

# Generation of Monoclonal Antibodies against S-Adenosylmethionine and Their Application in Diagnosing Liver Diseases

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## Abstract

S-AdenosylMethionine (SAM) hapten-carrier conjugates were used to immunize BALB/c mice. Two strains of hybridoma cell lines that stably produced high-titer monoclonal antibodies against SAM were obtained. The biological properties of these two antibodies were characterized. A competitive ELISA (cELISA) was established using these antibodies. The sensitivity and specificity were analyzed, and human serum samples were measured using the SAM cELISA kit. Very little cross-reactivity (<1%) was observed between the monoclonal antibodies and SAM analogues. These two antibodies are IgG2b-type antibodies. The affinity measured using the cELISA is  $5.75 \times 10^9$  L/mol for the 118-6 antibody and  $7.29 \times 10^{10}$  L/mol for the 84-3 antibody. According to the Immunohistochemistry (IHC) and flow cytometry results, normal liver cells contain much higher levels of SAM than liver cancer cells ( $p < 0.01$ ). The results from 81 healthy subjects and 99 patients with liver disease showed the following sensitivity using the 240 nM cutoff: 95.65% (44/46) for hepatitis; 95% (19/20) for cirrhosis; 92.86% (13/14) for carcinoma; 100% (19/19) for liver failure; 30.86% (25/81) for healthy subjects; the detection specificity is 69.14% (56/81). Using the 120 nM cutoff, the sensitivity was 80.43% for hepatitis; 85% for cirrhosis; 71.43% for carcinoma, 100% for liver failure; 3.70% for healthy subjects; the specificity is 96.30% (78/81). SAM, alanine transaminase and aspartate aminotransferase were compared in identifying liver diseases. Serum SAM allowed early detection of abnormal liver function and providing clinicians an important basis for a more accurate diagnosis of liver diseases than alanine transaminase and aspartate aminotransferase.

**Keywords:** S-Adenosylmethionine (SAM); Monoclonal Antibody; Competitive ELISA; Liver Disease Diagnosis

**Abbreviations:** Alanine Transaminase (ALT); Aspartate Aminotransferase (AST); Chronic Hepatitis B (CHB), Competitive ELISA (cELISA); Hematoxylin and Eosin (HE); Hepatocellular Carcinoma (HCC); Homocysteine (HCY); Immunohistochemistry (IHC); Keyhole Limpet Hemocyanin (KLH); L-Methionine(L-Met); Methionine Adenosine Transferase (MAT); Methylthioadenosine (MTA); Optical Density (OD); S-Adenosylhomocysteine(SAH); S-Adenosylmethionine(SAM).

## Introduction

S-adenosylmethionine (Ado Met or SAM-e), whose structure was first reported by Canton in 1952 is an active form of methionine and an important metabolic and physiological substance in the human body [1,2]. As a key molecule in methionine metabolism, SAM is directly involved in many important biological chemical reactions, such as methylation, transsulfuration and aminopropylation. It is the sole methyl donor for the methylation of nucleic acids, hormones, lipoproteins and neurotransmitters *in vivo*. DNA methylation plays important roles in embryonic development, growth, differentiation and death. Methyltransferase knockout mice do not survive [3]. SAM is used as a nutritional supplement and medication for the treatment of various diseases, such as depression, osteoarthritis and liver disorders [4-8].

The liver is a metabolic and detoxification organ, and hepatocytes are vulnerable to chemical substances, certain metabolites and pathogenic microorganisms, leading to liver cell damage. SAM is widely used to treat liver diseases [4]. SAM has significant effects on improving hepatitis and intrahepatic cholestasis [9]. SAM prevents hepatocellular carcinoma (HCC) in rats and inhibits the growth of liver cancer cells and is a therapeutic indicator for liver diseases [10-14]. SAM has been shown to control cell growth and death; a short-term decrease in SAM levels stimulates liver cell regeneration as a stress response, whereas a long-term SAM deficiency leads to malignant transformation of cells. Notably, SAM inhibits programmed cell death in normal hepatocytes but promotes apoptosis in hepatoma cells [14,15]. Therefore, SAM both protects against adverse effects of chemotherapy and is a therapy for hepatocellular carcinoma, which is very rare among chemotherapeutic medicines. Alcohol disrupts several processes in the normal cycle of methionine

metabolism, leading to progressive liver injury. In this process, the levels of S-AdenosylHomocysteine (SAH) and Homocysteine (HCY) are significantly elevated. Excess SAH significantly inhibits methyltransferase and increases endoplasmic reticulum-dependent programmed cell death and lipid synthesis, leading to increased fat deposition in liver cells, increased apoptosis, the accumulation of harmful proteins, changes in multiple cell signaling pathways, and the inevitable induction of progressive liver injury[15,16].

