Evaluation of oxidative stress markers in hospitalized patients with moderate and severe COVID-19

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Background. Clinical evidence suggests increased oxidative stress in COVID-19 patients and this worsened redox status could potentially contribute to the progression of the disease.

Objectives. To investigate the oxidative stress we have measured oxidative stress parameters, namely, PAT (total antioxidant power, iron reducing) and d-ROMs (plasma peroxides). Additionally we have investigated their correlation with the most frequently used clinical parameters CRP, LDH, and NLR in serum from moderate and severe COVID-19 patients hospitalized in a tertiary hospital.

Methods. PAT and d-ROMs were determined by analytical photometric metric method in serum from 50 hospitalized patients. For each of them, two samples were collected and analyzed immediately after collection seven days apart.

Results. All patients at admission had a much higher value for plasma peroxides and a significant correlation between oxidative stress parameters and CRP, LDH, and NLR. ($p < 0.05$), except for OS index (OSI) vs CRP in the severe group. At discharge, plasma peroxides were reduced and OSI was improved in the moderate group.

Conclusion. We consider that using OSI at the beginning of COVID-19 disease presents a valuable starting point for the general assessment of oxidative stress and hence enabling a better triage of the patients in terms of disease severity.

Key words: COVID-19, oxidative stress, plasma peroxides, antioxidant, disease severity.

What is new? What is important?

- Oxidative stress is increased in COVID-19 patients and this worsened redox status could potentially contribute to the progression of the disease.
- Continuous rising of oxidative stress index upon admission to hospital will more likely result in severe form of the disease or complications during hospitalization including death.
- Oxidative stress parameters analysis should be performed in primary healthcare institutions in order to determine oxidative stress earlier at disease onset.

INTRODUCTION

In the past year since the COVID-19 pandemic has been officially declared, scientists and clinicians throughout the world share their experiences in scientific publications extensively. This has aided availability of a more comprehensive laboratory data as well as several potential mechanisms behind COVID-19 pathogenesis have been proposed [1–8]. The global pandemic has questioned each health system even throughout the most powerful and

developed countries. We consider that data dissemination and its communication to the international scientific and health community will have large impact on developing and improving the strategies of the healthcare systems, especially those of the low income countries, in containing the virus and protection of the public health.

According to WHO, patients have been categorized in the range from asymptomatic or mild to moderate, severe or critical [1]. In general, SARS-CoV-2 virus attacks almost every system in the human body [9–11]. The outcome of the

disease in many patients is correlated with laboratory and clinical properties of a cytokine storm that triggers pro-inflammatory condition leading to excessive production of reactive oxygen species (ROS) that is linked with severe tissue damage. The link between oxidative stress and inflammation has been discussed previously elsewhere [12, 13].

Oxidative stress is a natural process that occurs during metabolism and plays an important role in maintaining balance in prooxidant-antioxidant levels and the homeostasis of cells, tissue and organs. During abnormal physiological conditions uncontrolled production of ROS such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$) and singlet oxygen (O_2) occurs that promote a cascade of biological events inducing pathological host responses. Disturbances in redox balance regarding excessive production of ROS observed in different inflammatory and viral diseases are discussed in details elsewhere [14–17].

Numerous publications in relation to the role of oxidative stress in viral infections such as respiratory syncytial virus, HIV, primary hepatotropic viruses (hepatitis B and hepatitis C) and herpes viruses such as Epstein Barr virus (EBV) have been published [18–20]. The general pathogenic mechanisms in connection to ROS production and infection are identical for all viruses. Essentially, viruses' leads to disturbance of redox homeostasis in infected cells and increased production of ROS in activated phagocytes [21–23].

In recently published reviews, authors focused on the effect of ROS on the pathophysiology of viral infection with SARS-CoV-2 and present the relevancy of relationship between viral infection, ROS production, oxidative stress and antiviral response [14–23].

Patients with comorbidities infected with SARS-CoV-2 have increased state of oxidative stress due to chronic illness and viral infection [6–9]. Therefore, the measurement of oxidative stress can be important in order to determine the progress of COVID-19 in terms of severity. Also, determination of oxidative stress can be considered as tool to support rational therapeutic decision in targeting the host response to viral aggression by using antioxidants, taking into account their action by interrupting the cytokine storm, oxidative stress and hematological abnormalities [12, 24–27].

