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# Serum neopterin for early assessment of severity of severe acute respiratory syndrome

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**Abstract**

Neopterin and C-reactive protein (CRP) concentrations were determined in serum samples from 129 severe acute respiratory syndrome (SARS) patients and 156 healthy blood donors. In the patients with confirmed SARS, an early neopterin elevation was detected already at the day of onset of symptoms and rose to a maximum level of 45.0 nmol/L 3 days after the onset. All SARS patients had elevated neopterin concentrations (>10 nmol/L) within 9 days after the onset. The mean neopterin concentrations were 34.2 nmol/L in acute sera of SARS patients, 5.1 nmol/L in convalescent sera, and 6.7 nmol/L in healthy controls. In contrast, the mean CRP concentrations in both acute and convalescent sera of SARS patients were in the normal range (<10 mg/L). Serum neopterin level in SARS patients was associated with fever period and thus the clinical progression of the disease, while there was no significant correlation between the CRP level and the fever period. Serum neopterin may allow early assessment of the severity of SARS. The decrease of neopterin level was found after steroid treatment, which indicates that blood samples should be collected before steroid treatment for the neopterin measurement.


*Keywords:* Coronavirus; CoV; Fever; Inflammation; Neopterin; Severe acute respiratory syndrome; SARS; SARS-CoV; Steroid

## Introduction

By the time of the initial outbreak of severe acute respiratory syndrome (SARS), there were 8437 cases diagnosed and 813 deaths attributed to SARS worldwide

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[1]. The disease first emerged in China, and proved to be highly contagious, spreading to Hong Kong, Taiwan, Singapore, the Philippines, Viet Nam, Canada, the United States, and other countries. In those countries that experienced the brunt of the outbreak, health care workers were a primary population at risk for contracting SARS. A novel coronavirus (SARS-CoV) was identified as the etiological agent of SARS [2–4] and the virus causes a similar disease in cynomolgous macaques [5].

In general, SARS begins with high fever, headache, an overall feeling of discomfort, and body aches. Some people also experience mild respiratory symptoms. After 2–7 days, SARS patients may develop a dry cough and short of breathing. The patients' clinical features varied from subclinical, mild to very severe situation, and their disease courses might be from a few days to weeks or months [6,7]. Treatment of SARS included antiviral drugs and steroid. It has been demonstrated that administration of high dose methylprednisolone [8] or corticosteroid [9] was associated with clinical improvement, especially in subsets of SARS patient with severe pneumonia. However, long-term side effects of high dose steroid have been reported [10]. Today, no clinical or laboratory judgment is accurately used for speculation of the disease progression or evaluating the need of steroid treatment.

During Th1-type immune response, e.g., triggered by a viral infection, 6-D-*erythro*-neopterin (molecular weight 253.2 Da) is generated and released in increased amounts by human macrophages upon activation by interferon-γ. Accordingly, determination of neopterin concentrations in body fluids is useful for the monitoring of cellular (=Th1-type) immune activation in various diseases such as infections, autoimmune diseases, malignant disorders, and to early detect allograft rejection episodes [11–19]. In particular, increased neopterin concentrations in blood or urine are an early and sensitive indicator for the presence of a broad panel of viral infectious diseases including human immunodeficiency virus type 1 (HIV-1) [11,20], and the degree of neopterin elevation, e.g., in patients with HIV-1 infection, is of predictive value [11,20]. During acute infections with HIV-1, cytomegalovirus or rubella, increased neopterin concentrations were observed before specific antibodies became detectable [15,21–23]. However, increased neopterin concentrations are not specific for virus infections.

Since almost all SARS cases are associated with pneumonia, an inflammation of the lung, the neopterin level in the early stage of SARS may be a sensitive indicator for estimation of the severity of the disease. To confirm our hypothesis, we detected concentration of neopterin in 306 serum samples collected from 129 confirmed SARS patients and 156 sera from healthy blood donors. Our results showed that the level of neopterin markedly increased in acute infection of SARS-CoV as early as the first day of the onset and returned to normal level in the convalescence period. It is also found that the higher level of the neopterin is associated with longer fever period in these patients. Nevertheless, neopterin levels in these patients did not relate to their specific antibody response.

