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El ácido fólico y la vitamina B_{12} como biomarcadores de morbilidad y mortalidad en pacientes con shock séptico

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ABSTRACT

Introduction and objective: a study was made of the folic acid (Fol) and vitamin B_{12} (B_{12}) serum concentrations in critical patients with septic shock upon admission and after three days of stay in the Intensive Care Unit (ICU), with an analysis of their association to inflammatory parameters and patient morbidity-mortality.

Methods: a prospective analytical study was made of 30 critically ill patients with septic shock. Demographic data, comorbidities, clinical information and severity scores were recorded. Data collected included serum Fol and B_{12} levels using the DxI® Autoanalyzer (Beckman Coulter) based on a competitive electrochemoluminescence immunoassay.

Results: mean serum Fol was within the reference range stipulated by the laboratory on the first day. Nevertheless, a total of 21.4 % of the patients had high Fol levels, with 14.2 % being Fol deficient. An association was observed between Fol ($p < 0.012$) status and 28-day mortality, and the number of days of mechanical ventilation, fraction of inspired oxygen (FiO_2) and fibrinogen increased in patients with higher Fol levels ($p < 0.05$). In addition, 85.7 % of cases had B_{12} levels above the reference values, with a correlation being observed between B_{12} and Fol.

Conclusions: this study proposes Fol as a novel morbidity-mortality biomarker in critical septic patients, and reinforces the usefulness of B_{12} as a morbidity biomarker. It is thus suggested that the measurement of Fol upon admission and over the first 72 hours of hospital stay could provide prognostic information about the clinical course and outcome of septic shock patients.

Keywords: Septic shock. Folic acid. Vitamin B_{12}. Morbidity. Mortality.

RESUMEN

Introducción y objetivo: se realizó un estudio de las concentraciones séricas de ácido fólico (Fol) y vitamina B_{12} (B_{12}) en

pacientes críticos con *shock* séptico al ingreso y después de tres días de estancia en la Unidad de Cuidados Intensivos (UCI), con un análisis de su asociación con los parámetros inflamatorios y la morbimortalidad de los pacientes.

Método: se realizó un estudio analítico prospectivo de 30 pacientes críticos con *shock* séptico. Se registraron datos demográficos, comorbilidades, información clínica y puntuaciones de gravedad. Los datos recopilados incluyeron los niveles séricos de Fol y B_{12} utilizando el autoanalizador DxI® (Beckman Coulter) basado en un inmunoensayo de electroquimioluminiscencia competitivo.

Resultados: la media de Fol sérico estuvo dentro del rango de referencia estipulado por el laboratorio el primer día. Sin embargo, el 21,4 % de los pacientes presentaban niveles altos de Fol y el 14,2 % presentaban deficiencia de Fol. Se observó una asociación entre el estado de Fol ($p < 0,012$) con la mortalidad a los 28 días, con el número de días de ventilación mecánica, con la fracción de oxígeno inspirado (FiO_2) y con el fibrinógeno, que aumentaron en los pacientes con niveles de Fol más altos ($p < 0,05$). Además, el 85,7 % de los casos tenían niveles de B_{12} por encima de los valores de referencia, observándose una correlación entre B_{12} y Fol.

Conclusiones: este estudio propone al Fol como nuevo biomarcador de morbimortalidad en los pacientes críticos con sepsis y refuerza la utilidad de la B_{12} como biomarcador de morbilidad. Por tanto, se sugiere que la medición de Fol al ingreso y durante las primeras 72 horas de estancia hospitalaria podría proporcionar información pronóstica sobre el curso clínico y el resultado de los pacientes con *shock* séptico.

Palabras clave: *Shock* séptico. Ácido fólico. Vitamina B_{12}. Morbilidad. Mortalidad.

INTRODUCTION

Septic shock is one of the major causes of mortality and morbidity in the Intensive Care Unit (ICU), and places a strong burden on healthcare resources (1,2). Septic shock is associated with a greater risk of mortality than sepsis alone, and with an in-hospital mortality rate of over 40 %, according to the Third International Consensus Definition for Sepsis and Septic Shock (1). Sepsis is characterized by tissue infiltration by polymorphonuclear cells (PMNs) and monocytes/macrophages, with excessive production of reactive oxygen species (ROS) (e.g., superoxide anions and hydrogen peroxide) and reactive nitrogen species (RNS) (e.g., nitric oxide) (3). Such free radicals produced in excess could amplify the inflammatory response in sepsis, acting as cell signal messengers, altering expression, and intervening in inflammatory immune modulation. These processes could provoke cell damage (to membranes, proteins and DNA), generally leading to cellular dysfunction, multiorgan failure, and eventual death of the septic patient. Inflammatory biomarkers are useful for the diagnosis of infections in the emergency care setting (4,5). More studies are required to help identify and understand the pathophysiological basis and biomolecular disorders that occur in this disease, as well as studying the evolution in the ICU, to help us understand, even predict a patient's clinical outcome in the future.

