke Monsen,[2] Per Magne Ueland,[3] and Jørn Schneede*[3] ([1] Laboratory of Clinical Biochemistry, Section of Clinical Pharmacology, and [2] Department of Pediatrics, Haukeland University Hospital, Bergen, Norway; [3] Locus for Homocysteine and Related Vitamins, University of Bergen, Bergen, Norway; * address correspondence to this author at: Laboratory of Clinical Biochemistry, Section of Clinical Pharmacology, Haukeland University Hospital, 5021 Bergen, Norway; fax 47-55-97-4605, e-mail bettina.marie.riedel@helse-bergen.no)

Low serum concentrations of vitamin B_{12} (cobalamin) have been observed in users of oral contraceptives (OCs) *(1)*, in women during pregnancy *(2)*, and in men treated with high doses of ethinylestradiol for prostate cancer *(3)*. Similar effects of hormone replacement therapy (HRT) have been noticed by some *(4)* but not all investigators *(5)*.

Serum cobalamin has low diagnostic accuracy as a marker of vitamin B_{12} status *(6)*. Because of the long-term consequences of cobalamin deficiency [see Ref. *(7)* for a review], it is important to investigate whether decreased total cobalamin in OC or HRT users is associated with other evidence of impaired cobalamin status, such as increased plasma concentrations of methylmalonic acid (MMA) and total homocysteine (tHcy) and decreased plasma concentrations of holo-transcobalamin (holoTC) *(8)*.

We performed a cross-sectional study on 264 female healthcare students and workers. All participants gave written informed consent, and the study was approved by the Regional Ethical Committee and by the Norwegian Social Science Data Services. Study groups were OC users (n = 54) and controls (n = 81; age range, 18–40 years) and HRT users (n = 51) and controls (n = 78; age range, 41–65 years). The most frequently used OCs were triphasic combination tablets containing the synthetic estrogen ethinylestradiol and the progestogens levonorgestrel or drospirenon [Trinordiol™ (Wyeth-Lederle) or Trionetta™ or Yasmin™ (Schering AG)]. The most commonly used HRTs were either tibolone (Livial™; Organon) or the naturally occurring estradiol in combination with the progestogen noretisterone (Trisekvens™, Kliogest™, or Activelle™; Novo Nordisk A/S). We did not collect data on menstrual cycle or menopause. Detailed characteristics of the study population are given in Table 1 of the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol51/issue4/.

Venous blood samples were collected over 6 months on a single occasion from each of the 264 consecutive nonfasting individuals. Blood samples used for tHcy measurements were immediately placed on ice, and EDTA-plasma was separated within 2 h after collection. For serum, blood was allowed to clot at room temperature for 30 min before centrifugation. The samples were kept at −80 °C until analysis, and the maximum storage time was 2.5 years.

Serum creatinine was analyzed by the Jaffe alkaline picrate method, and plasma MMA, plasma tHcy *(9)*, serum folate, whole-blood cell folate *(10, 11)*, serum cobalamin *(12)*, and plasma holoTC *(13)* were measured with the cited methods. A more detailed description of the methods is given in the online Data Supplement.

We used SPSS 10.0 for Windows NT 4.0 (SPSS Inc.) for statistical analyses, if not otherwise indicated. Mean values (range) are given for gaussian-distributed variables and median values (range) for variables showing skewed distributions. Means were compared by the Student *t*-test and medians by the Mann–Whitney *U*-test. Categorical data were compared between groups with the Fisher exact test. Logistic regression analysis was used to assess whether OC and HRT use independently affected plasma concentrations of MMA and tHcy after adjustment for age, serum creatinine, serum folate, number of cigarettes smoked, vitamin supplementation, and dietary factors, and results are reported as odds ratios (ORs) with 95%

confidence intervals (CIs). Bivariate relationships were evaluated by the Spearman correlation test. The dose–response relationships between cobalamin, holoTC, and metabolites were determined with gaussian generalized models *(14)*, as implemented in R *(15)*. This method generates a graphic presentation of the relationships and allows adjustment for covariates. P values ≤0.05 were considered significant.

