

Early Increase of Plasma Homocysteine in Sepsis Patients with Poor Outcome

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Moderate hyperhomocysteinemia is a well-established coronary risk factor that develops when dietary supply with folate and/or vitamin B₁₂ is inadequate. Recently, stimulated peripheral blood mononuclear cells were shown to produce homocysteine. Thus, the stimulated immune system may contribute to moderate hyperhomocysteinemia during certain diseases. Because multiple trauma and sepsis are accompanied by often strong inflammatory responses, we investigated whether hyperhomocysteinemia may develop in patients. Total homocysteine and cysteine concentrations were measured in 83 plasma specimens from 18 patients (14 men, 4 women; 15 posttrauma with sepsis and 3 with sepsis alone) every third day of follow-up. Finally results were compared with concentrations of cytokines tumor necrosis factor (TNF)- α and interleukin (IL)-6, the immune activation marker neopterin and the extent of tryptophan degradation as indicated by the kynurenine-to-tryptophan ratio (kyn/trp). Compared with baseline, average total homocysteine ($P < 0.05$, d 4–d 10) and cysteine ($P < 0.05$, d 7–d 13) concentrations increased during follow-up of patients. However, only the increase of homocysteine was related to the survival status: total homocysteine was significantly higher in nonsurvivors ($P < 0.05$, d 4 and d 10) than in survivors, whereas cysteine concentrations increased in both subgroups. Homocysteine correlated with kyn/trp but not with neopterin concentrations. Increase of total homocysteine is common in patients after trauma with unfavorable outcome. Because all patients received standardized enteral nutrition after the end of hypodynamic shock, inconsistent vitamin supply is unlikely to be the reason for hyperhomocysteinemia in some of the patients; rather, it is associated with a stronger proinflammatory response. Certainly, the number of patients in our study is still small and results can only be regarded as preliminary.

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INTRODUCTION

Moderate increase of total plasma homocysteine concentrations is a well-recognized cardiovascular risk factor, and hyperhomocysteinemia is considered to reflect inadequate dietary supply with B vitamins folic acid and/or vitamin B₁₂ (1–5). Accordingly, supplementation with B vitamins is able to slow down homocysteine accumulation. Among its enhancing effects on oxidation processes *in vitro*, homocysteine was found to be a potential proinflammatory compound that enhances production of specific cytokines and thus may contribute to the pathogenesis of cardiovas-

cular disease (2–4). Homocysteine can be trans-sulfurated to cysteine, the most abundant plasma thiol and precursor of the antioxidant glutathione. Whereas both thiols have been proposed as markers of increased cardiovascular risk, only total homocysteine was regarded as an independent predictor of coronary artery disease (CAD) (5).

Peripheral blood mononuclear cells have been observed to release increased amounts of homocysteine upon exposure to proinflammatory stimuli *in vitro* (6). Likewise, in patients with cardiovascular, neurodegenerative and autoimmune disorders, rather close associations have been

described between the concentrations of total homocysteine and immune activation and inflammation markers: for example, neopterin (7,8) and C-reactive protein (9), which themselves are significant predictors of cardiovascular risk (10–12). Neopterin concentrations serve as a reliable immune response marker because a great amount of neopterin is released by monocyte-derived macrophages and dendritic cells upon stimulation with Th1-type cytokine interferon- γ (13–15). It appears that in inflammatory conditions, moderate hyperhomocysteinemia could develop even when the supply of B vitamins is within the recommended range.

Because the clinical course in patients after multiple trauma with or without sepsis is often accompanied by strong inflammatory responses (16), we were interested to test whether hyperhomocysteinemia would develop in them although they received standard enteral

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nutrition with vitamin supplementation. Interestingly, no major study has investigated homocysteine concentrations in such patients thus far. We measured total homocysteine and cysteine concentrations in plasma of patients and compared it with earlier measured concentrations of neopterin and the extent of tryptophan degradation, which was expressed as the kynurenine-to-tryptophan ratio (kyn/trp) (17,18). Both biochemical changes indicate immune activation, and both are recognized to be closely associated with the outcome of patients after multiple trauma (17–21).