Folic acid (Fol) has not been evaluated in depth as a biomarker in critical patients. The present study was therefore designed to explore the behavior of Fol in the critically ill. The results of studies on serum Fol levels in critically ill patients and individuals with sepsis are subject to controversy. A study of critically ill patients (6) revealed deficient serum Fol levels in 65 % of patients. However, other authors have found Fol levels to be within the reference values in septic patients (7,8). Folic acid levels have been inversely correlated to the clinical severity of critically ill patients, and have been found to be lower in septic and febrile patients (9). Likewise, Fol has been shown

to contribute to the control of chronic inflammation in vitro through various mechanisms after inducing monocytes with lipopolysaccharides in vitro. The administration of a preparation with Fol, vitamin B_{12} and choline modified the levels of inflammatory molecules (10).

Vitamin B_{12} (B_{12}) is another key and essential nutrient that may be useful in defining the prognosis of critical patients (11). Deficiencies of some vitamins, including B_{12} and Fol, have been demonstrated in critical patients, suggesting the need for replacement measures (12). B_{12} has been claimed to have antioxidant properties that afford a glutathione (GSH) sparing effect. The underlying mechanism involves stimulation of the activity of methionine synthase and reaction with hydrogen and nitrogen free radicals. Manzanares et al. (13) proposed that high parenteral doses of B_{12} could benefit patients with septic shock. Moreover, Lin et al. (14) observed that the administration of intravenous B_{12} to patients during septic shock improved blood pressure. However, high levels of B_{12} are associated with more seriously ill critical patients (15). In contrast, elevated blood vitamin B_{12} levels have been associated with inflammatory diseases and poor prognosis in critically ill patients (16). Plasma vitamin B_{12} levels have also been associated with other acute phase biomarkers such as C-reactive protein (CRP), and with the Sequential Organ Failure Assessment (SOFA) score in critically ill patients (17,18).

The association between Fol concentration and in-hospital mortality in adult patients with septic shock has not been evaluated to date. The tentative use of Fol as a novel biomarker may allow early recognition and decreased severity of sepsis. Therefore, we initially aimed to evaluate Fol and B_{12} status in a sample of 28 patients with septic shock.

MATERIAL AND METHODS

Study design and patients

An analytical study was made of Fol and B_{12} levels and clinical parameters on day 1 and day 3 of ICU stay in critically ill patients with septic shock. Over a two-year period (September 2017 to May 2019), adult patients (≥ 18 years old) admitted to the ICU were systematically screened for study inclusion. The selection of patients was made in the ICU of the hospital. Patient diagnosis was established following the consensus criteria of septic shock (16) and according to the definition of the Third International Consensus Definition for Sepsis and Septic Shock (1). Patients over 18 years of age with sepsis and who had severe arterial hypotension unresponsive to fluid therapy were included. These patients did not receive Fol or B_{12} as oral, enteral, parenteral supplements or any combination of them. Control samples were obtained from healthy adult subjects with ages similar to those of the study cases, and presenting blood sample values within reference ranges.

The study was approved by the Ethics Committee of the University of Granada (Ref.: 248/CEIH/2015). Patients were admitted after providing their informed consent. The study was carried out according to the principles of the Declaration of Helsinki and also in abidance with the International Conference on Harmonization (ICH)/Good Clinical Practice (GCP) guidelines. The clinical and laboratory parameters of the patients were collected on day 1 and day 3 after admission to the ICU. Clinical parameters included the Acute Physiology and Chronic Health Evaluation II (APACHE II) score, the Sequential Organ Failure Assessment (SOFA) score, days of mechanical ventilation (DMV), ICU stay, and 28-day mortality rate.

Biochemical assessment

Fasting blood samples were drawn from the patients by venipuncture after the hemodynamic stabilization phase of admission and after three days of ICU stay: renal function (ions and creatinine), liver function (bilirubin), nutrition parameters (Fol and B_{12}), hematological and inflammatory parameters (lactic acid, fibrinogen, lactate

dehydrogenase [LDH], CRP and procalcitonin [PCT]) were measured by the hospital laboratory using standard techniques.