## Materials and methods

### *Study subjects*

To evaluate potential clinical significance of neopterin in SARS patients, we studied 129 cases admitted to 10 hospitals in Guangzhou China between January 19 and May 25, 2003. Seventy-three of them were health care workers from these hospitals. All cases had been diagnosed to have atypical pneumonia confirmed by chest X-ray examination and matched definitions for SARS described by the World Health Organization (WHO) and Centers for Disease Control and Prevention (CDC) [24,25]. Diagnosis of SARS was confirmed by serology and 78 of them also by virology. The clinical information of these SARS patients was summarized in Table 1.

### *Sample collection*

A total of 40 nasopharyngeal aspirate (NPA) and 89 throat swab (TS) specimens from SARS patients were collected for virus isolation and identification. The specimens were stored in viral transport medium at −80°C. At least pair sera were taken from each of these SARS patients for antibody, neopterin, and C-reactive protein (CRP) detections. Acute sera were collected within 9 days after the onset of the disease and convalescent sera were collected after 15 days of the onset. Additional 48 serum samples were subsequently obtained from 17 patients, so that a total of 306 serum samples from SARS patients were recruited in this study. Serum samples were also collected from 156

Table 1
Clinical information of SARS patients from Guangzhou hospitals in China

| | |
|---|---|
| Total numbers | 129 |
| Onset of SARS | January 19– May 25, 2003 |
| Hospital staff/community | 73/56 |
| Gender (male/female) | 43/86 |
| Age (year) | 18–53 |
| Duration of fever (mean ± SD, day) | 9.7 ± 3.6 |
| Contact history[a] (%) | 98 (75.9) |
| Shortness of breath (%) | 87 (67.4) |
| WBC count $10^9$/L (mean ± SD) | 6.6 ± 4.3 |
| Lymphocyte $10^9$/L (mean ± SD) | 0.9 ± 0.5 |
| Chest radiographic shadows (%) | 129 (100) |
| Impaired liver function test (%) | 33 (25.6) |
| SARS virus-specific antibody positive (%) | 129 (100) |
| SARS virus PCR positive (%) | 78 (60.5) |

[a] Definitive history of contact with known SARS patients.

healthy adults from blood banks of Red Cross in Hong Kong (109 sera) and Guangzhou (47 sera) as control. All serum samples were stored at −20°C.

*SARS-CoV isolation and identification*

The presence of SARS-CoV was identified by reverse transcription polymerase chain reactions (RT-PCR) as described [26,27]. Briefly, viral RNA was extracted from the NPA and TS samples with the RNesay Mini Kit (Qiagen, Chatsworth, CA). The first strand cDNA was synthesized using RNA H+ Reverse Transcriptase (Life Technologies Inc., MD) and random primers. Subsequently, PCR was carried out using a series of primers and PCR kit (Invitrogen Corp., CA). The NPA samples were also inoculated to fetal rhesus kidney (FRhK-4) cells for SCoV as described for SARS virus isolation and 3 strains of the virus were isolated and characterized [26].

*Detection of SARS-CoV-specific antibodies*

All sera were heat inactivated (56°C for 30 min) and tested in 1:10 dilution for the presence of anti-SARS virus antibodies using enzyme-linked immunosorbent assay (ELISA) and immunofluorescence assay (IFA). The ELISA was performed using ELISA kit for detection of SARS virus-specific IgG antibodies (Beijing GBI Bio-tech Co. Ltd, China) according to the manufacture's instruction. FRhK-4, Vero E6, or Vero cells were infected with human SARS CoV strain GZ50 [26], fixed with −20°C acetone for 30 min, and used as antigen for IFA to detect the virus-specific antibodies. Uninfected cells were applied to the experiments as negative controls and the IFA was carried out on as described [2,26,28,29]. For the positive sera, antibody titer was further determined by a serial of twofold titration.

