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ORIGINAL CONTRIBUTION

Methylmalonic acid values in healthy Dutch children

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Abstract *Background* Plasma methylmalonic acid (MMA) is a specific marker for functional cobalamin deficiency. This deficiency can give rise to non-specific but serious symptoms in childhood such as developmental delay, convulsions and failure to thrive and may even lead to irreversible neurological damage. *Aim of the study* To analyse plasma MMA concentrations in Dutch children and to evaluate possible factors influencing its concentration. *Methods* A number of 186 Dutch children aged 0–19 years were analysed cross-sectionally. Blood was collected to measure MMA, total homocysteine (tHcy), cobalamin (Cbl) and serum creatinine concentrations. In addition, information about medical history, age and sex was recorded. *Results* The geometric mean (GM) plasma MMA concentration was 0.17 μmol/l (95% CI 0.07–0.42) and the GM tHcy was 6.6 μmol/l (95% CI 3.1–13.9). There is a slight positive correlation between plasma MMA and age in children >1 year ($r = 0.211$, $P < 0.05$). Plasma MMA concentrations were significantly higher in children with low Cbl concentrations. No significant difference in MMA, Cbl, tHcy or creatinine concentrations between sexes could be observed. Regression analysis showed that Cbl was the strongest determinant of plasma MMA (regression coefficient −0.414, $P < 0.05$). The association between MMA and Cbl is stronger at increasing age (P for trend 0.045). *Conclusions* Plasma Cbl is the main determinant of MMA in this group of Dutch children. The strength of the association increased with increasing age.

Key words methylmalonic acid – cobalamin – children

Introduction

In mammalians, cobalamin (Cbl) is a cofactor for only two known enzymes: i.e. for methionine synthase in the remethylation of homocysteine to methionine as methylcobalamin (meCbl) and for methylmalonylCoA mutase in the formation of succinylCoA from L-methylmalonylCoA as adenosylcobalamin (adoCbl) [1]. Consequently, an intracellular Cbl deficiency may cause increased concentrations of total plasma homocysteine (tHcy) and methylmalonic acid (MMA), making them metabolic markers of Cbl

deficiency. Cbl values in serum or plasma can be normal despite evidence for functional deficiency [1, 7, 25, 34]. This is at least partly explained by the fact that about 80% of total plasma Cbl is bound to haptocorrin, which is not taken up by cells. The remaining 20% of total plasma Cbl is bound to transcobalamin and this complex is involved in transport and ultimately cellular uptake of Cbl. Plasma Cbl measurement shows the sum of both forms of Cbl, thus not necessarily reflecting the availability for cellular processes [34].

THcy is influenced by many environmental factors such as folate and cobalamin status, renal or thyroid function, age, sex, lifestyle factors such as smoking, coffee intake and several drugs as well as genetic factors including the MTHFR c.677 C > T polymorphism [9, 14, 31–33]. Plasma MMA is in addition to Cbl status only modulated by severe renal failure and is therefore a more specific marker of Cbl deficiency [18, 26, 27].

In recent studies evidence was obtained that Cbl deficiency is a relatively common finding in infants and children [3, 22, 29]. Cbl deficiency in childhood is not characterised by the classical symptoms of megaloblastic anaemia and neurological dysfunction as observed in adults, but can give rise to non-specific symptoms such as developmental delay, feeding problems and failure to thrive [3, 4, 16, 19, 23]. There is increasing evidence that low Cbl status in childhood may cause impairment of growth and development and may even lead to irreversible neurological damage [10, 15, 21].

Data on plasma MMA concentrations and its determinants in children aged 0–19 years are scarce. In a study performed by Bjorke Monsen et al. infants aged 6 weeks to 6 months had concentrations of MMA and tHcy that were higher and serum Cbl concentrations that were lower than in older children. In children above 12 months of age, median plasma MMA remained low and stable during puberty. Sex differences could not be observed. A strong correlation between Cbl and MMA and tHcy and MMA was found in both age groups [4]. Specker et al. reported markedly higher urinary MMA excretion in 62 infants compared to adults with the highest MMA excretion in breastfed infants of vegetarian mothers [29].

