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Routine determination of serum methylmalonic acid and plasma total homocysteine in Norway*

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Objective: To study the total number of combined analyses of methylmalonic acid (MMA) and total plasma homocysteine (tHcy) carried out during February 1998 at the Central Laboratory of Haukeland University Hospital. *Methods:* Laboratory data and requester comments of 2917 subjects in whom MMA was requested during February 1998, were retrieved from the laboratory information system (LIS). In 2520 cases, the results from the combined analyses of MMA and tHcy were available. Requester comments were registered in the LIS in 1084 cases. Results from additional laboratory analyses were accessible in about 10% of cases. *Results:* General practitioners requested MMA and tHcy on three main indications, i.e. low cobalamin, "control" and neurological symptoms. Metabolites were requested in twice as many women than men. Furthermore, MMA was requested in younger age groups of women compared with men. Plasma tHcy and MMA showed positive correlations with age and serum creatinine, and tHcy was generally 1–2 μmol/L higher in men than in premenopausal women. In cobalamin- (serum cobalamin > 300 pmol/L) and/or folate- (serum folate > 10 nmol/L) replete subjects, the average difference in MMA or tHcy according to the highest and lowest creatinine quartiles was 0.08 and 5–6 μmol/L, respectively. Different combinations of MMA and tHcy were evaluated by using a 5 × 5 matrix of predefined concentration intervals. Based on this matrix, cobalamin and folate deficiency could be excluded or the likely diagnoses proposed in 76% of cases. Cobalamin deficiency or folate deficiency was likely in about 7% and 12% of the subjects investigated, respectively. *Conclusions:* A combined analysis of MMA and tHcy provides complementary diagnostic information. When interpreting MMA and tHcy values, age, gender and renal function in particular must be taken into account. Typical combinations of MMA and tHcy concentration intervals could be proposed, which could either exclude deficiency or indicate likely diagnoses and/or influence of confounders.

Key words: General practice; homocysteine; methylmalonic acid; toutine test; vitamin B_{12}

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INTRODUCTION

Typical signs of cobalamin deficiency include low serum cobalamin concentrations combined with megaloblastic anaemia and myelopathy, which occur in the late stages of deficiency. Nowadays, cobalamin deficiency is usually diagnosed at an early stage, and these classical signs are rarely found. In clinical practice, subtle, non-specific symptoms prevail in cases where suspected cobalamin deficiency is one of many possible differential diagnoses. Traditional tests of cobalamin malabsorption, e.g. the Schilling test, may show normal results, and typical gastrointestinal findings may be missing. Moreover, up to 40% of patients may only present diffuse neurological symptoms without any haematological signs [1]. Such symptoms may not lead to the diagnosis of cobalamin deficiency. Still, early diagnosis is paramount since mild symptoms may finally develop into irreversible neurological damage.

Cobalamin supplementation is cheap and without major side effects. Even though the decision to start supplementation often implies life-long treatment, the main concern may not be over-treatment, but rather overlooking cobalamin deficiency, especially in the elderly.

There is no consensus between the different medical specialities on how to define cobalamin deficiency [2–6]. Ideally, general practitioners should have access to inexpensive, convenient and non-invasive tests that have sufficient sensitivity and specificity to establish an accurate diagnosis at an early stage. Until recently, there have been a limited number of laboratory tests that fulfilled these criteria.

Total homocysteine (tHcy) and methylmalonic acid (MMA) in serum or plasma are functional markers of cobalamin status. They may be used for the diagnosis and follow-up of cobalamin deficiency [7, 8]. MMA is not only a sensitive, but also a specific marker of cobalamin function, since apart from cobalamin deficiency, renal impairment, vascular volume depletion and rare inborn errors affecting the methylmalonate-CoA mutase activity are the only conditions known to cause elevation of MMA [9, 10]. tHcy is also increased in folate deficiency, and is used as an indicator of this condition [11–13]. Furthermore, elevated tHcy is also observed in subjects with unhealthy lifestyles and in pathophysiological conditions such as renal failure [14].

Determination of tHcy and MMA is relatively expensive and measurement of tHcy demands special sample handling procedures [15]. It is thus justifiable to question the cost/benefit relation of these tests [16, 17]. It is also important to know when and why general practitioners request tHcy and MMA [18], how they interpret the results [19, 20] and whether these tests have replaced traditional laboratory tests for vitamin B deficiency [21].

The present article reports on the total number of requests for combined MMA and tHcy during the course of one month in Norway and presents a picture of the spectrum of metabolite concentrations encountered by general practitioners.

