ИЗВЕШТАЈ ОД СПРОВЕДЕНО ИСТРАЖУВАЊЕ ВО ПОДРАЧЈЕТО НА ОКСИДАТИВЕН СТРЕС

1. Евалуација на маркери за оксидативен стрес како достапна анализа на Институтот за претклиничка и клиничка фармакалогија и токсикологија

Цели:

- Испитување на параметрите на оксидативен стрес кај пациенти со умерен и тежок облик на Ковид 19
- Испитување и докажување на корелација помеѓу параметрите на оксидативен стрес и често испитуваните клинички маркери CRP, д-димери, LDH и NLR кај Ковид-19 пациенти
- Испитување на цитокинскиот профил кај пациенти со тежок облик на Ковид-19
- Испитување и докажување на корелација помеѓу парамтерите на оксидативен стрес и селектираните цитокини

1.1.Евалуација на маркерите на оксидативен стрес кај пациенти со Ковид-19

Апстракт

Клиничките податоци сугерираат зголемен оксидативен стрес кај пациенти со Ковид-19 и овој влошен редокс статус потенцијално може да придонесе за прогресија на болеста. Со цел на евалуација на оксидативниот стрес, ги измеривме параметрите за оксидативен стрес, имено, PAT (вкупна антиоксидантна моќ) и d-ROM (плазма пероксиди). Покрај тоа ја проценивме нивната корелација со најчесто користените клинички параметри CRP, LDH, и NLR во серум кај умерени и тешко болни пациенти хоспитализирани при Инфективната клиника за фебрилни и заразни болести.

PAT и d-ROM беа утврдени со аналитичка фотометриска метрика во серум од 50 хоспитализирани пациенти. За секој од нив, по два примероци беа собрани и анализирани веднаш по собирањето во период од седум дена.

Кај сите пациенти при прием беше измерена многу висока вредност на плазма пероксиди и беше докажана значајна корелација помеѓу параметрите на оксидативниот стрес и CRP, LDH и NLR. (стр <0,05), освен за индексот на оксидативен стрес наспроти CRP кај тешко болните пациенти со Ковид-19. Во фазата на оздравување, забележан беше пад на плазма пероксидите и индексот на оксидативниот стрес беше подобрен кај пациентите со умерен облик на болеста.

Сметаме дека користењето на индексот на оксидавитен стрес на почетокот на болеста претставува важна почетна точка за општа проценка на оксидативниот стрес и оттука овозможува подобро класифицирање на пациентите во однос на сериозноста на болеста.

Клучни зборови: Ковид-19, оксидативен стрес, плазма пероксиди, антиоксиданс , сериозност на болеста

Abstract

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

Вовед

Во изминатата година откако официјално е прогласена пандемијата со Ковид-19, научниците и лекарите ширум светот опсежно ги споделуваат своите искуства во научните публикации. Ова ја помогна достапноста на поопширни лабораториски податоци, а исто така, предложени се неколку потенцијални механизми зад патогенезата на болеста. Глобалната пандемија го доведе во прашање секој здравствен систем, дури и во најмоќните и развиените земји.

Според СЗО , пациентите се категоризирани во опсег од асимптоматски или благи до умерени, тешки или критични. САРС-Cov-2 вирусот го напаѓа речиси секој систем во телото. Исходот на болеста кај многу пациенти е во корелација со лабораториските и клиничките својства на цитокинска бура што предизвикува про-воспалителна состојба која доведува до прекумерна продукција на реактивни видови на кислород (РОС) што е поврзана со сериозно оштетување на ткивото.

Оксидативниот стрес е природен процес кој се случува во текот на метаболизмот и игра една важна улога во одржување на рамнотежата на нивоата на прооксиданти – антиоксиданси и хомеостазата на клетките, ткивата и органите. Во абнормални физиолошки услови неконтролираното производство на РОС како што се супероксид анјонот, водород пероксидот, хидроксилниот радикал и молекуларниот кислород промовираат каскада од биолошки настани кои индуцираат патолошки одговори.

Кај пациенти со коморбидитети кои се инфицирани со САРС-Cov-2 евидентирана е состојба со зголемен оксидативен стрес поради хроничната болест и вирусна инфекција. Затоа, мерењето на оксидативниот стрес може да биде важно за да се одреди текот на болеста во однос на сериозноста. Исто така, евалуацијата на оксидативниот стрес може да се смета како алатка за поддршка на рационален пристап кон терапевтските одлуки преку користење на антиоксиданси, земајќи ги во предвид нивното дејство кон прекин на цитокинската бура како и намалувањето на оксидативниот стрес.

Корелацијата помеѓу оксидативниот стрес и сериозноста на болеста се уште не е целосно истражена и оттука недостасуваат експериментални податоци во клинички услови. Затоа, нашата студија се фокусираше на проценка на параметрите на оксидативниот стрес и нивната поврзаност со неколку најчесто користени биохемиски параметри кај хоспитализирани пациенти со Ковид-19. Ова потенцијално може да помогне во откривање на пациенти кои бараат посложен пристап за лекување. Нашата хипотеза е дека параметрите за оксидативен стрес може да се користат како дополнување на конвенционалните лабораториски анализи, како биомаркери за прогресија на болеста. За оваа цел се анализираше серум од 50 пациенти инфицирани со САРС-Cov-2 со добро воспоставена метода претходно користена во областа на предвидување на редокс рамнотежата. Резултатите од параметрите за оксидативен стрес беа во корелирани со CRP, LDH и NLR со цел да се потврди клиничката важност наовие маркери пошироко да се искористат од здравствените институции .

Материјал и методи

2.1. Дизајн

50 пациенти со Ковид-19 (31 маж и 19 жени) со средна возраст од 56 години (опсег од 18 до 79 години) хоспитализирани на Инфективната клиника за фебрилни и заразни

болести во Скопје во рок од 2 месеци се вклучени во оваа студија. Дијагнозата и класификацијата на Ковид-19 беа базирани врз основа на Упатството за клинички менаџмент на Ковид-19 издаден од страна на СЗО. Пациентите со умерена болест беа возрасни со клинички знаци на пневмонија, но без знаци на тешка пневмонија, вклучувајќи SpO2 > 90% . Тешките случаи дополнително исполнуваа еден од следниве услови: SpO2 < 90%, респираторна стапка>30 вдишувања во минута или присуство на тешка респираторна слабост.
Сите пациенти беа потврдени дека имаат SARS-CoV-2 инфекција со RT-PCR од примерок од назален и фарингеален брис. Само лабораториски потврдени случаи беа вклучени во студијата. Студијата беше одобрен од страна на Етичката комисија на Медицинскиот факултет. Истражувањето е извршена од страна на мултидисциплинарен група, вклучувајќи ги и клинички експерти во подрачјето на Ковид-19.