Assessment of folic acid and vitamin B_{12}

The patients had blood drawn on day 1 and day 3 of their stay in the ICU. The samples were processed immediately. They were centrifuged at 3500 rpm for 10 min at 4 °C and frozen at -80 °C until further analysis. Samples of healthy controls were processed in the same way as the case samples. Fol and B_{12} were measured using a DxI® Autoanalyzer (Beckman Coulter, CA, USA) employing a competitive electrochemoluminescence immunoassay for quantitative determinations. The reference values considered for Fol levels were 3.10 to 20.0 ng/mL and 116.0 to 513.0 pg/mL for B_{12}. The Fol analytical method involved binding of the Fol in the sample to a folate binding protein. The excess folate binding protein bound to a folate-alkaline phosphatase conjugate that in turn was bound to murine capture anti-antibodies coating paramagnetic particles. By applying a magnetic field in the reaction vessel, these particles were recovered; the light generated only by the molecules of the recovered particles was measured using a luminometer – the light generated being inversely proportional to Fol concentration in the sample.

The B_{12} analytical method involved the following steps: the proteins were first denatured with alkaline potassium cyanide and dithiothreitol. The B_{12} in the sample was then exposed to an intrinsic factor alkaline phosphatase conjugate and paramagnetic particles with anti-intrinsic factor were added to bind the excess conjugate. After applying a magnetic field and washing, only the intrinsic factor-alkaline phosphatase particles bounded to the paramagnetic particles were recovered; the remaining conjugate was measured with a luminometer, and the light generated was inversely proportional to the concentration of B_{12} in the sample.

Statistical analysis

The statistical analysis was performed using the SPSS version 21.0 statistical package (IBM SPSS, Armonk, NY, USA). Qualitative variables were reported as frequencies and percentages, while quantitative variables were shown as the mean ± standard deviation (SD). The assumption of normality was tested using the Shapiro-Wilk test. The chi-squared test was performed to calculate the frequencies of the variables between groups. The association between quantitative variables and mortality, and between the cases and controls, was explored by applying the Mann-Whitney U-test. Folic acid was stratified according to the median (6.20 ng/mL) into two groups (high and low Fol levels) and compared with the rest of the variables using the Mann-Whitney U-test. Comparison of the quantitative variables between day 1 and day 3 of admission was carried out using the Wilcoxon test in order to study the evolution of the critical patients with septic shock during ICU stay. Spearman's correlation coefficient was used to establish correlations between the primary outcomes and the inflammatory and clinical outcomes. Statistical significance was considered for $p < 0.05$.

RESULTS

Patient characteristics

A total of 30 patients admitted to the ICU with septic shock were enrolled after agreeing to participate in the study. However, two patients did not continue because their samples had to be discarded. Twenty-eight patients therefore were finally recruited for the study. Table I shows demographic and clinical characteristics of the patients, as well as the evolution over three days of ICU stay. The differences in mechanical ventilation data (PaO_2/FiO_2: partial oxygen arterial pressure/fraction of inspired oxygen; $PaCO_2$: partial pressure of carbon dioxide in arterial blood; PEEP: positive end-expiratory pressure, and C_{st}: static compliance) between the first and third day were not significant. The microorganisms causing infection were *Streptococcus*

(n = 3), *Acinetobacter* (n = 1), *Pseudomonas* (n = 1), *Campylobacter* (n = 1), *Clostridium* (n = 1), *Candida albicans* (n = 1) and *Escherichia coli* in the rest of the cases.

A majority of cases had underlying diseases such as cardiocirculatory diseases, hyperlipidemia, diabetes, chronic obstructive pulmonary disease, malignancy, etc. Two cases had hepatitis B virus (HBV), another human immunodeficiencyvirus (HIV) disease and another only hypothyroidism, which could interfere in the depletion of antioxidants.

As expected, the APACHE II and SOFA scores were high, with a significant decline in SOFA score ($p < 0.011$) during ICU stay. A total of 15 patients needed mechanical ventilation (53.6 %), and the mean number of days spent in the ICU was 7.04 ± 10.5. The 28-day mortality rate was 42.9 % (12 patients).

