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ScienceDirect
Metabolism Clinical and Experimental 56 (2007) 339–347
Metabolism
Clinical and Experimental
www.elsevier.com/locate/metabol

Elevated serum *S*-adenosylhomocysteine in cobalamin-deficient megaloblastic anemia

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Received 20 June 2006; accepted 26 October 2006

Abstract

Impaired methylation due to accumulation of *S*-adenosylhomocysteine (SAH) may contribute to the pathophysiology of cobalamin-deficient anemia. We assayed serum *S*-adenosylmethionine (SAM), SAH, total homocysteine (tHcy), and methylmalonic acid (MMA) in 15 subjects with cobalamin-deficient megaloblastic anemia and compared results with those of 19 subjects with anemia/pancytopenia due to other causes. Cobalamin-deficient subjects had a median hematocrit level of 20% and mean cell volume of 111.7 fL. The median serum cobalamin level was 37 pg/mL, MMA 3030 nmol/L, and tHcy 62.0 μmol/L. SAH was elevated in 13 of 15 subjects (median, 42 nmol/L) and the median SAM value was normal (103 nmol/L), but SAM/SAH ratio was low (2.5). The SAH was higher and SAM/SAH ratio was lower in cobalamin-deficient subjects compared with those with other anemias after excluding 4 patients with renal insufficiency. SAM concentrations were not low in cobalamin deficiency. Cobalamin injections corrected anemia, MMA, tHcy, SAM/SAH ratio, and SAH. Some hematologic variables were inversely correlated with SAH and cobalamin but not tHcy or MMA. In conclusion, serum SAH is elevated in cobalamin-deficient subjects with megaloblastic anemia and corrects with parenteral cobalamin therapy.

1. Introduction

Severe megaloblastic anemia due to cobalamin (vitamin B_{12}) or folate deficiency is uncommon yet important to recognize because other causes of macrocytic pancytopenia rarely have curative therapy. Assays of the cobalamin-dependent metabolites are useful adjuncts to serum vitamin levels in diagnosing deficiency [1-3] because virtually every patient with cobalamin-deficient megaloblastic anemia has elevated levels of serum and/or urine methylmalonic acid (MMA) and total homocysteine (tHcy) [1,2]. One coenzyme form, methylcobalamin, is a cofactor for methionine synthase, which is shown in Fig. 1. Homocysteine is methylated to methionine, as N_5-methyltetrahydrofolate is demethylated to form tetrahydrofolate, the precursor of cofactors for the synthesis of thymidine and purines as well as other reactions of 1-carbon metabolism [4]. Methionine is a precursor of *S*-adenosylmethionine (SAM), which is used in many SAM-dependent methylations important in the synthesis of creatine, phospholipids, and neurotransmitters and in DNA and RNA methylation reactions [5]. *S*-Adenosylhomocysteine (SAH) is the resulting product and can be cleaved by SAH hydrolase to form homocysteine. Homocysteine can also be condensed with serine by cystathionine β-synthase. The flow of methionine metabolism is regulated by the availability of SAM because SAM is an activator of cystathionine β-synthase [6]. SAM also inhibits methylenetetrahydrofolate reductase (MTHFR), the enzyme that converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate [6]. A SAM-dependent methylation of glycine removes excess methyl groups and is inhibited by 5-methyltetrahydrofolate [7]. Homocysteine is also methylated by betaine homocysteine methyltransferase (BHMT) with products of methionine and *N*,*N*-dimethylglycine. Both *N*,*N*-dimethylglycine and *N*-methylglycine

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doi:10.1016/j.metabol.2006.10.009