2.2. Клинички карактеристики и лабораториски податоци

Демографски карактеристики, медицинска историја, клинички симптоми и знаци, истовремена примена на лекови, исходот, како и лабораториските анализи беа добиени од медицинската историја на пациентите. Лабораториската проценка се состоеше од: комплетна крвна слика, биохемиски анализи, коагулациски профил, анализи на артериски крвни гасови и инфламаторни маркери
Критериумите за отпуштање од Клиниката беа: клиничко подобрување на физикалниот преглед, подобрување на оксигенација, телесна температура во нормала најмалку три дена и подобрување на инфилтрацијата на белите дробови (докажано со рендгенски преглед на градите).

2.3. Метод за одредување на d - ROM, PAT и индекс на оксидативен стрес

PAT (вкупна антиоксидантна моќност) и d - ROM (плазма пероксиди) беа измерени на FRAS5 аналитички фотометриски систем со употреба на брз кит REDOX. Два примерока беа собрани и анализирани веднаш по собирањето со разлика од седум дена. Првиот примерок беше земен при приемот во клиниката. Референтните нормални вредности на d-ROM s и PAT се 250-300 U. Carr (1 U. Carr = 0.08 mg H202 / dL) и 2200-2800 U. Carr, соодветно. Индексот на оксидативен стрес (OSI) ги прикажува информациите добиени од брзиот тест d-ROM и тестот PAT кој автоматски се пресметува со наменскиот спектрофотометар FRAS5 со нормални референтни вредности помали од 40 дадени од производителот (H&D srl, 43124 Парма, Италија).

2.4 Статистичка анализа

Податоците беа опишани како број и/или процент, или средна и опсег или просечна и стандардна девијација (СД) или стандардна грешка на просекот (СЕМ), каде што е соодветно. Разликите помеѓу групите беа откриени со помош на т-тестот, Ман-Витни или АНОВА, проследени со повеќекратниот тест за споредба на Холм- Сидак, со $p<0.05$ за статистички значајно. За споредба помеѓу групите, ние користевме вредности на оксидативен стрес кај здрави лица (12 мажи и 8 жени, просечна возраст од 54 години) со негативен RT-PCR кои беа во опсегот на веќе објавени референтни нормални вредности

како што е наведено во делот 2.3. Сите анализи се направени со користење на статистичката програма GraphPad 9 (САД).

3.0 Резултати

За да ја испитаме додадената вредност на OSI, направивме анализа на споредби со некои често применети лабораториски маркери (CRP, LDH и NLR) кои се користат за да се предвиди текот на болеста. Нашата хипотеза е дека кај пациенти каде вредноста на OSI постојано се зголемува по приемот во болница, најверојатно ќе развијат тешка форма на болеста или компликации за време на хоспитализација, вклучително и смрт. Во спротивно, ако вредноста на OSI се намалува, пациентот треба да има побрзо закрепнување и периодот на хоспитализација треба да се намали. Затоа, за да се потврди нашата хипотеза ги анализиравме умерените и тешко болните пациенти заразени САРС-Cov-2 пациенти хоспитализирани на Универзитетската клиника инфективни и фебрилни состојби во нашата земја.

3.1. Демографија и клинички карактеристики на пациентите

Од 50 пациенти, 30 пациенти припаѓаат на тешка група и 20 од нив беа класифицирани како умерени случаи. Просечната возраст на умерената група (52.05 ± 12.84 години) беше малку пониска од просечната возраст на тешката група (58.0 ± 9.94 години), но не беше забележана значајна разлика во однос на овој параметар помеѓу групите ($p > 0.05$, t-тест). Просечното време од почетокот на симптомите до приемот во болница беше 10.52 + 2.33 дена (опсег 7-16 дена). Повеќето пациенти (60% во умерена група и 76.7% во тешка група) имале основни медицински состојби при приемот. Најчесто пријавувани коморбидитети беа хипертензија, дијабетес и хронични срцеви заболувања. Дополнително, просечниот број на коморбидитети кај секој пациент беше малку поголем кај тешката група 1.7 + 0.56 (опсег 1-3), отколку кај умерената група 1.42 + 0.52 (опсег 1-2). Најистакнатите и вознемирувачки симптоми пријавени од пациентите при прием беа висока телесна температура (80%), диспнеа (64%), малаксаност (62%) и кашлица (56%).

Во умерената група при прием, беа забележани абнормални вредности за CRP, LDH, гликоза и NLR. Средната вредност за CRP беше 44.1 ± 6.46 наспроти 7.16 ± 1.96, LDH беше 280.3 ± 25.27 наспроти 283.4 ± 59.19 и NLR беше 6.47 ± 1.64 наспроти 5.09 ± 1.73 при прием и отпуштање од Клиниката, соодветно. Во тешката група на пациенти беа забележани абнормални вредности за нивоата на CRP , LDH , CK, ALT, AST, глукоза, WBC и NLR . Средната вредност на CRP, LDH, CK, ALT и NLR во тешката група на пациенти беше повисока во споредба со умерената група пациенти при прием и отпуштање од Клиниката.

3.2 Асоцијација помеѓу параметрите за оксидативен стрес, CRP, LDH и NLR

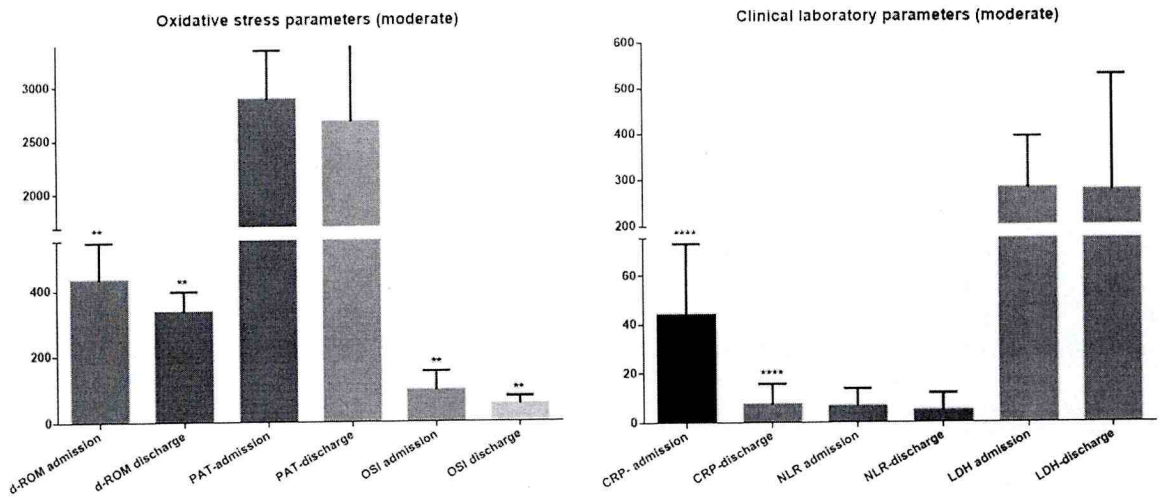
Пациентите кои припаѓаат на двете групи при приемот имаат многу повисока вредност за плазма пероксидите и затоа го зголемуваат нивото на оксидативен стрес во споредба со претходно утврдените вредности за овие параметри кај здрави лица од нашата лабораторија (види Табела подолу).

