S-Adenosylmethionine Levels in the Diagnosis of *Pneumocystis carinii* Pneumonia in Patients with HIV Infection

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Background. S-adenosylmethionine (AdoMet) is a key molecule involved in methylation reactions and polyamine synthesis. *Pneumocystis carinii* are unable to synthesize this molecule and have been shown to scavenge this metabolic intermediate from the plasma of rats during active infection. A prior study involving humans strongly suggested that low levels of plasma AdoMet are sensitive and specific indicators of acute infection.

Methods. From March 2004 through January 2006, we collected plasma AdoMet levels from patients with human immunodeficiency virus (HIV) infection and either confirmed *Pneumocystis carinii* pneumonia (PCP), confirmed pulmonary tuberculosis, or confirmed bacterial pneumonia. We compared levels in patients with PCP with those in patients with other diseases and also monitored changes in levels during treatment of PCP.

Results. Initial AdoMet levels were significantly lower in patients with PCP, and there was no overlap between the groups. Among patients with PCP, levels of AdoMet increased with successful treatment.

Conclusions. Measurement of plasma AdoMet levels in patients with HIV infection who have pulmonary infections can identify those with PCP.

Although the frequency of *Pneumocystis carinii* pneumonia (PCP) in patients with HIV infection has decreased dramatically in developed nations, it remains common, especially among patients who are unaware of their HIV infection status [1]. It also continues to be a significant burden in Africa and in developing countries [2].

In practice, the diagnosis of PCP is often made presumptively on clinical grounds, but because of the inadequate specificity of clinical criteria, the risk of toxicity with empirical treatment, and the morbidity resulting from incorrect diagnosis, a definitive diagnosis is essential [3]. The mainstay of definitive PCP diagnosis continues to be the histologic demonstration of *P. carinii* cysts and trophozoites obtained from sputum induction, bronchoalveolar lavage, or transbronchial biopsy specimens. Often, histologic tests cannot be performed at the time of presentation; therefore, there is considerable interest in a rapid, noninvasive diagnostic test.

S-adenosylmethionine (AdoMet) is a critical biochemical intermediate involved in methylation reactions and polyamine synthesis [4]. *Pneumocystis* species have a very unusual requirement for exogenous AdoMet, because they are unable to synthesize this compound. This was first found in cultured *P. carinii* [5], then confirmed by showing that plasma from rats infected with *P. carinii* is >99% AdoMet depleted [6]. *Pneumocystis* species only attach to the type I pneumocytes in the lung, but the total plasma depletion is possible because of the 500:1 concentration gradient between the intracellular and extracellular space [7]. This striking reduction is consistent with in vitro data. On the basis of the measured consumption rate of AdoMet per *P. carinii* cell in vitro, the peak number of *P. carinii* cells per rat, and the rough approximation that 20% of the weight of a rat is extracellular fluid, we found that *P. carinii* can consume an amount of AdoMet that is equal to the total amount

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of AdoMet in the extracellular fluid of a rat in just 36 min [8].

With the exception of *P. carinii,* a rickettsia, and an aberrant *Amoeba* species, every organism studied thus far is capable of AdoMet synthesis. Because of this relatively unique phenomenon, we felt that measurement of plasma AdoMet levels had potential as a minimally invasive and reliable diagnostic marker of PCP. A preliminary study was done that found profoundly reduced AdoMet levels in adults with confirmed and clinically diagnosed PCP and a return to levels comparable with those in healthy control subjects after successful treatment [9]. Because of the limited number of patients with confirmed PCP and the inclusion of comparison patients with conditions unlikely to be confused with PCP, there was only a limited ability to generalize the observations. The current study reproduces and broadens those preliminary results.