Since data on plasma MMA concentrations and predictors are scarce and in the light of the suggested high prevalence of Cbl deficiency in children, we analysed plasma MMA concentrations in a group of Dutch children and evaluated possible determinants. These data are relevant to explore the prevalence of Cbl deficiency among children and infants and to evaluate possible long-term effects of cobalamin deficiency.

Subjects and methods

MMA was measured in 186 healthy Dutch children. Blood samples were collected in 1997 as described before [2]. In short, older children (11–19 years) were recruited as healthy volunteers, whereas younger children (<11 years) were recruited in a hospital setting. When blood was drawn for diagnostic or follow-up reasons, children and/or their parents were asked to donate some blood in the same venipuncture session. Exclusion criteria were closure defects, overt renal, thyroid and/or liver dysfunction, use of folate antagonists, malignancies, hormonal therapy, objectively defined occlusive vascular disease and closure defects such as cleft lips and spina bifida. Information about medical history, smoking behaviour and puberty features was obtained from medical records or by questionnaire. The study was approved by the local medical ethics committee and informed consent was obtained from parents and/or children depending on their age.

Blood samples were drawn by venipuncture. Three milli-liter EDTA vacutainers were used for the collection of plasma for tHcy and MMA measurement. In newborns and infants, 1 ml EDTA microtainers obtained from capillary blood samples were used. The EDTA samples were immediately placed on ice and centrifuged at 2000×*g* for 10 min within 4 h. The plasma was separated and stored at −20°C until analysis. If possible, blood was drawn into 5 ml heparin containing vacutainers for analysis of cobalamin and creatinine.

THcy levels were determined in EDTA plasma by an automated high-performance liquid chromatography method with reverse-phase separation and fluorescent detection (Gilson 232–401 sample processor, Spectra Physics 8800 solvent delivery system and Spectra Physics LC 304 fluorometer) as described by Fiskerstrand et al. [12] with some modifications [24]. Plasma MMA was determined by a new LC/MS-MS method as described by us [6]. After deproteinization by ultra filtration an acidified aliquot of the eluate was injected into the HPLC system for separation of MMA and succinic acid and subsequently MMA was analysed by ESI-MS/MS. Calibrations between 0.1 and 1.0 μM exhibited consistent linearity and reproducibility. The lower limit of detection for plasma MMA was 0.1 μM (signal to noise ratio ≥3). The intra- and inter-assay CVs of ten determinations of a plasma sample were 1.5 and 6.7%, respectively, at a mean concentration of 0.29 μM. Inter-assay CVs of 25 determinations of a low, medium and high concentration (0.22, 0.45 and 0.94 μM MMA, respectively) were 8.3, 5.9 and 4.6%, respectively. The mean recovery of MMA added to plasma was 100%. Plasma Cbl was determined by using Dualcount Solid Phase

Boil Radioasssay (Diagnostic Products, Los Angeles, CA, USA).

Statistical analysis was performed using SPSS-software package (version 12.0.1). The distributions of MMA and Cbl were skewed, so logarithmic transformations were applied to normalize these distributions. Inverse transformations were computed to describe geometric means and 95% confidence intervals. Differences between two groups were tested by t-test analyses where appropriate. We used a multivariate linear regression model to study the simultaneous influence of variables on plasma MMA concentration and to observe possible interactions between individual determinants. Plasma MMA was the dependent variable, and age, creatinine and Cbl were the independent variables. The beta-coefficient expresses the change in logarithmically transformed plasma MMA that is related to a one unit change in logarithmically transformed plasma Cbl or creatinine (a 1% change in the x variable correlates with a beta% change in the y variable). Possible interaction between the variables was studied by adding interaction terms into the model. All statistical analyses were performed in children above 1 year of age.

Results

Study population

A total number of 186 children were evaluated in this study (92 boys, 94 girls). The median age of this group was 11.3 years (range 0.01–19.3). The distribution of plasma MMA was skewed to the right in this population of healthy Dutch children. The concentrations of the variables measured (MMA, tHcy, Cbl, creatinine) are summarised in Table 1. None of the children used medication possibly interfering with the studied variables. Pubertal features (menstruation and/or axillary hair growth) were present in 54 children aged 10–19 years. No significant difference in MMA, Cbl, tHcy or creatinine concentrations was found between children with or without these features. Six children smoked. This subgroup was too small to study differences between smoking and non-smoking children. In the whole study population, no significant differences in MMA, Cbl, tHcy or creatinine concentrations between sexes could be observed.