Biochemical parameters

The descriptive statistics and comparative bivariate analysis between the biochemical parameters upon admission and on the third day of ICU stay are shown in table II. In general, abnormal laboratory parameters were observed in our 28 cases of septic shock. Acute markers of inflammation and infection such as CRP and PCT were found to be above the reference values. At follow-up a statistically significant decrease was found for lactic acid, PCT, hemoglobin and platelet count on the third day ($p < 0.05$). Serum Fol and B_{12} levels showed no significant changes over the three days of ICU stay.

Table III shows the serum Fol and B_{12} levels of the patients with septic shock and the healthy controls. A total of 84 serum samples from healthy patients were used as controls. There were statistically significant differences in serum Fol and B_{12} levels between the two groups, with higher values among the cases ($p < 0.047$ and $p < 0.001$, respectively). No significant differences were found for Fol and B_{12} in our group of cases during ICU stay. However, the chi-squared test revealed that 21.4 % and 28.6 % of the patients were deficient in

Fol, and that 14.2 % and 14.3 % of the patients presented high Fol values, on the first and third day of stay, respectively. Moreover, a total of 85.7 % of the patients presented high levels of B_{12} at the beginning of the study, versus 92.3 % of the patients after three days of ICU stay.

Table IV in turn describes the association between serum Fol and B_{12} levels and in-hospital morbidity-mortality. The serum Fol levels in the patients who died were compared with the serum Fol levels in the patients who survived: those who died had significantly higher levels of Fol on the first day of ICU stay ($p < 0.017$). In addition, there were significant differences in lactic acid concentration and platelet count between the patients who died and those who survived. No differences were observed in the case of B_{12}, CRP, PCT, LDH, fibrinogen, leukocytes or hemoglobin.

Table V shows the matrix correlations between folic acid and B_{12} and clinical outcome and severity markers. A statistically significant correlation was recorded between Fol and days of mechanical ventilatión (DMV) ($r = 0.459$; $p < 0.05$) on the first day of ICU stay. However, no statistically significant differences were observed between Fol versus SOFA, Fol versus APACHE II, or Fol versus days of stay in the ICU. After associating Fol and B_{12} with other acute phase parameters, we found statistically significant correlations between Fol versus fibrinogen ($r = 0.382$; $p < 0.045$) and Fol versus B_{12} ($r = 0.374$; $p < 0.05$) on the first day of ICU stay. A correlation was also observed between Fol on the first day and Fol on the third day of ICU stay ($r = 0.939$; $p < 0.001$).

Figure 1 shows DMV, fraction of inspired oxygen (FiO_2) and fibrinogen levels in patients with low and high serum Fol levels. The results showed the subjects with higher serum Fol levels to have more DMV ($p < 0.012$), higher percentages of FiO_2 ($p < 0.012$) and higher fibrinogen levels ($p < 0.008$).

DISCUSSION

The aim of the present study was to investigate Fol and B_{12} behavior in critical care patients with septic shock upon admission and after three days in the ICU, and to assess their association to inflammatory parameters and patient morbidity-mortality. Our main findings were that the Fol levels were high in nearly one-third of the patients, being positively associated to mortality and to clinical outcomes such as the number of days of mechanical ventilation, FiO_2 and fibrinogen. Moreover, serum B_{12} levels were seen to be elevated in patients with septic shock.

An association between low Fol levels and mortality has been documented in the literature in patients with cardiovascular diseases and cancer (19). To the best of our knowledge, the present study is the first one to associate Fol levels with morbidity and mortality in patients with septic shock. However, no association between Fol and mortality was observed on the third day, possibly due to hemodynamic stabilization during ICU stay, and also because deceased patients were no longer included in the evaluation on the third day. In a previous study (7), Fol and B_{12} were measured in patients with severe sepsis, and no significant differences were observed with respect to the healthy control group. Another study (6) found 65 % of the critical patients admitted to the ICU to have decreased Fol levels (< 3.40 ng/mL), with a more pronounced decrease 24 hours after admission. Similarly, B_{12} and Fol were measured in 102 patients with severe sepsis, with results similar to our own, recording higher levels of B_{12} and levels of Fol within reference ranges (8). When Fol was analyzed in septic patients and compared with healthy individuals, no significant differences were found between the two groups (7,8), as also occurred in our study. A sample of 105 critical patients was studied, and 19 % were found to have Fol deficiency (< 2.70 ng/mL) upon admission to the ICU, with the observation of a negative correlation between Fol levels and the clinical severity of patients, and the identification of lower Fol levels in septic and febrile patients (9). In our study, the average Fol level on