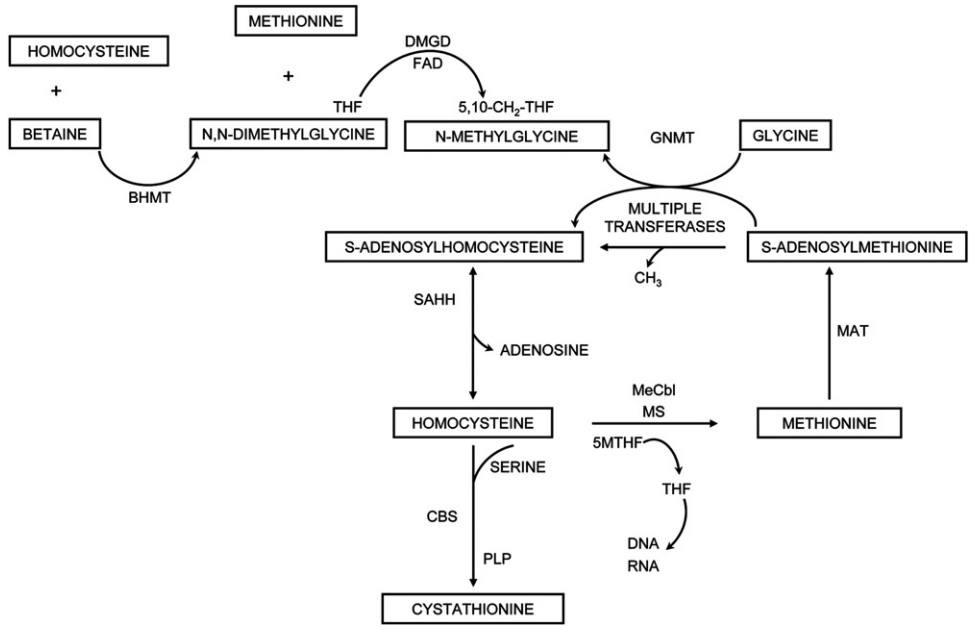

Fig. 1. Pathways of methionine metabolism. MS indicates methionine synthase; MAT, methionine adenosyltransferase; GNMT, glycine *N*-methyltransferase; SAHH, *S*-adenosylhomocysteine hydrolase; CBS, cystathionine β-synthase; PLP, pyridoxal phosphate; THF, tetrahydrofolate; MeCbl, methylcobalamin; FAD, flavin adenosine dinucleotide; DMGD, dimethylglycine dehydrogenase.

are cleared by using tetrahydrofolate as a cofactor [5]. Previous investigations of subjects with severe cobalamin deficiency largely due to pernicious anemia have shown that tHcy and MMA are invariably elevated [1,2], cystathionine is usually elevated, [2,8] and *N*,*N*-dimethylglycine is frequently elevated [2,9], whereas methionine and *N*-methylglycine concentrations are generally maintained [2,9]. In megaloblastic anemia due to folate deficiency, tHcy [1,2], cystathionine [2,8], *N*,*N*-dimethylglycine, and *N*-methylglycine [2,9] are usually elevated. MMA is in the reference range in folate deficiency.

Studies in animals with nitrous oxide–induced cobalamin inactivation have suggested that SAH concentrations in tissues are increased and SAM concentrations may be decreased with an altered SAM/SAH ratio [10]. Such abnormalities could be of pathophysiological importance because SAH is an inhibitor of most methylation reactions. It is also possible that a deficiency of SAM could result in deficient methylation of critical compounds and cause some of the abnormalities seen in cobalamin or folate deficiency [11]. We recruited subjects with severe cobalamin-deficient megaloblastic anemia and measured serum SAM and SAH [12] as well as the other vitamin-related metabolites both before and (in a subgroup) after treatment with cobalamin.

2. Methods

The patients were recruited from the Leonor Hospital in Sorocaba City, Sao Paulo, Brazil, between March and May, 2001. Patients were eligible if they had macrocytic red blood cells, anemia, and/or other cytopenias. The routine testing for hematologic abnormalities was performed in Brazil and included the determination of the hemoglobin, hematocrit, mean corpuscular volume (MCV), white blood cell count, absolute neutrophil count, and platelet count with an STK Coulter counter (Beckman Couter, Fullerton, CA). Serum cobalamin was measured by IMx System (Abbott Laboratories, Abbott Park, IL) and serum and red blood cell folate by ionic capture methodology (Abbott Laboratories). Routine serum chemistries were determined by the clinical laboratory. Blood was also collected for methionine metabolites. Whole blood was allowed to clot on ice and then centrifuged to separate serum and stored frozen at −80°C. These samples were sent to the laboratory in Denver, Colorado, by overnight express on dry ice and were still frozen on arrival. MMA, tHcy, cystathionine, 2-methylcitric acid, methionine, *N*,*N*-dimethylglycine, and *N*-methylglycine were determined by stable isotope dilution and capillary gas chromatography-mass spectrometry as previously described [8,9,13]. The reference ranges had been previously determined on 60 blood donors aged 18 to 65 years in the United States and were as follows: MMA, 73 to 271 nmol/L; tHcy, 5.4 to 13.9 μmol/L; cystathionine, 44 to 342 nmol/L; 2-methylcitric acid, 60 to 228 nmol/L; methionine, 13 to 45 μmol/L; *N*,*N*-dimethylglycine, 1.4 to 5.3 μmol/L; and *N*-methylglycine, 0.6 to 2.7 μmol/L [8,9,13]. Cobalamin deficiency was defined as serum cobalamin level less than 350 pg/mL, serum MMA greater than 271 nmol/L, and MMA greater than 2-methylcitric acid value [13]. Pure folate deficiency was defined as serum folate concentration less than 5 ng/mL, tHcy greater than 13.9 μmol/L, and MMA 271 nmol/L or less.

SAM and SAH were quantitated by stable isotope dilution and liquid chromatography-mass spectrometry [12].