In an attempt to delineate possible age dependency of the biochemical variables we calculated geometric means of MMA, Cbl, tHcy and creatinine in different age categories (Fig. 1a–d). In children above 1 year of age, GM Cbl concentrations decreased whereas tHcy and creatinine concentrations increased with increasing age. There were positive correlations between logarithmically transformed MMA, tHcy and creatine and age ($r = 0.21$, $r = 0.63$, $r = 0.68$, $P < 0.05$, respectively). Cbl correlated negatively with age ($r = -0.53$, $P < 0.05$).

Associations between MMA and Cbl and between tHcy and Cbl are nearly the same ($r = -0.348$, $P < 0.001$ and $r = -0.409$, $P < 0.001$, respectively). No correlation between MMA and creatinine was found (Pearson coefficient 0.072, $P = 0.377$).

Children with plasma Cbl concentrations below 250 pmol/l (25th percentile) showed significantly higher mean plasma MMA concentration compared to those with higher plasma Cbl concentrations (GM plasma MMA 0.18 versus 0.25 μmol/l, respectively, $P = 0.001$). Children with Cbl concentrations below and above 200 pmol/l (10th percentile) also showed significant different GM MMA of 0.25 and 0.17 μmol/l, respectively ($P = 0.003$).

Multiple linear regression

Multiple linear regression analysis was performed to elucidate the main predictors of plasma MMA. In this analysis, Cbl was found to be the strongest determinant of plasma MMA; a 1% decrease in logarithmically transformed plasma Cbl resulted in a 0.414% increase in logarithmically transformed plasma MMA. The regression coefficient for the association between Cbl and MMA corrected for age and creatinine was −0.432 ($P < 0.0001$). Cbl alone explained 12% of the variance in plasma MMA concentrations. When Cbl, age, sex and creatine were added to the model, Cbl explained 19%, whereas the other factors each explained less than 0.1%.

Interaction between age and Cbl was studied by adding an interaction term age × Cbl to a linear regression model describing the continuous relation-

Table 1 Concentrations of variables Concentrations of variables are expressed as percentiles, geometric mean (GM) and 95% confidence intervals (95% CI). *MMA* methylmalonic acid (μmol/l), *THcy* total homocysteine (μmol/l), *Cbl* cobalamin (pmol/l), *Creat* creatinine (μmol/l), *N* number of children

	Number	GM	95%CI	10th	25th	50th	75th	90th
MMA	186	0.17	0.07–0.42	0.11	0.13	0.17	0.23	0.30
THcy	184	6.6	3.1–13.9	3.8	5.6	6.9	8.3	10.0
Cbl	161	339	139–824	200	250	330	455	592
Creat	151	65	39–111	46	59	67	78	87