METHODS

Patients and study design. We conducted a prospective, observational, nonrandomized, comparative study from March 2004 through January 2007. We screened patients prospectively by reviewing daily hospital admissions to identify HIV-infected patients with diagnoses of pneumonia or presence of fever. Pharmacy data were also reviewed daily for patients newly initiating therapy with trimethoprim-sulfamethoxazole, pentamidine, or antibiotics in use for treatment of community-acquired pneumonia. We enrolled adults with HIV infection and clinical pneumonia within 36 h after starting treatment for their infections. If these patients were subsequently found to have confirmed PCP, bacterial pneumonia, or pulmonary tuberculosis, they were included in the final study. Patients were excluded from enrollment if they could not give informed consent or if they had received treatment for >36 h prior to enrollment.

All patients had 5 cc of blood collected at study enrollment (day 0) and, while hospitalized, on days 3, 5, and 7 and prior to hospital discharge. In some cases, there were slight variations from the schedule. It was initially planned to enroll 40 patients in each group, but because of slow accrual, an interim analysis was performed after a total enrollment of 56 patients.

Case definitions. Patients with confirmed PCP had acute symptoms, such as shortness of breath, cough, fever, dyspnea on exertion, and demonstrable cysts or trophozoites seen on stained specimens from induced sputum or bronchoalveolar lavage. Patients with confirmed bacterial pneumonia also had some combination of the above pulmonary symptoms, abnormal chest radiograph findings, and blood cultures that were subsequently positive for pulmonary pathogens. Patients with pulmonary tuberculosis all had pulmonary symptoms, abnormal chest radiograph findings, and sputum cultures subsequently positive for *Mycobacterium tuberculosis.*

Laboratory analysis. Plasma was separated by centrifugation at 1000 g for 15 min at 4°C and transferred to labeled 1.5-mL screw-top tubes. For every 80 μL of plasma, 20 μL of 10% perchloric acid was added to precipitate the proteins. The sample was clarified by centrifugation at 5000 *g* for 10 min, and the supernatant was stored for up to 7 days at −20°C.

AdoMet was assayed by high-performance liquid chromatography. For precolumn derivatization, 5 μL of internal standard (5 μg 1,7-diaminoheptane/mL [−1]) and 45 μL of borate buffer (0.2 M sodium borate, 1 mM EDTA; Ph, 8.8) were added to 30 μL of plasma extract. After mixing, 20 μL of AccQ.Fluor reagent (Waters) was added and mixed. High-performance liquid chromatography conditions were as described elsewhere [8]. For each specimen, 3 aliquots of plasma were analyzed. AdoMet concentrations of the samples were determined using the high-performance liquid chromatography AdoMet peak area, and interpolation from a standard curve was obtained by analyzing solutions containing known amounts of AdoMet. For quality control, batches of samples included plasma with known amounts of AdoMet. The lower limit of the AdoMet assay was 5 nM. The precolumn derivatization with AccQ.Fluor tag takes ~15 min, and the high-performance liquid chromatography gradient is 30 min. Therefore, the whole test takes <1 h.

Statistical considerations. Given the fact that, in our preliminary study, there was no overlap between AdoMet levels in patients with PCP and in patients with other conditions, sample size decisions focused on establishing reasonable confidence limits for sensitivity and specificity. We hypothesized that the ultimate sensitivity would be 95% and that the specificity would be 99%. Based on these assumptions and based on Monte Carlo sampling from binomial distributions, a sample size of 40 patients per group would have an 80% power to yield a sensitivity estimate >92.5% and a specificity estimate of 100%.

RESULTS

We enrolled a total of 56 patients in the study, including 28 patients with PCP and 28 patients in a non-PCP group (19 with bacterial pneumonia and 9 with tuberculosis). In addition to the initial AdoMet levels, we obtained serial measurements for 26 of 28 patients in the PCP group and 27 of 28 patients in the non-PCP group.

Of the 28 patients with proven PCP, 27 had abnormal chest radiograph findings. Thirteen showed interstitial infiltrates, 11 had bilateral alveolar infiltrates, and 3 had radiographs that were interpreted as showing congestion. The single patient with PCP and normal chest radiograph findings was a person with a 2-week history of dyspnea and a partial pressure of oxygen in the arterial blood of 62 mm Hg at admission to the hospital.