MATERIALS AND METHODS

Patients

Plasma specimens were obtained from 18 patients (14 men, 4 women; Acute Physiology and Chronic Health [APACHE] II score, 18.9 ± 6.75 , 8–34; Injury Severity Score [ISS] for trauma, 39 ± 13.1 , 18–57; 15 posttrauma with sepsis and 3 with sepsis alone) every third day during 12–14 d of follow-up (Table 1). The first sample was drawn within 24 h after admission to the intensive care unit (ICU). Patients were admitted to the ICU of either the Medical University of Vienna or Lorenz Boehler Trauma Center, Vienna. The etiologies of trauma were motor vehicle accidents in 12 patients, attempted suicide in 2 patients, and occupational blunt trauma in 1 patient. Average length of stay in the ICU was 25.3 ± 20.1 d. During follow-up, six patients died on d 7, 10, 14, 17, 26 and 37. The cause of death was multiple organ failure (MOF) in five patients and cerebral death in one patient. Sepsis was defined according to American College of Chest Physicians/Society of Critical Care Medicine criteria (22). For statistical analyses, specimens were divided into five groups: one specimen collected on days 1–2 of each patient was referred to group “day-1,” days 3–5 to group “day-4,” days 6–8 to group “day-7,” days 9–12 to group “day-10” and days 13–14 to group “day-13.” In total, every patient contributed three to five specimens to the total number of 83 sera ana-

Table 1. Comparison of characteristics between survivors and nonsurvivors.^a

| | Survivors | Nonsurvivors | P value |
|------------------------------------------------|-----------------|-----------------|--------------------|
| Number of patients | 11 | 7 | |
| Age, y | 39.8 ± 16.3 | 53.7 ± 21.8 | 0.141 ^b |
| Sex, F/M | 2/9 | 2/5 | 0.605 ^c |
| Main diagnosis trauma with sepsis/sepsis alone | 9/2 | 6/1 | 0.829 ^c |
| Length of stay in ICU, d | 32.0 ± 20.9 | 17.7 ± 14.8 | 0.137 ^b |
| Received inotropes, Y/N | 10/1 | 7/0 | 0.412 ^c |
| Comorbidities, Y/N | 2/9 | 1/6 | 0.829 ^c |
| Apache II score | 16.9 ± 6.1 | 21.7 ± 7.1 | 0.154 ^b |
| Trauma score (ISS) | 35.2 ± 10.9 | 44.8 ± 14.9 | 0.170 ^b |
| SOFA score | 12.1 ± 1.8 | 14.0 ± 1.8 | 0.049 ^b |
| Organ dysfunction/failure, Y/N | | | |
| Renal | 1/10 | 5/2 | 0.006 ^c |
| Hepatic | 5/6 | 7/0 | 0.017 ^c |
| Pulmonary | 4/7 | 6/1 | 0.040 ^c |
| Cardiac | 2/9 | 2/5 | 0.605 ^c |

^aData are mean \pm SD unless stated otherwise.

^bStudent *t* test.

^cChi-quadrat test.

lyzed. All patients received standard enteral nutrition via gastric tube and vitamin supplementation (1700 kcal/d, 100 g/d amino acids and 750 mg/d vitamins including 0.4 mg/d folate, 0.006 mg/d cyanocobalamin and 5.5 mg/d pyridoxine hydrochloride) within the first 24 h after admission to the ICU. APACHE II scores, Sequential Organ Failure Assessment (SOFA) scores and ISS trauma scores are given in Table 1.

The study was performed according to the Helsinki declaration. The protocol was approved by the local ethics committee, and written consent was granted by the next of kin.