Табела. Параметри на оксидативен стрес (PAT, d-ROM and OSI) во серум.

Параметар	PAT(U. Carr) ± SEM	d-ROM(U. Carr) ± SEM	OSI± SEM
Умерена група (n=20)	2887±102.1	431.2±25.25	94.2±13.9
прием vs отпуст	p=0.0004, t-test	p=0.0001, t-test	p=0.0001, t-test
	2673±160.6	334.3±13.87	52.25±5.60
	p=0.1325, t-test	p=0.0002, t-test	p=0.0001, t-test
Тешка група (n=30)	2801±85.86	413±17.28	84.03±8.86
прием	p=0.0008, t-test	p=0.0001, t-test	p=0.0001, t-test
Тешка (n=6)	2652±148.8	325.3±22.46	43.40±10.87
оздравени	p=0.0043, t-test	p=0.0019, t-test	p=0.005, t-test
Тешка (n=9)	2186±233.2	462.4±28.04	107.8±18.20
починати	p=0.2497, t-test	p=0.0001, t-test	p=0.0001, t-test
Здрава група (n=20)	2406±71.55	271±5.590	21±2.527

3.2.1 Умерена група пациенти

Меѓу умерената група пациенти (n =20) при приемот се покажа добра корелација помеѓу сите испитани параметри (d-ROM s , PAT, OSI, CRP, LDH и NLR) ($R2 = 0.9425$, $p < 0.05$, ANOVA). Овие резултати се графички прикажани на Слика 1, каде што беше откриена значајна разлика за CRP, d-ROM и OSI ($p<0.05$, ANOVA). Покрај тоа, значајна корелација беше забележана помеѓу сите параметри при отпуштање на пациентите од Клиниката ($R2 = 0.9383$, $p> 0.05$, ANOVA) освен за d-ROM-от со LDH и со NLR и CRP ($p> 0.05$) .



Слика. Графички приказ на параметрите на оксидативен стрес (a) и клинички лабораториски параметри (b) кај пациентите со умерена слика на Ковид-19 (n=20) при прием и отпуштање од Клиниката. **p<0.05, ****p<0.0001

Податоците за пациентите со тежок облик на болеста во детали се достапни во веќе објавената публикација дадена во прилог.

4.0 Дискусија

До сега, експерименталните податоци за мерење на оксидативниот стрес кај Ковид-19 пациенти се лимитирани, се фокусиранна ретроспективна анализа на податоци. Нашата студија придонесува кон клиничките докази дека оксидативниот стрес е зголемен кај пациенти со Ковид-19 и дека влошениот редокс статус потенцијално може да придонесе за прогресија на болеста.

Нашите резултати покажуваат дека и во двете групи на пациенти (умерени и тешки) имаат многу повисока вредност за плазма пероксиди во однос на здравата популација и со тоа и зголемен ниво на индексот на оксидативен стрес. Ова е во согласност со наодите објавени од Мухамед и сор., Пинцемаил и сор., и Аламдари и сор., каде е покажано зголемено ниво на оксидативен стрес кај пациенти со Ковид-19.

Дополнително, во студија на Полоников, прикажано е дека пациентите со умерена и тешка форма на Ковид-19 имале пониско ниво на глутатион и повисоки реактивни видови кислород (РОС). Иако неговите анализи се извршени на мала големина на примерок (т.е. 4 пациенти), авторот сугерира дека недостатокот на глутатион може да биде важен фактор што го подобрува оксидативното оштетување предизвикано од САРС-КоВ-2 и води кон прогресија на болеста.

Статистичката разлика забележана во двете подгрупи меѓу тешките пациенти во однос на второто мерење на оксидативниот стрес може да се придонесе за прекумерните нивоа на РОС и на тој начин предизвикува каскада на патолошки настани. Ова би резултирало со зголемен оксидативен стрес водејќи кон развој на тешка форма на болеста, дури и откажување на повеќе органи и смрт.

Би сакале да наведеме дека студијата има неколку ограничувања. Прво, ова беше едноцентрична студија во терциерна болничка институција која ги прифаќа најсложените случаи од целата земја. Покрај тоа, повеќето пациенти, веќе се третираат дома или во секундарна здравствена заштита. Општо земено, беше забележано влошување на состојбата поради доцна упатување во терциерна клиника. Авторите сметаат дека оваа анализа треба да се изврши во примарните здравствени установи со цел да се утврди индексот на оксидативен стрес порано на почетокот на болеста. Второ, сметаме дека треба да се користи почест распоред за земање крв (или барем во временски период пократок од 7 дена), особено за пациенти со висок ризик. Исто така , не добивме информации за дополнителна терапија и нутритивен статус пред хоспитализација, што исто така може да има влијание врз вкупната антиоксидантна моќ. Оттука, во понатамошните студии ќе се фокусираме на потенцијалната придобивка од разните антиоксиданси како агенси кои можат да го намалат оксидативниот стрес и потенцијално да го подобрат исходот на болеста. Потребна е студија на повеќе центри, со поголема големина на примерок што ќе вклучува здрави лица и пациенти со различни степени на сериозност на болеста, за дополнително да се разјасни улогата на параметрите на оксидативниот стрес во Ковид-19.