MATERIALS AND METHODS

Patients and study design

In February 1998, we performed an audit among general practitioners in Norway on the diagnostic procedures of vitamin B_{12} deficiency with special reference to MMA. The results of this audit are published elsewhere [22, 23]. In the present study, we retrieved from the laboratory information system (LIS) the total number of MMA analyses ($n = 2917$) carried out at the Laboratory of Clinical Biochemistry (LKB), the central laboratory of Haukeland University Hospital in Bergen, Norway, during the same month the audit was carried out. MMA and tHcy were requested by general practitioners in $>99\%$ of cases. In 2520 of cases, data from concomitant tHcy analyses were available. Data on age and gender were recorded in all subjects. In addition, we retrieved all comments registered in the LIS in connection with the MMA/tHcy requests. In the few cases where several comments were provided, only the comment most relevant for vitamin diagnostics was included in the analysis. Moreover, in about 10% of cases, results from concomitant or recent (<2 weeks) analyses of serum cobalamin, serum folate, RBC folate and serum creatinine were available.

Sample handling and biochemical analyses

Blood sampling and sample preparation, including centrifugation, are usually performed at the primary healthcare centres. Request forms for tHcy and MMA contain detailed instructions. The serum/plasma samples are usually shipped at room temperature by mail and reach the laboratory within less than two days.

Plasma tHcy, which includes free and protein-bound Hcy forms, was determined by modification of an automated procedure based on derivatization with monobromobimane, followed by high performance liquid chromatography (HPLC) and fluorescence detection [24, 25]. The between-day coefficient of variation is about 3%. The laboratory uses a cut-off value of 15 μmol/L as the upper reference limit for plasma tHcy in subjects older than 12 years [26]. In children under 12 years of age, the cut-off value is 10 μmol/L. Cut-off values are not further differentiated according to age or gender.

Serum MMA was measured either by capillary electrophoresis (CE) [27] or by gas chromatography mass spectrometry (GCMS) [28]. The between-day coefficients of variation for the CE- and GCMS method were 5–10%, and 3–10%, respectively, for MMA concentrations within a range of 0.12–0.57 μmol/L. Both of the MMA methods are intercalibrated and subjected to external standardization, using the same calibrators. The inter-assay variation was $<14\%$. The local reference range for MMA is 0.05–0.26 μmol/L without further age- or gender differentiation.

Both the tHcy and MMA methods are regularly subjected to a Scandinavian quality assessment programme [29].

Statistics and data analyses

All patient data were anonymous. Requester comments were retrieved from the LIS and categorized into nine different groups. All skewed variables were log-transformed. One-way ANOVA analysis was performed to compare geometric means between the different diagnostic groups. Gender differences were evaluated using the Mann-Whitney U test. Tests were two-tailed, and a p-value <0.05 was considered statistically significant. Curve estimations for evaluating the influence of renal function on tHcy and MMA values were performed according to the S-curve model implemented in the SPSS statistical package. The SPSS statistical program, version 10.0 for Macintosh, was used for all data analyses.

RESULTS

Study population

We investigated all the MMA analyses ($n=2917$) carried out during February 1998. In 2520 cases (86.4%) the results from tHcy determinations were also available (Table I). Concurrent, additional biochemical parameters were retrieved in about 10% of subjects. In total, results from 330 cobalamin, 275 serum folate, 161 RBC folate and 302 creatinine analyses were obtained from the LIS database of Haukeland University Hospital (Table I). All parameters showed positive skewedness, with the exception of age, which was negatively skewed. Significant gender differences were found for age, tHcy and creatinine, but not for MMA, cobalamin, serum or R.B.C. folate (Table I).

Age and gender

MMA and tHcy tests were requested in twice as many women as in men. There were marked gender differences in the frequency distribution of tHcy and MMA requests according to age (Fig. 1). Requests for tHcy and MMA tests were more common in the female younger age groups than in the male younger age groups, and the frequency distribution indicated three fairly distinct peaks at 26, 50 and 75 years of age. In males, there was a unimodal distribution, which peaked at 78 years of age, and only a few tHcy and MMA tests were performed in men younger than 40 years.

Clinical comments of requesters

In 1084 cases (43.0%), the general practitioners provided comments on the request forms. These comments were classified into nine categories (Table II). The predominating indications for tHcy and MMA testing were "neurological symptoms", "low cobalamin"

TABLE I. Characteristics of the study population.