In OC users, median concentrations of both serum cobalamin and plasma holoTC were ~25% lower than in controls (Table 1). However, there was no difference between OC users and controls in the relationship between plasma holoTC and total serum cobalamin expressed as the holoTC/cobalamin ratio, and in plasma MMA, plasma tHcy, serum folate, whole-blood folate, and serum creatinine (Table 1). The lower concentrations of total cobalamin and holoTC were not accompanied by signs of functional cobalamin deficiency as judged by plasma MMA (<0.26 μmol/L) and plasma tHcy (<15.0 μmol/L) values within the reference intervals for >98% of OC users. Notably, no controls and only one OC user had biochemical signs (cobalamin, 67 pmol/L; holoTC, 12 pmol/L; tHcy, 63.1 μmol/L; MMA, 0.34 μmol/L) of cobalamin deficiency. However, OC use increased the risk of having plasma MMA in the highest quartile [>0.16 μmol/L; OR (95% CI), 3.5 (1.3–9.5); $P = 0.01$] but was not associated with plasma tHcy in the highest quartile [>9.2 μmol/L; OR (95% CI), 1.2 (0.4–3.8); $P = 0.8$].

We investigated the relationships of plasma holoTC and serum cobalamin with plasma concentrations of MMA and tHcy in the young women, using a gaussian generalized model that produces dose–response curves adjusted for age, serum creatinine, and the daily number of cigarettes smoked (Fig. 1). In women not taking OCs, the relationship of plasma holoTC and serum cobalamin with plasma MMA was weak and nonsignificant, whereas in OC users, both analytes showed a strong, inverse correlation with plasma MMA, particularly at low concentrations. Opposite interrelationships according to OC use were seen for plasma tHcy: strong negative associations were observed in the control group, whereas essentially no relationships were found in OC users (Fig. 1). The relationships depicted in Fig. 1 were supported by Spearman correlation analyses; the Spearman correlation coefficients are listed in Table 2 of the online Data Supplement.

In HRT users, the concentrations of blood markers were not significantly different from in controls (Table 1), and we found no association between HRT use and the risk of having plasma tHcy in the highest quartile (>10.1 μmol/L), holoTC in the lowest quartile (<61 pmol/L), or serum cobalamin in the lowest quartile (<281 pmol/L). However, HRT use lowered the risk of having plasma MMA in the highest quartile [>0.19 μmol/L; OR (95% CI), 0.31 (0.11–0.93); $P = 0.04$].

Low serum cobalamin attributable to OC use has been described by others *(1, 16)*, whereas low plasma holoTC in OC users has not been reported previously. The proportional decreases in both cobalamin markers may indicate that cobalamin bound to haptocorrin and to TC was equally affected in OC users. However, we have previously shown that total TC is not significantly lower in OC users *(17)*, whereas others have found an OC-induced decrease in haptocorrins *(18)*. Hence, the mechanism for the observed decrease in plasma holoTC is not readily apparent.

Plasma MMA values in OC users have not been published before, and earlier studies indicated that urinary MMA concentrations did not differ between OC users and controls *(18)*. Plasma tHcy has been reported to not be influenced by OC use [see Ref. *(1)* and references therein], which agrees with our data. This could be attributable to the Hcy-lowering effects of female sex hormones *(19)*, which may antagonize the tHcy increase secondary to impaired cobalamin status. Alternatively, female sex hormones may cause intracellular cobalamin redistribution favoring supply of cobalamin as a cofactor for methionine synthase at the expense of the methylmalonyl-CoA mutase reaction. Such cobalamin redistribution may explain the observed increase in the risk of having MMA values in the highest quartile [>0.16 μmol/L; OR (95% CI), 3.5 (1.3–9.5); $P = 0.01$], the strengthening of the relationships of plasma holoTC and serum cobalamin with plasma MMA, and the weakening of the relationships with plasma tHcy

Table 1. Vitamins, metabolites, plasma holoTC, and serum creatinine in relation to hormone therapy.[a]