*Neopterin assay*

Serum neopterin was determined using a commercially available ELISA (ELItest® Neopterin-Screening, BRAHMS Diagnostica, Berlin, Germany) according to the manufacturer's instructions, having a sensitivity of 2 nmol neopterin/L. The reference level for healthy controls was set at 10 nmol/L.

*C-reactive protein assay*

CRP concentrations in serum samples were determined using a modified conventional enzyme-linked immunosorbent assay of the antigen capture type (sandwich ELISA). Both catcher and detector monoclonal antibodies were purchased from HyTest Ltd. in Finland. The catcher antibodies directed to CRP were coated on 96-well microtiter plates (Immulon 2 HB Flat Bottom, 3455, Dynex Technologies Inc.) in 0.1 mol/L carbonate buffer, pH 9.4 at 4°C overnight. All further steps were performed at room temperature in PBT (10 mM phosphate-buffered saline, pH 7.4, supplemented with 0.1% (wt/vol) bovine plasma albumin (BSA) and 0.05% (vol/vol) Tween-20). Between each step, the plate was washed five times with PBT. After coating and washing, 100 μL of sample or standard containing CRP (HyTest Ltd., Finland) was incubated for half an hour with gentle shaking, allowing the CRP to bind to the antibodies attached to the plates. The plate was then washed and 100 μL of detector antibodies conjugated with horseradish peroxidase was again incubated for half an hour with gentle shaking. Then, 100 μL of substrate mixture (Arista biologicals Inc., PA, USA) containing 3,3′,5,5′-tetramethylbenzidine (TMB) and hydrogen peroxide ($H_2O_2$) was added to each well. The enzyme reaction was stopped within 10 min with 50 μL of 2 M sulphuric acid,

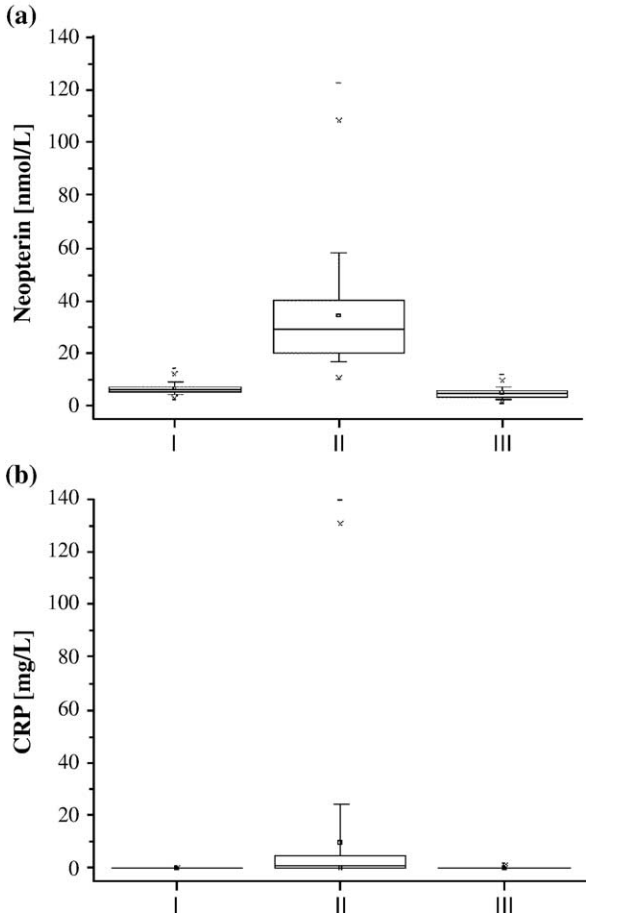


Fig. 1. Serum neopterin and C-reactive protein (CRP) concentrations in different groups of the population. (a) Neopterin concentrations as determined by ELItest® Neopterin-Screening and (b) CRP concentrations as determined by in-house ELISA in (I) healthy subjects and in (II) acute sera or (III) convalescent sera of SARS patients. The lines inside the boxes denote medians; the boxes indicate the interval between the 25th and 75th percentiles. The whiskers denote the interval between the 10th and 90th percentiles. The small squares (□) denote the means and the dashes (-) denote the maximum and minimum values. The difference in neopterin concentrations between the groups is statistically significant ($P < 0.001$, Mann–Whitney test).

and the absorbance at 450 nm was measured using a microtiter plate reader (Dynatech Medical Products Limited, Guernsey). The calibration curve was linear between 0 and 10 mg/L. The detection limit of the assay was 0.2 mg/L and obtained less than 10% in inter- and intra-assay verifications. The reference level for healthy controls was set at 10 mg/L.