Table 1
Laboratory and clinical variables in 15 cobalamin-deficient subjects

Case No.	Age (y)	Sex	MMA (nmol/L)	tHcy (μmol/L)	SAM (nmol/L)	SAH (nmol/L)	SAM/SAH ratio	Methionine (μmol/L)	Cobalamin (pg/mL)	Folate use[a]	Hct (%)	Tx Hct (%)	MCV (fL)	Tx MCV (fL)
1	48	M	13900	197.8	93	58	1.6	9.2	26	–	18.3	50.5	130.3	89.3
2	77	F	12300	134.0	164	66	2.5	14.8	34	–	27.3	34.8	122.5	89.7
3	43	F	8730	76.3	234	59	4.0	14.4	0	+	11.2	38.6	119.2	89.2
4	77	M	6600	119.2	91	36	2.5	31.1	66	–	34.8	38.9	111.7	98.5
5	19	F	3160	70.6	65	26	2.5	13.8	91	–	33.9		104.1	
6	58	F	3150	117.3	131	65	2.0	10.3	25	+	18.0	45.1	100.4	82.1
7	40	M	3100	107.0	144	47	3.1	12.9	20	–	18.5	47.5	116.9	86.8
8	38	F	3030	44.3	65	42	1.6	17.2	20	+	20.0		109.6	
9	65	M	1880	56.8	104	29	3.6	21.2	89	+	24.6	34.5	56.1	63.4
10	56	F	1810	55.9	94	50	1.9	15.8	82	+	18.7	38.1	128.1	89.6
11	15	F	1550	62.0	96	42	2.3	13.4	0	–	15.7		113.2	
12	29	F	1090	55.1	103	41	2.5	13.4	34	+	17.4	41.8	112.0	95.8
13	68	M	833	8.1	222	48	4.6	15.3	212	+	26.6		65.0	
14	39	F	373	7.6	104	18	5.8	40.1	185	–	32.9	34.3	75.9	80.5
15	56	M	299	13.5	88	29	3.0	22.2	252	–	38.0		81.2	
Mean (SD)	48.5 (19.2)		4120 (4310)	75.0 (52.3)	120 (51)	44 (14)	2.9 (1.2)	17.7 (8.2)	77 (79)		23.7 (8.1)		103.0 (22.9)	
Median (reference range)	48		3030 (73-271)	62.0 (5.4-13.9)	103 (71-168)	42 (8-26)	2.5 (4.4-12.4)	14.8 (13-45)	34 (>350)		20.0 (>36 F, >40 M)		111.7 (80-100)	

Hct indicates hematocrit; Tx Hct, Hct after parenteral cobalamin treatment; Tx MCV, MCV after parenteral cobalamin treatment.

[a] Folate use for 1 week to 1 year before metabolite measurements.

In 48 healthy subjects from the United States, reference range ±2 SD for SAM was 71 to 168 and for SAH was 8 to 26 nmol/L; SAM/SAH ratio was 4.4 to 12.4. Subjects were genotyped for the thermolabile variant of MTHFR C677T by polymerase chain reaction-restriction fragment length polymorphism [14]. The common variant at C677T was designated as follows: CC, wild type; CT, heterozygous; and TT, thermolabile homozygous variant.

The treatment of the subjects with various diagnoses followed the usual clinical practice at Leonor Hospital. Empiric folic acid therapy in varying doses had been given to 7 subjects for 1 week to 1 year before being seen at Leonor Hospital and before the blood collections for vitamin and related metabolites. Those determined to be cobalamin deficient received cyanocobalamin injections. Ten cobalamin-deficient subjects returned to the hospital after treatment and had repeat determinations of the blood counts and metabolites. Patients with iron deficiency anemia or other anemias had appropriate treatment. The protocol was approved by the institutional review board in Brazil and at the University of Colorado Health Sciences Center.

The various variables were analyzed with SPSS-based software version 10.0 (SPSS, Chicago, IL). Differences in means for continuous variables across 2 categories were evaluated with the t test and Levene test for equality of variances. A P value $<.05$ was considered significant. Paired t tests were used to compare pre- and posttreatment variables. The Spearman ρ correlation coefficient was calculated between variables.

3. Results

Thirty-seven subjects with macrocytosis, anemia, neutropenia, or thrombocytopenia were evaluated prospectively during the study period. There were 13 whites, 5 blacks, 17 mixed race, 1 Asian, and 1 unknown race. Fifteen met our diagnosis of cobalamin deficiency because MMA was elevated and serum cobalamin was less than 350 pg/mL. One subject met our diagnosis of folate deficiency because of serum folate less than 5 ng/mL and tHcy greater than 13.9 μmol/L. Two other subjects probably had impaired folate status. In one, serum folate was low and tHcy was 13.9 μmol/L, but cystathionine was clearly elevated to 521 nmol/L. The other subject had a borderline serum folate level of 5.0 ng/mL but tHcy was 19.8 μmol/L. All 3 with impaired folate status also had low serum cobalamin, although MMA ranged only from 183 to 255 nmol/L; thus, they did not meet our cobalamin deficiency definition. Eighteen subjects with anemia and 1 with isolated neutropenia were also evaluated and did not meet the criteria for cobalamin deficiency. These 19 subjects are referred to as the group with "other anemia."

