

Blood Determinations of *S*-Adenosylmethionine, *S*-Adenosylhomocysteine, and Homocysteine: Correlations with Diet¹

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Abstract

An increasing number of both clinical and experimental studies have shown an association between deficiencies of the dietary sources of physiological methyl groups and cancer formation. The critical metabolic intermediate in a determination of methylation status is *S*-adenosylmethionine (SAM), the body's chief physiological methyl donor. The present study examined the erythrocyte levels of SAM and of its demethylated metabolite *S*-adenosylhomocysteine (SAH) in 66 normal subjects (33 men and 33 women), whose blood had been drawn at days 0, 7 and 14 of an experimental period during which they were fed a fixed diet. The plasma levels of homocysteine (HCys) were also determined in the same individuals at the same time points. In addition, the subjects had completed a food frequency questionnaire (FFQ) describing their usual dietary habits before being placed on the dietary regimen. The blood levels of SAM, SAH, and HCys were compared with the dietary intakes of folate, vitamin B₆, fats, and calories, both prior to using the FFQ and during the experimental period. The results indicated that the intraindividual differences were very low, but the interindividual differences were large for the values of SAM, SAH, SAM:SAH ratios, and HCys. Interestingly, the blood levels of SAM and HCys were higher in men than in women and generally showed the expected correlations with folate intake *i.e.*, positive for SAM and negative for HCys. The intakes of folate (276 μg/days) and B₆ (1.87 mg/days) during the 2-week experimental period were relatively low compared with the usual intakes of these vitamins (375 and 2.06 mg/day for folate and B₆, respectively) but correlated well with each other during both periods of

the study. Surprisingly, both men and women showed a significant rise in erythrocyte SAM:SAH ratios as a function of age. In addition, the combined results from men and women, even adjusted for gender, showed significant correlations between HCys and both weight and body mass index. On the other hand, during the experimental period of the study, blood SAM levels were inversely correlated with the intakes of both fat and calories when the data for both men and women were combined and adjusted for gender. The blood determinations of SAM and related compounds showed a high degree of reproducibility over time and thus appear to provide a practical marker of methylation status for the assessment of cancer risk from dietary, environmental, and genetic factors.

Introduction

It is now well established that deficiencies of the major dietary sources of methyl donors, methionine and choline, lead to the formation of liver cancer in rodents (1–5). Although not so widely studied as the liver tumors, extrahepatic tumor formation has also been increased in carcinogen-treated animals fed diets low in the methyl donors methionine, choline, and folic acid (6–8). Several plausible mechanisms have been proposed to explain the enhancing effects of dietary methyl deprivation on carcinogenesis (9–13). The most widely investigated, however, has been the idea that dietary methyl insufficiency results in abnormal DNA methylation subsequent to the formation of a physiological methyl insufficiency *in vivo* (9, 14–17). A critical metabolite in the investigation of this hypothesis is SAM,³ the body's chief physiological methyl donor (18). The metabolic interactions of the methyl intermediates are described in Fig. 1.

It has been known for decades that cancer patients are folic acid deficient (19) and that the incidence of gastric carcinoma is elevated in patients with pernicious anemia (20). Pernicious anemia is the clinical manifestation of a deficiency of vitamin B₁₂, a vitamin that, with folic acid, is responsible for the *de novo* synthesis of methyl groups. Several recent human studies have also linked deficiencies of folic acid, vitamin B₁₂, and methionine with increased risk of cancer in various organs. These include the colon (21–23), the breast (24), the cervix (25), the lung (26), the stomach (27), the pancreas (28), and multiple myeloma (27). The question thus arises as to whether such deficiencies exert their activities through diminished

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³ The abbreviations used are: SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine; HCys, homocysteine; FFQ, food frequency questionnaire; Usual, diet intake during the preliminary period before the controlled dietary period; Exp, diet intake during the controlled, experimental period of the study; HPLC, high-performance liquid chromatography; BMI, body mass index.