the third day was lower than on the first day, but higher in those patients who died during ICU stay. We believe that the Fol levels increased in more severe patients in response to decreased antioxidant status (20), since folic acid has the ability to suppress ROS (21,22). In fact, an in vivo study (21) found that supplementation with Fol could prevent apoptosis as induced by oxidative stress, reducing ROS levels, through negative regulation of vascular peroxidase 1 as a consequence of changes in DNA methylation. Also, the in vitro administration of Fol has been shown to suppress hypoxia-induced inflammation (22). Moreover, a study found that the administration of Fol, vitamin B_6 and B_{12} significantly increased fibrinogen levels in women at increased risk of cardiovascular disease (23). This consequently would confirm our observation of higher fibrinogen levels in patients with high Fol levels ($p < 0.008$).

Some of the cases in this study (12 of them) developed acute kidney injury as shown in the mean creatinine at ICU admission shown in table II. These patients have been on continuous renal replacement therapy. Altered micronutrient status has been found to be common in patients with acute common insufficiency, including Fol (24). Furthermore, it has been described in a recent study that 33 % of the critical ill patients investigated with continuous renal replacement therapy had serum Fol deficiency (25). This could explain the 14 % of Fol deficiencies in our study.

A number of studies have also associated B_{12} levels with other inflammatory biomarkers and patient morbidity-mortality (11,12,18,26,27). In a study on critically ill patients, B_{12} levels were seen to be significantly linked to inflammatory markers such as CRP on day one and two of ICU admission, and to severity parameters such as the SOFA score during patient stay in the ICU (18). Another study also confirmed the association between B_{12} and CRP in critically ill patients (12). Therefore, B_{12} could be considered a predictor of patient morbidity. Other studies (11,26) have supported the association between high levels of B_{12} and morbidity and mortality

among critically ill patients, with higher B_{12} concentrations being found in those who died versus the survivors. Recently, elevated plasma B_{12} levels have been associated with an increased risk of all-cause mortality in the general population of The Netherlands (27). No association between B_{12} and mortality was observed in our study, however.

High plasma levels of B_{12} have been linked to functional deficiency of B_{12} (28). High plasma B_{12} concentrations may be a consequence of low levels of B_{12} within the cell, caused by an efflux of B_{12} from the cell towards the plasma compartment. Vitamin B_{12} functional status can only be measured by the enzymatic activity of cobalamin-dependent enzymes within the cell (28). In this study, B_{12} was considered a marker of plasma inflammation regardless of B_{12} levels within the cell. This explanation could also be applied to Fol. Thus, the fact that Fol was elevated in plasma in some patients does not guarantee folic acid functionality, since cell deficiency of Fol may actually exist.

The SOFA score is the gold standard for assessing severity in patients with sepsis in the ICU. However, groups of potential biomarkers evaluated jointly can increase diagnostic performance as well as morbidity-mortality prognostic yield compared to use of the SOFA score alone. Such biomarkers include PCT (29). Lactic acid is contemplated in the definition of sepsis and septic shock in the 2016 Consensus (1). Similarly to our own study, another paper has suggested lactic acid to be a predictor of mortality in patients with infection in the emergency care setting (30). In short, this biomarker has proven useful in the diagnosis, prognosis and evolution of septic patients (1,30,31).

Since methionine is necessary for the synthesis of DNA, both folic acid and B_{12} contribute to the production of leukocytes and red blood cells, which are necessary for defense against infection. Figure 2 shows the dependence of Fol and B_{12} upon the synthesis of methionine, since these vitamins favor the conversion from homocysteine to methionine. Furthermore, a study (32) found metabolites of

methionine (S-adenosylmethionine and S-adenosylhomocysteine) to be elevated and related to the sepsis mortality. On the other hand, the administration of group B vitamins (including B_{12} and Fol) has been shown to decrease homocysteine levels (33). In another study, the authors believed that dietary differences were unlikely to explain the route of homocysteine, but rather that hyperhomocysteinemia causes a prothrombotic disorder that alters the coagulation-anticoagulation balance, which is related to patient prognosis (7). In our study, we found an association between Fol and fibrinogen, and between thrombocytopenia and mortality. In another study, a relationship was observed between coagulopathy and organic dysfunction, since the authors found a correlation between the SOFA score and thrombocytopenia (34). As seen in figure 2, homocysteine is not available for synthesizing the cysteine necessary for glutathione, since through B_{12} and Fol it goes on to synthesize the methionine needed for cell development. It has also been observed that vitamin B_6 is decreased in patients with inflammation (35-37). In fact, another study (20) documented a positive correlation between B_{12} and glutamic acid in patients with septic shock. Accordingly, we attempted to explain the importance of the alteration of these two vitamins – Fol and B_{12} – since many pathways on which they depend are altered, and this may cause patient clinical status to worsen.