	Female			Male			
	n	Mean	Median (10th–90th percentiles)	n	Mean	Median (10th–90th percentiles)	p-value*
Age, years	1965	58.9	61 (29–84)	952	62.5	66 (37–84)	<0.01
Cobalamin (pmol/L)	224	529	330 (189–1299)	106	497	312 (163–742)	0.386
S–Folate (nmol/L)	185	10.6	9.3 (4.8–16.9)	90	9.2	8.9 (3.9–14.3)	0.211
E–Folate (nmol/L)	101	379	324 (165–588)	60	339	307 (177–578)	0.584
Creatinine (μmol/L)	198	85	83 (68–106)	104	101	99 (80–124)	<0.01
Homocysteine (μmol/L)	1703	12.3	10.6 (6.9–19.5)	817	14.6	12.4 (8.6–21.7)	<0.01
Methylmalonic acid (μmol/L)	1965	0.25	0.18 (0.11–0.34)	952	0.29	0.18 (0.12–0.38)	<0.291

*Mann-Whitney test for differences between genders.

FIG. 1. Frequency distribution of the total number of combined methylmalonic acid and homocysteine requests at Haukeland University Hospital in relation to age and gender during February 1998.

and "control". These three annotations accounted for about 76% of all requester comments. Results from tHcy and MMA analyses were not significantly different in subjects with and without requester comments; $p > 0.4$ (Table II).

We compared tHcy and MMA values according to comment category using ANOVA (Table III). Among women, subjects with reported "anaemia" had significantly higher tHcy values, compared with the rest of the female study population. The same tendency was observed for MMA. Furthermore, females with "gastrointestinal symptoms" had significantly lower tHcy concentrations in relation to all other females in the study population. In males, only subjects with the comment "low cobalamin" had significantly

TABLE II. Requester comments and number of subjects with elevated metabolite values.

Requester comment	n (% of total)	Subjects with tHcy > 15.0 μmol/L n (% within category)	Subjects with MMA > 0.26 μmol/L n (% within category)
Anaemia	68 (6.3)	17 (26)	20 (29)
Neurological symptoms	244 (22.5)	54 (24)	40 (16)
Elevated homocysteine	3 (0.3)	2 (67)	1 (33)
Low cobalamin	323 (39.3)	78 (25)	76 (24)
Low folate	14 (1.3)	5 (36)	2 (14)
Control	248 (22.9)	51 (22)	42 (17)
Cardiovascular symptoms	45 (4.2)	11 (28)	14 (31)
Gastrointestinal symptoms	40 (3.7)	3 (8)	4 (10)
Other comments	99 (9.1)	17 (19)	21 (21)
Total	1084 (100)	238 (23)	220 (20)
No comment*	1833	356 (24)	379 (21)

MMA = methylmalonic acid; tHcy = total plasma homocysteine.
*Homocysteine and MMA values were not significantly different in subjects where requester comments were supplied compared with those where comments were not supplied (Mann-Whitney *t*-test, p = 0.944 and 0.377, respectively).

higher values for tHcy and MMA than the rest of the male study population.

Metabolite values and bivariate correlations

Using 15.0 μmol/L and 0.26 μmol/L as cut-off values for tHcy and MMA, respectively, we found tHcy elevated in 594 out of 2520 (23.6%) of cases, while elevated MMA values were registered in 599 out of 2917 subjects (20.1%).

The distribution of tHcy and MMA concentrations showed a strong positive skewness. The tHcy and MMA values according to age and gender are depicted in Figure 2. Both tHcy and MMA increased with age; total Hcy was generally higher in males. The gender differences in median tHcy concentrations ranged from 1 to 3 μmol/L between the different age groups. No significant gender differences were observed for MMA.

TABLE IIIa. One way ANOVA analysis of differences in mean tHcy values in relation to requester comments.