*Statistical analysis*

The data are indicated as mean ± SD. The plot of neopterin concentrations versus time after admission is presented as means ± SEM. The Mann–Whitney test was used to assess statistically significant differences. The level of significance was set at $P < 0.05$.

## Results

*Neopterin level raised at early stage of SARS patients*

Serum neopterin level raised in SARS patients as early as on the first day of the onset of symptoms (>10 nmol/L), reaching a mean concentration of 34.2 ± 20.0 nmol/L (mean ± SD, ranged from 10.3 to 122.5 nmol/L) in 129 acute sera (Fig. 1). In contrast, serum neopterin levels were normal (<10 nmol/L) either in 129 convalescent sera of SARS patients (5.1 ± 1.9 nmol/L) or serum samples from healthy blood donors (6.7 ± 2.0 nmol/L). Median serum neopterin concentration was fivefold higher in acute sera of the SARS patients than in convalescent sera (25.8 vs. 5.0 nmol/L; $P < 0.001$, Fig. 1), and fourfold higher than those in healthy blood donors (25.8 vs. 6.4 nmol/L; $P < 0.001$, Fig. 1). On the other hand, the mean CRP concentration in acute SARS sera was 9.6 ± 26.2 mg/L, which was below the reference level (<10 mg/L). Also, serum CRP levels in convalescent SARS sera were very similar to those in healthy subjects.

*Release kinetics of neopterin*

As shown in Fig. 2, the neopterin concentration was elevated on day 1 of the onset (15.7 ± 8.8 nmol/L, mean ± SEM) and reached the peak value of 45.0 ± 27.5 nmol/L on day 3. However, the CRP concentration was slightly elevated only on day 4 (14.9 ± 9.6 mg/L). The serum neopterin maintained at high levels (>33 nmol/L) up to day 8 of the onset, but returned to normal level (<10 nmol/L) after 11 days of the onset and maintained at low level through out the study period of 6 months.

*Higher neopterin level associated with longer fever period*

To analyze the clinical significance of increased neopterin in early stage of SARS patients, we compared the patients' neopterin level in different days (1–8 days) after the onset and their fever period. We found that the higher neopterin level of patients had, the longer fever period they experienced (Fig. 3). The patients with longer fever period also appeared severer disease progression, including higher fever, severer shortness of breath, longer hospitalized period, and more complications (Table 2). The results suggest that higher neopterin level may be an early sign of the patients suffering from severer SARS. In contrast,