The present study has several strengths and limitations. As strengths, to our knowledge, it is the first to report on the association between Fol levels and morbidity-mortality in patients with septic shock. With regard to the limitations, the measurement of biomarkers in serum might not reflect their status within the cell. On the other hand, although we would have obtained greater statistical power with a larger sample size, it should be taken into account that the study group was limited to patients with septic shock in a very serious and labile state – a fact that made it difficult to recruit a greater number of patients.

CONCLUSIONS

In sum, this study contributes a possible novel biomarker – folic acid – which could be useful for the prognosis of morbidity-mortality during ICU stay in critical patients, with B_{12} levels acting as a biomarker of morbidity. Further studies are needed to elucidate the behavior and response of FoI in critically ill patients with septic shock during ICU stay.

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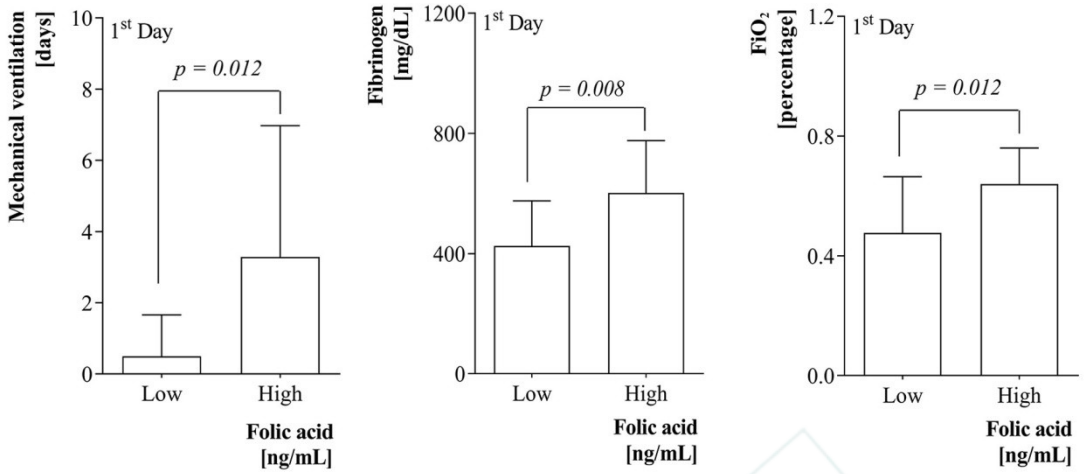

Fig. 1. Days of mechanical ventilation (DMV), fibrinogen and FiO_2 according to serum folic acid (FiO_2: fraction of inspired oxygen. A *p*-value less than 0.05 was considered statistically significant).