The correlation between oxidative stress and the disease severity is still not completely investigated and hence there is a lack of experimental data in a clinical setting. Therefore, our study focused on assessment of oxidative stress parameters and their correlation with several most commonly used biochemical parameters among hospitalized COVID-19 patients. This could potentially help in early detection patients that require a more complex treatment approach. Our hypothesis is that oxidative stress parameters could be used in addition to conventional laboratory analyzes, as biomarkers for disease progression. For this purpose we have analyzed serum from 50 patients infected with SARS-Cov-2 by well-established photometric fast analytical method previously used in the field of redox balance prediction. The results of oxidative stress parameters were correlated with CRP, LDH and NLR in order to confirm the clinical importance of these markers to be exploited by the health institutions more extensively.

MATERIAL AND METHODS

Study design (Patients)

A total of 50 patients with COVID-19 (31 males and 19 females) with a mean age of 56 years (range from 18 to 79 years) hospitalized at the University Clinic for Infectious Diseases and Febrile Conditions, Skopje, Republic of North Macedonia within a period of 2 months were included in this study. The diagnosis and classification of COVID-19 were based on the Interim Guidance for Clinical Management of COVID-19 issued by the WHO [1]. Patients with moderate disease were adults with clinical signs of pneumonia, but no signs of severe pneumonia, including $SpO_2 \geq 90\%$ on room air. Severe cases additionally met at least one of the following conditions: $SpO_2 < 90\%$ on room air, respiratory rate >30 breaths/minute or presence of severe respiratory distress.

All patients were confirmed to have SARS-CoV-2 infection by real-time reverse transcriptase-polymerase chain reaction assay (RT-PCR) from nasal and pharyngeal swab specimen. Only the laboratory confirmed cases were included in the study. The study was approved by the local ethics committee (Ethics Committee of the Faculty of Medicine, University of Ss Cyril and Methodius, Skopje, Republic of North Macedonia,

No #03-366/7). The work was performed by a multidisciplinary group, including clinical experts in COVID-19 management.

Clinical characteristics and laboratory data

Demographic characteristics, medical history, clinical symptoms and signs, concomitant medication, outcome data, as well as laboratory analyzes were obtained from the patients' medical records. Laboratory assessment consisted of: complete blood cell count, blood biochemistry, coagulation profile, arterial blood gas analyses and inflammation markers (only selected laboratory parameters are presented in this article).

The discharge criteria consisted of: clinical improvement on physical examination, improvement of oxygenation, body temperature back to normal at least for three days and an improvement of lung infiltration (proved by chest X-ray examination).

Method for determination of d-ROMs, PAT and Oxidative stress index

PAT (total antioxidant power, iron reducing) and d-ROMs (plasma peroxides) were measured on FRAS5 analytical photometric system by using REDOX fast kit (made of 50 individual d-ROMs fast tests and 50 individual PAT tests, H&D srl, 43124 Parma, Italy), upgraded by H&D srl but initially developed by Mauro Carratelli. [28,29]. Two samples were collected and analyzed immediately after collection seven days apart. The first sample was taken on admission at the clinic. The procedure was done according to the producers' guidelines for the both, d-ROMs and PAT tests.

The d-ROMs and PAT are reported in equivalents of H_2O_2 and ascorbic acid, respectively. The d-ROMs and PAT reference normal values are 250–300 U. Carr (1 U. Carr = 0.08 mg H_2O_2 /dL) and 2200–2800 U. Carr, respectively. Oxidative stress index (OSI) presents information obtained from d-ROMs fast test and the PAT test that is automatically calculated by the dedicated spectrophotometer FRAS5 with normal reference values less than 40 given by the manufacturer (H&D srl, 43124 Parma, Italy).

Statistical analysis

Data were described as number and/or percentage, or median and range or mean and standard deviation (SD) or standard error of mean (SEM), where appropriate. Differences between

groups were explored using the t-test, Mann-Whitney or ANOVA followed with Holm-Sidak's multiple comparison test, where appropriate. A p-value less than 0.05 was considered significant. For purpose of comparison between groups, we have used oxidative stress values of healthy individuals (12 males and 8 females, mean age 54) with negative RT-PCR that were in the range of already published reference normal values as stated in section 2.3. All analyses were made using the statistical program GraphPad 9 (USA).