Table 1 General clinical and dietary data during the preliminary (Usual) and the experimental (Exp) periods of the study

	Men	Women
No. of subjects	33	33
Age (yr)	40.5 ± 1.2 ^a	38.4 ± 1.3
Body weight (kg)	79.4 ± 1.5	61.2 ± 1.6 ^b
BMI	24.5 ± 0.3	22.0 ± 0.5 ^b
Caloric intake (KCal/day)		
Usual	2074 ± 110	1522 ± 82 ^b
Exp	2908 ± 45 ^c	2096 ± 33 ^{b,c}
Fat intake (g/day)		
Usual	79.0 ± 6.4	57.4 ± 3.5 ^b
Exp	116.6 ± 1.7 ^c	86.4 ± 1.3 ^{b,c}
Folate (μg/day)		
Usual	392 ± 42	358 ± 34
Exp	325 ± 7	227 ± 4 ^{b,c}
Vitamin B ₆ (mg/day)		
Usual	2.19 ± 0.19	1.92 ± 0.18
Exp	2.13 ± 0.02	1.60 ± 0.02 ^b

^a Mean ± SE.

^b Significantly different from the corresponding value in men ($P < 0.05$).

^c Significantly different from the corresponding value during the Usual period ($P < 0.05$).

a 50-μl sample of plasma, 100 μl of internal standard, 50 μl of reduction reagent, and 100 μl of derivatization reagent. The mixture was incubated for 5 min at 50°C and cooled for 5 min at 4°C. Then 100 μl of precipitation reagent were added, and after mixing, the tube was centrifuged for 5 min at 10,000 × *g*. Using the mobile phase provided by Bio-Rad at a flow rate of 0.7 ml/min and the Bio-Rad Micro-Guard Cartridge and Analytical Cartridge recommended, 20 μl of the supernatant were injected. The column compartment of the Hewlett-Packard 1090 HPLC was adjusted to 45°C. The excitation wavelength on the Hewlett-Packard 1046A Fluorescence detector was set at 385 nm with the emission wavelength at 515 nm. The retention time for homocysteine was 3 min, with a run time of 5 min. Standards were provided by Bio-Rad. Both procedures gave comparable results.

Statistical Methods. Data on blood parameters were analyzed as a repeated measure design, *i.e.*, three measurements on each subject collected at 0, 1, or 2 weeks relative to the start of the experimental diet (41). Among subjects, each analysis was adjusted for a gender difference; within subjects, each analysis was adjusted for a time effect and for a time by sex interaction. Both trends and departures from linear trends were determined for these time effects. The correlations reported were Pearson's. Computations were done on a personal computer using SYSTAT 8.0 (SPSS Science, Chicago, IL). Unless otherwise specified, correlations using the combined data from men and women were adjusted for sex. The statistical significance of differences between group means were determined by *t* tests. All results were regarded as statistically significant when $P < 0.05$.

Results

Subject Characteristics and Diets. The subjects of this study consisted of 33 men and 33 women, with the men ranging in age from 27 to 58 years (mean, 40.5 ± 1.2 years) and the women from 27 to 62 years (mean, 38.4 ± 1.3 years; Table 1). The men weighed an average of 79.4 ± 1.5 kg (range, 56.8 to 104.5 kg); the women weighed an average of 61.2 ± 1.6 kg, with a range from 43.2 to 79.5 kg. The BMIs of the two sexes were fairly similar: 24.5 ± 0.3 for the men and 22.0 ± 0.5 for

the women (Table 1). There were two diets consumed during the period described in these investigations. The first was individual subjects' preliminary, unrestricted (Usual) diet, which was described by the FFQ and the salient features of which are listed in Table 1. The second was the experimental (Exp) diet, consisting of a defined experimental diet used for the 2-week period of investigation during which the blood was drawn (days 7 and 14; Ref. 33). Table 1 illustrates the average daily fat and calorie intake in both men and women provided by each of the diets. As expected, the men consumed more of both diets than did the women (Table 1); the Exp diet provided more fats and calories than did the Usual diet (Table 1). Regression analysis of the combined data (unadjusted) from both men and women showed that the BMI was positively related to the intake of fat and calories, regardless of whether the Usual or the Exp diet was consumed ($P < 0.01$ in each case except for BMI *versus* calories in the Usual period, where $P = 0.025$).