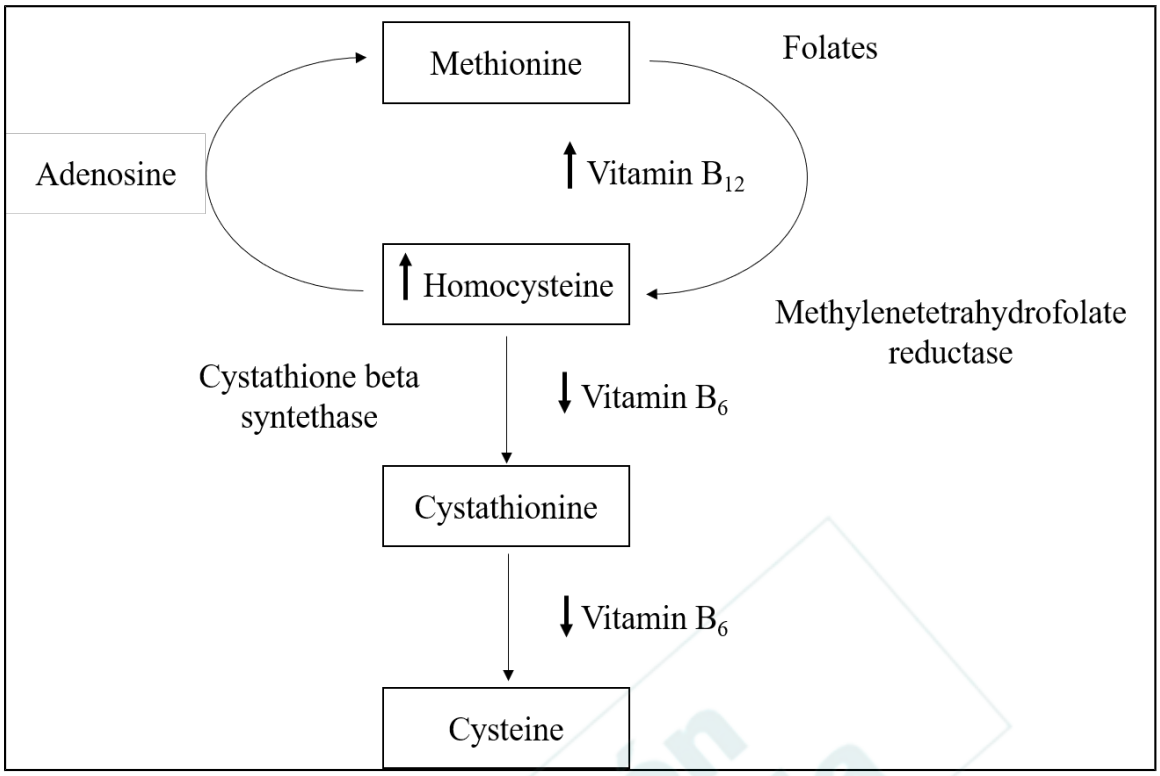

Fig. 2. Homocysteine cycle. In the present study, Fol and B_{12} were seen to be consumed in the synthesis of methionine. On using homocysteine, and when vitamin B_6 is decreased, little cysteine is produced. The latter molecule forms part of the structure of glutathione – a primary molecule for protection against the oxidative stress generated in these patients.

Table I. Demographic and clinical characteristics and evolution over three days of ICU stay in critically ill septic shock patients

	1st day (*n* = 28) (mean ± SD)	**3rd day (*n* = 14) (mean ± SD)**	***p*-value**
Age (years)	61.9 ± 14.1	-	-
Male, number (%)	22.0 (78.6 %)	-	-
SOFA score	12.40 ± 2.60	8.88 ± 4.40	p < 0.05
APACHE II score (range)	22.0 (17.0-27.0)	-	-
SBP (mm Hg)	67.1 ± 15.9	79.6 ± 10.9	p < 0.05
FiO_2 (%)	0.56 ± 0.17	0.40 ± 0.14	p < 0.001
Etiology of sepsis (number of subjects)			
Abdominal	14.0	**-**	**-**
Respiratory	8.00	**-**	**-**
Urinary	6.00	**-**	**-**

Values are expressed as mean ± standard deviation (SD), as ranges or percentages. SOFA: Sequential Organ Failure Assessment; APACHE: Acute Physiology and Chronic Health Evaluation; SBP: systolic blood pressure; FiO_2: fraction of inspired oxygen. A *p*-value less than 0.05 was considered statistically significant.

Table II. Biochemical parameters and their evolution over three days of ICU stay in patients with septic shock

	1st day (*n* = 28) (mean ± SD)	**3rd day (*n* = 14) (mean ± SD)**	**Reference values**	***p*-value 1st day vs. 3rd day**
Lactic acid (mmol/L)	4.72 ± 1.98	2.39 ± 2.17	0.60-2.50	p < 0.05
Sodium (mmol/L)	137.0 ± 7.2	136.8 ± 82.0	136.0-146.0	ns
Potassium (mmol/L)	4.26 ± 0.91	3.92 ± 0.71	3.50-5.10	ns
Anion gap (mmol/L)	12.1 ± 4.3	7.3 ± 10.8	7.00-16.0	ns
Creatinine (mg/dL)	2.99 ± 1.47	2.35 ± 1.64	0.67-1.20	ns
Total bilirubin (mg/dL)	2.37 ± 3.04	2.79 ± 3.07	0.30-1.20	ns
Fibrinogen (mg/dL)	513 ± 183	514 ± 290	200-350	ns
LDH (U/L)	620 ± 473	1286 ± 2131	110-295	ns
CRP (mg/L)	35.1 ± 28.9	46.7 ± 53.5	0.02-5.00	ns
Procalcitonin (ng/mL)	75.5 ± 59.3	42.6 ± 65.9	< 0.50	p < 0.05
Leukocytes (x 10^3/µL)	15.3 ± 17.9	13.3 ± 68.4	3.5-10.5	ns
Hemoglobin (g/dL)	11.2 ± 2.6	9.4 ± 2.1	11.0-17.0	p < 0.001
Platelets (x 10^3/µL)	122.3 ± 96.0	86.8 ± 59.4	120.0-450.0	p < 0.05
INR (ratio)	2.01 ± 1.40	1.90 ± 2.39	0.80-1.16	ns
aPTT (sec)	49.2 ± 30.3	40.8 ± 12.4	26.0-37.0	ns