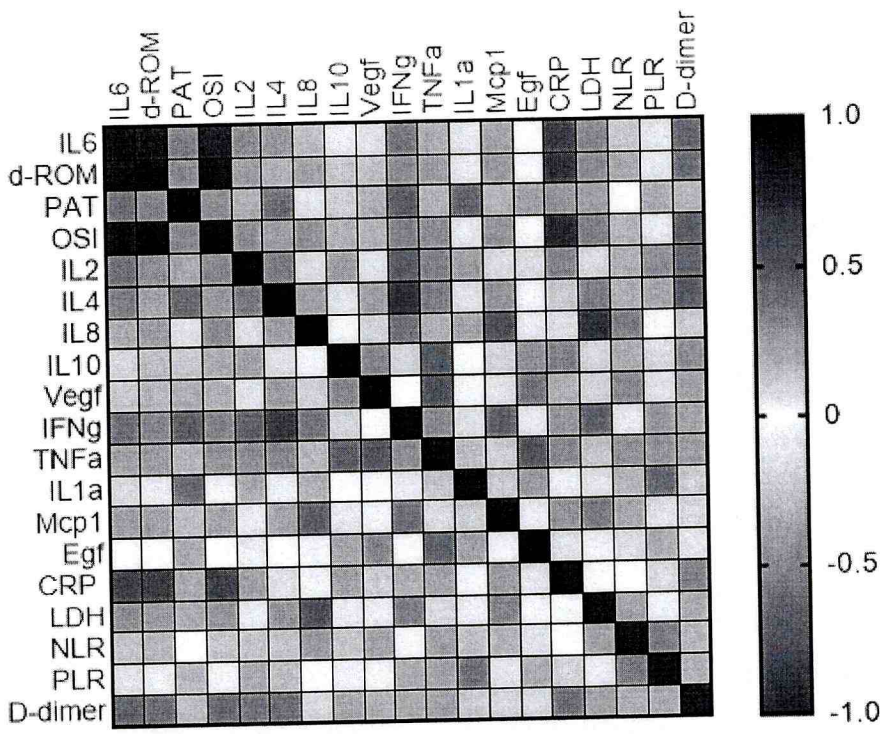
1.2. Презентација на цитокинскиот профил во однос на оксидативниот стрес

Исто така, кај селектирани пациенти беше испитана корелацијата помеѓу сет на цитокини (IL2, IL4, IL6, IL8, IL10, VEGF, IFN -γ , TNF -α , IL - 1 α , MCP-1 и EGF) и маркерите на оксидативен стрес Резултатие се прикажани во Табелата подолу.

Табела. Лабораториски анализи кај пациенти со тежок облик на Ковид-19

Parameter	Severe COVID-19 patients mean±SEM (n=14)	Not infected individuals mean±SEM (n=20)	p (t-test)
IL-6 (pg/mL)	250.1±39.07	2.135±0.453	**0.0001**
IL-2 (pg/mL)	4.426±2.177	2.005±0.402	0.2818
IL-4 (pg/mL)	1.936±0.268	1.956±0.137	0.3150
IL-8 (pg/mL)	108±19.79	7.159±1.298	**0.0001**
IL-10 (pg/mL)	11.14±4.551	0.916±0.219	**0.0001**
VEGF (pg/mL)	530.7±147.1	27.04±4.708	**0.0001**
IFN-g (pg/mL)	1.487±0.745	0.389±0.082	0.3889
TNF-a (pg/mL)	5.223±0.751	3.646±0.757	0.090
IL-1a (pg/mL)	0.4614±0.263	0.2153±0.0422	0.7210
MCP-1 (pg/mL)	891±92.35	89.61±12.18	**0.0001**
EGF (pg/mL)	65.37±17.46	24.28±5.367	**0.0318**
d-ROM (U.Carr)	448.8±30.37	271±5.590	**0.0001**
PAT (U.Carr)	3048±100.1	2406±71.55	**0.0001**
OSI	107.7±14.38	21±2.527	**0.0001**
CRP (mg/L)	144.7±21.38	2.1±0.05	**0.0001**
LDH (IU/L)	823.4±80.02	156±20.31	**0.0001**
NLR	17.08±2.058	1.5±0.02	**0.0001**
PLR	538.2±85.09	113±10.35	**0.0001**
D-dimer (ng/mL)	2688±499.1	225±22.75	**0.0001**
WBC (x10^3µL)	14±2.004	6.1±1.365	**0.0019**
ALT (U/L)	51.93±7.171	28.96±2.658	**0.0018**
AST (U/L)	61.210±7.283	30.56±3.487	**0.0002**

Имено, статистички значајно разлика ($p < 0.05$, t-тест) е забележана за IL-6, IL-8, IL-10, VEGF, MCP-1 и EGF кај пациенти со CAPC-CoV-2, додека IL- 2, IFN- γ, TNF- α и IL-1 α беа зголемени, но оваа разлика не беше значајна во споредба со поединците без инфекција со CAPC-CoV-2($p<0.05$, t-тест). Важно откритие на оваа пилот студија е дека параметрите на оксидативниот стрес, d-ROM (448.8 ± 30.37 U.Carr), индексот на оксидативен стрес (107.7 ± 14.38) и PAT (3048 ± 100.1 U.Carr) беа значително повисоки ($p<0.05$, t-тест) кај тешки пациенти со Ковид-19 во споредба со неинфицираните лица. Покрај тоа, ние ја испитавме корелацијата помеѓу испитаните цитокини, параметрите за оксидативен стрес и CRP, LDH, PLR, D-димер и NLR. Преку користење на приказ на 'heatmap' потврдена е позитивна и значајна корелација помеѓу сите цитокини и параметрите на оксидативниот стрес (d-ROM, PAT и OSI), освен негативна корелација помеѓу IL-10 и вкупниот антиоксиданс капацитет, PAT. Не значајна корелација е евидентирана помеѓу индексот OS и IL-8 ($r=0.3762$, $p=0.8552$) и помеѓу d-ROM и VEGF ($r=0.2156$, $p=0.999$). IL-6 покажа најсилна корелација со сите маркери на оксидативниот стрес, d-ROM ($r=0.9725$, $p=0.0001$), PAT ($r=0.5000$, $p=0.0001$) и индекс на оксидативниот стрес ($r=0.9593$, $p=0.012$).



Слика. Spearman r прикажани како heatmap помеѓу цитокините, параметрите на оксидативност стрес и често користените клинички биомаркери во Ковид-19.

Подеталните резултати и дискусија се прикажани во објавената публикација. Оваа пилот студија покажува добра корелација помеѓу панелот на тестирани цитокини и параметрите на оксидативниот стрес измерени со брза фотометриска метода што може да се користи на почетокот на болеста за да се предвиди дали Ковид-19 ќе се развие во тешка форма. Презентираните резултати ќе придонесат за поддршка на доказите дека синдромот на цитокинска бута лежи како имунопатогенеза за време на инфекција

со САРС- КоВ-2 и со користење на параметрите за оксидативен стрес лекарите можат да обезбедат навремени и рани интервенции кај пациенти со Ковид-19.

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

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Running head: Oxidative stress in COVID-19 patients

Abstract

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.

Keywords: 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_20_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_20_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 ±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± 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.

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 7^{th} 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.

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 sulfhydryl 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 showed 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 statsul 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 nalaize 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 observant o corelație semnificativă cu CRP, LDH și NLR. La externare valoarea peroxizilor plasmatici a fost mult redusă.
Concluzii: Folosind indexul stresului oxidativ (OSI) la debutul COVID-19 poate reprezenta un punct de pronire important în evaluarea general a stresului oxidative și astfel să putem avea un triaj bun din punctul de vedere al serverității bolii.

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Declaration of interest: The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be constructed as a potential conflict of interest.