Methods

Plasma obtained from venous blood samples was kept cold, and aliquots were frozen at -70°C until used. Total homocysteine and cysteine concentrations were measured by HPLC, monitoring fluorescence at 385 nm and 515 nm emission wavelengths, after precipitation of plasma protein and reduction of homocysteine and cysteine with Tris-(2-carboxylethyl)-phosphine and derivatization with ammonium-7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (23). Neopterin concentrations were measured by ELISA with a detection limit of 2 nmol/L (BRAHMS,

Hennigsdorf, Germany), and kyn/trp was determined by HPLC, results that have been described (17,18). Serum levels of tumor necrosis factor (TNF)- α and interleukin (IL)-6 were measured earlier with Immulite semiautomated chemiluminescent immunoassay analyzer (DPC, Los Angeles, CA, USA); detection limits were 1.7 pg/mL for TNF- α and 2 pg/mL for IL-6 (17). All laboratory measurements were performed in a blinded manner without knowledge about the diagnoses or disease status of patients.

Statistical Analysis

Nonparametric statistics were applied for calculations, because not all the data sets showed normal distribution. For comparison of grouped data, Friedman test and Wilcoxon signed ranks test were used. Associations between parameters were tested for by using Spearman rank correlation coefficients (*rs*). The Statistical Package for the Social Sciences (version 14 SPSS, Chicago, IL, USA) was used.

RESULTS

Results of Pooled Data

Average homocysteine concentrations in all specimens were (mean \pm SD) 9.59 ± 7.67 $\mu\text{mol/L}$, ranging between 2.0 and

39.8 $\mu\text{mol/L}$; 10 of 83 measurements (12%) revealed homocysteine concentrations above the upper limit of normal (15 $\mu\text{mol/L}$) (24). Average cysteine concentrations were $157 \pm 51.5 \mu\text{mol/L}$ (range 45–297 $\mu\text{mol/L}$) and thus all well below the upper limit of normal (347 $\mu\text{mol/L}$) (25). Average neopterin concentrations were $19.7 \pm 21.7 \text{ nmol/L}$ (range 4.00–116 nmol/L) and thus significantly elevated compared with healthy controls (17,18); 15 of 83 specimens (82%) had neopterin concentrations above the upper limit of normal (8.7 nmol/L) (15). Average kyn/trp was $123 \pm 120 \mu\text{mol/mmol}$, ranging between 28.7 and 810 $\mu\text{mol/mmol}$, and was significantly higher than in healthy controls (18).

Results of Follow-Up Comparisons

Homocysteine. Compared to baseline, total homocysteine concentrations increased during follow-up of patients ($P < 0.05$ on d 7 and d 10; Figure 1). Thereafter total homocysteine concentrations declined and returned to baseline on d 13. When splitting patients into two groups according to survival status, higher total homocysteine levels compared with baseline were found during follow-up of patients who died ($P < 0.05$ on d 4, d 7 and d 10; Figure 2), whereas in survivors homocysteine concentrations did not differ from baseline at any time. In agreement, higher total homocysteine concentrations were found in nonsurvivors than in survivors ($P < 0.05$ on d 4 and d 10; Figure 2), and the frequency of homocysteine concentrations $\geq 15 \mu\text{mol/L}$ was significantly higher in nonsurvivors (22.6%) than in survivors (5.8%; $\chi^2 = 6.78$, $P < 0.01$).

Cysteine. Cysteine concentrations were low at study start and increased during follow-up of patients (Figure 1). Unlike homocysteine, cysteine levels increased in survivors and nonsurvivors to a similar extent but still remained within the normal range (Figure 2). Cysteine concentrations reached a plateau from d 7 and did not return to baseline later. There was no significant difference of