RESULTS

In order to investigate the added value of OSI we have performed comparisons analysis with some frequently applied laboratory markers (CRP, LDH and NLR) used to predict the course of the disease. Our hypothesis is that in patients where the value of OSI is continuously increasing upon admission to hospital are more likely to develop severe form of the disease or complications during hospitalization including death. Otherwise, if OSI value is decreasing the patient should have a quicker recovery and the hospitalization period should be reduced. Therefore, to confirm our hypothesis we have analyzed moderate and severe SARS-CoV-2 infected patients at a tertiary health clinic i.e., the University Clinic for Infectious Diseases and Febrile Conditions in our country.

Demographics and clinical characteristics of patients

The demographic characteristics of the 50 patients are given in Table 1. Among them 30 patients belong to severe group and 20 of them were classified as moderate cases. The mean age of the moderate group (52.05 ± 12.84 years) was slightly lower than the mean age of the severe group (58.0 ± 9.94 years), but no significant difference was observed in terms of this parameter between the groups ($p > 0.05$, t-test). The average time from onset of symptoms to hospital admission was 10.52 ± 2.33 days (range 7–16 days).

Most of the patients (60% in the moderate group and 76.7% in the severe group) had underlying medical conditions at admission. The most frequently reported comorbidities were hypertension, diabetes and chronic cardiac disease. Additionally, the average number of comorbidities in each patient was slightly higher in the severe group 1.7 ± 0.56 (range 1–3), than in the moderate group 1.42 ± 0.52 (range 1–2).

The most prominent and disturbing symptoms reported by the patients on admission were high body temperature (80%), dyspnea (64%), malaise (62%) and cough (56%).

All patients received standard of care, and most of them were treated with antibiotics, oxygen therapy, anticoagulants and corticosteroids. Additionally, other symptomatic and supportive therapy was applied, if necessary, upon clinician's judgement for each patient.

The mean value of all clinical laboratory parameters upon admission and discharge are presented in Table 2. In the moderate group on admission, abnormal values for CRP, LDH, glucose and NLR were observed. The mean value $\pm$ SEM for CRP was 44.1 ± 6.46 vs 7.16 ± 1.96 , LDH was 280.3 ± 25.27 vs 283.4 ± 59.19 and NLR was 6.47 ± 1.64 vs 5.09 ± 1.73 at admission and discharge, respectively. In the

severe group of patients abnormal values for CRP, LDH, CK, ALT, AST, glucose, WBC and NLR levels were observed. The mean value of CRP, LDH, CK, ALT and NLR in the severe group were higher when compared to the moderate group of patients upon admission and discharge.

Association between oxidative stress parameters, CRP, LDH and NLR

The results (mean $\pm$ SEM) for d-ROMs, PAT and OSI are presented in Table 3 for the moderate and severe group of patients. Patients belonging to both groups at admission have much higher value for plasma peroxides and therefore increased oxidative stress level in comparison with previously determined values for these parameters in healthy individuals from our laboratory.

Table 1
Demographic and baseline characteristics of COVID-19 patients

	All patients (n=50)	Moderate group (n=20)	Severe group (n=30)
Gender (M/F)	31/19	9/11	22/8
Age (year) (mean $\pm$ SD)	55.57 $\pm$ 11.47	52.05 $\pm$ 12.84	58.0 $\pm$ 9.94
Days from disease onset to admission (mean $\pm$ SD)	10.52 $\pm$ 2.33	10.35 $\pm$ 2.18	10.63 $\pm$ 2.46
Patients with medical conditions (%)	35 (70%)	12 (60%)	23 (76.7%)
Number of medical conditions (mean $\pm$ SD)	1.6 $\pm$ 0.54	1.42 $\pm$ 0.52	1.7 $\pm$ 0.56
Co-existing medical conditions (%)			
Hypertension	18 (36%)	4 (20%)	14 (46.7%)
Diabetes	12 (24%)	3 (15%)	9 (30%)
Chronic cardiac disease	7 (14%)	2 (10%)	5 (16.7%)
Haematological disease	4 (8%)	2 (10%)	2 (6.7%)
Thyroid disease	3 (6%)	2 (10%)	1 (3.3%)
Urology disorders	3 (6%)	1 (5%)	2 (6.7%)
Chronic gastrointestinal disease	2 (4%)	1 (5%)	1 (3.3%)
Chronic lung disease	1 (2%)	/	1 (3.3%)
Other chronic conditions	3 (6%)	1 (5%)	2 (6.7%)
Outcome (recovered/ deceased)	26/ 24	20/ 0	6/ 24