Dietary Folate and Vitamin B₆. Table 1 also shows the differences in folate intakes between men and women ingesting the Usual and the Exp diets. As shown in Table 1, there is no significant difference between men and women in their average daily folate intakes while they are on the Usual diet. In both men and women, the Usual folate intake appeared to be greater than Exp folate (Table 1), but this difference may partially reflect differences in the methods by which vitamin intakes were determined (self-assessment *versus* food intake). The 37% decline in folate intake by women shifting from the Usual diet to the Exp diet was found to be statistically significant by a paired *t* test ($P < 0.001$); the corresponding 17% decline in folate intake by men was not significant ($P = 0.12$). The folate intake of 227 μg/day by women during the Exp period was particularly low.

The average daily intake of vitamin B₆ as a function of gender and dietary regimen was also examined. The only differences noted in the daily intake of vitamin B₆ were the lower intake of this vitamin in women compared with men during both the Usual and the Exp periods of the study (Table 1). The folate and B₆ intakes were very closely correlated, regardless of whether the data from men and women were analyzed together or separately, with *r* ranging from 0.652 to 0.999, and $P < 0.001$ in all cases. Because of the more defined conditions of the Exp portion of the study, the intakes of both folate and vitamin B₆ were shown by regression analysis to be strongly and positively associated with the ingestion of both fats and calories during this period ($r = 0.680$ to 0.999 , $P < 0.001$ in all instances); correlations between the consumption of fats and calories and that of folate and B₆ during the Usual period of the study were less extensive and significant than those seen during the Exp portion of the study (data not shown).

SAM, SAH, and HCys. Fig. 2 illustrates the reproducibility of the individual values for erythrocyte SAM, SAH, and SAM:SAH ratios as well as for plasma levels of HCys. Each panel plots the determination of each parameter at Exp week 2 *versus* the corresponding individual value at week 0. As can be seen from Fig. 2, the individual values of SAM, SAH, SAM:SAH ratios, and plasma HCys taken at different times varied widely among individuals but were quite uniform within each individual subject. The variation among individuals is illustrated by the following ranges in the average individual values for each parameter: SAM, 1.00–3.19 μM; SAH, 0.11–0.59 μM; and SAM:SAH ratios, 2.3–22.1; and HCys, 5.34–26.11 μM. Fig. 2 depicts the linearity in SAM, SAH, SAM:SAH, and HCys values at week 2 compared with those seen at week 0 (Fig. 2, A–D, respectively; $r = 0.761$ to 0.919 , $P < 0.001$, in all cases). Although not presented here, similar plots of the corresponding