3.1. Metabolites in cobalamin-deficient megaloblastic anemia

Table 1 shows individual data from the 15 cobalamin-deficient subjects arranged in descending order of elevation of serum MMA. The megaloblastic anemia was frequently severe, with median hemoglobin of 6.9 g/dL and hematocrit of 20%. The MCV was greater than 100 fL in 11 of the 15 subjects and the median value was 111.7 fL. The median white blood count was only 3.3 × 10^9/L with a median absolute neutrophil count of 1.8 × 10^9/L (Table 2). Six of the subjects had an absolute neutrophil count of fewer than 1.5 × 10^9/L. The median platelet count was 171 × 10^9/L; however, 7 subjects had fewer than 120 × 10^9/L. The serum cobalamin values were markedly low, with a median value of 34 pg/mL. The median serum MMA was 3030 nmol/L and was greater than 5000 nmol/L in 4 subjects and between 1000 and 5000 in 8 subjects. Elevations in tHcy were also extreme with a median value of 62.0 μmol/L. The values were greater than 100 μmol/L in 5 of the 15 subjects and between 40 and 100 μmol/L in 7 subjects. Three subjects had tHcy of 13.9 μmol/L or less, although MMA was elevated. The SAH was elevated in 14 of the 15 subjects and the median value was 42 nmol/L. SAH was at the upper level of the reference range (26 nmol/L) in one of the subjects. The median value for SAM was 103 nmol/L and only 2 subjects had low (<71 nmol/L) values. The median value for cystathionine was 630 nmol/L and all but 4 individuals were elevated over our previously determined reference range (Table 2).

The median 2-methylcitric acid value (reference range, 60-228 nmol/L) was also increased to 589 nmol/L and was elevated in all but 2 individuals. The median methionine value was 14.8 μmol/L, which was in the lower part of the reference range (13.1-45 μmol/L), and 3 of the cobalamin-deficient subjects had low values. The median N,N-dimethylglycine value of 5.5 μmol/L was near the top of

Table 2
Additional laboratory and metabolic variables in 15 patients with cobalamin deficiency

Variable	Mean (SD)	Median	Patient range[a]	Reference range[b]
Hemoglobin (g/dL)	7.9 (2.7)	6.9	4-12.6	12-17
White blood count (× 10^9/L)	4.6 (3.8)	3.3	1.9-17.2	4.5-11.0
Absolute neutrophil count (× 10^9/L)	2.7 (3.4)	1.8	0.6-14.7	3.0-5.8
Platelets (× 10^9/L)	215 (162)	171	51-564	150-450
Cystathionine (nmol/L)	737 (371)	630	306-1410	44-342
2-Methylcitrate (nmol/L)	841 (650)	589	167-2537	60-228
N-Methylglycine (μmol/L)	1.5 (0.5)	1.3	0.9-2.5	0.6-2.7
N,N-Dimethylglycine (μmol/L)	5.6 (2.5)	5.5	3.1-13.0	1.4-5.3
Ferritin (μg/L)	227 (265)	188	9-967	20-250
Creatinine (mg/dL)	0.8 (0.3)	0.8	0.4-1.5	<1.4
Serum urea nitrogen (mg/dL)	29 (11)	26	15-56	<30
Serum folate (ng/mL)	13.7 (5.2)	13.0	6.7-24	>5.0

[a] The actual range of values in the 15 cobalamin-deficient subjects is shown.

[b] Generally accepted normal values are shown except for the cobalamin-dependent metabolites, which were determined as in "Methods."

our previously determined reference range (1.4-5.3 μmol/L) and was elevated above the reference range in 8 of the 15 subjects.

Seven cobalamin-deficient subjects received oral folic acid therapy before the phlebotomy for intervals of 1 week up to 1 year. The serum and red blood cell folate values were not significantly different in those pretreated with folate or not. The mean hematocrit and hemoglobin levels were lower, 19.5% vs 28.6% ($P = .023$) and 6.4 vs 9.7 g/dL ($P = .011$), respectively, in those who were treated. *N*-Methylglycine trended lower in those treated, 1.3 vs 1.6 μmol/L ($P = .08$). The other metabolites and variables were not significantly different.

The serum SAH was elevated in the 1 subject who met our diagnosis of folate deficiency. This subject also had macrocytic anemia, elevated levels of tHcy, *N*,*N*-dimethylglycine, and *N*-methylglycine, consistent with folate deficiency. The SAM/SAH ratio was low (2.8) in this subject and in another possible folate-deficient subject, although SAH was normal in the latter.

There were 4 cobalamin-deficient subjects (cases 9, 13, 14, and 15 in Table 1) who likely also had iron deficiency based on serum ferritin values of 9 to 17 μg/L in 3 of them and low MCV of 65.0, 75.9, and 81.2 fL. Ferritin was not available for the fourth subject; however, MCV was 56.1 fL in this subject. Cases 14 and 15 had only mild cobalamin-deficient metabolic abnormalities but did meet our diagnosis of cobalamin deficiency. Cases 9 and 13, however, had MMA values that overlapped those of the other cobalamin-deficient patients. Hemoglobin electrophoresis was performed on samples from 14 of the subjects and showed normal findings except a small increase in hemoglobin F in one and sickle trait in another subject. No subject appeared to have β-thalassemia; however, electrophoresis was not available for the subject with the severely depressed MCV of 56.1 fL. It therefore appeared that the low MCV values in 3 of the subjects with cobalamin deficiency were probably related to low iron status rather than coexisting β-thalassemia.