		Females				Males			
		N	Total range	GM	p-value*	n	Total range	GM	p-value*
Anaemia	Yes	47	4.7–83.9	12.6	0.040	18	3.5–39.0	12.6	0.728
	All other	1620	3.5–83.6	11.1		777	4.4–110.2	13.1	
Neurological symptoms	Yes	151	5.1–44.6	11.2	0.810	75	4.4–33.1	12.3	0.201
	All other	1516	3.5–83.9	11.2		720	3.5–110.2	13.2	
Low cobalamin	Yes	221	3.5–83.6	11.5	0.297	89	5.4–91.2	14.4	0.025
	All other	1446	3.5–83.9	11.1		706	3.5–110.2	12.9	
Gastrointestinal symptoms	Yes	19	3.6–13.6	8.7	0.011	17	7.1–17.9	11.5	0.212
	All other	1648	3.5–83.9	11.2		778	3.5–110.2	13.1	
Cardiovascular symptoms	Yes	16	7.0–23.5	11.2	0.945	22	7.4–24.7	12.7	0.737
	All other	1651	3.5–83.9	11.2		773	3.5–110.2	13.1	
Diffuse symptoms	Yes	85	5.2–31.3	10.4	0.132	40	4.4–33.1	12.7	0.629
	All other	1582	3.5–83.9	11.2		755	3.5–110.2	13.1	
Control	Yes	144	4.3–51.4	10.9	0.432	84	5.4–66.8	13.1	0.989
	All other	1559	3.5–83.9	11.2		733	3.5–110.2	13.1	
All comments	Yes	665	3.5–83.9	11.2	0.750	341	3.5–91.2	13.1	0.818
	None	1002	3.5–56.1	11.1		454	4.8–110.2	14.2	

GM = geometric mean; tHcy = total plasma homocysteine.
*p-values were calculated based on log-transformed data.

TABLE IIIb. One way ANOVA analysis of differences in mean MMA values in dependence of requester comments.

		Females				Males			
		N	Total range	GM	p-value*	n	Total range	GM	p-value*
Anaemia	Yes	48	0.07 – 23.74	0.22	0.073	20	0.08 – 0.35	0.20	0.907
	All other	1917	0.05 – 12.07	0.19		932	0.04 – 30.20	0.20	
Neurological symptoms	Yes	163	0.08 – 1.68	0.19	0.472	81	0.05 – 0.97	0.19	0.249
	All other	1802	0.05 – 23.74	0.19		871	0.04 – 30.20	0.20	
Low cobalamin	Yes	227	0.06 – 12.07	0.20	0.329	96	0.09 – 6.47	0.24	<0.01
	All other	1738	0.05 – 23.74	0.19		856	0.04 – 30.20	0.20	
Gastrointestinal symptoms	Yes	21	0.06 – 12.07	0.20	0.886	19	0.08 – 0.54	0.16	0.104
	All other	1944	0.05 – 23.74	0.19		933	0.04 – 30.20	0.20	
Cardiovascular symptoms	Yes	21	0.13 – 0.52	0.23	0.135	24	0.08 – 0.90	0.20	0.851
	All other	1944	0.05 – 23.74	0.19		928	0.04 – 30.20	0.20	
Diffuse symptoms	Yes	92	0.08 – 1.66	0.18	0.366	43	0.08 – 0.97	0.18	0.236
	All other	1873	0.05 – 23.74	0.19		909	0.04 – 30.20	0.20	
Control	Yes	161	0.06 – 0.61	0.19	0.359	87	0.05 – 1.04	0.19	0.222
	All other	1804	0.05 – 23.74	0.19		865	0.04 – 30.16	0.20	
All comments	Yes	714	0.05 – 23.74	0.19	0.759	370	0.05 – 6.47	0.20	0.858
	None	1251	0.05 – 9.80	0.19		582	0.04 – 30.20	0.20	

GM = geometric mean; MMA = methylmalonic acid.
*p-values were calculated based on log-transformed data.

The number of tHcy values above the reference limits varied according to age. Notably, rather high frequencies (up to 20%) of elevated tHcy (>15.0 μmol/L) were found in the younger age groups (16 – 30 years), especially in women. In middle-aged groups (31 – 40 years) elevated metabolite concentrations were less common (Fig. 2, data not shown). Among the oldest subjects (>85 years) about 50% of the subjects had elevated metabolite levels. Further stratification according to age groups indicates that median tHcy values in the age groups 16 – 20, 21 – 25 and 26 – 30 years actually were higher than in the age groups 31 – 35 and 36 – 40 in both genders (Fig. 2, upper panel). In contrast, median MMA values were relatively stable until the age of 55 (with the exception of a few subjects in the youngest age group of 0 – 10 years) and then increased with age in both genders (Fig. 2, lower panel).

A 5×5 matrix depicting the frequencies within certain concentration intervals of the 2520 combined tHcy and MMA analyses is presented in Table IV. Both parameters were within normal range in 65.5% of cases (cells *A1-3* and *B1-3*), but elevated in 8.7% of subjects (cells *C4-5*, *D4-5* and *E4-5*). In 14.8% of cases, only tHcy (*A4-5* and *B4-5*), but not MMA, was above the cut-off value, whereas 11.0% of subjects exhibited an isolated MMA elevation, (*C1-3*, *D1-3* and *E1-3*).