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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±SD)	55.57±11.47	52.05±12.84	58.0±9.94
Days from disease onset to admission (mean±SD)	10.52±2.33	10.35±2.18	10.63±2.46
Patients with medical conditions (%)	35 (70%)	12 (60%)	23 (76.7%)
Number of medical conditions (mean±SD)	1.6±0.54	1.42±0.52	1.7±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 ± SEM

Parameter	Moderate (n=20) Admission	Moderate (n=20) Discharge	Severe (n=30) Admission	Severe (n=6) *Discharge recovered*	Severe (n=9) *Died after 2nd sample*	Reference values
CRP (mg/L)	44.1 ± 6.46	7.16 ± 1.96	113 ± 17.85	20 ± 9.39	127 ± 33.49	0-10
LDH (IU/mL)	280.3 ± 25.27	283.4 ± 59.19	800 ± 60.27	484.6 ± 204.2	827.2 ± 130.6	120-246
CK (U/L)	91.47± 16.88	60.24 ± 12.23	272 ± 60.67	184 ± 101.6	228.9 ± 131.5	30-170
ALT(U/L)	49.31 ± 15.46	75.39 ± 14.60	94.76 ± 36.82	107.2 ± 70	38.67 ± 14.72	10-52
AST(U/L)	45.63 ± 9.49	44.06 ± 7.71	81.59 ± 18.65	36 ± 15.62	33.33 ± 5.43	10-47
Glucose (mmol/L)	12.68 ± 3.62	8.52 ± 1.45	16 ± 3.82	5.84 ± 0.97	9.09 ± 1.42	3.9-5.6
Creatinine (µmol/L)	57.44 ± 2.84	60.32 ± 3.73	58.27 ± 3.44	58.40 ± 9.34	51.11 ± 6.56	62-133
Urea (mmol/L)	4.76 ± 0.54	5.16 ± 0.65	7.38 ± 0.39	6.06 ± 1.31	7.00 ± 0.74	1.7-8.3
Hemoglobin (g/L)	128.1 ± 2.62	130.1 ± 3.34	137.8 ± 2.83	126.2 ± 10.78	139.9 ± 4.45	115-180
RBC ($x10^3\mu L$)	4512 ± 28.91	4543 ± 101.2	4776 ± 113.6	4358 ± 410.1	4862 ± 1.49	4000-5500
WBC ($x10^3\mu L$)	7.54 ± 0.88	9.07 ± 0.82	24.7 ±10.35	11.88 ±0.87	22.41 ± 2.87	4.0-11.0
Platelets ($x10^3\mu L$)	343.1 ± 34.78	377 ± 33.04	292.4 ± 22.24	241.2 ± 47.26	284.7 ± 25.30	150-400
NLR	6.47 ± 1.64	5.09 ± 1.73	20.23 ± 2.06	5.92 ± 0.99	44.72 ± 10.49	≤ 3.0

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

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

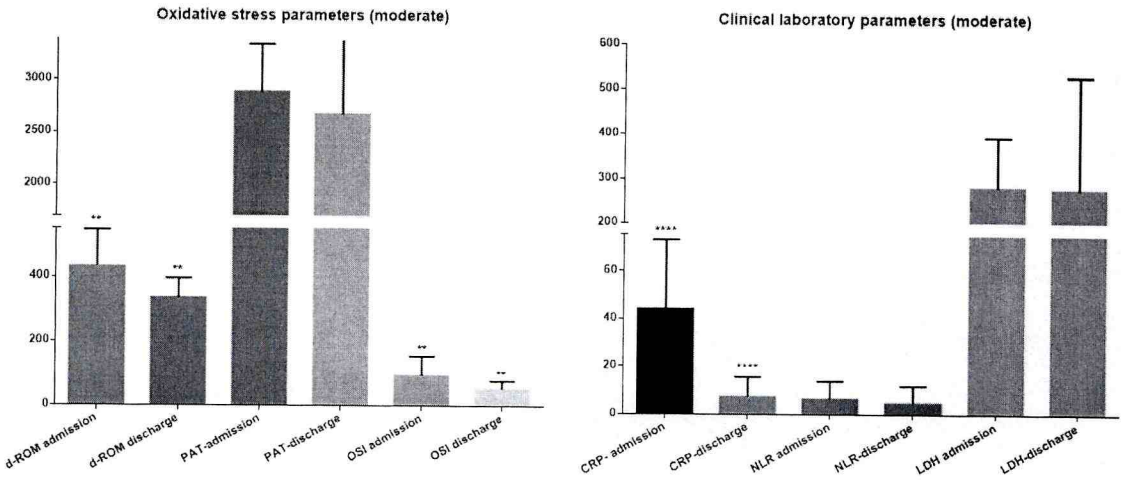

Figure 1a. Graphical presentation of oxidative stress parameters (a) and clinical laboratory parameters (b) in the moderate group of patients (n=20) at admission and at discharge. **p<0.05, ****p<0.0001

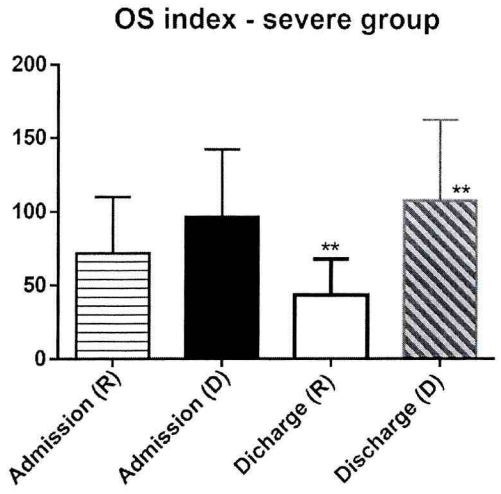

Figure 2. Graphical presentation of OSI for the two subgroups of COVID-19 patients with different outcome within the severe group. R-recovered (n=6), D-died (n=24). **p<0.05

editorial@f1000research.com
Wed 7/28/2021 5:44 PM
To: Marija Petrusevska

Dear Marija

Presentation of cytokine profile in relation to oxidative stress parameters in patients with severe COVID-19: an observational pilot study
Petrushevska M, Zendelovska D, Atanasovska E, Eftimov A and Spasovska K

We have now accepted your article for publication in F1000Research. It will be sent to the typesetters and a member of the Production team will send you a proof in due course.

One of our editorial team will be assisting you with the peer review process of your article, and will be your main contact once the article is published.

Best wishes,

David
The Editorial Team, F1000Research

RESEARCH ARTICLE

Presentation of cytokine profile in relation to oxidative stress parameters in patients with severe COVID-19: an observational pilot study [version 1; peer review: awaiting peer review]

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Abstract

Introduction: COVID-19 can be worsened by hyper-production of cytokines accompanied by increased level of oxidative stress. The aim of this study was to investigate the correlation between a set of cytokines and the markers of the oxidative stress.