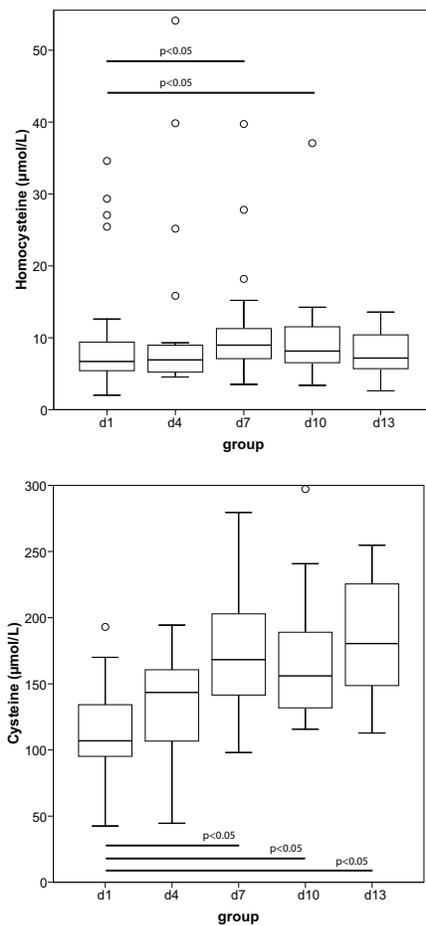


Figure 1. Box and whisker plots of total homocysteine (upper) and cysteine (lower) concentrations in 18 ICU patients developing sepsis during follow-up for 2 wks (box plots are shown; P values indicate differences from baseline; paired rank test).

cysteine concentrations between survivors and nonsurvivors at any time point (Figure 2).

Neopterin. Neopterin concentrations significantly increased during follow-up of patients throughout and were significantly higher in nonsurvivors compared with survivors (17,18). However, whereas mean homocysteine levels reached a plateau between d 4 and d 10 and declined to almost baseline concentrations on d 13 (Figure 2), mean neopterin concentrations continued to increase until end of follow-up (Figure 2).

Interleukin (IL)-6. IL-6 concentrations were higher in nonsurvivors, reaching significant differences on d 7 and d 13.

Tumor necrosis factor (TNF)- α . TNF- α concentrations stayed at baseline in survivors and reached significant differences versus those of nonsurvivors on d 1, d 7, d 10 and d 13.

Tryptophan degradation. Kyn/trp significantly increased during follow-up until d 10, followed by a slight decline (d 13). At all time points, kyn/trp was significantly higher in nonsurvivors compared with survivors (17).

Correlations between Variables

There was no significant correlation between total homocysteine and neopterin concentrations (Figure 3), either in the whole group of patients or at any time point in the subgroups. By contrast, total cysteine concentrations correlated significantly with neopterin concentrations ($r_s = 0.386$, $P < 0.001$; $n = 83$). This was true also in the subgroups of nonsurvivors ($r_s = 0.623$, $P < 0.001$; $n = 31$) and survivors ($r_s = 0.499$, $P < 0.001$; $n = 52$). In the whole data set, the tryptophan degradation status as expressed by kyn/trp correlated significantly with homocysteine ($r_s = 0.400$, $P < 0.001$; $n = 83$) (Figure 3) but not with cysteine concentrations ($r_s = 0.086$, NS). No such correlation was found between concentrations of homocysteine and specific cytokines TNF- α and IL-6 (not shown); however, both correlated with kyn/trp and neopterin as was shown earlier (18).

When patients were split into two groups by survival status, only in the nonsurvivors did there exist significant correlations of homocysteine with kyn/trp ($r_s = 0.402$, $P = 0.01$) but not in the group of survivors. Finally, there was a significant correlation between neopterin concentrations and kyn/trp (0.543, $P < 0.001$) but not between homocysteine and cysteine concentrations.

No significant correlations between homocysteine concentrations and ISS, Marshall or SOFA scores were observed.