Alterations in methionine metabolism that involve changes in the plasma SAM level occur in chronic liver diseases. The downregulated Methionine Adenosine Transferase (MAT) in liver diseases leads to a decrease in the SAM. One study demonstrated that patients with HCC had higher plasma SAM concentration than did patients with Chronic Hepatitis B (CHB) and normal controls[17]. This might be explained by the possibility that circulating SAM is produced by both abnormal and normal liver cells. Damaged liver cells showed a decrease in SAM levels, but normal liver cells produced more SAM by functional compensation. Meanwhile when liver cells were damaged or were dying, intracellular SAM was released into the plasma, which contributed to the higher plasma SAM level observed. Thus, the time when plasma samples were obtained for the measurement of SAM may impact the results [18-20].

Although many studies have examined the roles of SAM in liver damage, relevant research on SAM as a diagnostic marker for liver diseases has been rather limited due to limited and impractical methods for quantifying SAM levels in clinical samples. In this study, we successfully generated monoclonal antibodies against SAM and applied them to studies of liver pathogenesis and to measurements of SAM levels in serum samples from patients with various liver diseases using a competitive enzyme-linked immune-absorbent assay (cELISA) to explore its diagnostic value of liver diseases.

## Materials and Methods

### Reagents and samples

SAM, SAH, adenosine, L-methionine (L-Met), ADP, ATP and Methylthioadenosine (MTA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The L02, HepG2 and myeloma cell lines (SP2/0) are routinely maintained in our laboratory. RPMI-1640 (Thermo-Fisher Scientific, Waltham, MA, USA). BALB/c mice (Hunan Slack Jingda Experimental Animal Co., Changsha, China). HRP-conjugated goat anti-mouse IgG (Southern Biotech, Birmingham, AL, USA). ELISA kits for SAM (Arthurs Bios stems, CA, USA). Eight liver cancer samples and 3 liver failure samples were obtained from the Central South University Laboratory, and 6 liver cancer samples were obtained from the Department of Oncology, General Hospital of Jixi Mining Group. The remaining samples were obtained from the Department of Infectious Diseases, the First Affiliated Hospital of Xiangya, and Central South University. Samples were also collected from eighty-one healthy volunteers.

## Methods

### Antigen preparation

SAM was diazotized to form an analogue hapten with the chemical name 5'-N-methyl, 5'-N-butyryl-5'-deoxyadenosine (aza-SAM, AdaM), and Keyhole Limpet Hemocyanin (KLH)-aza-SAM was prepared as follows: 17.5mg of KLH, and 5ml of a freshly prepared 100mM PBS solution, pH 8.25 were added together. The KLH solution was placed in a 4°C with vigorous stirring. AdaM-NHS was formed by mixing 15mg of AdaM, 21.7mg of N,N'-dicyclohexylcarbodiimide (DCC) and 7.2 mg of N-hydroxysuccinimide (NHS). The solid mass dried under a vacuum. Approximately 1.5 ml dry dimethylformamide (DMF) was added to the flask under a nitrogen stream, and the flask was sealed. The solution was stirred overnight. AdaM-NHS was slowly added in 10 µl aliquots every few minutes. After 150 µl was added, the conjugation mixture became cloudy. One milliliter of DMSO was added to aid dissolution. Upon the addition of an additional 50 µl of AdaM-NHS, the mixture again became cloudy. Sonication in a water bath was applied for 5 min after each of 5 additions of 10 µl of AdaM-NHS. After 150 µl of AdaM-NHS in DMF were added, the mixture was solicited for 20 min. The pool was dialyzed against PBS (10 mM PB, 150 mM NaCl, pH 7.4) for 2 days.

### Immunization

The immunogen KLH-Aza-SAM was mixed with an equal volume of Freund's complete adjuvant (Sigma) and used to subcutaneously immunize BALB/c mice. After 2 weeks, the same amounts of antigen and Freund's incomplete adjuvant were mixed thoroughly and injected subcutaneously. This process was repeated a few times at an interval of 2 weeks.

### Specificity

Both SAM monoclonal antibodies were tested in a cELISA with SAM and SAM analogues, such as SAH, L-Met, ADP, ATP and MTA, as competitive antigens to evaluate the specificity. A 100-fold excess of SAM analogues compared to SAM was used to competitively bind to the limited amount of anti-SAM antibodies.