Methods: The levels of cytokines IL-2, IL-4, IL-6, IL8, IL-10, VEGF, IFN-γ, TNF-α, IL-1α, MCP-1 and EGF were determined by using High Sensitivity Evidence Investigator™ Biochip Array technology. The oxidative stress parameters (d-ROM, PAT, OS index) were measured in serum on FRAS5 analytical photometric system.

Results: IL-6, IL-8, IL-10, VEGF, MCP-1 and EGF were significantly higher ($p<0.05$) in the patients with severe COVID-19 with increased levels of IL-2, IFN-g, TNF-a and IL-1α. The d-ROM, OS index, and PAT were significantly higher ($p<0.05$) in severe COVID-19 patients. IL-6 demonstrated the strongest correlation with all of the markers of the oxidative stress, d-ROM ($r=0.9725$, $p=0.0001$), PAT ($r=0.5000$, $p=0.0001$) and OS index ($r=0.9593$, $p=0.012$). Similar behavior was evidenced between IFN-g and d-ROM ($r=0.4006$, $p=0.0001$), PAT ($r=0.6030$, $p=0.0001$) and OS index ($r=0.4298$, $p=0.012$).

Conclusion: The oxidative stress markers show good correlation with the tested cytokines which can be measured at the beginning of the disease in a primary care setting to predict the course of COVID-19.

Keywords

oxidative stress, COVID-19, cytokines

Open Peer Review

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Any reports and responses or comments on the article can be found at the end of the article.

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1. Introduction

Cytokine storm syndrome has been widely discussed and proposed as one of the underlying aetiologies of respiratory failure in patients infected with SARS-CoV-2. Pro-inflammatory cytokines play a key role in large number of respiratory viral infections by activation of the adaptive immune response and, when this response is not controlled, it can lead to involvement of the lung tissue in the course of ARDS or can result in severe damages of multiple organs. For example, following influenza viral infection, an excessive amount of reactive oxygen species (ROS) is produced in several tissues including alveolar epithelium and endothelium[1] for which induced expression of cytokines through activation of Toll-like receptors (TLR3, TLR7 and TLR8, retinoic acid inducible gene I and members of NOD-like receptor family) stand in the background of the pathogenesis.[2,3] Oxidative stress is typical for infection of human respiratory syncytial virus,[4] rhinoviruses,[5] and many other viruses. This has been discussed in previously published reviews[7-12] and as well, several experimental studies suggest that cytokine storm correlated with direct tissue injury and lead to unfavourable prognosis of severe form of the COVID-19 disease.[7] Briefly, particularly high levels of IL-6, IL-10, IL-2R and TNF-α have been reported in patients with severe form of the disease[13,14] although other authors suggest that more cytokines, such IL-1β, IL-1RA, IL-8, IL-18 are included in the COVID-19 pathogenesis.[7,13,14]

Authors have suggested that the innate immune response follows same pathway for SARS-CoV-2 infection.[1,8] Namely, ROS is a strong ligand and a direct mediator in the NLPR3 (inflammasome) trigger. Moreover, NF-κB, which is activated by ROS, triggers transcriptional levels of NLPR3 are enhanced by TLR and NLR ligands. This means that the inflammasome is increased by ROS either directly or indirectly.[8,13] To the addition of ROS, H_2O_2 activates NF-κB to produce inflammatory cytokines.[15] Hyperproduction of IL-6, TNF-α, IL-1β, IP-10, GCSF, MCP-1, MIP1-α/CCL3 and elevated blood ferritin are also observed in patients infected with SARS-CoV-2.[7,16]

For this purpose, and in the light to share more experimental data as evidence to the suggested pathogenesis of COVID-19 with the scientific community, we have utilized a highly standardized cytokine assay to measure plasma levels of 11 inflammatory cytokines potentially associated as key factors with the cytokine storm syndrome. Afterwards, we have investigated which of these cytokines involved in the cytokine storm of COVID-19 show good association/correlation with the oxidative stress markers determined with fast and inexpensive photometric analytical method. Moreover, the relation between the cytokines, oxidative stress markers and the most commonly used inflammation-related biomarkers (CRP, D-dimers, PLR, NLR and LDH) in severe form of the disease was investigated.

2. Methods

2.1 Study design, patients profile and data collection

52 patients with COVID-19 were hospitalized at the University Clinic for Infectious Diseases and Febrile Conditions, Skopje, Republic of North Macedonia at the beginning of the pandemic within a period of 1 month. 14 patients classified with severe COVID-19 (nine males and five females) with a mean age of 58.36 years (range from 36 to 71 years) 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 WHO. Severe cases in addition to severe pneumonia 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). Severe form of COVID-19 as primary exposure variable, demographic characteristics, medical history, clinical symptoms and signs, concomitant medication, outcome data, as well as laboratory analyzes were obtained from the patients' medical records were other predictor variables. The study flow chart is shown in Figure 1. 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) and complies with the STrengthening the Reporting of Observational studies in Epidemiology (STROBE) statements for reporting of observational trials.[17]

2.2 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 a FRAS5 analytical photometric system (H&D, Italy). Samples were collected and analyzed immediately after hospital admission. The instructions of the manufacturer were followed for the both tests. The d-ROM and PAT are reported in equivalents of H_2O_2 and ascorbic acid, respectively. Oxidative stress index (OSI) presents information obtained from d-ROMs Fast test and the PAT test that is automatically calculated by the manufacturer's software (OB manager, FRAS5, H&D, Italy) with normal reference values less than 40.

2.3 Cytokines profile assay

The High Sensitivity Evidence Investigator™ Biochip Array technology (Randox Laboratories, GB) was used to perform simultaneous quantitative detection of multiple analytes from a single patient sample (14 SARS-CoV-2 infected and 20 non-infected individuals).

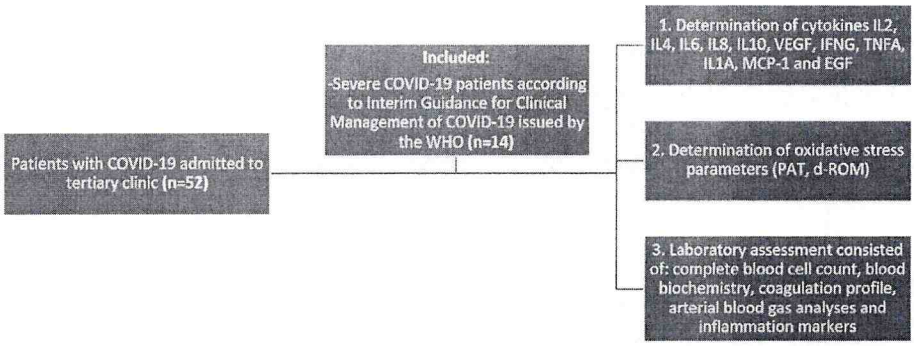

Figure 1. Flow-chart of the study.