DISCUSSION

Increasing total homocysteine concentrations were observed in patients after

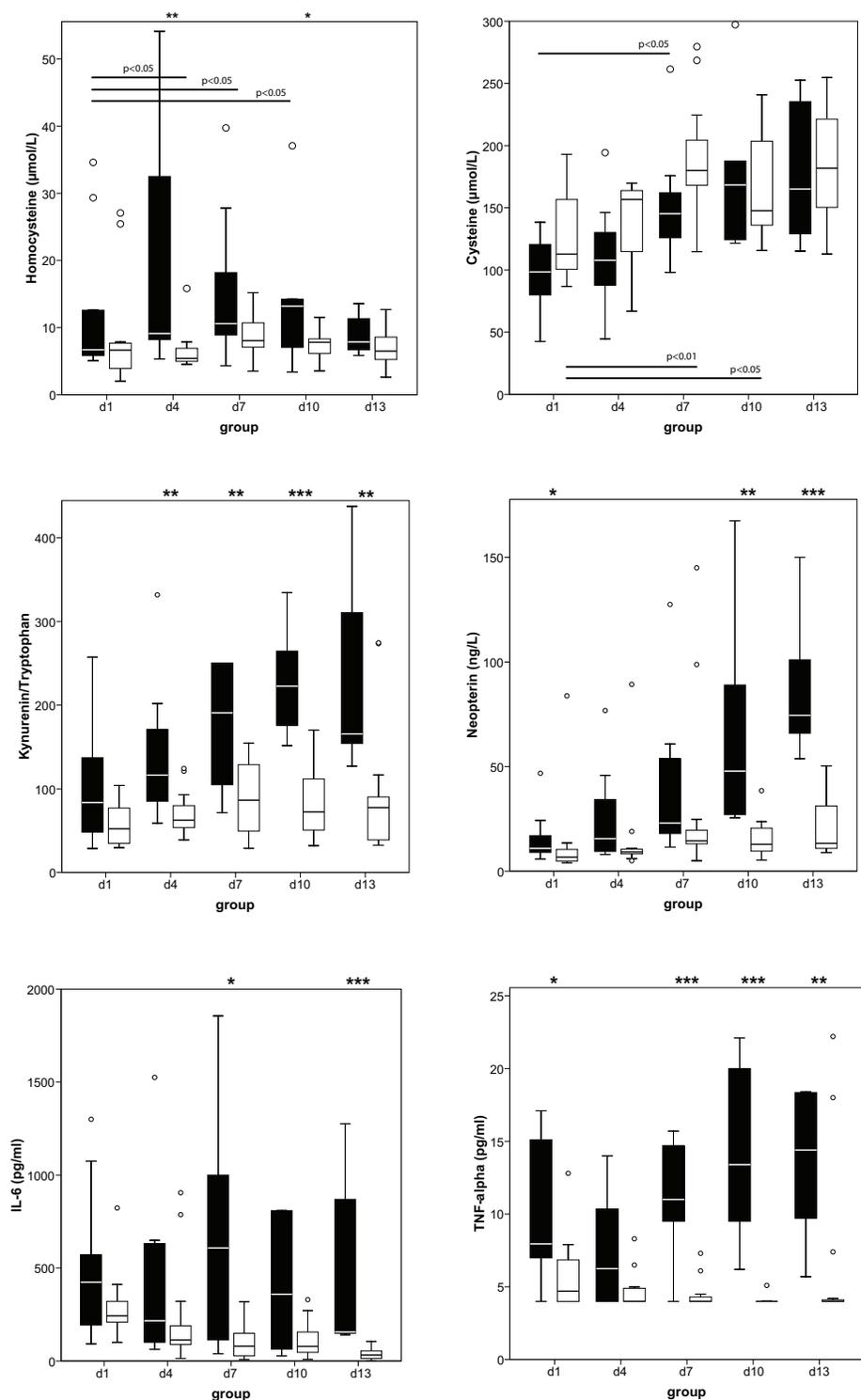


Figure 2. Box and whisker plots of total homocysteine (upper left), cysteine (upper right), neopterin (middle right), IL-6 (lower left) and TNF- α (lower right) concentrations and the kynurenine to tryptophan ratio (middle left) in 18 intensive care unit patients developing sepsis (9 survivors, white boxes; 8 nonsurvivors, black boxes) during follow-up for 2 wks (box plots are shown; *P* values indicate significant differences from baseline and asterisks between survivors and nonsurvivors: **P* < 0.05, ***P* < 0.01, ****P* < 0.001).