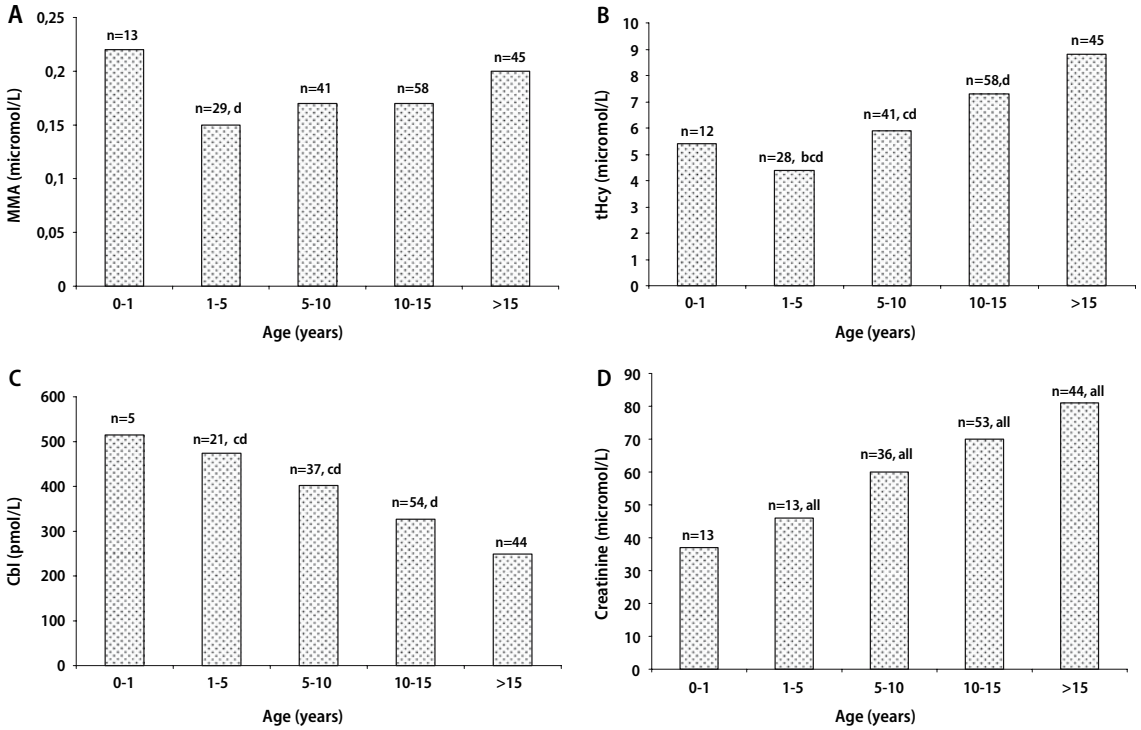

Fig. 1 The geometric mean concentrations of plasma MMA (**A**), serum tHcy (**B**), plasma Cbl (**C**) and creatinine (**D**) amongst different age groups. *Characters* indicate significant differences between age categories ($P < 0.05$). Character *a* symbolizes a significant difference with geometric mean of children aged 1–5 years, character *b* with children aged 5–10 years, character *c* symbolizes a signifcant different geometric mean from age category 10–15 years and character *d* from children above 15 years of age

Table 2 Linear regression model for plasma MMA stratified for age Linear regression model for plasma MMA versus plasma Cbl stratified for different age groups. Data for MMA and Cbl were log transformed because of skewed distribution. Results are expressed as regression coefficients with 95% confidence intervals in parenthesis. The beta-coefficient represents the percentage of change in logarithmic transformed plasma MMA following a one-unit change in logarithmically transformed Cbl

	1–5 years	5–10 years	10–15 years	>15 years
Cbl	−0.04 (−0.5 to 0.4)	−0.35 (−0.7 to −0.03)*	−0.34 (−0.6 to −0.06)*	−0.64 (−0.99 to −0.3)*
Cbl, creat	0.02 (−0.7 to 0.8)	−0.33 (−0.7to 0.006)*	−0.35 (−0.6 to −0.07)*	−0.64 (−0.99 to −0.3)*
Number	21	37	54	44

* = P value < 0.05

ship between plasma MMA and Cbl with increasing age ($P = 0.032$). Because we observed a significant interaction between Cbl and age we calculated regression coefficients for the relationship between MMA and Cbl stratified to age (Table 2). We observed negative relationships between plasma MMA and Cbl for all age groups who become stronger with increasing age (P for trend 0.045 in children >1 year of age).

Discussion

Both MMA and tHcy are metabolic markers of an intracellular Cbl deficiency. Because tHcy is influenced by many factors, especially folate status and kidney function, MMA is considered to be more sensitive and specific [18, 27]. In view of the high prevalence of Cbl deficiency among the elderly and the mounting evidence of a high prevalence of Cbl deficiency in infants and children, extensive knowledge on plasma MMA concentrations in the normal population and factors that influence this concentration is warranted [5, 7, 22, 29].