### Immunohistochemistry analysis

Traditional IHC technique was used with 1:200 anti-SAM antibody. The slides were sequentially stained with diaminobenzidine and hematoxylin. After dehydration, clearing and sealing, the slides were observed and photographed.

### Flow cytometry analysis

At 4°C for 30 min and washed with PBS. The SAM monoclonal antibody was added and incubated for 1.5h; the suspension was The L02 and HepG2 cell lines were cultured in vitro. After digestion with trypsin, a cell suspension containing at least  $1 \times 10^5$  cells was prepared, fixed centrifuged and the supernatant was discarded. The secondary antibody was added and incubated with the cells for 30 min in the dark. The suspension was centrifuged,

rinsed, re-suspended in 500 ml of PBS and mixed well for testing.

### Determination of SAM

The cELISA was used to quantitatively analyze SAM levels in serum samples using a SAM ELISA kit. The SAM standards were 0-960nM. The standard curve was generated and used to calculate the SAM concentrations in the samples.

### Statistical analysis

A paired chi-square test and SPSS software were used to analyze the data;  $p < 0.05$  was considered statistically significant.

## Results

### Selection of positive hybridoma cell lines

Spleen cells from the immunized mice were fused with SP2/0 cells, and hybridoma cell lines secreting anti-SAM antibodies were screened and named 118-6 and 84-3. The antibody subclass assessment indicated that clones 118-6 and 84-3 were both of the IgG2b subtype. The relative affinity constants of clones 118-6 and 84-3 measured using the ELISA were  $5.75 \times 10^9$ L/mol and  $7.29 \times 10^{10}$ L/mol, respectively. The titers of the supernatant and ascites of hybridoma cells were 1:6400 and 1:51200 for 118-6. The titer of 84-3 was higher than the titer of 118-6.

### Sensitivity

Standards were configured to detect SAM in the 0–100 nM range. The minimum SAM concentration detected was 1.6–3nM with phosphate buffer, as shown in (Table 1). The lowest detectable concentration of SAM using this method was 2–4nM in phosphate buffer. The detection limit was about 10–15nM using a serum-like matrix (data not shown).

### Specificity

After mixing L-Met, ATP and (MAT) together, the chemical reaction leading to the formation of SAM and PPI/Pi/Pi took place under appropriate conditions. As shown in (Figure 1), the anti-SAM monoclonal antibody competitively bound to the antigen in a dose-dependent manner in the cELISA used to measure the SAM. When the MAT-catalyzed enzymatic reaction did not reach equilibrium under the conditions used, as more SAM product was formed, a stronger inhibition of the cELISA reaction was observed. When the MAT-catalyzed reaction reached equilibrium after approximately 60 min, the inhibition of the cELISA reaction was not substantially enhanced (reached plateau phase). When the MAT concentration increased, the increased SAM production was seen through enhanced competitive inhibition in the cELISA. These results indicated the anti-SAM monoclonal antibody specifically binds to physiologically produced SAM.

**Table 1:** OD450 values measured using varying amounts of coating antigen

	SAM standard (nM)			PLL-aza-SAM					
	0.2 µg/ml			0.15 µg/ml			0.1 µg/ml		
Blank	0.0498	0.056	0.0522	0.0476	0.0471	0.0482	0.0754	0.0515	0.0476
0	1.4709	1.4344	1.4072	1.215	1.2484	1.2651	1.0844	1.1231	1.0633
6.25	1.365	1.3257	1.2673	1.0789	1.0734	1.0842	0.879	0.9122	0.8995
25	1.1769	1.1498	1.1015	0.8231	0.844	0.8334	0.6209	0.6561	0.6679
50	1.023	0.9856	0.9577	0.6428	0.6362	0.6005	0.4517	0.4555	0.5187
100	0.7687	0.7532	0.7292	0.4292	0.4203	0.4427	0.3036	0.3103	0.3071
Sensitivity	3.0 nM			1.7 nM			1.6 nM		