Table V presents an overview of Spearman correlation coefficients between the different test variables. Plasma tHcy showed a highly significant, inverse relationship with cobalamin, serum folate and RBC folate, while significant, positive correlations were observed with MMA, creatinine and age. MMA showed an inverse correlation with cobalamin, and was positively associated with creatinine and age.

In addition, RBC folate showed a positive association with serum folate and cobalamin, and creatinine was positively correlated with age (Table V). Notably, age was not related to cobalamin, serum folate or RBC folate, despite the fact that the prevalence of vitamin deficiencies is expected to increase with age.

Renal function

A substantial number of subjects had elevated creatinine concentrations (Table I), which were positively related to both serum MMA and plasma tHcy (Table V). We tried to assess the effect of renal function on the metabolite concentrations independent of vitamin status by plotting MMA or tHcy versus the vitamin levels at different creatinine quartiles (Fig. 3).

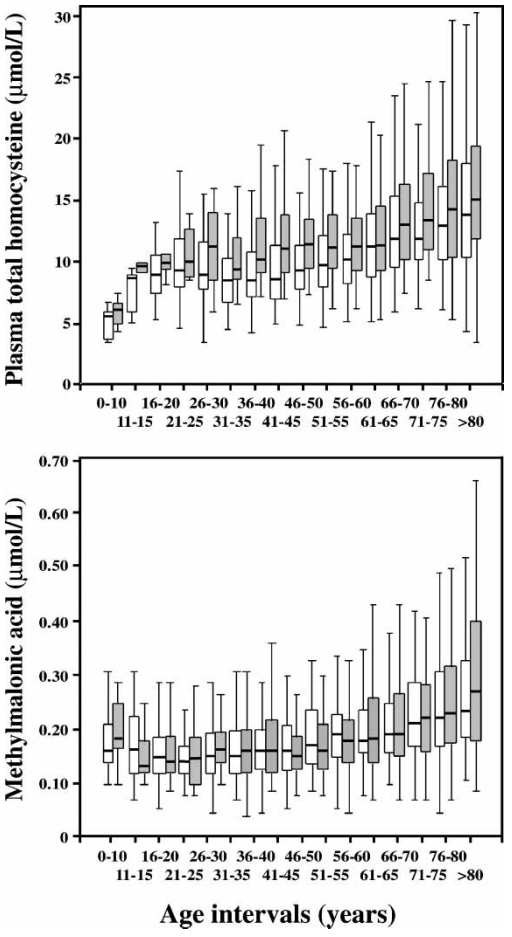

FIG. 2. Changes in total homocysteine and methylmalonic acid concentrations according to age and gender. Clustered box-plot depicting median values, interquartile range and extreme values of total plasma homocysteine and serum methylmalonic acid according to age and gender. The white boxes indicate female gender, the shaded boxes male gender.

The results from this analysis were implemented in Table IV. The MMA and tHcy increment (0.08 and 5 μmol/L, respectively) corresponding to average concentration changes between the creatinine quartiles at cobalamin concentrations $>300-400$ μmol/L (Fig. 3) was added to the laboratory cut-off values for MMA and tHcy to obtain the supposed upper limit of metabolite concentrations (20 μmol/L for tHcy and 0.34 μmol/L for MMA) below which confounding due to impaired renal function might be possible. The lower limits of this "borderline" interval were somewhat arbitrarily set at 13 μmol/L for tHcy and 0.20 μmol/L for MMA based on the observed average MMA/tHcy values and standard deviations at high concentrations of cobalamin as well as recent publications on changes of tHcy cut-off values in vitamin optimized adults [30]. The "borderline metabolite interval", indicated by the grey-shaded area in Table IV, harbours 8% ($n=209$) of the study population.

Table VI illustrates the number of subjects in the different creatinine quartiles in relation to the four possible combinations of tHcy and MMA categories, using 15.0 and 0.26 μmol/L as cut-off values. The Pearson chi-squared test showed significant deviations from the expected counts in the different cells of the table ($p<0.001$). Subjects with the highest creatinine quartile were over-represented among cases with a combined elevation of MMA and tHcy values, and in subjects with isolated tHcy elevation. Fewer subjects than expected with elevated MMA, but normal tHcy, had signs of impaired renal function.

The 209 subjects with "borderline" tHcy/MMA combinations (grey-shaded area, Table IV) had a significantly higher chance of belonging to the highest creatinine quartile (≥ 101 μmol/L) than all other subjects outside the grey-shaded area (Pearson's chi-squared test, $p<0.001$, data not shown).