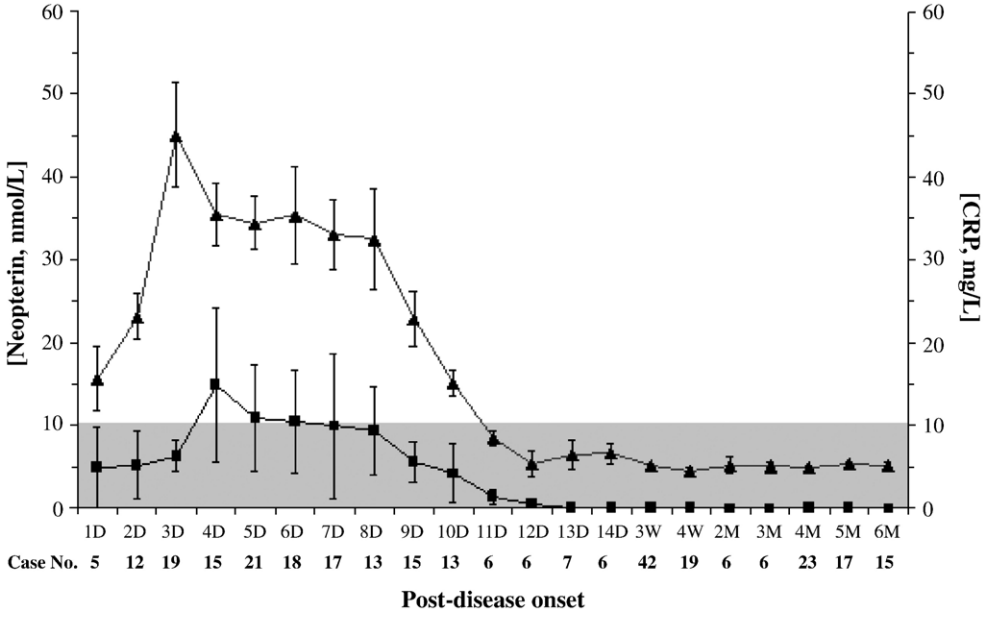


Fig. 2. Serum neopterin (▲) and CRP (■) concentrations (mean ± SEM) in 129 SARS patients after the onset of symptoms expressed as days (D), weeks (W), and months (M); the normal ranges of neopterin and CRP levels are <10 nmol/L and <10 mg/L, respectively (grey area).