Seven patients in the PCP group and 5 in the non-PCP group received new diagnoses of HIV infection at study entry. Of the patients with previously known HIV infection, 8 in the PCP group and 7 in the non-PCP group were receiving antiviral drugs. There were 2 patients in the PCP group receiving PCP

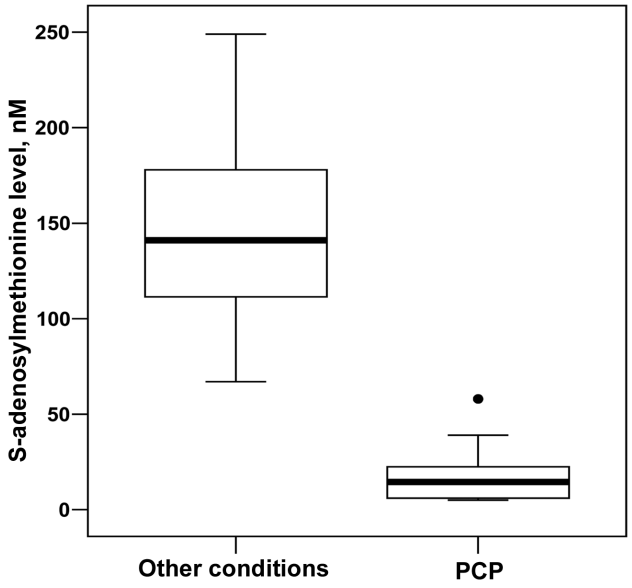

Figure 1. Initial levels of S-adenosylmethionine in patients with *Pneumocystosis carinii* pneumonia (PCP) and other pulmonary infections. Horizontal bars indicate the mean value, and vertical bars indicate the standard deviation.

prophylaxis (1 with atovaquone and 1 with trimethoprim-sulfamethoxazole).

The 2 groups were similar in age (mean age ± SD, 43.0 ± 6.6 years for the PCP group and 41.3 ± 7.8 years for the non-PCP group). Patients in the PCP group had lower $CD4^+$ cell counts than did patients in the non-PCP group (mean $CD4^+$ cell count ± SD, 33.4 ± 29.4 cells/mm^3 [interquartile range, 9.5–43 cells/mm^3] vs. 181.5 ± 195.9 cells/mm^3 [interquartile range, 46.5–219 cells/mm^3]; $P < .001$, by Mann-Whitney *U* test).

Patients with PCP were mildly to severely ill, with a median A-a gradient of 88 (interquartile range, 46–127). Three patients had gradients >400. Among the 28 patients with PCP, additional pathogens were identified for 3 patients, as follows: 1 had pneumococcal bacteremia, 1 had salmonella bacteremia, and 1 had cryptococcemia. Among the 28 patients in the non-PCP group, 9 had tuberculosis. Sixteen had pneumonia due to *Streptococcus pneumoniae,* 2 had pneumonia due to *Haemophilus influenzae,* and 1 had pneumonia due to *Staphylococcus aureus.* The patient with *S. aureus* pneumonia also had endocarditis.

The day 0 plasma AdoMet concentrations are shown in figure 1. Of the 28 patients with PCP, 7 (25%) had undetectable concentrations. The median AdoMet level in the PCP group was 14.5 nM (range, <5 nM to 58 nM), whereas in the control group, the median AdoMet level was 141 nM (range, 67–249 nM). There was no overlap between the 2 groups, and for breakpoints between 59 nM and 66 nM, the sensitivity, specificity, and predictive values were all 1.0. Binomial confidence limits for sensitivity and specificity, given a sample size of 28, were 0.88 and 1.0.

We performed serial measurements of plasma AdoMet for both groups of patients. Patients in the control group showed only minor variations in levels during the course of treatment, with no consistent pattern noted. Among the patients with PCP, initially low plasma concentrations of AdoMet increased with effective treatment in 25 (96%) of 26 patients (figure 2).