3.2. Thermolabile MTHFR and cobalamin deficiency

The genotype for MTHFR C677T polymorphisms was available for 14 of the cobalamin-deficient patients. There were 7 of each genotype, CC and CT. The serum cobalamin and the platelet count were significantly higher in those with the CT status. The serum SAH trended lower in those with the CT status. In the other anemia group there were 10 CC, 6 CT, and 1 TT genotype. Hematocrit and hemoglobin levels were lower in those with the CT rather than the CC status. One of the possible folate-deficient subjects had the TT variant.

3.3. Serum metabolites in cobalamin-deficient patients compared with those with other causes of anemia

The clinical variables and mean (SD) of the serum metabolites are shown in Table 3 for the cobalamin-deficient patients compared with the 19 patients with other apparent causes of anemia or cytopenias.

The mean values are also shown for 15 other anemia subjects after excluding the 4 subjects who had renal insufficiency, as defined by serum urea nitrogen level greater than 60 mg/dL. These 4 had serum urea nitrogen values ranging from 60 to 85 mg/dL and serum creatinine level of 0.9, 1.6, 1.8, and 2.8 mg/dL. Only 2 cobalamin-deficient subjects had evidence of mild renal insufficiency with serum creatinine of 1.5 mg/dL in one and serum urea nitrogen of 56 mg/dL in another.

The MCV, MMA, and tHcy levels were lower in the other anemia subjects compared with those with cobalamin deficiency, as expected (Table 3). The serum SAM was not different in the 2 groups. The SAH was not significantly lower in the total group of subjects with other anemia because of the elevated values in the 4 subjects with renal failure whose values ranged from 56 to 124 nmol/L. When these 4 subjects were removed from the analysis, the mean SAH was significantly higher in the cobalamin-deficient subjects (44 vs 26 nmol/L, $P = .004$). Additional analysis of SAH values excluding the 2 cobalamin-deficient subjects with borderline renal insufficiency and the 4 other anemia subjects was also significant ($P < .005$, data not shown). Anemia may have been related to renal failure with erythropoietin deficiency in these 4 other anemia subjects, although it was not investigated further. The SAM/SAH ratio was also significantly lower in cobalamin-deficient subjects compared with the subjects with other anemia. Serum MMA was normal in all of the subjects in the other anemia group except one who had a value of 869 nmol/L with a simultaneous serum cobalamin concentration of 454 pg/mL and, thus, did not meet our diagnosis for cobalamin deficiency. He had undergone gastric surgery, had normal serum creatinine, and appeared to have iron deficiency, with an MCV of 56.7 fL and ferritin of 29 μg/dL.

Table 3
Mean (SD) metabolites and other variables in cobalamin-deficient subjects vs those with other causes of anemia

	Cobalamin deficient (n = 15)	Other anemia (n = 19)	P*	Other anemia[a] normal renal status (n = 15)	P**
Hematocrit (%)	23.7 (8.1)	27.1 (7.2)	NS	28.1 (6.6)	NS
MCV (fL)	103.1 (22.9)	87.9 (17.3)	.035	86 (18)	.029
Cobalamin (pg/mL)	77 (79)	666 (512)	<.001	580 (429)	<.001
MMA (nmol/L)	4120 (4310)	226 (166)	.004	225 (187)	.004
tHcy (μmol/L)	75.0 (52.3)	9.4 (4.0)	<.001	8.6 (3.9)	<.001
SAM (nmol/L)	120 (51)	127 (62)	NS	104 (40)	NS
SAH (nmol/L)	44 (14)	37 (29)	NS	26 (16)	.004
SAM/SAH ratio	2.9 (1.2)	4.1 (1.3)	.008	4.4 (1.2)	.002

NS indicates not significant.

[a] The subjects with poor renal function (serum urea nitrogen ≥60) were excluded.

* *P* value comparing cobalamin-deficient vs other anemia by *t* test.

** *P* value comparing cobalamin-deficient vs other anemia without the 4 subjects with renal insufficiency.

Because his cobalamin value was greater than our predetermined cut point, we could not include him in the cobalamin-deficient group, but it is extremely likely that cobalamin deficiency was the cause of his elevated MMA value. The tHcy was 13.9 μmol/L or greater in 3 other anemia subjects; however, serum folate was not decreased and the MMA and serum cobalamin values did not meet our criteria for deficiency. One of these 3 subjects had macrocytic anemia, 1 normocytic, and 1 microcytic.

Microcytic anemia likely due to iron deficiency was present in 8 of the subjects with other anemia, of whom 5 had low serum ferritin ranging from 2 to 15 μg/L, 1 had 29 μg/L, and 2 had unknown values. There were 6 subjects with other anemia with pancytopenia and macrocytosis who most likely had bone marrow failure syndromes. One of these subjects was diagnosed 3 months later with acute leukemia. Two of those with pancytopenia also had elevated serum urea nitrogen and creatinine levels.