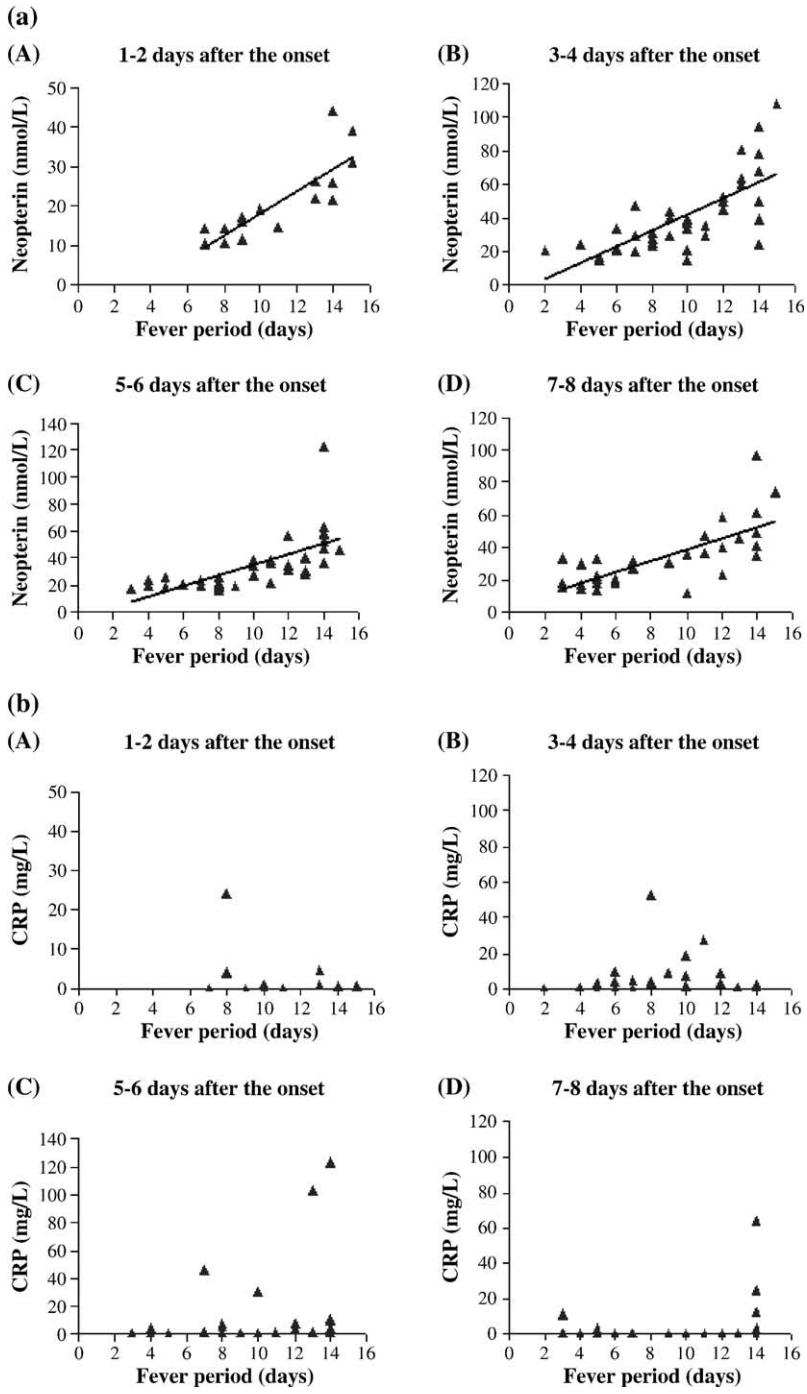


Fig. 3. Relationship between the fever periods of SARS patients and (a) serum neopterin or (b) serum CRP concentrations detected (A) 1–2 days, (B) 3–4 days, (C) 5–6 days, and (D) 7–8 days after the onset.

there was no correlation between the serum CRP levels and the fever period. In this study, all SARS patients were recovered and discharged from hospitals.

*Neopterin level not related to antibody level of SARS patients*

Since the neopterin is an indicator of cellular immune response in various diseases [12,16,20], we further compared the levels of neopterin and SARS-CoV-specific antibodies in these patients. The results did not show significant correlation between neopterin level and antibody level determined by IFA in the patients (Fig. 4).

*Steroid therapy suppressed neopterin production in SARS patients*

When comparing neopterin concentrations between the acute sera taken before the SARS patients received steroid

Table 2
Relationship between fever period and other clinical status of SARS patients

| Fever period (mean days ± SD) | Number of patients | Ventilatory assistance | | Hospitalization* (mean days ± SD) |
|---|---|---|---|---|
| | | Number (%) | Mean days ± SD | |
| 2–7 (5.2 ± 1.5) | 40 | 20 (50) | 5.7 ± 3.8 | 15 ± 6.9 |
| 8–11 (9.3 ± 1.1) | 44 | 33 (75) | 11.5 ± 6.1 | 19 ± 8.6 |
| 12–15 (13.5 ± 0.9) | 45 | 43 (96) | 16.2 ± 7.7 | 25 ± 9.2 |

* All patients in this study cohort were recovered from SARS and discharged from hospitals.

treatment and after they were treated with steroid, it was found that the neopterin levels in acute sera obtained before the patients accepted steroid were significantly higher than that collected from the patients who had been treated with steroid on the same day after the onset (Fig. 5). The results showed that the treatment of steroid might suppress the generation of neopterin in SARS patients. Therefore, blood samples should be collected before steroid treatment for the neopterin measurement.

## Discussion

This is the first study to show increase of serum neopterin concentrations in SARS patients as early as the first day after the onset of symptoms. There is a significant difference in neopterin concentrations between 129 acute SARS sera (34.2 ± 20.0 nmol/L, mean ± SD) and either 129 convalescent SARS sera (5.1 ± 1.9 nmol/L) or 156 healthy blood donors (6.7 ± 2.0 nmol/L). All SARS patients had elevated neopterin concentrations (>10 nmol/L) within 9 days after the onset. In contrast, the mean CRP concentrations in both acute and convalescent SARS sera were found below the reference level (<10 mg/L). The kinetics of neopterin concentrations based on the 129 SARS patients indicates that an early neopterin elevation can be detected already 1 day after the onset of symptoms and quickly reaches its peak level 3 days after the onset. The neopterin concentration maintained at high level up to 8 days after the onset to prevent false negative result. This observation fits well to data obtained from other virus infections such as HIV [21,23], Rubella [22], or also Ebola [30] and it agrees with results from, e.g., the experimental infection of rhesus macaques with simian immunodeficiency virus (SIV) [31]. Data available primarily from studies with HIV-1 infection [20,32] imply that a predictive value of neopterin concentrations might exist also in patients with SARS, those with lower neopterin concentrations being more likely to recover than others. Notably, behavior of neopterin concentrations in acute SARS patients is similar to cytomegalovirus (CMV) or rubella infection [15,22] but differs considerably from patients with HIV-1 or animals with SIV infection [21,23,31]: like in SARS patients, acute CMV and rubella infection causes a sharp increase of neopterin concentrations which is followed by seroconversion and a drop of neopterin concentrations into the range of healthy controls. By