DISCUSSION

The AdoMet assay reliably separated patients with PCP from patients without PCP. Therefore, we have confirmed the results of our prior study [9] showing that plasma AdoMet levels are depleted in patients with PCP and that these levels return to normal with treatment. Although levels of AdoMet in our current study are generally higher than those that we encountered in our previous study, the difference between the 2 groups remains the same.

We have also addressed a number of potential complications involved with applying this test. First, coinfection is not a confounding problem. There were 3 patients in the PCP group who had concurrent blood stream infections, and each patient had reduced plasma AdoMet concentrations. Second, the AdoMet levels were reliable even with low-grade or early infections with PCP. One of the patients in the PCP group had only a single cyst noted at physical examination, but his initial AdoMet concentration was suppressed to 26 nM and returned to normal with treatment. Another patient presented with abnormal chest radiograph findings, but his only symptom was fever. He had no cough or dyspnea and underwent bronchoscopic examination for suspected malignancy. His biopsy specimen revealed PCP, and he improved with trimethoprim-sulfamethoxazole monotherapy. His initial plasma AdoMet

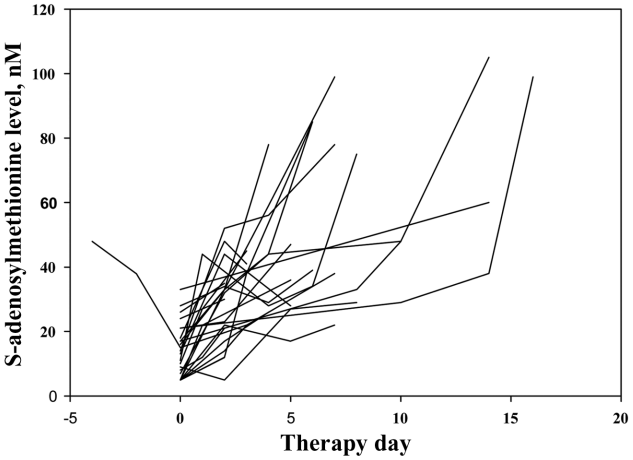

Figure 2. Recovery of S-adenosylmethionine levels with treatment of *Pneumocystosis carinii* pneumonia.

level decreased over a 5-day period from 48 nM to 15 nM (figure 2); at the end of this period, trimethoprim-sulfamethoxazole therapy was started, and the patient's AdoMet level then increased toward normal. Similarly, a third patient was admitted to the hospital for mental status changes without any pulmonary symptoms. He had abnormal chest radiograph findings and was persistently febrile while hospitalized. Bronchoscopic examination and histologic examination of biopsy specimens showed *Pneumocystis carinii.* His fevers only resolved after treatment with trimethoprim-sulfamethoxazole. His initial AdoMet level was 32 nM.

In another case, we found that the AdoMet levels were suppressed even when the findings of a bronchoscopic examination were negative. A young woman with asthma and AIDS ($CD4^+$ cell count, 97 cells/mm^3) was admitted to the hospital for possible PCP, complicated by asthma. At hospital admission, her initial AdoMet level was suppressed (21 nM), but a bronchoalveolar lavage specimen had negative findings. The patient developed respiratory failure and was treated with both trimethoprim-sulfamethoxazole and asthma medications. Eight days later, a second bronchoscopic examination (with biopsy) confirmed the diagnosis of PCP. The patient's AdoMet level at that time was 29 nM. She made a complete recovery, and 16 days after enrollment, her AdoMet level was 99 nM.

The 1 patient with PCP who did not have increasing AdoMet levels during her hospital stay was discharged from the hospital against medical advice on hospital day 8 with continued fever and pulmonary symptoms. She was readmitted to the hospital with respiratory failure (requiring intubation) 1 week later.