Six subjects with other anemia received folic acid treatment before phlebotomy for metabolite levels. The serum and red blood cell folate were significantly higher, 12.1 vs 7.9 ng/mL ($P = .018$) and 1490 vs 448 ng/mL ($P = .025$), in those treated, and the tHcy was significantly lower, 5.8 vs 11.2 μmol/L ($P = .001$). The serum SAH trended lower in those treated, 21 vs 42 nmol/L ($P = .079$). Hematologic variables were not different.

3.4. Correlations between variables in the cobalamin-deficient subjects

Correlations were determined between variables for the entire group of cobalamin-deficient patients (n = 15) and for some variables in the subgroup of cobalamin-deficient patients excluding those with apparent coexisting iron deficiency (n = 11). For all 15 subjects, age was correlated with creatinine as expected (Spearman ρ, 0.600; $P = .018$) but not with other metabolites.

S-Adenosylhomocysteine was correlated with SAM (0.523, $P = .045$), methylcitric acid (0.533, $P = .041$), cystathionine (0.623, $P = .013$), tHcy (0.544, $P = .036$), and MMA (0.535, $P = .040$), and inversely with methionine (−0.552, $P = .033$), cobalamin (−0.535, $P = .040$), and *N*-methylglycine (−0.607, $P = .017$). SAM was correlated only with SAM/SAH ratio (0.560, $P = .030$). tHcy, MMA, cystathionine, and methylcitric acid were all mutually correlated ($P < .001$). Methionine was directly correlated with cobalamin (0.581, $P = .023$) and indirectly with tHcy (−0.542, $P = .037$) and cystathionine (−0.468, $P = .078$). The white blood cell count was directly correlated with cobalamin (0.513, $P = .050$).

Correlations between metabolic variables and the other hematologic parameters were done on the 11 subjects thought to have "pure" cobalamin deficiency. The creatinine was directly correlated with hematocrit (0.608, $P = .047$) and hemoglobin (0.637, $P = .035$). Hematocrit was correlated with cobalamin (0.700, $P = .016$), trended with methionine (0.551, $P = .079$), and inversely trended with SAM (−0.556, $P = .076$) with similar findings for hemoglobin. The white blood cell count and absolute neutrophil count had strong inverse correlations with SAH (−0.683, $P = .021$ and −0.672, $P = .023$, respectively). The correlation of SAH with hematocrit was not significant (−0.337, $P = .311$).

The hemoglobin and hematocrit trended inversely with SAH (−0.478, $P = .099$, and −0.506, $P = .078$, respectively) when the correlations were adjusted for serum ferritin in the entire group of 15 cobalamin-deficient patients. MCV became inversely correlated with SAM/SAH ratio in the same analysis (−0.657, $P = .015$).

3.5. Response to treatment in cobalamin-deficient megaloblastic anemia

There were 10 patients who were available for investigation after treatment with cyanocobalamin injections. The individual and mean pre- and posttreatment values, shown in Tables 1 and 4, demonstrate an excellent correction of hematologic abnormalities in most cases. There were dramatic increases in mean hematocrit (82%), white blood cells (77%), and absolute neutrophil count (115%), and a decrease in MCV (20%). One subject with combined iron and cobalamin deficiency remained severely microcytic. Mean tHcy decreased 12-fold and MMA decreased 34-fold. Cystathionine and methylcitric acid values were corrected.

The mean SAH decreased by nearly 50%, and SAM was unchanged. The SAM/SAH ratio, however, was increased significantly. SAH remained elevated at 51 nmol/L after treatment in 1 subject who had elevated serum urea nitrogen. The SAM/SAH ratio improved in this subject, 2.5 to 4.0, because the SAM increased from 164 to 203 nmol/L after treatment. In contrast to serum SAH, the tHcy decreased from 134 to 7.6 μmol/L and MMA decreased from 12,300 to 139 nmol/L in this subject. Like

Table 4
Mean (SD) values before and after cobalamin treatment in 10 cobalamin-deficient patients

	Before treatment	After treatment	P*
Hematocrit (%)	22.2 (7.5)	40.4 (5.7)	.001
Hemoglobin (g/dL)	7.5 (2.5)	14.0 (2.2)	<.001
MCV (fl)	107.3 (23.8)	86.5 (9.7)	.004
White blood cells (× 10^9/L)	3.4 (0.9)	6.0 (3.4)	.025
Absolute neutrophil count (× 10^9/L)	1.7 (0.6)	3.6 (2.4)	.023
Platelets (× 10^9/L)	165 (128)	219 (66)	.092
tHcy (μmol/L)	92.7 (53.6)	7.7 (3.2)	.001
MMA (nmol/L)	5300 (4860)	154 (63)	.009
SAH (nmol/L)	47 (16)	25 (11)	.001
SAM (nmol/L)	126 (45)	114 (41)	.563
SAM/SAH (ratio)	3.0 (1.3)	4.9 (1.3)	.006
Cystathionine (nmol/L)	886 (363)	265 (169)	<.001
Methylcitric acid (nmol/L)	1050 (708)	145 (79)	.002
Methionine (μmol/L)	18.3 (9.9)	23.2 (8.8)	.200
N-Methylglycine (μmol/L)	1.6 (0.5)	1.6 (0.6)	.747
N,*N*-Dimethylglycine (μmol/L)	5.4 (1.4)	5.2 (2.5)	.766