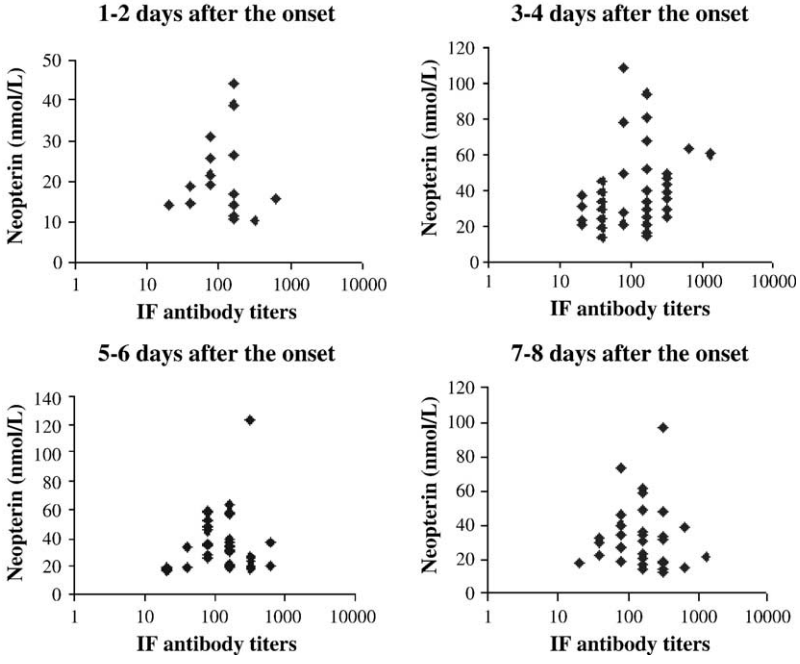


Fig. 4. Relationship between serum neopterin concentrations detected 1–2 days, 3–4 days, 5–6 days, and 7–8 days after the onset and the antibody levels in convalescent sera (collected after 15 days of the onset) of these SARS patients determined by IFA.

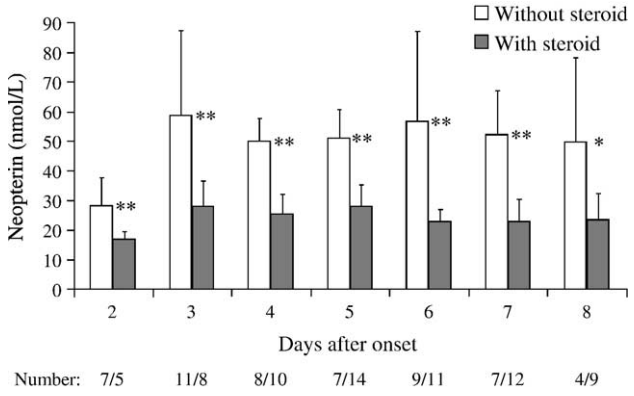


Fig. 5. Serum neopterin level (mean ± SD) in the patients with ($n$ = 69) or without ($n$ = 53) steroid therapy when the serum samples were collected (*$P$ < 0.05, **$P$ < 0.01; the number of patients in each group is indicated in the bottom line).

contrast, in the majority of patients with acute HIV-1 infection the drop of neopterin concentrations after seroconversion does not reach the normal range, elevated neopterin concentrations in the subsequent asymptomatic phase indicating continuous presence of virus and its replication albeit at a low level. Thus, normal neopterin concentrations in convalescent SARS patients suggest absence of vial replication.