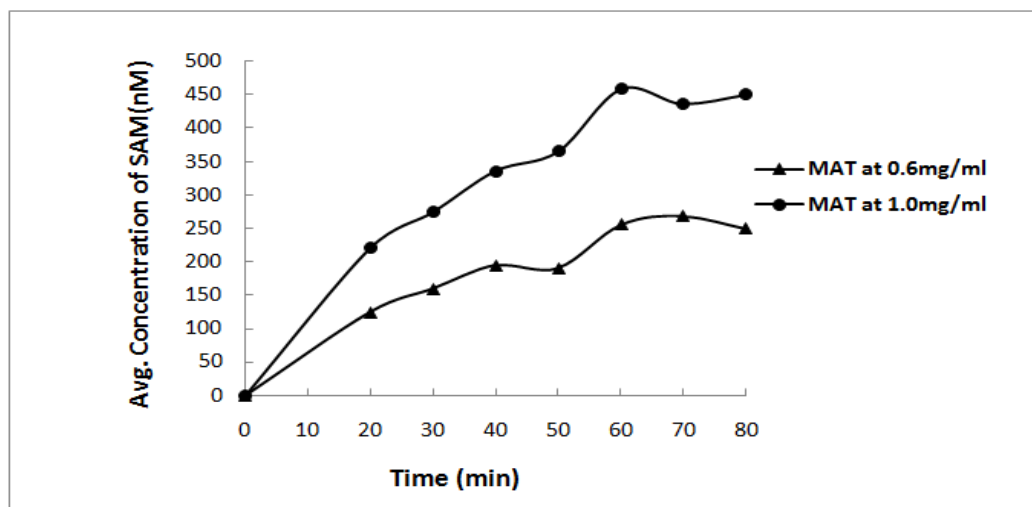
The cross-reactivity of the 84-3 with SAH, HCY, L-Met, ADP, ATP and MTA was studied. Only SAM competitively bound to the 84-3; the other six analogs did not (Figure 2). The cross-reactivity was less than 1%. These analogues do not competitively bind to anti-SAM monoclonal antibodies at a dose more than 100-fold higher than that of the dose of S-adenosylmethionine.

Spleen cells from the immunized mice were fused with SP2/0 cells, and hybridoma cell lines secreting anti-SAM antibodies were screened and named 118-6 and 84-3. The antibody subclass assessment indicated that clones 118-6 and 84-3 were both of the IgG2b subtype. The relative affinity constants of clones 118-6 and 84-3 measured using the ELISA were  $5.75 \times 10^9$ L/mol and  $7.29 \times 10^{10}$ L/mol, respectively. The titers of the supernatant and ascites of hybridoma cells were 1:6400 and 1:51200 for 118-6. The titer

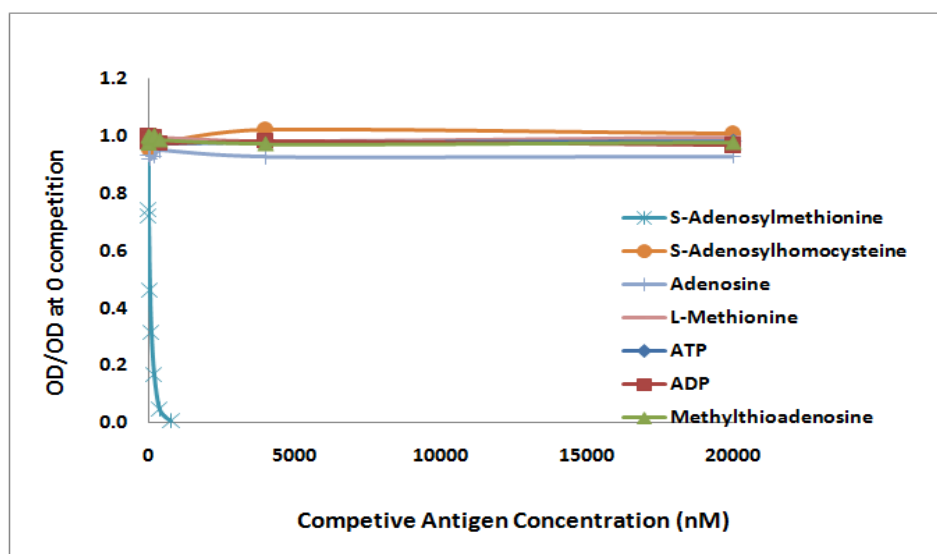
of 84-3 was higher than the titer of 118-6.