We evaluated plasma MMA concentrations in a cohort of Dutch children and studied possible determinants of plasma MMA. Regression analysis showed that Cbl was the main determinant of plasma MMA concentrations. The strength of the negative association between plasma MMA and Cbl becomes stronger with increasing age. Plasma MMA levels were significantly different between children with normal and

low Cbl concentrations. No gender difference was found for MMA, Cbl, tHcy or creatinine for the whole group. There were positive associations between plasma MMA, tHcy and creatinine with age in the children older than 1 year.

We observed a skewed MMA distribution in our population of Dutch children. GM MMA concentrations were highest in infancy. This is in line with the results from Bjorke Monsen et al. who observed the highest MMA concentrations in children aged 6 weeks to 6 months in 173 newborns [3]. In their study, MMA concentrations strongly correlated with Cbl, showing low median Cbl and high median MMA in infants during the first 6 months [3]. This contrasts with our data that show the highest MMA concentrations, but also the highest Cbl concentrations in the youngest children. Since we only have a very limited number of children in this age category with both variables known ($n = 5$), these data should be interpreted cautiously. The higher plasma MMA concentrations in infancy could also be caused by immaturity of liver and kidney function, propionate production by gut bacteria or other, yet unknown factors [17, 28, 30].

Classic cobalamin deficiency is a disease most often observed in the elderly and which usually presents with megaloblastic anaemia and/or neuropathy. There is no definite cut-off value defined for low Cbl levels but in general, patients with Cbl concentrations less than 120–200 pmol/l are considered deficient. Several authors have emphasised the importance of markers of Cbl status like plasma MMA since a functional Cbl deficiency (=decreased intracellular availability) can exist in spite of normal or low-normal Cbl concentrations [1, 7, 25, 34]. The importance of an adequate Cbl status in infancy and childhood is more and more recognized [2, 3, 10, 16, 18, 21, 23, 26, 27, 29] as well as the possible clinical sequelae of functional Cbl deficiency that may have a major impact on children's health in the general population.

Impaired Cbl status in infancy and childhood can cause a variety of common and non-specific symptoms such as failure to thrive, developmental delay or arrest, convulsions and may even lead to irreversible neurological sequelae [3–5, 10, 15, 19, 21]. No haematological symptoms need to be present. 10 to 15% of apparently healthy infants from omnivorous mothers, showed increased plasma and/or urinary MMA concentrations in studies performed by Bjorke Monsen, Minet and Specker [3, 22, 29]. In most infants this increased plasma MMA concentration is accompanied by an increase in tHcy concentrations and/or decrease in Cbl concentrations reflecting most probably not an immature system but a true functional Cbl deficiency. Our observation that children with elevated MMA also have decreased Cbl supports this theory.

This high proportion of suspected Cbl deficiency in infants is an alarming observation considering the role of Cbl in the development of the central nervous system. Irreversible neurological sequelae have been described [21]. Causes of functional Cbl deficiency in infants and children are, amongst others, decreased intrauterine storage (placental disorders, low Cbl status of the pregnant woman), postnatal decreased dietary intake (vegetarian or macrobiotic diet, breastfed infants of Cbl deficient women) and absorption problems [3, 11, 25].

We observed a significant difference in GM MMA between the 1–5 years old and the children above 15 years as well as a slight positive correlation between MMA and age, which contrasts with the observations of Bjorke Monsen et al. [4, 5] who found no effect of age on median plasma MMA values in children above 1 year of age, although a decline in Cbl levels was found. The relationship between Cbl with age in children has been described before [2, 4, 20].

As expected, plasma MMA and Cbl were strongly inversely correlated, confirming data from literature [4, 5]. The strength of the negative association increased with increasing age. As anticipated, it appeared that the association between MMA and Cbl is the strongest in the lowest Cbl concentrations.

To summarize, we studied plasma MMA concentrations in a cohort of Dutch children. In the youngest age group (<1 year), plasma MMA values show the greatest variation and highest values. Plasma Cbl is the main determinant of MMA in this Dutch study group, especially in those individuals >1 year of age. The strongest correlation exists in the eldest age group (>15 years of age). There is a slight increase with age in the children older than one year of age. We suggest thinking of functional Cbl deficiency at a wide range of clinical symptoms in infants and children. Plasma MMA should be the main marker for further evaluation.

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