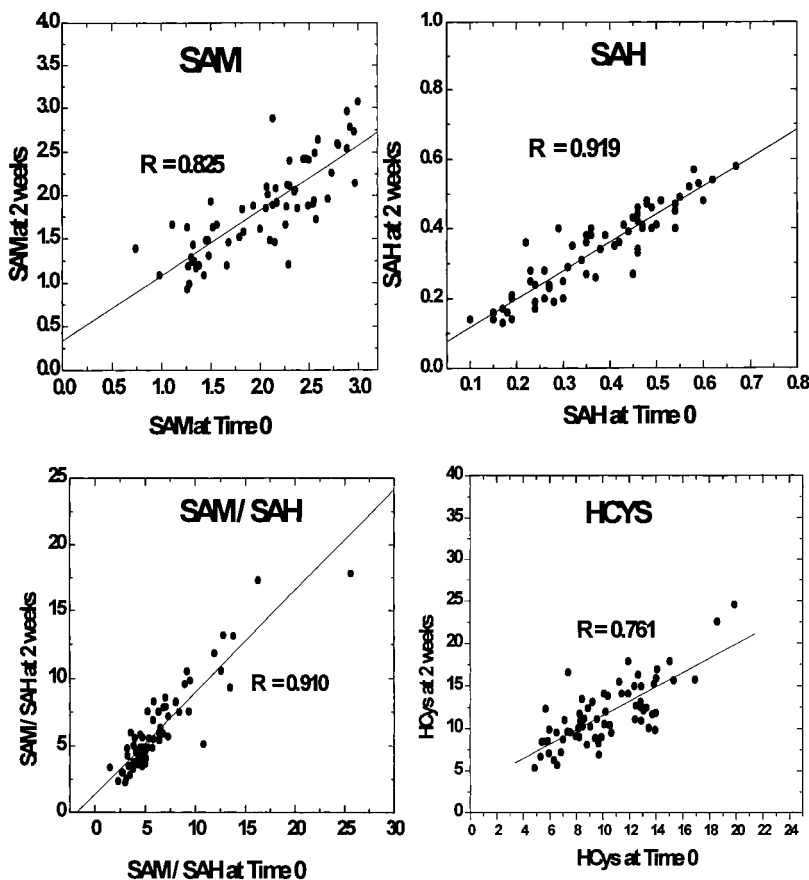


Fig. 2. The individual values SAM, SAH, SAM/SAH ratios and HCys determined at Exp week 2 plotted versus the corresponding value at time 0. The RBC SAM and SAH and the plasma HCys were all determined by HPLC, as described in "Materials and Methods"; the blood samples drawn immediately prior to (Time 0) and at the end of the 2-week Exp period. The final values from the individual subjects are plotted against the corresponding initial value from the same subject.

values at week 1 versus those at week 0 gave virtually identical results to those seen at week 2.

Table 2 reports the average values for SAM, SAH, SAM:SAH ratios and HCys in men and women at each of the time points investigated. Significant differences between men and women were seen in their blood levels of SAM, SAM:SAH ratios, and HCys throughout the study. The average blood SAM level in women was $\sim 40\%$ less than in men (1.63 ± 0.08 versus $2.28 \pm 0.06 \mu\text{M}$), and accordingly, the average SAM:SAH ratio in men was found to be 60% greater than that seen in women (7.80 ± 0.75 versus $4.86 \pm 0.32 \mu\text{M}$; Table 2). Consistent with previous observations (42, 43) were the elevated levels of HCys in men compared with those in women (12.0 ± 0.7 versus $10.0 \pm 0.5 \mu\text{M}$; Table 3). The decreases in SAM and SAH and the increases in HCys over the 2-week experimental period were statistically significant for both men and women ($P < 0.01$). Table 3 presents the estimates from the repeated measures analysis of the differences between the men and women as well as the time trends for each of the parameters over the experimental period. The changes over time are consistent with a constant slope. Aside from the gender differences in average levels that were noted above, the time trends for men and women were not statistically different.

Correlations between SAM, SAH, and HCys Levels and Vitamin Intakes. With the normal subjects used in the present studies, the intakes of folate and vitamin B₆ exerted greater effects on the blood levels of HCys than on those of SAM and SAH (Table 4). As expected, HCys levels were inversely correlated with the daily intakes of both vitamin B₆ and of folate

during the Usual period of the study (Table 4). These correlations were seen when the data from men only were analyzed or when they were combined with those from women; they were also valid whether the vitamin intakes were calculated as average daily intakes or as daily intakes/kg body weight. However, the negative correlations between HCys and dietary folate and B₆ were not seen with women. The relation seen in the Usual period between HCys and vitamin B₆ intake was maintained during the Exp period of the study but only when the daily B₆ intake was calculated as on a kg⁻¹ basis; no negative correlation between HCys and folate could be seen during the Exp period. No significant effect of folate or B₆ intake on SAM levels during the Usual period of this study could be observed. SAM and SAM:SAH ratios were positively correlated with the intakes of both vitamins during the Exp period of the study, when the combined, unadjusted data from men and women were examined (Table 4). These correlations were not seen when the vitamin intakes were determined per kg body weight or when the combined data were adjusted for sex. These observations appear to be attributable to the facts that SAM levels are higher in men than in women and that, during the EXP period of the study, the men consumed more B₆ and folate than did the women. SAH levels exhibited no significant correlation with the intakes of folate or of B₆ during either period of the study. One final unexpected finding was made in these studies; Exp SAM was negatively associated with dietary folate when the adjusted data from men and women were combined ($r = -0.250$, $P < 0.05$). The possible cause of this observation is described below.