### Affinity

An ELISA method was employed to evaluate affinity of antibody-antigen binding. Different amounts of antigen were coated onto micro-titer strips for ELISA with a series of diluted antibodies. When using 0.1 mg/ml coating antigen, the half maximum OD was observed at an approximately 1:130000. The antibody concentration was  $[Ab] = (1\text{mg/ml}/160000\text{g/mol})/130000 = 4.807 \times 10^{-11}$  M. When using 0.05 mg/ml coating antigen, the half maximum OD was observed when the 84-3 was diluted to approximately 1:70000. The corresponding antibody concentration was  $[Ab]_t = (1\text{mg/ml}/160000\text{g/mol})/70000 = 8.92 \times 10^{-11}$  M.  $n = (0.1 \text{ mg/ml})/(0.05 \text{ mg/ml}) = 2$ .  $K_a = (n-1) / 2 \times (n[Ab] - [Ab]_t) = 7.29 \times 10^{10}$  L/mol.



**Figure 1:** Dose-dependent competition was detected in a sample using a cELISA (SAM from a sample competed with the coated SAM hapten and bound to the HRP-conjugated 84-3 antibody). The sample is the product of the following biochemical reaction: MAT was added to Met and ATP in the appropriate buffer at 37°C. The competition indicated that the 84-3 antibody specifically bound physiologically produced SAM. The data came from the averages of two experiments.



**Figure 2:** Results from the cELISA with the 84-3 anti-SAM monoclonal antibody; 0.1 µg/ml of a SAM coating standard (Cat # ACT00201) was coated onto 96-well plates. Serial dilutions of a SAM standard (Cat # AST00201), SAH, L-Met, MTA, ADP, and ATP, as well as a 1:35000 dilution of the 84-3 antibody were added. An HRP-conjugated rabbit anti-mouse IgG antibody was used to develop the color.

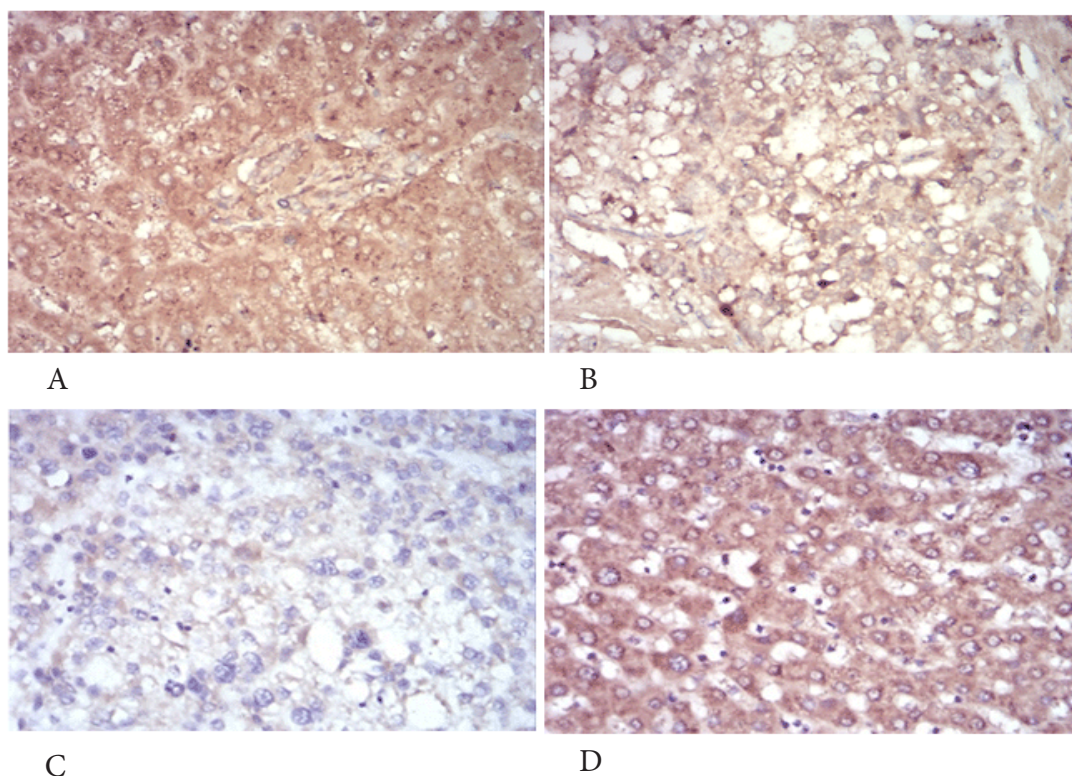
### Applications using SAM antibodies

Indirect immunohistochemistry showed substantial positive staining in normal liver tissues (Figures 3A,3C), with positive brown staining observed in cells. In hepatoma tissues (Figures 3B,3D), the same procedure did not produce much brown staining, indicating during carcinogenesis SAM was decreased. The 84-3 antibody was diluted at 1:200, incubated with normal L02 hepatocytes and HepG2 HCC cells and fluorescein-conjugated secondary antibody was added. The fluorescence intensities were

analyzed by flow cytometry. The geometric mean of fluorescence was 178.22±34.63 in HepG2 and 274.70±12.11 in L02 cells in three experiments. Thus, the SAM level in cancer cells was lower than the level in normal cells. The average fluorescence signal in HepG2 cells was significantly reduced compared to that in L02 cells ( $p < 0.05$ ).