Mortality was low. Of the 58 patients in the study, 3 (5.2%) died. A single patient with pneumococcal pneumonia and severe wasting disease died on hospital day 4 due to respiratory failure. She had the lowest AdoMet level (68 nM) in the non-PCP group. Two patients in the PCP group died; 1 patient died of a complication of bronchoscopic examination, and 1 patient with severe wasting disease died on hospital day 43 after all care was withdrawn at the family's request. This second patient completed 21 days of trimethoprim-sulfamethoxazole therapy, and his AdoMet levels were increasing steadily before phlebotomies were halted.

It is likely that disease severity affected study recruitment. Some of the most severely ill patients that we assessed for inclusion were intubated very early in the course of their illness and could not be enrolled in the study. There was no overall relationship between A-a gradient and decreased AdoMet levels; however, among 3 patients with gradients >400, 2 had undetectable levels of AdoMet.

Beyond the problem of concurrent infection, there are other questions regarding the assay and its possible use. First, are there other diseases that will affect AdoMet levels? Unpublished data from our preliminary study [9] showed markedly elevated plasma AdoMet levels in 4 patients who had coexisting malignancies. This led to a recently published study [10] by one of the authors (S.M.) that showed consistently elevated plasma AdoMet levels in patients with untreated pulmonary malignancies. Also, patients with end-stage renal disease who are receiving hemodialysis have been shown to have increased plasma levels of AdoMet [11]. Studies regarding cirrhotic liver disease have shown consistently lower AdoMet levels in both the plasma and intracellular space [12–14]. We feel that it is unlikely that advanced liver disease with cirrhosis would be mistaken for acute pneumonia or PCP.

Second, are AdoMet levels affected by other therapies? The B vitamins have been studied extensively, because they are the source of coenzymes in the methylation pathways, producing AdoMet from methionine, which, in turn, is produced from homocysteine. Vitamins B2 and B6 have been shown to affect intracellular levels of AdoMet [15, 16] but, along with vitamin B12, have been shown to have no effect on plasma levels [17]. Oral intake of AdoMet, along with methionine loading, has also been shown to increase plasma AdoMet levels [18, 19]. AdoMet has been studied for its effects on depression, and it has been shown that treatment with desipramine increases plasma levels to almost the same levels as oral AdoMet [20]. Patients with Parkinsons's disease who are treated only with levodopa will also have increased plasma AdoMet levels [21]; however, when a dopa decarboxylase inhibitor is added, the plasma levels decrease to as low as 35 nM [22], which is within the range of positive values found in this study.

Third, are there any better rapid tests being proposed? Recently, Tasaka et al. [23] compared 4 serum markers (lactic dehydrogenase, C-reactive protein, KL-6 antigen, and β-D-glucan levels) as diagnostic tests for PCP. Only the β-D-glucan level had any utility, with a sensitivity of 92% and specificity of 86%. Tasaka et al. [23] observed 295 consecutive patients, of whom 57 (19%) had proven PCP. In that population, the positive predictive value was only 0.61, whereas the negative predictive value was 0.98. Thus, the β-D-glucan assay will not perform well in persons with small to moderate prior probabilities of pneumocystosis. Furthermore, because β-D-glucan is present in many fungi, it is likely that the specificity of its measurement will prove to be even lower than reported as additional patient populations are studied.

Finally, can the assay be performed by a standard hospital laboratory? At present, the assay of plasma AdoMet has a cost of ~$300 per sample. This should be reducible by economies of scale and standardization; however, most community hospitals will not have the laboratory equipment on which the test is performed. Therefore, the test would most likely be performed by referral laboratories.

We conclude that our study confirms that reduced levels of plasma AdoMet are sensitive and specific indicators of active

PCP. With adequate delimitation of the lower limits of the normal range, the assay can be used as a rapid and reliable diagnostic test for PCP. Furthermore, serial measurements of AdoMet can be used to monitor the progress of treatment. We believe measurement of serum AdoMet to be a significant advance from current diagnostic methods, and the challenge is now to develop an accessible assay

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Potential conflicts of interest. All authors: no conflicts.

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