	OC (age range, 18–40 years)		HRT (age range, 41–65 years)	
Markers	**Users (n = 54)**	**Controls (n = 81)**	**Users (n = 51)**	**Controls (n = 78)**
Serum cobalamin, pmol/L	254[b] (67–477)	354 (168–711)	331 (159–623)	349 (146–1209)
Plasma holoTC, pmol/L	55[b] (12–108)	74 (31–218)	82 (44–179)	80 (26–1541)
holoTC/cobalamin ratio	0.21 (0.07–0.43)	0.21 (0.10–0.60)	0.26 (0.12–0.52)	0.23 (0.11–1.27)
Plasma MMA, μmol/L	0.14 (0.10–0.34)	0.13 (0.08–0.26)	0.16 (0.09–0.29)	0.17 (0.09–0.40)
Plasma tHcy, μmol/L	7.8 (4.8–63.1)	7.1 (4.1–21.2)	8.4 (5.2–19.0)	8.6 (5.6–16.0)
Serum folate, nmol/L	13.3 (2.7–60.7)	15.7 (6.5–96.8)	16.4 (5.4–87.4)	16.2 (5.1–51.2)
WB folate[c], nmol/L	251 (81–485)	240 (74–585)	268 (86–506)	256 (79–843)
Serum creatinine, μmol/L	76 (66–87)	74 (60–90)	78 (62–97)	76 (60–108)

[a] Data shown as median (range).
[b] $P < 0.01$.
[c] WB folate, whole-blood cell folate.

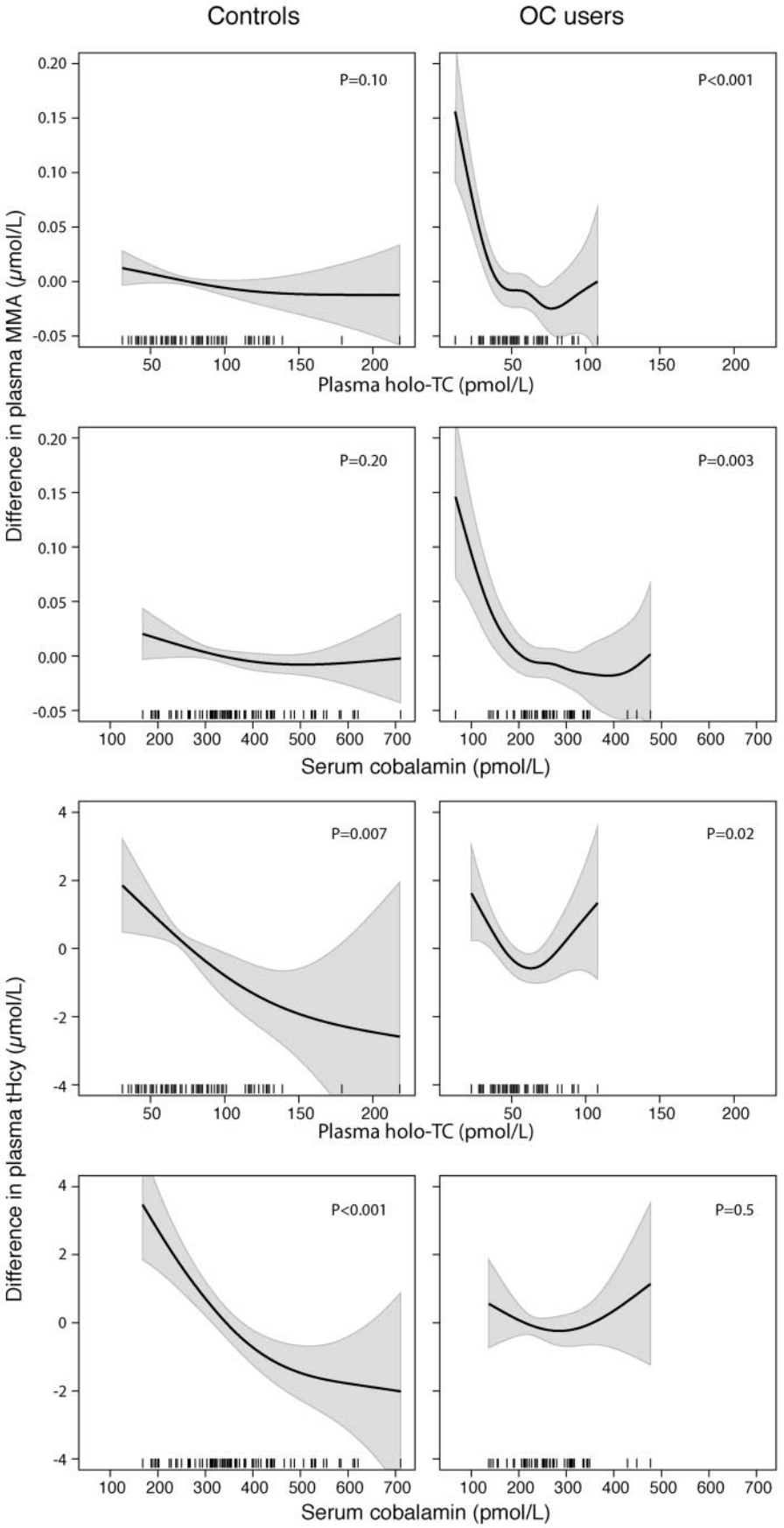
Controls
OC users
P=0.10
P<0.001
P=0.20
P=0.003
P=0.007
P=0.02
P<0.001
P=0.5
Difference in plasma MMA (μmol/L)
Difference in plasma tHcy (μmol/L)
Plasma holo-TC (pmol/L)
Serum cobalamin (pmol/L)