### Measurement of SAM levels in serum samples

SAM levels were measured in serum samples from 81 healthy individuals and 99 patients with different liver diseases. SAM



**Figure 3:** IHC results for paraffin-embedded liver tissue sections and a tissue chip. The 118-6 anti-SAM antibody was diluted 1:200 and incubated with normal liver tissues (A and C) and liver cancer tissues (B and D). C-D shows the results from a tissue chip. C: normal adjacent tissue to the sample shown in D; D: HCC(T2N0M0, II) from M39 (male, 39 years old). E and F show the results for paraffin-embedded liver tissue sections. All pictures were captured at 400x magnification.

levels were significantly higher ( $p < 0.05$ ) in the normal serum samples than those from patients (Table 2). Since the SAM level in human blood is influenced by diet, gender, age and diseases, standard deviation (SD) between samples could be bigger. The average (Avg) SAM of normal subjects was  $386.66 \pm 212.20$  (Avg $\pm$ SDnM),  $101.42 \pm 83.12$  in hepatitis,  $104.96 \pm 82.63$  in liver cancers,  $92.95 \pm 62.41$  in cirrhosis, and  $66.46 \pm 29.77$  in liver failure (deficiency). The SAM levels were sorted at thresholds of 120nM and 240nM. At the 240-nM threshold, 25 having SAM levels below 240nM (positive), with a detection or positive rate of 30.86% (false positive rate). The SAM levels of 44 patients with hepatitis (95.65%) were less than 240nM. Only 1 of 20 patients with liver cirrhosis and 1 in 14 patients with liver cancer had serum SAM levels greater than 240nM (negative), and the positive rates were 95% and 92.86%, respectively. Therefore, the probability of detecting liver diseases (sensitivity) was high. If the threshold was 120nM, only 3 normal human serum samples contained SAM less than 120nM, with a detection rate of 3.70% (positive rate). Lowering the detection threshold significantly reduced the false positive rate, thereby specificity (probability that normal serum was detected above threshold, i.e., negative) increased from 69.14% to 96.30%, the sensitivities were also decreased. Thirty-seven of the 46 patients with hepatitis (80.43% positive) had serum SAM levels below 120nM. Ten of 14 patients with HCC (71.43% positive) had a SAM below 120nM. Seventeen

of the 20 patients with liver cirrhosis (85% positive) had a SAM below 120nM. The detection rate for the 19 patients with liver failure was 100% with any of the thresholds.

We compared the use of SAM in identifying liver diseases to that of alanine transaminase (ALT) and aspartate Amino transferase (AST), and found SAM has advantages compared with these markers (Table 3): (1) the AST marker was negative in 11 patients diagnosed with liver diseases, but the SAM levels were positive, regardless of whether the 120nM or 240nM threshold was used. Another 11 patients were detected in the range of 50-100U/L as weak positive results; 8 of them had SAM levels below 120nM (positive results), and all were positively detected when the 240nM threshold was used. (2) Two patients diagnosed with liver diseases were negative by ALT, but were positively detected by SAM regardless of whether the 120nM or 240nM threshold was used. Another 21 patients with a range of 40-100U/L were reported as weak positive results; 18 of them had SAM levels below 120nM, and 20 of them were reported as positive by SAM when the 240nM threshold was used. (3) For the combination of AST and ALT, two patients with liver diseases were not identified by either AST or ALT but were detected by SAM at a threshold of 120nM. The use of AST or ALT alone had a higher risk of false negative results. Among 16 patients with weak positive by AST or ALT, 15 displayed SAM levels less than 120nM, and all SAM levels