Table 2
Clinical laboratory parameters expressed as Mean $\pm$ SEM

Parameter	Moderate (n=20) Admission	Moderate (n=20) Discharge	Severe (n=30) Admission	Severe (n=6) Discharge recovered	Severe (n=9) Died after 2 nd sample	Reference values
CRP (mg/L)	44.1 $\pm$ 6.46	7.16 $\pm$ 1.96	113 $\pm$ 17.85	20 $\pm$ 9.39	127 $\pm$ 33.49	0–10
LDH (IU/mL)	280.3 $\pm$ 25.27	283.4 $\pm$ 59.19	800 $\pm$ 60.27	484.6 $\pm$ 204.2	827.2 $\pm$ 130.6	120–246
CK (U/L)	91.47 $\pm$ 16.88	60.24 $\pm$ 12.23	272 $\pm$ 60.67	184 $\pm$ 101.6	228.9 $\pm$ 131.5	30–170
ALT (U/L)	49.31 $\pm$ 15.46	75.39 $\pm$ 14.60	94.76 $\pm$ 36.82	107.2 $\pm$ 70	38.67 $\pm$ 14.72	10–52
AST (U/L)	45.63 $\pm$ 9.49	44.06 $\pm$ 7.71	81.59 $\pm$ 18.65	36 $\pm$ 15.62	33.33 $\pm$ 5.43	10–47
Glucose (mmol/L)	12.68 $\pm$ 3.62	8.52 $\pm$ 1.45	16 $\pm$ 3.82	5.84 $\pm$ 0.97	9.09 $\pm$ 1.42	3.9–5.6
Creatinine (μ mol/L)	57.44 $\pm$ 2.84	60.32 $\pm$ 3.73	58.27 $\pm$ 3.44	58.40 $\pm$ 9.34	51.11 $\pm$ 6.56	62–133
Urea (mmol/L)	4.76 $\pm$ 0.54	5.16 $\pm$ 0.65	7.38 $\pm$ 0.39	6.06 $\pm$ 1.31	7.00 $\pm$ 0.74	1.7–8.3
Hemoglobin (g/L)	128.1 $\pm$ 2.62	130.1 $\pm$ 3.34	137.8 $\pm$ 2.83	126.2 $\pm$ 10.78	139.9 $\pm$ 4.45	115–180
RBC ($\times 10^3 \mu$ L)	4512 $\pm$ 28.91	4543 $\pm$ 101.2	4776 $\pm$ 113.6	4358 $\pm$ 410.1	4862 $\pm$ 1.49	4000–5500
WBC ($\times 10^3 \mu$ L)	7.54 $\pm$ 0.88	9.07 $\pm$ 0.82	24.7 $\pm$ 10.35	11.88 $\pm$ 0.87	22.41 $\pm$ 2.87	4.0–11.0
Platelets ($\times 10^3 \mu$ L)	343.1 $\pm$ 34.78	377 $\pm$ 33.04	292.4 $\pm$ 22.24	241.2 $\pm$ 47.26	284.7 $\pm$ 25.30	150–400
NLR	6.47 $\pm$ 1.64	5.09 $\pm$ 1.73	20.23 $\pm$ 2.06	5.92 $\pm$ 0.99	44.72 $\pm$ 10.49	$\leq$ 3.0

Table 3
Oxidative stress parameters (PAT, d-ROM and OSI) in serum samples. Results are presented as mean $\pm$ SEM