DISCUSSION

This article describes the routine use of MMA and tHcy for the assessment of vitamin B_{12} and folate status in clinical practice. In more than 99% of cases, general practitioners were responsible for the request. These samples represent the total number of routine MMA analyses performed during February 1998 in Norway.

Study design and limitations

The study design did not allow us to compare metabolite values in subjects with a certain requester comment with a presumed healthy control group. We therefore compared subjects with a specific requester comment category with the rest of the study population. This approach may, however, underestimate real associations

TABLE IV. Number of subjects in different concentration intervals of total homocysteine and methylmalonic acid.

		tHcy ≤10.0 *1*	tHcy 10.1–13.0 *2*	tHcy 13.1–15.0 *3*	THcy 15.1–20.0 *4*	tHcy ≥20.1 *5*	Total
MMA 0–0.20	A	732	414	165	151	99	1561
MMA 0.21–0.26	*B*	150	134	55*	72*	52	463
MMA 0.27–0.34	*C*	60	66	32*	50*	37	245
MMA 0.35–0.75	*D*	27	39	28	50	50	194
MMA ≥0.76	*E*	7	9	8	13	20	57
Total		976	662	288	336	258†	2520

MMA = methylmalonic acid; tHcy = total plasma homocysteine.
*The grey-shaded area indicates the number of subjects with "borderline" metabolite concentrations where it is especially important to exclude impaired renal function as a potential confounder.
†29 subjects had total homocysteine concentrations >40.0 μmol/L (1.2%). Of these subjects, 14 individuals (5.6‰) had MMA concentrations <0.26 μmol/L.

TABLE V. Spearman correlation coefficients for metabolites, B-vitamins, creatinine and age.*

	THcy	MMA	Cbl	S-folate	RBC-folate	Creatinine
MMA	0.313 (2520)†					
Cbl	−0.184 (237)†	−0.261 (330)†				
S-folate	−0.421 (195)†	−0.009 (275)	0.076 (234)			
RBC-folate	−0.288 (129)†	−0.103 (161)	0.243 (112)‡	0.414 (108)†		
Creatinine	0.449 (248)†	0.267 (302)†	−0.020 (186)	−0.091 (148)	0.061 (97)	
Age	0.413 (2520)†	0.364 (2917)†	−0.085 (330)	−0.062 (275)	0.043 (161)	0.289 (302)†

MMA = methylmalonic acid; tHcy = total plasma homocysteine.
*Number of individuals in parentheses.
†$p < 0.01$.
‡$p < 0.05$.

between requester comments and concentrations of metabolites.

Comments were supplied in about 40% of the requests, but not all comments unequivocally described the clinical indications for requesting an MMA test. An example of an ambiguous comment is "control", where it remains unclear whether "control after treatment" or "control as a secondary test" is meant. Furthermore, no information was available about co-morbidity and medication. Remarkably enough, MMA and tHcy values did not show significant differences in subjects with and without requester comments (Table III).

A major limitation of the present study is the fact that in the majority of cases, clinical information and results from concurrent analyses of other relevant biochemical parameters were not available. A thorough clinical evaluation and interpretation of the vitamin status in the individual case were thus not possible. However, as we simultaneously performed an audit among a representative selection of general practitioners, we were able to make qualified assumptions on how general practitioners interpreted the results.

Age and gender

The requests for MMA and tHcy analyses showed a distinct distribution according to age and gender. In women, MMA/tHcy was more commonly requested in younger age groups, as compared to men. The frequency distribution of the number of metabolite requests in women reveals three rather distinct peaks. The first