100 μL of plasma was used in biochip carriers, following by incubation on thermo-shaker for 1 hour at 37°C and 370 rpm and 16–20 hours incubation at 4°C. Afterwards, carry out of two wash cycles and 300 μL conjugate was added into each well followed by another incubation of 1 hour at 37°C and 370 rpm. At the final step after twice washing the carriers, fluorescent dye was added to carriers according to protocol and carriers were captured by Evidence Investigator Array. Results were processed automatically using EvInvest software and levels of cytokines IL-2, IL-4, IL-6, IL-8, IL-10, VEGF, IFN-γ, TNF-α, IL-1α, MCP-1 and EGF were calculated as pg/mL.

2.4 Statistical analysis

Exposure variables were summarized using descriptive statistics. 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 followed by Mann–Whitney where appropriate. A p-value less than 0.05 was considered significant. For purpose of control and comparison between groups, we have analyzed samples of 20 healthy individuals with negative RT-PCR test for SARS-CoV-2 (12 males and eight females, mean age 54). Spearman r coefficient of correlation was performed. All analyses were made using the statistical program GraphPad Prism 9 (USA) (RRID:SCR_000306); an open-access alternative is JASP (RRID:SCR_015823).

3. Results

3.1 Demographics and laboratory findings

All 14 patients with a mean age of 58.36 years had severe form of the disease. The average time from onset of symptoms to hospital admission was 10.52±2.33 days (range 7–16 days). All of them had underlying medical conditions at admission. The most frequently reported comorbidities were hypertension, diabetes and chronic cardiac disease. The most prominent and disturbing symptoms reported by the patients on admission were high body temperature (80%), dyspnea (64%), malaise (62%) and cough (56%). The mean value of all clinical laboratory parameters upon hospitalization are presented in Table 1. Abnormal values for CRP, LDH, PLR, D-dimer and NLR were observed. The mean ± SEM value for CRP was 144.7 ± 21.37 mg/L, LDH was 823.4 ± 80.02 IU/L, PLR was 538.2 ± 85.09, NLR was 17.08 ± 2.058, and D-dimer was 2688 ± 499.1 ng/mL. All 14 patients had increased values for ALT, AST and WBC in comparison to the individuals not infected with SARS-CoV-2. The observed statistically difference between the two groups was significant in all cases ($p < 0.05$).

3.2 Cytokine profile, oxidative stress parameters and commonly used biomarkers

As presented in Table 1, 11 cytokines (including chemokines and growth factors) were analyzed in 14 patients infected with SARS-CoV-2 with severe form of the disease and these values were compared with individuals without SARS-CoV-2 infection. In this comparison, statistically significant increase ($p < 0.05$, t-test) was observed for IL-6, IL-8, IL-10, VEGF, MCP-1 and EGF in the SARS-CoV-2 patients, while IL-2, IFN-γ, TNF-α and IL-1α were increased but this difference was not significant when compared to the individuals without SARS-CoV-2 infection ($p < 0.05$, t-test). Important finding of this pilot study is that the parameters of the oxidative stress, d-ROM (448.8 ± 30.37 U.Carr), OS index (107.7 ± 14.38) and PAT (3048 ± 100.1 U.Carr) were significantly higher ($p < 0.05$, t-test) in severe COVID-19 patients when compared to the not infected individuals (Table 1). Moreover, we have investigated the correlation among the investigated cytokines, the oxidative stress parameters and CRP, LDH, PLR, D-dimer and NLR. The Spearman r coefficient of correlation between all these parameters is presented as a heat-map on Figure 2. The heat-map confirmed a positive and significant correlation between all cytokines and the parameters of the oxidative stress (d-ROM, PAT and OSI), except a negative correlation between IL-10 and the total antioxidant capacity, PAT. The correlation was not considered to be significant between OS index and the IL-8 ($r = 0.3762$, $p = 0.8552$) and between d-ROM and VEGF

Table 1. Laboratory findings in severe COVID-19 patients and non-infected individuals expressed as mean ± SEM.

Parameter	Severe COVID-19 patients mean ± SEM (n = 14)	Not infected individuals mean ± SEM (n = 20)	p (t-test)
IL-6 (pg/mL)	250.1 ± 39.07	2.135 ± 0.453	**0.0001**
IL-2 (pg/mL)	4.426 ± 2.177	2.005 ± 0.402	0.2818
IL-4 (pg/mL)	1.936 ± 0.268	1.956 ± 0.137	0.3150
IL-8 (pg/mL)	108 ± 19.79	7.159 ± 1.298	**0.0001**
IL-10 (pg/mL)	11.14 ± 4.551	0.916 ± 0.219	**0.0001**
VEGF (pg/mL)	530.7 ± 147.1	27.04 ± 4.708	**0.0001**
IFN-g (pg/mL)	1.487 ± 0.745	0.389 ± 0.082	0.3889
TNF-a (pg/mL)	5.223 ± 0.751	3.646 ± 0.757	0.090
IL-1a (pg/mL)	0.4614 ± 0.263	0.2153 ± 0.0422	0.7210
MCP-1 (pg/mL)	891 ± 92.35	89.61 ± 12.18	**0.0001**
EGF (pg/mL)	65.37 ± 17.46	24.28 ± 5.367	**0.0318**
d-ROM (U.Carr)	448.8 ± 30.37	271 ± 5.590	**0.0001**
PAT (U.Carr)	3048 ± 100.1	2406 ± 71.55	**0.0001**
OSI	107.7 ± 14.38	21 ± 2.527	**0.0001**
CRP (mg/L)	144.7 ± 21.38	2.1 ± 0.05	**0.0001**
LDH (IU/L)	823.4 ± 80.02	156 ± 20.31	**0.0001**
NLR	17.08 ± 2.058	1.5 ± 0.02	**0.0001**
PLR	538.2 ± 85.09	113 ± 10.35	**0.0001**
D-dimer (ng/mL)	2688 ± 499.1	225 ± 22.75	**0.0001**
WBC ($\times 10^3 \mu L$)	14 ± 2.004	6.1 ± 1.365	**0.0019**
ALT (U/L)	51.93 ± 7.171	28.96 ± 2.658	**0.0018**
AST (U/L)	61.210 ± 7.283	30.56 ± 3.487	**0.0002**

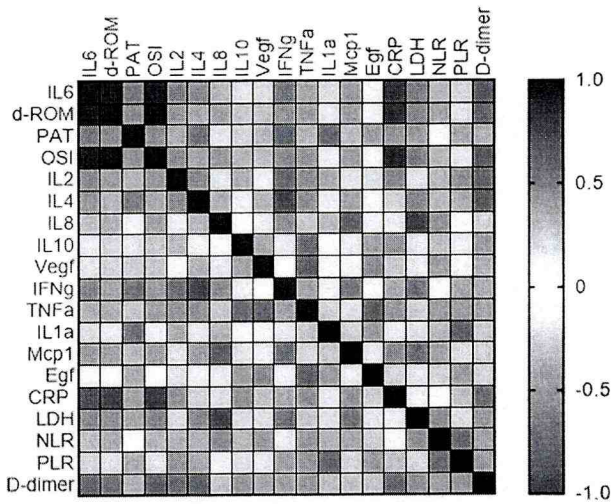

Figure 2. Spearman r presented as a heatmap between the investigated cytokines, oxidative stress parameters, and commonly used biomarkers in COVID-19.