Fig. 1. Dose–response relationships of plasma holoTC and serum cobalamin with the metabolic markers plasma MMA and plasma tHcy, according to OC use.

The curves were obtained by additive gaussian generalized models. The models include age, serum creatinine, and smoking. The *solid lines* are the estimated dose–response curves, and the *shaded areas* are the 95% CIs. The *P* values indicate the significance of the smooth terms.

in OC users (Fig. 1; also see Table 2 in the online Data Supplement).

In HRT users, we found no significant differences in serum cobalamin or in plasma holoTC, MMA, and tHcy compared with controls. To our knowledge, there are currently no published data on the effects of HRT on plasma holoTC, and studies on serum cobalamin have not established a clear pattern *(4, 5)*. We observed that HRT use decreased the risk of having plasma MMA in the highest quartile but was not associated with significant changes in plasma MMA (Table 1). In another study investigating postmenopausal women >60 years, HRT users had significantly lower plasma MMA than nonusers *(5)*. Oral HRT lowered *(20)* or did not affect plasma tHcy *(4)*. Our findings indicate at least that use of HRT has no negative effect on cobalamin status.

In conclusion, both serum cobalamin and holoTC are lower by 25% in younger women taking OCs, but this is not associated with significantly higher concentrations of the metabolic markers of impaired cobalamin status, plasma MMA and plasma tHcy. This may suggest redistribution rather than depletion of intracellular cobalamin. Such hormonal effects may weaken the diagnostic utility of total cobalamin and holoTC. Further studies are warranted to decide whether OC users with marginal cobalamin status are prone to develop cobalamin deficiency. HRT use had no noticeable effect on circulating cobalamin, holoTC, or the metabolic markers.

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Effects of Preanalytical Factors on the Molecular Size of Cell-Free DNA in Blood, *K.C. Allen Chan,[1] Sze-Wan Yeung,[2] Wing-Bong Lui,[1] Timothy H. Rainer,[2] and Y.M. Dennis Lo[1*]* ([1] Department of Chemical Pathology and [2] Accident and Emergency Medicine Academic Unit, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, New Territories, Hong Kong Special Administrative Region, China; * address correspondence to this author at: Department of Chemical Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, New Territories, Hong Kong Special Administrative Region, China; fax 852-2194-6171, e-mail loym@cuhk.edu.hk)

Recently, plasma DNA analysis has been increasingly explored for different clinical diagnostic purposes. Previous studies have shown that the concentration of plasma

Supplemental data: Methods

Serum creatinine was determined by the Jaffe alkaline picrate method for the Hitachi 917 system (F. Hoffmann-La Roche Ltd.). The total imprecision was 3.6 %. Plasma MMA and tHcy were analyzed by a modification of a gas chromatographic-mass spectrometry method [Husek, 1995 #17]. The CVs for the MMA and tHcy assays were 3.2 % and 1.5 %, respectively. Serum and WB-folate were determined by a *Lactobacillus casei* microbiologic assay [O'Broin, 1992 #364], and serum cobalamin was determined by a *Lactobacillus leichmannii* microbiological assay [Kelleher, 1991 #118] on a microtitre plate platform [Molloy, 1997 #112], adapted to a robotic workstation (Microlab AT plus 2; Hamilton Bonaduz AG). The total imprecisions of the analyses for folate and WB-folate were 6.3 % and 17 %, respectively, and for cobalamin 6.7 %. Plasma holoTC was measured by holoTC RIA (Axis-Schield ASA) [Ulleland, 2002 #97]. The total imprecision was 12 % at a mean plasma concentration of 14 pmol/L, and 6 % at a mean plasma concentration of 67 pmol/L.