Table 2 The comparative levels of SAM and SAH and of SAM:SAH ratios in the erythrocytes of men and women^a

	Time (wk)	Gender	
		Men	Women
SAM ^b	0	2.40 ± 0.05	1.69 ± 0.09 ^c
	1	2.30 ± 0.07	1.61 ± 0.08 ^c
	2	2.16 ± 0.06 ^d	1.62 ± 0.08 ^c
SAH ^b	0	0.38 ± 0.03	0.38 ± 0.02
	1	0.36 ± 0.03	0.38 ± 0.02
	2	0.34 ± 0.02	0.35 ± 0.02
SAM:SAH ^b	0	7.84 ± 0.82	4.90 ± 0.36 ^c
	1	7.98 ± 0.82	4.78 ± 0.35 ^c
	2	7.56 ± 0.66	4.90 ± 0.32 ^c
HCys	0	11.2 ± 0.6	9.3 ± 0.5 ^c
	1	12.2 ± 0.5	9.8 ± 0.7 ^c
	2	12.7 ± 0.5	10.8 ± 0.7 ^c
		12.0 ± 0.5 ^e	10.0 ± 0.7 ^c

^a Experimental details on the patients and protocols.

^b SAM, SAH, and HCys are expressed as the means of the μ molar concentrations \pm SE and SAM:SAH ratios as the means \pm SE.

^c Significantly different from the corresponding value seen in men ($P < 0.01$).

^d Significantly different from the corresponding 0-time mean ($P < 0.01$).

^e Average.

Effects of Age, Weight, and Calories. The age, weight, and fat and caloric intake of the subjects all had significant associations with the blood levels of the methyl compounds investigated. Principal among these were: (a) the correlations between body weight and BMI and HCys; (b) the effects of age on HCys and on SAM:SAH ratios; and (c) the effects of fat and Kcal intake on Exp SAM levels. Blood SAM:SAH ratios increased as a function of age ($P < 0.01$), regardless of whether the data were adjusted for sex. HCys levels appeared to increase in blood as a function of age ($P < 0.05$), but only when the data were not adjusted for gender. Plasma HCys levels were positively associated with two clinical parameters commonly associated with increased cancer risk: weight and BMI ($P < 0.01$ and <0.05 , respectively) for men and women combined. SAM levels exhibited a relatively complex set of correlations with the intake of fats and calories. When the combined, unadjusted data were used in the calculations, SAM levels increased positively with the intake of dietary fat and of Kcal ($r = 0.407$, $P < 0.01$ in each instance). However, when the same data were adjusted for gender, slight but significant negative correlations between SAM levels and the intakes of fat and Kcal ($r = -0.257$, $P < 0.04$, in each case) were noted. It thus appears that the dietary content of fats and calories may alter the availability of physiological SAM.

Discussion

Three major findings were made in this study: (a) the blood levels of SAM, SAH, and HCys in a sample of 66 normal subjects were consistent both within the same individuals and with previously published values; (b) among different individuals, blood SAM, SAM:SAH ratios and HCys exhibited a broad range of values but still reflected differences in diet; and (c) these intermediates appear to constitute useful biomarkers to study the susceptibility to and progression of cancer in humans. The erythrocyte levels of SAM that we have reported here, 2.28 ± 0.06 for men and $1.63 \pm 0.08 \mu$ M for women, fall within the previously published ranges for blood SAM values (30, 32,

Table 3 Repeated measures analyses of the differences between males and females and the time trends for HCys, SAM, SAH, and SAM:SAH ratios over the experimental period

	Difference between males and females ^a	Slope ^b
HCys	2.08 ± 0.87 ^c	1.48 ± 0.34 ^c
SAM	0.65 ± 0.11 ^c	-0.19 ± 0.04 ^c
SAH	0.01 ± 0.03	-0.04 ± 0.01 ^c
SAM:SAH	2.94 ± 0.81 ^c	-0.14 ± 0.19

^a SAM, SAH, and HCys are expressed as the differences in μ M concentration \pm SE, and SAM:SAH ratios are expressed as the differences in values \pm SE.

^b Change in units/week \pm SE.

^c Significantly different from 0.

Table 4 Significant correlations between the blood levels of SAM, SAH, and SAM:SAH ratios and homocysteine versus the dietary levels of folate and vitamin B₆ during the preliminary (Usual) and the experimental (Exp) portions of the study^a

Blood parameter and vitamin ^a	Period	Sex	n	r	P
HCys	Usual ^b	M	33	-0.522	<0.01
		M + F	66	-0.377	<0.01
B ₆ ^c	Exp ^b	M	33	-0.352	<0.05
		M + F	66	-0.254	<0.05
Folate	Usual	M	33	-0.508	<0.01
		M + F	66	-0.431	<0.01
SAM	Exp	M + F (Unadjusted)	66	0.438	<0.01
		M + F (Unadjusted)	66	0.386	<0.01
SAM/SAH	Exp	M + F (Unadjusted)	66	0.271	<0.05
		M + F (Unadjusted)	66	0.303	<0.05

^a Unless otherwise specified, the correlations were calculated on the basis of total daily intake of each vitamin.