Parameter	PAT(U. Carr) $\pm$ SEM	d-ROM(U. Carr) $\pm$ SEM	OSI $\pm$ SEM
Moderate (n=20)	2887 $\pm$ 102.1	431.2 $\pm$ 25.25	94.2 $\pm$ 13.9
admission vs discharge	p=0.0004, t-test	p=0.0001, t-test	p=0.0001, t-test
	2673 $\pm$ 160.6	334.3 $\pm$ 13.87	52.25 $\pm$ 5.60
	p=0.1325, t-test	p=0.0002, t-test	p=0.0001, t-test
Severe (n=30)	2801 $\pm$ 85.86	413 $\pm$ 17.28	84.03 $\pm$ 8.86
Admission	p=0.0008, t-test	p=0.0001, t-test	p=0.0001, t-test
Severe (n=6)	2652 $\pm$ 148.8	325.3 $\pm$ 22.46	43.40 $\pm$ 10.87
Discharge recovered	p=0.0043, t-test	p=0.0019, t-test	p=0.005, t-test
Severe (n=9)	2186 $\pm$ 233.2	462.4 $\pm$ 28.04	107.8 $\pm$ 18.20
Died after 2 nd sample	p=0.2497, t-test	p=0.0001, t-test	p=0.0001, t-test
Healthy individuals (n=20)	2406 $\pm$ 71.55	271 $\pm$ 5.590	21 $\pm$ 2.527

Moderate group of patients

Among the moderate cases ($n=20$) on admission good correlation was demonstrated between all investigated parameters (d-ROMs, PAT, OSI, CRP, LDH and NLR) ($R^2=0.9425$, $p<0.05$, ANOVA). We have also performed additional comparison analysis for these parameters between the two measurements on admission and on 7th day of hospitalization (further in the manuscript refers to term 'discharge'). These results are graphically presented in Figure 1, where significant difference was detected for CRP, d-ROMs and OSI ($p<0.05$, ANOVA). This could possibly reflect the overproduction of ROS and patients' poor antioxidant system in the pathogenesis of SARS-CoV-2 infection which has been already proposed and is in line with acute inflammation process as confirmed by the presented laboratory parameters (Table 2). Additionally, a significant correlation was observed between all parameters at discharge ($R^2=0.9383$, $p>0.05$, ANOVA) except for d-ROMs with LDH and CRP with NLR ($p>0.05$).

Severe group of patients

Among 30 patients from the severe group, only 6 of them recovered and were discharged, whereas 24 had deterioration of their condition and died. Nine of them died later than 7 days after hospital admission and we managed to obtain the second blood sample for analyze, but

15 patients were deceased prior the second blood sample could be taken. ANOVA summary test was performed for all 30 severe patients at admission. The differences among means of tested parameters were statistically significant ($R^2=0.9080$, $p < 0.0001$). Additionally, Holm Sidak's multiple comparison test showed a significant difference between all investigated parameters ($p<0.05$) except OSI vs CRP, OSI vs NLR and CRP vs NLR on admission ($p>0.05$). Among the severe patients that died and those who recovered and were discharged, a statistically significant difference was observed between these two subgroups in terms of the second OSI measurement (Figure 2) ($p<0.05$, Mann-Whitney). This can contribute to the excessive levels of ROS and thus triggers a cascade of pathological events (tissue damage, thrombosis, RBC dysfunction) that would result in increased oxidative stress and will contribute to developing a severe form of the disease even multi organ failure and death. Patients that died versus those who recovered from the severe group had CRP values 127 ± 33.49 vs 20 ± 9.39 , LDH values 827.2 ± 130.6 vs 484.6 ± 204.2 and NLR values 44.72 ± 10.49 vs 5.92 ± 0.99 , respectively. This high values of investigated clinical parameters additionally contributed to the increased oxidative stress and may help to identify high risk patients early in the course of the disease and prevent further disease's complication.

Figure 1. Graphical presentation of oxidative stress parameters (a) and clinical laboratory parameters (b) in the moderate group of patients ($n=20$) at admission and at discharge. ** $p<0.05$, **** $p<0.0001$

Figure 2. Graphical presentation of OSI for the two subgroups of COVID-19 patients with different outcome within the severe group. R-recovered (n=6), D-died (n=24). **p<0.05

DISCUSSION

Until now, little experimental data on the measurement of the oxidative stress in COVID-19 patients have been reported, with research groups focusing more on retrospective data analysis, clinical features of the disease or review articles [3, 7, 14, 15]. Our study contributes to clinical evidences that oxidative stress is increased in COVID-19 patients and this worsened redox status could potentially contribute to the progression of the disease. Gadotti *et al.* have presented clinical data of 77 patients where they have assessed the production of hydrogen peroxide, defense antioxidants (total antioxidant capacity, reduced and oxidized glutathione, glutathione s-transferase) and oxidative damage (MDA, carbonyl and sulphydryl which as analytical methods are time and material consuming [30]. Their results demonstrated that hospitalization was prolonged in those patients who had high serum leukocytes count and high CRP level. However, they did not demonstrate a correlation between the oxidative stress parameters and the severity of disease due to difficulties in data collection and limitation of access to medical records.