($r = 0.2156$, $p = 0.999$). IL-6 demonstrated strongest correlation with all of the markers of the oxidative stress, d-ROM ($r = 0.9725$, $p = 0.0001$), PAT ($r = 0.5000$, $p = 0.0001$) and OS index ($r = 0.9593$, $p = 0.012$). Alongside, similar behavior was evidenced between IFN-γ and d-ROM ($r = 0.4006$, $p = 0.0001$), PAT ($r = 0.6030$, $p = 0.0001$) and OS index ($r = 0.4298$, $p = 0.012$). We further investigated the correlation between the cytokines and CRP as one of the most commonly used biomarkers, where the strongest one was observed with IL-6, IL-8, MCP-1 and IFN-γ. Moreover, in terms of correlation, investigated inflammatory cytokines IL-2, IL-4, IL-6, IL-8, IL-10, VEGF, IFN-γ, TNF-α, IL-1α and MCP-1 showed a strong positive correlation between each other, except between IL-6 and EGF (Figure 2).

4. Discussion

Cytokines including chemokines and growth factors together with lipid metabolites are among the main factors of immune cell function and their differentiation, hence upon their dysregulation various diseases can arise.[7-9,12] Herein, we share our results to give an add-on to the clinical evidences that oxidative stress is increased in patients with severe form of COVID-19 and that the measured oxidative stress parameters had shown a good correlation with the cytokines and the commonly used laboratory biomarkers. This pilot study focused on the possibility to utilize the oxidative stress parameters (d-ROM, PAT and OS index) as a fast and inexpensive prognostic tool for disease progression and potentially predict the outcome of COVID-19 in patients. Several retrospective studies and reviews have been published where abnormal levels of cytokines involved in the adaptive immunity (IL-2, IL-4) or pro-inflammatory cytokines and interleukins (IFNs, IL-1, IL-6, IL-10 IL-17 and TNF-α) were reported.[7,13,18,19]

Our study revealed that several cytokines and biomarkers were significantly increased in infected SARS-CoV-2 patients with severe form of the disease in comparison to those who were not, which was accompanied with coagulopathy as determined by deterioration of the platelet related parameters (PLR, D-dimer, IL-6) and MCP-1 as thrombosis related indicator. Huang *et al.* (2020) reported that MCP-1 levels were much higher in critical ICU patients and additionally that the platelet count was lower in those patients that do not survive.[19] Patients from our study were all with severe form of COVID-19 and all of them had died during hospitalization. Moreover, in our patients several of the cytokines had been increased more than 10-fold above the levels of the non-infected that we considered as a baseline. It is worth noting, the statistically significant increase of the VEGF levels more than 10-fold that can be related to the essential role of VEGF in endothelial cell activation by binding to cell surface VEGF receptors. VEGF up-regulation was observed in several viral infections and it has been investigated as a target for potential therapy development.[20] In addition, Huang *et al.*, report higher levels of VEGF in hospitalized COVID-19 patients.[19]

The strong correlation between the investigated cytokines (including chemokines and growth factors), the oxidative stress parameters and some of the commonly used biomarkers (CRP, D-dimers, NLR, PLR) are in line with the proposed cytokine storm as underlying mechanism of the infection. The cytokine storm syndrome occurs when large numbers of leukocytes are activated and release a high concentration of proinflammatory cytokines, with IL-6, IL-10, IFN, MPC-1, IL-1, IL-2 and IL-8 being the foremost. Generally, SARS-CoV-2 infection is associated with oxidative stress, the proinflammatory state, cytokine production, and cell death demonstrated by increase in ROS levels and an alteration of antioxidant defense during the infection.[11,21]

Even though limited published data are available, we believe that SARS-CoV-2 in line with other RNA viruses triggers oxidative stress by disturbing the pro-antioxidant–antioxidant balance.[8,22,23] We have demonstrated the significantly higher level of the d-ROM and OS index values in the infected patients with SARS-CoV-2 when compared with those who were not infected, supporting the hypothesis that viral infection will increase the oxidative stress and complicate the course of the disease. Whilst we consider that the OS index value presents an important parameter that we can have an impact on against COVID-19, by supplementation with antioxidants especially when there is applicable knowledge for several nutraceuticals/vitamins (vitamin C, vitamin D, curcumin, selenium, quercetin and other polyphenols) with proven anti-inflammatory, antioxidant and antiviral capacity.[24,25]

There are several limitations of the study besides being a single-center experience and a pilot study with only severe and critically ill patients. The herein presented patients were hospitalized at the beginning of the global pandemic when no specific and official guidelines were issued and available to assist the need for hospitalization. They had symptoms developed several days prior being hospitalized, however we believe that these symptoms were not life threatening and the hyper-inflammatory phase was at its beginning stage which is deemed by the obtained levels of the cytokines and the oxidative stress index. Nevertheless, further studies concerning COVID-19 patients with high levels of d-ROMs and OS index are warranted to determine whether supporting antioxidant therapy can reduce the possibility for the fatal outcome of the critically ill COVID-19 patients.

5. Conclusion

This observational pilot study demonstrates a good correlation between the panel of tested cytokines and the parameters of the oxidative stress measured by a fast photometric method that could be used at the beginning of the disease to predict whether COVID-19 will develop in severe form. The presented results will contribute to support the evidences that the cytokine storm syndrome lies as an immunopathogenesis during SARS-CoV-2 infection and by using the oxidative stress parameters (d-ROM, PAT, OS index) physicians can provide timely and early interventions in COVID-19 patients.

Author contributions

MP, DZ, EA contributed to the conception and design of the study. MP and DZ contributed to the oxidative stress parameters analyses, collated the data for the study, and completed all statistical analysis of data. MP wrote the first draft of the manuscript. AE performed the cytokine assay. KS and EA contributed to the clinical evaluation and medical data collection from the COVID-19 patients. All authors read and approved the final version of the manuscript.

Data availability

Underlying data

DataDryad: Underlying data for 'Presentation of cytokine profile in relation to oxidative stress parameters in patients with severe COVID-19: an observational pilot study'. https://doi.org/10.5061/dryad.gf1vhhmqg.

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