Values are expressed as mean ± standard deviation (SD). LDH: lactate dehydrogenase; CRP: C-reactive protein; INR: international normalized ratio; aPTT: activated partial thromboplastin time. A *p*-value less than 0.05 was considered statistically significant. ns: not significant.

Table III. Serum folic acid and vitamin B_{12} levels in patients with septic shock and healthy controls

	Controls (*n* = 84) (mean ± SD)	**Cases 1st day (*n* = 28) (mean ± SD)**	**Cases 3rd day (*n* = 14) (mean ± SD)**	***p*-value 1st day**	***p*-value 3rd day**
Folic acid (ng/mL)	8.71 ± 3.16	9.61 ± 7.86	7.49 ± 7.08	p < 0.05	p < 0.05
Vitamin B_{12} (pg/mL)	466 ± 152	976 ± 511	1119 ± 192	p < 0.001	p < 0.001

A *p*-value less than 0.05 was considered statistically significant.

Table IV. Association between biochemical parameters and 28-day mortality in septic shock patients

	1st day (*n* = 28)			**3rd day (*n* = 14)**		
	Survivors (mean ± SD)	**Non-survivors (mean ± SD)**	***p*-value**	**Survivors (mean ± SD)**	**Non-survivors (mean ± SD)**	***p*-value**
Folic acid (ng/mL)	6.48 ± 5.33	13.80 ± 8.93	p < 0.05	7.04 ± 6.63	9.10 ± 10.1	ns
Vitamin B_{12} (pg/mL)	1000 ± 533	943 ± 500	ns	1141 ± 421	1363 ± 238	ns
Platelets (x 10^3/µL)	141 ± 88	96 ± 103	p < 0.05	104 ± 55	29 ± 26	p < 0.05
Lactic acid (mmol/L)	3.94 ± 1.77	5.76 ± 1.82	p < 0.05	1.45 ± 0.36	5.23 ± 2.98	p < 0.05
CRP (mg/L)	40.0 ± 36.2	27.9 ± 10.5	ns	35.6 ± 36.9	72.6 ± 85.8	ns
PCT (ng/mL)	72.0 ± 57.8	80.3 ± 63.8	ns	47.0 ± 69.3	4.90 ± 3.30	ns
LDH (U/L)	630 ± 578	607 ± 323	ns	713 ± 910	3767 ± 4208	ns
Fibrinogen (mg//dL)	482 ± 146	557 ± 223	ns	523 ± 322	489 ± 203	ns
Leukocytes (x 10^3/µL)	15.9 ± 10.5	14.2 ± 25.8	ns	13.3 ± 4.5	13.2 ± 12.6	ns
Hemoglobin (g/dL)	11.5 ± 2.3	11.0 ± 3.0	ns	9.4 ± 2.1	9.7 ± 2.5	ns

CRP: C-reactive protein; PCT: procalcitonin; LDH: lactate dehydrogenase. A *p*-value less than 0.05 was considered statistically significant. ns: not significant.

Table V. Matrix correlations between folic acid and vitamin B_{12} and clinical outcome and severity markers

	Fol 1st day (*n* = 28)	**Fol 3rd day (*n* = 14)**	**B_{12} 1st day (*n* = 28)**	**B_{12} 3rd day (*n* = 14)**
SOFA	ns	ns	ns	ns
APACHE	ns	-	ns	-
DMV (day)	$p < 0.05$	ns	ns	ns
Stay (day)	ns	ns	ns	ns
Fibrinogen	$p < 0.05$	$p < 0.05$	ns	ns
Folic acid	-	$p < 0.05$	$p < 0.05$	ns

SOFA: Sequential Organ Failure Assessment; APACHE: Acute Physiology and Chronic Health Evaluation; DMV: days of mechanical ventilation. A *p*-value less than 0.05 was considered statistically significant. ns: not significant.