* P value for paired samples t test is shown.

the SAH value, cystathionine remained elevated at 610 nmol/L and methylcitric acid was also slightly over the reference range at 232 nmol/L (the latter 3 metabolites very sensitive to impaired renal status). The MCV declined from 122 to 89.7 fL over the same interval, confirming a hematologic response to cobalamin therapy.

Only 1 subject still had elevated tHcy after treatment (14.6 μmol/L), although the value had decreased from the extreme value of 197.8 μmol/L. A different subject had a modest elevation in MMA of 303 nmol/L after treatment.

The MCV was decreased to less than 100 fL in every case and remained quite low in a microcytic subject at 63.4 fL. Four of the subjects remained mildly anemic including the 2 with the lowest MCV values who probably had coexisting iron deficiency. The white blood cell count and absolute neutrophil count remained low in the 1 subject mentioned above with the elevated SAH after treatment. One patient with a clear response of metabolites, MCV, and hematocrit after treatment continued to be thrombocytopenic after treatment.

4. Discussion

We have prospectively assayed serum SAH and SAM/SAH ratio for the first time in patients with well-documented cobalamin-deficient megaloblastic anemia and determined that SAH is increased, SAM/SAH ratio is decreased, and these abnormalities correct after parenteral cobalamin treatment. Many of the subjects had severe anemia accompanied by very low serum cobalamin values and often extreme elevations of tHcy and MMA levels. Therefore, this cohort was suitable for testing the hypothesis that SAM deficiency or altered methylation ratio occurs in cobalamin-deficient megaloblastic anemia. Another strength of our investigation was that the samples were carefully collected, stored and shipped frozen, and assayed promptly so that artifactual increases in SAH and decreases in SAM, which we have previously described [12], did not occur. Although we found that SAH was elevated approximately twice normal in cobalamin deficiency, our surprising finding was that serum SAM was not decreased and did not change significantly in the 10 subjects who also had values obtained after treatment. In fact, inspection of Table 1 shows that some of the subjects had SAM values that were high or high normal. We also found a surprising inverse relationship between hemoglobin and SAM. An important hypothesis explaining the pathophysiology of cobalamin or folate deficiency has been the depletion of SAM, which is a crucial substrate for the methylation of DNA and neurotransmitters, formation of creatine and phospholipids, and many other important reactions [11]. We are not able to provide data to substantiate this hypothesis. Other investigators recently found that SAM was not low in cobalamin-deficient subjects [15]. The archived samples in that report were not suitable for SAH assay; thus, SAM/SAH ratio was not reported [15].

In contrast, high SAH concentrations may be important in the pathophysiology of megaloblastic anemia because SAH correlated inversely with white blood count and hematocrit (after adjustment for ferritin) in our cobalamin-deficient cohort, in contrast to MMA and tHcy. The elevation of SAH in the subjects could be just a marker for the level of inhibition of methionine synthase in cobalamin deficiency with a resulting decrease in production of methionine and an accumulation of homocysteine. SAH hydrolase actually favors the synthesis of SAH, and only the removal of homocysteine and adenosine keeps the reaction going in the catabolic direction under usual physiological conditions [6]. That tHcy was not a predictor of the hematologic variables suggests that the build up of SAH is detrimental, independent of its relationship with tHcy. Because SAH is an inhibitor of many methyltransferases, it may have other detrimental effects [17]. For example, we found a direct relationship between serum creatinine and hematocrit, which was unexpected. It is well known that renal insufficiency causes anemia, which could have caused an inverse relationship. However, because this group of cobalamin-deficient subjects had normal renal function, lower serum creatinine level could reflect decreased creatine synthesis. Approximately 75% of daily SAM-dependent methylations are used in the formation of creatine from guanidinoacetic acid [5]. The positive relationship between hematocrit and creatinine in this study could be interpreted as showing that in severe cobalamin deficiency, the inhibition of creatine formation by SAH is physiologically important.