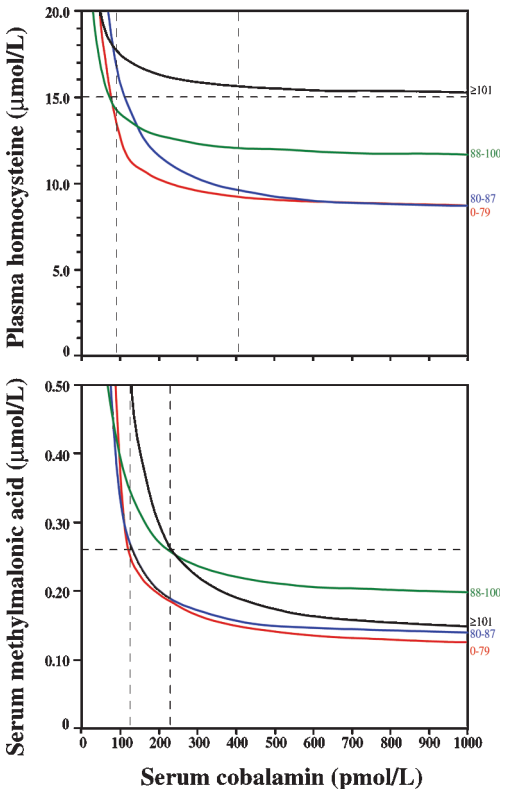

FIG. 3. Plots of total plasma homocysteine and serum methylmalonic acid concentrations versus serum cobalamin at different creatinine quartiles. The red line indicates the lowest creatinine quartile ($\leq$79 µmol/L), the blue line the second quartile (80–87 µmol/L), the green line the third quartile (88–100 µmol/L), and the black line the highest creatinine interval ($\geq$101 µmol/L). The dashed horizontal lines indicate the cut-off values of total homocysteine and methylmalonic acid, 15.0 µmol/L and 0.26 µmol/L, respectively. The dashed vertical lines indicate changes in the inflection points between the different creatinine quartiles.

peak could reflect increased frequency of MMA/tHcy testing in women of childbearing age, while the second peak might be related to menopause. The large number of MMA and tHcy analyses among older men and women may indicate that general practitioners are aware of the high prevalence of cobalamin and other vitamin B deficiencies in the elderly [31].

Indications for requesting MMA and THcy

The most frequent indications for requesting MMA were "low cobalamin" or "neurological symptoms". This is in line with the findings of Hvas *et al*. [18].

The ANOVA analysis showed only a few significant differences in the geometric mean values of tHcy and MMA according to comment categories (Table III). Notably, in the category "low cobalamin", only men had significantly higher tHcy and MMA concentrations than the rest of the study population. This could partly be attributed to the fact that many women may have low circulating cobalamin due to the effects of hormones [32].

The lack of statistically significant differences between the comment categories suggests that either the predictive values of the tentative diagnoses and indications given by the general practitioner were low, or that real associations were underestimated owing to the lack of a healthy reference group. In addition, the study may have lacked statistical power because many of the comment categories had a low number of cases.

Concentrations of MMA and THcy

Metabolite values increased according to age, but showed relatively higher median tHcy values in the 16–30 years age group, particularly in women (Fig. 2). This age profile contrasts with the continuous increase of tHcy with age consistently reported in the general population [33]. The relatively high prevalence of elevated tHcy in the younger age groups is probably associated with impaired folate status.

Given the gender differences in the frequency distribution of tHcy and MMA requests in our study (Fig. 1) and the observed age and gender influence on metabolite values (Fig. 2), our findings strongly advocate the establishment of age- and gender-specific reference limits for MMA and tHcy.

Renal function as confounder

In addition to age, renal function is one of the strongest determinants of tHcy and MMA [7, 34–36] (Table V).

MMA correlated with both cobalamin and creatinine, and the correlation coefficients were of equal magnitude (Table V). MMA may be subjected to glomerular filtration and active tubular secretion like other carboxylic acids [37], in addition to passive tubular re-absorption [7].

TABLE VI. Cross-table over the number of subjects with tHcy and MMA values above/below the cut-off values according to creatinine quartiles.

Quartiles of creatinine		MMA ≤ 0.26 Thcy ≤ 15.0	MMA ≤ 0.26 tHcy > 15.0	MMA > 0.26 THcy ≤ 15.0	MMA > 0.26 THcy > 15.0	Total
0 – 79 μmol/L	Count	55	4	8	3	70
	Expected	46.0	11.3	8.5	4.2	70
80 – 87 μmol/L	Count	45	8	7	1	61
	Expected	40.1	9.8	7.4	3.7	61
88 – 100 μmol/L	Count	40	8	11	3	62
	Expected	40.8	10.0	7.5	3.8	62
> 101 μmol/L	Count	23	20	4	8	55
	Expected	36.1	8.9	6.7	3.3	55
Total	Count	163	40	30	15	248
	Expected	163	40	30	15	248

MMA = methylmalonic acid; tHcy = total plasma homocysteine.
Pearson chi-squared test: p = < 0.001.

A substantial contribution of the kidneys to the overall MMA clearance is thus likely [38].

In individual cases, it is often difficult to discern the selective influence of renal dysfunction and vitamin deficiency on metabolite values [38]. From the graphs in Figure 3, we were able to quantify the average tHcy and MMA increments resulting from renal impairment. These estimates were used to define borderline concentration intervals and to evaluate potential renal influence at various combinations of tHcy and MMA (Tables IV and VI). About 8% of the study population had borderline metabolite concentrations where renal dysfunction may be responsible (Table IV). However, in subjects with sole elevation of MMA, impaired renal function is less likely to be a confounder (Table VI).