**Table 2:** Detection of different liver diseases by SAM levels in serum samples

Group	Case#	SAM (nM)		SAM = 240 nM cutoff		SAM = 120 nM cutoff	
		Avg	SD	Sensitivity <sup>2</sup>	Specificity <sup>2</sup>	Sensitivity <sup>1</sup>	Specificity <sup>1</sup>
Normal	81	386.66	216.20	30.86% (25/81)		3.70% (3/81)	
Hepatitis	46	101.42	83.12	95.65% (44/46)	69.14% (56/81)	80.43% (37/46)	96.30% (78/81)
Cancer	14	104.96	82.63	92.86% (13/14)	69.14% (56/81)	71.43% (10/14)	96.30% (78/81)
Cirrhosis	20	92.95	62.41	95.00% (19/20)	69.14% (56/81)	85.00% (17/20)	96.30% (78/81)
Liver failure	19	66.46	29.77	100% (19/19)	69.14% (56/81)	100% (19/19)	96.30% (78/81)

Avg: average; SD: standard deviation;  
 Specificity<sup>2</sup>: the probability that an individual without the disease is screened negative (SAM < 240 nM);  
 Sensitivity<sup>2</sup>: the probability that an individual with the disease is screened positive (SAM < 240nM);  
 Specificity<sup>1</sup>: the probability that an individual without the disease is screened negative (SAM < 120 nM);  
 Sensitivity<sup>1</sup>: the probability that an individual with the disease is screened positive (SAM < 120 nM).

**Table 3:** Comparison of SAM levels and other liver biomarkers

Single comparison	SAM (nM)				Single comparison	SAM (nM)			
AST	> 240	120-240	< 120	total	ALT	> 240	120-240	< 120	total
< 50	0	0	11	11	< 40	0	0	2	2
50-100	0	3	8	11	40-100	1	2	18	21
> 100	2	6	32	40	> 100	1	7	31	39
total	2	9	51	62	total	2	9	51	62
AST sensitivity	81.70%				ALT sensitivity	96.70%			
SAM sensitivity	96.10%				SAM sensitivity	96.70%			
Agreement	79.00%				Agreement	93.50%			
Combined comparison	SAM								
AST or ALT	> 240			120-240	<120	total			
AST < 50 and AST < 40	0			0	2	2			
AST ≤ 50 and 40 < ALT < 100	0			0	8	8			
50 < AST < 100 and ALT ≤ 100	0			1	7	8			
AST > 100 or ALT > 100	2			8	34	44			
total	2			9	51	62			

were less than 240nM. (4) Among the 44 cases diagnosed as liver diseases by both AST and ALT, 42 were also detected by SAM. Two patients with liver diseases were not detected by SAM (256.05nM and 262.35nM), but were still below the average normal level of 386.66 nM and was randomly related to the SD factor. In summary, SAM levels are more accurate and useful for identifying liver diseases than AST or/and ALT since SAM marker has less false negative results. SAM levels are useful in identifying functional liver damage at an early stage when other common liver function markers, such as AST or ALT, cannot. Early diagnosis is always ideal for quickly and effectively treating liver diseases, leading to

timely recovery of liver function and preventing further damage to the liver.

We used SAM antibodies to stain sections of normal and cancer tissues (paraffin-embedded) using IHC. Positive staining was shown as brown areas. The blue areas show the HE-counterstained nuclei. (Figure 3A, 3C) show mostly cytoplasmic and relatively less nuclear SAM-specific staining in normal liver cells. (Figure 3B, 3D) show hepatocellular carcinoma tissue sections that were stained side-by-side using normal adjacent liver tissue (Figure 3A, 3C). As SAM is a small metabolite with highly dynamic levels, the results may vary with the method and time at which

tissue slides were fixed and prepared. Various cancer types, cases and samples may also yield different results as each case is unique in terms of stage and type, the patient's overall health, treatments, which may contribute to IHC result variation.

## Discussion

SAM is involved in methylation, transsulfuration, aminopropylation, gene regulation, the development of nerve conduction and detoxification, etc. Its roles in clinical applications are being extended. SAM relieves depression and chronic pain, enhances tissue regeneration and contributes to the repair of inflammation; e.g. SAM metabolite glutathione detoxifies free radicals. SAM improves dopamine levels in the brain and relieves symptoms of depression [8,9,1]. Significant progress has been made in using SAM to treat liver diseases, which is considered ideal for treating liver diseases by promoting recovery from liver damage, cirrhosis and other liver disorders [10,11,1,21,22]. However, SAM levels in blood samples have not been used to identify or diagnose liver diseases.