Supplemental Data Table 1. Demographic data of the study population.[a]

	Oral contraceptives, age 18-40 years		Hormone replacement, age 41-65 years	
	Controls (n = 81)	Users (n = 54)	Controls (n = 78)	Users (n = 51)
Variables				
Age, years	29 (20-40)	24 (18-40)	49 (41-65)	53 (41-62)
Body mass index, kg/m^2	23 (18-40)	22 (17-31)	24 (18-31)	25 (19-40)
Cigarettes/day	4.1 (0-22)	3.5 (0-20)	1.9 (0-20)	1.4 (0-20)
Vitamin supplements[b]				
No [n (%)]	63 (78)	40 (74)	61 (78)	38 (75)
Yes [n (%)]	18 (22)	14 (26)	17 (22)	13 (25)
Meat intake				
< 3 times/week [n (%)]	20 (25)	13 (24)	35 (45)	16 (32)
≥ 3 times/week [n (%)]	60 (75)	41 (76)	42 (55)	34 (68)
Dairy product intake				
< 3 times/week [n (%)]	10 (14)	2 (6)	7 (9)	5 (11)
≥ 3 times/week [n (%)]	61 (86)	34 (94)	67 (91)	42 (89)

[a] Data are mean (range), if not otherwise indicated.

[b] Vitamin supplements contained both cobalamin and folate.

Numbers in certain columns may deviate from the total number of participants due to incomplete data from questionnaires.

Supplemental Data Table 2. Spearman correlation coefficients for vitamins and markers of cobalamin status in relation to hormone therapy.

Oral contraceptives, age 18-40 years

	Controls (n=81)						Users (n=54)					
Parameters	Serum cobalamin	Plasma holoTC	Plasma MMA	Plasma tHcy	Serum folate	WB-folate	Serum cobalamin	Plasma holoTC	Plasma MMA	Plasma tHcy	Serum folate	WB-folate
Plasma holoTC	0.683[b]						0.389[b]					
Plasma MMA	-0.162	-0.183					-0.214	-0.377[b]				
Plasma tHcy	-0.405[b]	-0.337[b]	0.045				-0.105	-0.261	-0.038			
Serum folate	0.110	0.300[b]	-0.069	-0.612[b]			0.096	0.157	-0.010	-0.603[b]		
WB-folate	0.233[c]	0.139	-0.168	-0.574[b]	0.599[b]		0.114	0.098	-0.228	-0.304[c]	0.421[b]	
Serum creatinine	0.010	-0.040	0.092	0.294[b]	-0.254[c]	-0.209	0.086	0.085	-0.036	0.065	-0.080	-0.068

Hormone replacement therapy, age 41-65 years

	Controls (n=78)						Users (n=51)					
Parameters	Serum cobalamin	Plasma holoTC	Plasma MMA	Plasma tHcy	Serum folate	WB-folate	Serum cobalamin	Plasma holoTC	Plasma MMA	Plasma tHcy	Serum folate	WB-folate
Plasma holoTC	0.694[b]						0.556[b]					
Plasma MMA	0.003	-0.153					-0.233	0.045				
Plasma tHcy	-0.203	-0.289[c]	0.189				-0.066	-0.167	0.182			
Serum folate	0.270[c]	0.270[c]	0.017	-0.470[b]			0.104	0.057	-0.182	-0.510[b]		
WB-folate	0.180	0.267[c]	-0.074	-0.317[b]	0.615[b]		-0.051	-0.080	-0.146	-0.502[b]	0.766[b]	
Serum creatinine	0.033	0.070	0.301[b]	0.338[b]	-0.133	-0.098	0.018	-0.055	0.371[b]	0.411[b]	-0.097	-0.115

[a]WB-folate, whole blood cell folate

[b]P<0.01

[c]P<0.05