Our results show that both group of patients (moderate and severe) have much higher value for plasma peroxides and therefore increased OSI level on admission in comparison with the values oxidative stress parameters in healthy individuals from our laboratory. This is in accordance with

the findings published by Muhammad *et al.* [31], Pincemail *et al.* [32] and Alamdari *et al.* [24] that have shown increased levels of oxidative stress in COVID-19 patients.

Additionally, Polonikov [33] observed that patients with moderate and severe COVID-19 illness had lower levels of glutathione and higher reactive oxygen species (ROS) and ROS/reduced glutathione ratio in plasma than patients with moderate disease. Although the measurements were performed on a small sample size (i.e. 4 patients), the author suggests that glutathione deficiency could be an important factor that enhances the SARS-CoV-2 induced oxidative damage and leads to disease progression.

The statistical difference observed in the two subgroups among the severe patients in terms of the second measurement of the oxidative stress can be contributed to the excessive levels of ROS and thus triggers a cascade of pathological events. This would result in increased oxidative stress and will contribute to developing a severe form of the disease even multi organ failure and death [31–33].

Herein, authors would like to state that the study has several limitations. First, this was a single-center study at a tertiary hospital institution that accepts the most demanding cases from the whole country. Additionally, most of the patients were already treated either at home or in secondary health-care setting. In general, condition deterioration was observed due to late referral to the tertiary clinic. Authors consider that this analysis should be performed in primary

healthcare institutions in order to determine OSI earlier at disease onset. Second, we consider that a more frequent blood sampling schedule should be employed (or at least in a time period shorter than 7 days), especially for high-risk patients. Also, we did not obtain information about the supplemental therapy and nutritional status before hospitalization which could also have impact on the PAT (total antioxidant power). Hence in further studies we will focus on the potential benefit of various antioxidants as agents that might reduce the oxidative stress and potentially improve the disease outcome. A multi-center study, with bigger sample size that will include healthy individuals and patients with various degrees of disease severity is needed to further clarify the role of the oxidative stress parameters in COVID-19.

CONCLUSION

In conclusion, we have presented results for oxidative stress parameters d-ROM, PAT and OSI and their association with several frequently used clinical laboratory parameters (CRP, LDH and NLR) in patients infected with SARS-CoV-2 divided in two groups: moderate and severe. Patients which recovered had lower values for the free radicals and OSI in comparison with the values obtained at the beginning of hospitalization. Since in all groups increased oxidative stress was observed, we consider that using OSI at the beginning of COVID-19 disease presents a valuable starting point for the general assessment of oxidative stress and hence enabling a better triage of the patients in terms of disease severity.

Introducere. Datele clinice sugerează faptul că stresul oxidativ ar putea înrăutăți statusul redox, fapt ce ar putea duce la o progresie a bolii. Obiectivul studiului a fost de a investiga parametrii stresului oxidativ PAT (puterea totală antioxidantă) și d-ROM (peroxizii plasmatici) la pacienții cu forme moderate și severe de COVID-19. În plus a fost urmărită asocierea cu analize uzuale de laborator (CRP, LDH și NLR).

Metode. PAT și d-ROM au fost evaluate prin metode analitice fotometrice din serul a 50 de pacienți spitalizați cu COVID-19. 2 probe au fost prelevate, imediat la internare și la 7 zile distanță.

Rezultate. Toți pacienții cu forme severe aveau la internare valori mult crescute ale peroxizilor plasmatici și s-a observat o corelație semnificativă cu CRP, LDH și NLR. La externare valoarea peroxizilor plasmatici a fost mult redusă.

Concluzii. Folosirea indexul stresului oxidativ (OSI) la debutul COVID-19 poate reprezenta un punct de pornire important în evaluarea generală a stresului oxidativ și astfel se poate avea un triaj bun al severității bolii.

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