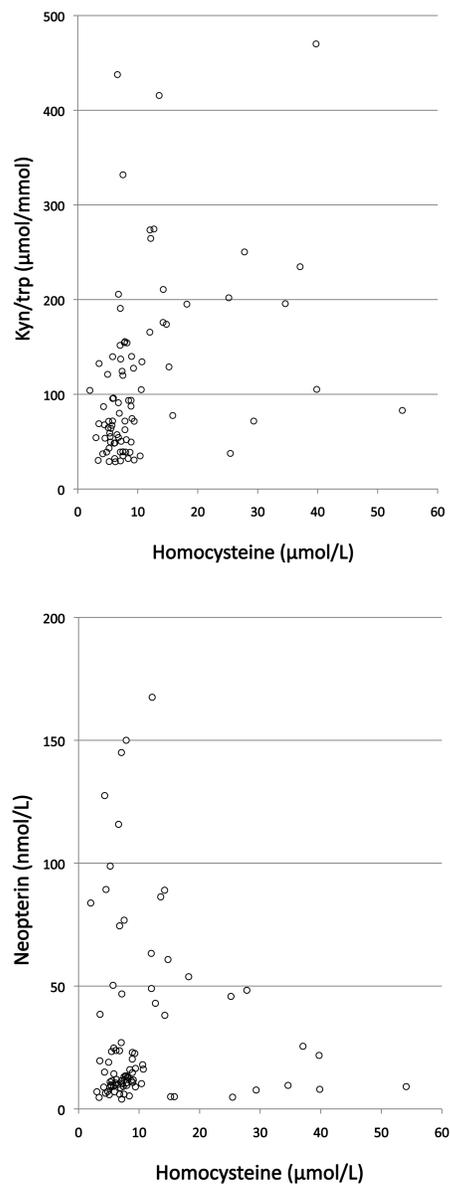


Figure 3. Association of homocysteine concentrations with the kynurenine to tryptophan ratio ($r_s = 0.400$, $P < 0.001$; upper graph) and with neopterin concentrations ($r_s = 0.096$, not significant; lower graph).

multiple trauma with sepsis and in patients with sepsis alone. However, the increase of total homocysteine was almost exclusively observed in nonsurvivors, whereas in survivors homocysteine concentrations remained stable and low. Accordingly, an increase of total homocysteine concentrations was significantly

associated with death during the follow-up period. Thus, monitoring of total homocysteine concentrations in an ICU patient posttrauma with sepsis or sepsis alone appears to be a suitable parameter for supporting risk assessment in patients at least in the first 4 days after admission to the ICU.

Folate deficiency is considered to be primary in the development of hyperhomocysteinemia. Moreover, because several pro-atherogenic actions are ascribed to high total homocysteine levels, insufficient supply with dietary folate is regarded as a crucial and primary event in the epidemic of cardiovascular diseases (2–4). Because all patients received standardized enteral nutrition and vitamin supplementation, distinct B-vitamin supply is unlikely to explain the different development of homocysteine levels in survivors and nonsurvivors.

The development of hyperhomocysteinemia in this particular situation seems to be independent from differences in dietary B-vitamin supply, because all patients received standard enteral nutrition and vitamin supplementation, and thus supply with amino acids or vitamins did not differ between patients. Thus, dietary differences are unlikely to explain different courses of homocysteine levels in patients, and the predictive value of hyperhomocysteinemia in our patients suggests a relationship to the inflammation status of patients. Homocysteine concentrations may increase when B-vitamin deficiency develops more rapidly in those patients with adverse outcome, which is known to be associated with a stronger proinflammatory response (16). After 4 days, no further increase of homocysteine levels was observed; after 2 weeks, homocysteine concentrations also returned to baseline in the patients who died later. The decline of homocysteine concentrations could relate to the dropping out of patients who died and had high homocysteine. However, it could also indicate that the vitamin deficiency is transient and is at least

partly compensated for by enteral nutrition. Certainly it is a crucial limitation of this study that vitamin concentrations were not monitored in the patients. Also, impaired renal function is common in trauma patients with sepsis and in patients with sepsis alone and could influence homocysteine concentrations. However, in patients with unfavorable prognosis, it usually accelerates until multiple organ failure and death. Notably, in contrast to neopterin concentrations (18), total homocysteine did not increase until the end of follow-up as would be expected.