^b For the Usual portion of the study, the blood levels of the methyl intermediates at day 0 were compared with the vitamin intakes during this period; in the Exp portion of the study, the levels of the methyl intermediates at days 7 and 14 were averaged for corresponding regression analysis.

^c Intake is calculated as mg/kg/day.

36, 44–47). The present study also appears to be the first demonstrating a gender difference in blood SAM levels. As was the case with SAM, the erythrocyte levels of SAH (30, 48) and the plasma levels of HCys (31, 49) matched those reported previously.

Although the blood levels of SAM, SAH, and HCys varied considerably among individuals, they were remarkably consistent within the same subject. The interindividual variation of each parameter was still sufficiently small to permit the detection of minor differences caused by diet, particularly by the change in diet from the Usual to the Exp period. The high intraindividual reproducibility raises the prospect that determinations of blood SAM, SAH, and HCys may serve to detect changes in a single individual's diet, chemical exposure, or health that impact on methyl group availability. It is interesting that among the three parameters studied, HCys was the most sensitive to the dietary content of vitamin B₆ and of folate with these normal subjects. The correlations between blood levels of HCys and deficiencies of vitamins B₆ and B₁₂ and of folate have been well described previously (24, 31, 42). Another novel finding made in this investigation is the rise in SAM:SAH ratios as a function of age in both men and women. As far as we are aware, this is the first clinical study in which SAM and SAH have been measured over a period of weeks.

Clinical analyses of SAM, SAH, and HCys appear to constitute a useful tool in examining populations at increased risk of developing cancer attributable to alterations in methyl availability. Dietary or metabolic deficiencies of the physiological methyl group precursors in humans have now been linked to increased risk of several types of cancer. These include: multiple myeloma (27) and carcinoma of the colon (21), breast (24), stomach (27), and pancreas (28), as well as to the formation of preneoplastic lesions of the lung and cervix (25, 26). Metabolic insufficiency of physiological methyl groups has also been produced by exogenous agents known to be associated with increased cancer risk in humans, such as arsenic and alcohol (50–53). Genetic polymorphisms of methylenetetrahydrofolate reductase and catechol-*O*-methyltransferase, each of which modifies SAM bioavailability (54, 55), have been associated with altered risk of colorectal cancer and breast cancer in humans (23, 56, 57). In this regard, it is worth noting that women, who in the present studies had lower levels of SAM and SAM:SAH ratios than did men, showed an elevated risk of developing multiple myeloma in cases of vitamin B₁₂ deficiency (27) and of breast cancer formation with the consumption of even moderate amounts of alcohol (58). Further studies are required to establish the utility of blood determinations of SAM, SAH, and homocysteine in assessing cancer risk. For example, in the present investigation with normal subjects, no correlations could be seen between the blood levels of SAM and SAH and those of HCys. Studies on patients with renal failure (48, 59) have shown clear correlations between elevated blood levels of HCys and those of SAH and/or SAM. More recent results have extended these positive correlations between blood levels of HCys and of SAH and have shown a negative correlation between plasma HCys and lymphocyte DNA hypomethylation (60–62). The present investigation needs to be extended to subjects at increased risk of developing tumors, particularly of those tumors known to be associated with dietary deficiencies of the methyl group precursors. In this regard, the other biomarkers such as DNA hypomethylation and uracil misincorporation, known to be associated with experimental carcinogenesis by dietary methyl (63) or folate (64) deficiency, may prove useful adjuncts in a screen for cancer risk.

Finally, among the more intriguing observations made in these investigations were the correlations between the blood parameters of methylation and those of energy intake. The plasma HCys was positively associated with body weight and BMI, whereas the SAM levels were negatively associated with fat and calorie intake. The resulting hypothesis, *i.e.*, that the increase in cancer risk associated with elevated body weight, BMI, fat, and calorie intake is partially mediated through methyl deficiency is thus a reasonable one. Limited experimental results support this proposal (65–67), and further studies along this line are warranted.

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