Higher neopterin concentration, but not the CRP concentration, in SARS patients was associated with longer fever period and thus severer course of disease. This shows that neopterin can be an early indicator of the severity of SARS. The clinical progression of SARS is described as a tri-phasic pattern [2]. Increased neopterin levels of SARS patients in phase I prior to seroconversion indicate increased activity of the cellular immune system, which is primarily due to the effect of viral replication and cytolysis. The symptoms generally improve after a few days. As the disease progresses into phase II, the neopterin levels return to the reference range after seroconversion. The lung damage at this phase is related to immunopathological damage as a result of an exuberant host response, rather than uncontrolled viral replication. Therefore, there was no correlation between neopterin levels and antibody titers. Recurrence of fever, onset of diarrhea, oxygen desaturation, and shifting radiographic shadows occur. Phase III is characterized by acute respiratory distress syndrome (ARDS) necessitating ventilatory support. Patients may develop nosocomial sepsis during this phase of end-organ damage and severe lymphopenia. To minimize both the risk of progression to the chronic phase of ARDS and the side effects of steroid treatment, early assessment of the severity of SARS is critical for the treatment design. Using neopterin to quantify the severity of SARS, patients can receive proper medical treatment at an early stage, thus producing a good recovery rate, contributing considerable economic gains for both patients and hospitals and minimizing the risk of infection transmission.

Serum neopterin levels were significantly lower in the SARS patients with steroid therapy than in those without such treatment (Fig. 5). This implies that neopterin level may be a useful marker of inflammatory activity in SARS. Proinflammatory cytokines released by stimulated macrophages in the alveoli have a prominent role in pathogenesis of SARS, resulting in cytokine dysregulation [33]. Using steroids in the treatment of SARS patients is to modulate this cytokine response and prevent a fatal outcome. However, steroids may lead to permanent suppression of the cellular immune reactions associated with the T cells and thus to an increased susceptibility to infection by opportunistic germs. By stratifying patients according to the severity of the disease, suitable dosage of steroid can be applied to minimize the major side effects. Interestingly, the use of neopterin monitoring to classify patients for steroid therapy has been already suggested for patients with sarcoidosis [34].

Neopterin concentrations in healthy controls (mean ± SD: 6.7 ± 2.0 nmol/L) were comparable to data from the literature (5.3 ± 2.7 nmol/L [12]) albeit slightly higher. However, the number of specimens from healthy controls tested in this study is still low. Interestingly, neopterin concentrations in sera collected from convalescent patients (5.3 ± 2.2 nmol/L) even better fit to the healthy European controls.

In patients with chronic renal failure the concentration of neopterin in serum is very likely to be high and elevates over a longer period of time [35]. Thus, caution must be taken when using neopterin for screening of SARS in case of renal insufficiency.

Further studies are required to validate and refine the optimal serum neopterin cutoff values for diagnosis and prognosis, and future work should address relationships with neopterin levels among patients with SARS and non-SARS pneumonias, effects of treatment, and morbidity outcomes.

Technologic advances may further enhance the usefulness of neopterin measurement in acute infection. At present the use of neopterin ELISA allows the results to be available at least 2 h after sampling. On the other hand, measurement of neopterin concentrations can also be performed in urinary specimens. Thus, the development of a one-step rapid test for quantitative neopterin analysis in urine sample may allow this time to be reduced further, to 15 min. It is a noninvasive test for monitoring patients presenting with SARS symptoms.

## Conclusion

This study is the first to report neopterin as an early marker for assessment of the severity of SARS. Elevated neopterin level can be detected as early as the first day after the onset of symptoms and all patients had elevated neopterin concentrations (>10 nmol/L) within 9 days after

the onset. SARS is an entirely new emerging disease and its clinical course varies widely. Due to the extremely contagious nature of the disease, a single undetected case may lead to widespread transmission of SARS. Testing of hundreds of suspected SARS cases are required during each day of an outbreak. Thus, a more sensitive, simple, and rapid noninvasive test must be developed, particularly if an effective treatment regime is available in the future.

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