We have previously found that methionine was not low in subjects with severe cobalamin deficiency [2], and the data in this cohort confirm that finding for the entire group. We previously did find lower methionine and SAM in pregnant Brazilian women who had low cobalamin status, although a diet deficient in animal protein probably was the cause of both conditions [16]. The serum methionine values in the Brazilian subjects studied here and previously [16] appeared to be somewhat lower than values in a reference range derived in the United States, which may reflect differences in the quantity of dietary animal protein consumed. It is likely that methionine concentrations, however, can be maintained in the cobalamin-deficient subject by both dietary intake of protein and by hepatic synthesis of methionine by BHMT [6]. The high *N*,*N*-dimethylglycine values in these deficient subjects suggests that BHMT synthesis of methionine was high. Because serum and presumably hepatic methionine concentrations are maintained in cobalamin deficiency, it is not surprising that the serum SAM concentrations were maintained. Unexplained, however, is that a number of the cobalamin-deficient subjects in this investigation actually had *elevated* concentrations of SAM. The inverse correlation of hematocrit and SAM was also unexpected. Investigations of hepatic methionine adenosyltransferase activity in cobalamin deficiency would be interesting, although not feasible in human subjects.

Although most subjects had normal SAM concentrations, the elevation in SAH resulted in a decreased SAM/SAH ratio, which corrected after treatment. The elevated SAH and altered SAM/SAH ratio could be important because SAH is a potent product inhibitor of most SAM-dependent methyltransferases. There are at least 39 mammalian SAM-dependent methyltransferases involved in reactions of DNA, RNA, lipid, and small molecule, and in protein methyl transfer reactions [17]. The activity of an erythrocyte repair protein, L-isoaspartate (D-aspartate) *O*-methyltransferase, has been studied in patients with renal failure and found to be inhibited by the buildup of SAH in such patients [18]. It would be of interest to study this enzyme in subjects with severe cobalamin-deficient megaloblastic anemia.

One of the most striking features of cobalamin- and folate-deficient megaloblastic anemia is the death of the immature red cell precursors in the bone marrow before release, which has been termed "ineffective erythropoiesis" [19]. It appears that many of the erythrocyte precursors undergo intramedullary apoptosis, which has been postulated to be due to the inability of the cells to replicate and repair DNA due to the lack of thymidylate synthesis [19]. It is also possible that accumulation of the methylation inhibitor SAH could contribute to apoptosis. In one report, DNA fragmentation was more severe with treatment using a SAH hydrolase inhibitor combined with extra homocysteine [20]. Experimental evidence is also accumulating that apoptosis of erythroid precursors in experimental folate deficiency is due to DNA damage resulting from thymidylate deficiency and purine synthesis [21].

We were not able to demonstrate an effect of the common thermolabile mutation of MTHFR in our small population. There were only 3 of the 34 individuals who were homozygous for the TT polymorphism. We studied many persons with African ancestry who are known to have a low prevalence, and the racial mix of the studied population was probably responsible for the low frequency [22].

One limitation of our study is that we assayed serum SAM and SAH instead of tissue levels, and our previous research has shown that the concentration of these compounds are highly dependent on renal status [12]. This is an inherent problem of performing clinical research because it is extremely unlikely that hepatic concentrations of these compounds will be measured in humans with cobalamin deficiency. A strength of our investigation is that most of the subjects had clear, unequivocal evidence of severe cobalamin-deficient megaloblastic anemia. The subjects who received empiric folate therapy also had severe megaloblastic anemia and can be said to have pure cobalamin deficiency because folate status had been previously corrected. The dramatic rise in hematocrit level, fall in MCV level, and normalization of MMA and tHcy levels in the subjects available for follow-up further confirmed the specificity of the diagnosis. Another strength of our investigation is that renal function was normal in most of the subjects so that elevations of SAM and SAH due to impaired clearance of these metabolites were less of a diagnostic problem. Furthermore, our conditions of sample preparation and storage likely minimized artifactual increases in SAH because prolonged storage and room temperature incubations increase SAH and decrease SAM [12]. Because these subjects did not have low serum SAM, it can be concluded that serum SAM is not decreased in severe cobalamin deficiency. It is possible that liver SAM concentrations could have been decreased, but this will be difficult to study in humans. Although SAH is elevated, the increase in SAH and the decrease in the methylation ratio is modest compared with the increases in tHcy and MMA seen in severe cobalamin deficiency. It seems likely that serum SAH assays will have limited diagnostic use for cobalamin deficiency for that reason. Elevated SAH has been found in renal insufficiency in past investigations [12,23] and was seen in our investigation. One subject with clear normalization of MMA and tHcy after cobalamin treatment had elevated SAH after treatment likely related to renal insufficiency. It appears that SAH is more likely to be elevated in renal insufficiency than MMA or tHcy, and thus again may be a less specific diagnostic test for cobalamin deficiency. Our studies provide a background for future investigations of perturbations of SAM-dependent methyltransferases in megaloblastic anemia and possibly in renal failure–associated anemia.

Acknowledgments

This study was supported by grant Proc 00/12467/0 from the Fundação de Amparo à Pesquisa do Estado de São Paulo, Brazil, and grant AG-09834 from the National Institutes of Health.

We thank Drs Marcelo G. Cliquet and Fátima C. Minari from the Division of Hematology, Pontificia Universidade Católica de São Paulo, for referring their patients; Bev Raab and Carla Ray for expert technical assistance; and Theresa Martinez for secretarial support.

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