The probability of detecting diseases (sensitivity) was very high at the high threshold of 240nM, but the false positive rate was also higher (30.86%). Using the lower threshold of 120 nM, the detection rate for healthy individuals was 3.70% (the false positive rate was lower), but sensitivity for hepatitis, cancer and cirrhosis were also reduced. The accuracy of diagnosis may be improved through comprehensive consideration of the two thresholds. If a detected value fell between 120-240 nM and was near 240nM, it might be likely to represent a normal individual, followed by patients with HCC and hepatitis. If a value was between 120-240nM and near 120nM, it might be more likely to represent hepatocellular carcinoma, followed by hepatitis and cirrhosis.

Nineteen of 20 patients with cirrhosis had SAM lower than 240nM (detection rate 95%); SAM level in the remaining one patient was 272.35nM, slightly above the threshold but less than the average normal serum level. SAM levels in 13 of 14 patients with HCC were less than 240nM (detection rate 92.8%). The SAM level in the remaining one patient was 297nM, less than the normal average. The SAM levels in all patients with liver failure were less than 240nM. The SAM levels in the 81 normal serum samples were significantly higher than in patients with liver disease, with average level of 386.66nM, and 20 of 81 samples had SAM levels less than 240nM. A larger number of samples were needed to obtain more accurate false positive and false negative rates. In addition, due to potential differences between patients and healthy volunteers, the most persuasive method was to compare SAM levels in serum samples collected at multiple time points from the same case. Thus, SAM levels would be measured and dynamically evaluated multiple times, contributing to the timely capture of any changes in liver function or occurrence of disease.

Dysfunction in SAM metabolism is very common in liver diseases. When liver damage occurs, the methionine cycle is

destroyed and the SAM level is reduced. Oxidative stress and large amounts of oxygen free radicals are produced, leading to lipid peroxidation in liver cells. In addition, immune cells from the liver (e.g., Kupffer cells, monocytes and macrophages) produce a variety of cytokines, such as tumor necrosis factor, interleukin-6 (IL-6) and IL-1. These cytokines will further damage liver cells and thereby increase the probability that normal liver cells undergo apoptosis and necrosis. The serum SAM level reflects liver damage. Therefore, the significance of measuring SAM clinically is important. Measuring SAM concentrations in blood samples will not only aid in the detection of liver diseases but also serve as a warning signal for liver damage that can be found earlier than by AST and ALT. By monitoring SAM levels, patients could be diagnosed earlier, thereby facilitating early and effective treatment to prevent further damage to the liver.

Traditional methods to measure SAM were HPLC and LC-MS/MS, which require costly equipment, are time-consuming, laborious and specially trained professionals to operate the equipment. The ability to measure SAM in biological samples has been a huge challenge due to its instability. The specificity, sensitivity and accuracy of the HPLC method are poor. The main limitations of HPLC and LC-MS/MS to measure SAM are the sample extraction, pretreatment, and lengthy measurement processes, which involve high temperatures and solvent steps that may cause changes in the molecule being measured, especially the very unstable SAM. A simple, convenient, specific, accurate and inexpensive detection method is required to measure SAM concentrations. In this study, SAM concentrations were quantified using a cELISA. The 118-6 and 84-3 anti-SAM monoclonal antibodies prepared from cell lines in this study have high sensitivity, specificity and affinity, and the established cELISA method was suitable for the quantitative measurement of serum samples obtained from patients with various liver diseases. The immunoassay is simple, fast, sensitive and specific, does not need specialized or expensive equipment, and can be performed by any lab technician. The methylation index can be determined simply, quickly and accurately, which is of great significance to allow researchers to obtain a deeper understanding of biochemical metabolic pathways. These pathways are essential for studies of various organisms (including fauna and flora) to explore the root causes of disease development and assist in diagnosis and therapy.

Ademetionine or Transmeti, which use SAM as the active ingredient, are routinely used as liver-protective medicines in China, some Asian-Pacific countries and Eastern Europe. Based on the efficacy of these medicines, SAM is clearly important for normal liver function, and reduced SAM level is a common factor in liver damage and diseases. Thus, serum SAM can reflect the severity or status of liver diseases.

## Ethics approval and consent to participate

The studies were approved by The Ethics Committee of Central South University.

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Administrative Committee of Changsha Economical and Technological Developmental Zone

## Ethics approval and consent to participate

The studies were approved by The Ethics Committee of Central South University.

## Author contribution

Xiujian Hao: study concept and design; analysis and interpretation of data; writing the manuscript; critical revision of the manuscript for important intellectual content; study supervision; Jianguo Sun, Yan Huang and Wenting Li: sample collection; Jing Hao, HuimingRen, Yaxia Zhou, Jiazhi Xia and Wenna Zhang: acquisition of data and statistical analysis.

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