In vitro, activated peripheral blood mononuclear cells were found to release increased amounts of homocysteine (6). Because death of patients after multiple trauma is often associated with the development of sepsis and a proinflammatory cytokine storm (16), it is likely that the increase of total homocysteine is a consequence of these immunopathological changes. The increase of homocysteine concentrations could relate to alterations in the body's redox status, which relates to clinical outcomes. Activated immunocompetent cells, such as macrophages, release ample amounts of reactive oxygen species (ROS) as part of their antimicrobial armature (26). When ROS wipe out antioxidant systems, so-called oxidative stress is developing. Vitamin cofactors of homocysteine-converting enzymes, and especially the active cofactor forms such as 5,6,7,8-methylenetetrahydrofolate, are rapidly destroyed upon oxidation and cannot be recycled (27). Consequently, during conditions of overwhelming production of oxidizing compounds, it can be assumed that the intake of essential antioxidant vitamins can become insufficient for proper function of enzymes, and hyperhomocysteinemia may develop even when vitamin supply is normal. Thus, folate deficiency can emerge when dietary demand is increased, especially during inflammatory conditions (28).

Proinflammatory stimuli such as interferon- γ are potent stimuli not only

for ROS formation (26), but also for neopterin production and tryptophan degradation in human macrophages (29). In stimulated peripheral blood mononuclear cells *in vitro*, the production of neopterin and of homocysteine occurs in parallel (6). Likewise, in several clinical conditions, significant positive correlations were observed between concentrations of total homocysteine and neopterin (6,8,30), and in patients with coronary artery disease, significant inverse associations were observed between concentrations of neopterin and antioxidant compounds, such as vitamins C and E, and lycopene (31) or serum selenium content (32). Like the increase of homocysteine concentrations, the increase of neopterin levels was also associated with nonsurvival (17). However, no correlation between neopterin and homocysteine concentrations was observed in this study, whereas the correlation between homocysteine and tryptophan degradation was significant (Figure 3). Standardized enteral nutrition may have disturbed the parallel development of neopterin and total homocysteine levels in patients. Such a conclusion fits observations made in patients with cardiovascular (9) and neurodegenerative (33) diseases, in whom a correlation between neopterin and total homocysteine was seen at baseline but was rapidly lost when B-vitamin supplementation was initiated. It is interesting to note that the development of homocysteine concentrations and kyn/trypt were very similar, showing an early rise in the nonsurvivors followed by a decline from day 10 on, whereas neopterin concentrations continuously increased until the end of the observation period. Not only homocysteine conversion pathway but also kynurenine catabolism requires the presence of B vitamins (34). Thus, parenteral nutrition that includes B-vitamin supplementation may be able to slow down accumulation of homocysteine and kynurenine but cannot interfere with neopterin production pathways.

In our study, average total cysteine concentrations increased in patients of both groups, survivors and nonsurvivors, and they were significantly associated with neopterin levels. Enteral nutrition and vitamin supplementation may be involved in the increase (normalization) of cysteine levels during follow-up of patients after multiple trauma with sepsis or in patients with sepsis alone, and it is independent of the fate of the patient. The background for the association between neopterin and total cysteine concentrations remains unexplained, but a similar relationship has already been described in patients with stable coronary artery disease (35).

Our study shows that distinct patterns of total homocysteine increases may develop in patients after trauma with sepsis or in patients with sepsis alone rather independently from vitamin supply. Enteral nutrition and vitamin supplementation via gastric tube of ICU patients after trauma and sepsis or with sepsis alone allows full control of vitamin intake and thus can rule out that dietary influences may have contributed to the distinct development of total homocysteine concentrations in survivors and nonsurvivors. Immune system stimulation and inflammatory response in patients likely underlie the transient increase of total homocysteine levels.

The number of patients in our study is still small, and vitamin measurements were not performed. So results can only be regarded as preliminary and need to be confirmed in larger cohorts of patients. Despite this limitation, our study suggests that in patients with trauma and sepsis or patients with sepsis alone, the underlying immunopathogenetic mechanisms which go along with immune activation and inflammation may contribute to the development of moderate hyperhomocysteinemia because of an increased demand of B vitamins. As a consequence, B-vitamin supplementation may need to be increased in inflammatory conditions.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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