In the majority of subjects with tHcy, values > 20 μmol/L and/or MMA values > 0.34 μmol/L (elevated metabolites outside the grey-shaded area, Table IV), the metabolite elevation is unlikely to be explained by impaired renal function.

Tentative diagnoses

Both tHcy and MMA are continuous parameters, expected to increase with development of B_{12} deficiency, but also partly under the influence of confounding factors such as impaired renal function, age and gender. The assumption of a distinct, uniform cut-off value to distinguish between adequate vitamin status and deficiency – irrespective of the investigated population – thus seems to be inadequate. Our study suggests that the information contained in the combined determination of tHcy and MMA is complementary (Table IV).

In cobalamin deficiency, both tHcy and MMA are expected to be elevated [39]. In contrast, isolated elevation of tHcy without increase of MMA usually indicates impaired folate metabolism [39]. Thus, MMA may help to differentiate between folate and cobalamin deficiency in subjects with elevated tHcy. In addition to renal insufficiency, these deficiencies represent the three major causes of hyperhomocysteinaemia [8].

Based on the considerations above, the following tentative diagnoses can be made: 732 individuals (29.1%, cell *A1* in Table IV) had a combination of low MMA (< 0.20 μmol/L and low tHcy (< 10.0 μmol/L), which essentially excludes cobalamin and folate deficiency [39]. In 1430 individuals (56.7%, cells *A1-2* and *B1-2*), the likelihood of cobalamin and folate deficiency is low, irrespective of age and gender.

In the 302 subjects with tHcy > 15.0 μmol/L, but normal MMA, and who were outside the grey-shaded area (cells *A4 – 5* and *B5*), impaired folate status is the most likely diagnosis [39]. Among these subjects, 29 individuals had tHcy concentrations > 40.0 μmol/L. Fourteen of the subjects with tHcy > 40.0 μmol/L had in addition MMA concentrations below 0.26 μmol/L. According to the findings from the Hordaland study, the latter 14 subjects have a > 90% chance of being homozygous for the C677T MTHFR polymorphism in combination with reduced folate status [40].

Theoretically, all conditions that may cause

MMA elevations (mainly cobalamin deficiency and renal insufficiency) are also expected to increase tHcy. However, in 244 subjects (cells *C1–2*, *D1–3* and *E1–3*) only MMA but not tHcy was elevated. There are several possible reasons for this discrepant metabolic profile. Results from recent studies indicate that subjects adhering to a vegetarian lifestyle and high folate intake may exhibit normal tHcy values despite nutritional cobalamin deficiency [41]. The same is true in subjects with impaired cobalamin status erroneously treated with folic acid [42]. Similarly, the effects of impaired renal function on tHcy may be suppressed by a high folate intake, while MMA values remain unaltered [43]. MMA may also have higher diagnostic sensitivity compared to tHcy [39]. Lastly, MMA may be elevated during pregnancy, while tHcy tends to be lowered [44, 45]. Thus, a substantial proportion of these 244 subjects could suffer from cobalamin deficiency despite having tHcy within the normal range.

In the 16 subjects (0.6%, cells E*1–2*) with low tHcy but grossly elevated MMA ($\geq$0.76 μmol/L), falsely elevated MMA values due to small-bowel bacterial overgrowth [46] or analytical interference should be ruled out.

In 170 cases (C*5*, D*4–5* and E*4–5*), corresponding to 6.8% of the investigated population, both tHcy and MMA were elevated and outside the grey-shaded area. In these cases cobalamin deficiency is very likely.

CONCLUSION

General practitioners requested MMA and tHcy on three main indications, ''low cobalamin'', ''control'' and ''neurological symptoms''. The number of MMA/tHcy requests in the investigated population showed strong age- and gender variations. Associations of metabolite values with age and gender were in accordance with the existing literature. Bivariate analyses indicated that both metabolites were positively associated with renal function. The strongest correlations were seen for tHcy. The information contained in the combined analysis of MMA and tHcy is complementary. Rather than relying on strict cut-off values of a single laboratory parameter, an integrated evaluation of MMA and tHcy in certain concentration intervals, taking into account potential confounders, should be made. This information, together with additional clinical and laboratory data, should enable cobalamin/folate deficiency to be confirmed or ruled out in the majority of cases.

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