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THE RELATIONSHIP BETWEEN OXIDATIVE STRESS AND INFLAMMATION ACCORDING TO COVID-19 SEVERITY

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ACE2: angiotensin-converting enzyme 2

ARDS: acute respiratory distress syndrome

AST: aspartate aminotransferase

ATP: adenosine triphosphate

BMI: body mass index

COPD: chronic obstructive pulmonary disease

CRP: C-reactive protein

CVD: cardiovascular disease

DAMPs: damage-associated molecular patterns

E: envelope protein

ELISA: enzyme-linked immunosorbent assay

ESR: erythrocyte sedimentation rate

FP: fusion peptide

GGT: gamma-glutamyl transferase

GOT: aspartate aminotransferase

GPT: alanine aminotransferase

ICU: intensive care unit

IFN-I: type 1 interferon

IL: interleukin

Kb: kilobases

LDH: lactate dehydrogenase

M: membrane protein

MAVS: mitochondrial antiviral signalling

mt-DNA: mitochondrial DNA

mt-ROS: mitochondrial ROS

N: nucleocapsid protein

OxPHOS: oxidative phosphorylation

PTE: pulmonary thromboembolism

RBM: receptor binding motive

RNA: ribonucleic acid

ROS: rea**ctive oxygen species**

S: spike protein

SARS-CoV-2: severe acute respiratory syndrome coronavirus 2

ssRNA+: positive sense, single-stranded RNA

TFAM: mitochondrial transcription factor A

TMPRSS2: transmembrane protease factor A

TNF-α: tumor necrosis factor-alpha

TOS: total oxidant status

WHO: world health organization

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ABSTRACT

Upon SARS-CoV-2 infection, patients may suffer diverse clinical manifestations, which translates into different COVID-19 severity degrees. COVID-19 severity is closely related to the inflammatory and oxidative stress. Higher levels of inflammation are associated with a poor prognosis in COVID-19. However, the quantification of Reactive Oxygen Species (ROS) levels and their correlation with inflammation based on different COVID-19 severity remains unclear. The study cohort was comprised of 42 COVID-19 patients classified into mild, moderate and severe according to symptomatology. Plasma samples were analysed to measure pro-inflammatory cytokines related to altered mitochondrial function (IL-6, IL-1β and TNF-α) and Total Oxidant Status (TOS). Our findings revealed significantly higher levels of IL-6, IL-1β and TOS in severe patients, although no correlation was detected between these three cytokines and TOS. Furthermore, both TOS and IL-6 were significantly positively correlated with age and weight, while IL-6 was also higher in male patients, independently of COVID-19 severity. They correlated significantly with other clinical parameters, such as Lactate dehydrogenase (LDH), C-Reactive Protein (CRP), neutrophil or lymphocyte count. These results show that severe COVID-19 is characterized by heightened inflammation and oxidative stress, with demographic factors such as age, sex or weight influencing disease severity.

1. INTRODUCTION

The emergence of COVID-19 posed a great challenge for global health due to its rapid spread and infectious nature. The first outbreak was in December 2019, several cases of pneumonia with an unidentified etiology were reported in Wuhan, China, most of them clustered in the Huanan market. It quickly reached pandemic status, declared by the World Health Organization (WHO) on January 30th, 2020. Later, in March 2020, it escalated to the status of a global pandemic (1,2). Since then, almost 800 million cases have been reported, and over 7 million people have died worldwide (3).

1.1 SARS-CoV-2 etiology

COVID-19 is caused by a virus from the *Coronaviridae* family, originally named 2019-nCoV and later changed to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) after phylogenetic analysis by the International Committee on Taxonomy Viruses. They found that this new virus was related to SARS-CoV, a previously existing virus that provoked a SARS outbreak in 2003 (4).

SARS-CoV-2 has a zoonotic origin, meaning it likely originated in animals and adapted to infect humans. This theory is supported by the fact that SARS-CoV-2 has a 96% genomic similarity to a bat-derived betacoronavirus identified in 2013 (RaTG13). Furthermore, the receptor-binding motif (RBM), key to host infection, of COVID-19 displays considerable sequence homology with β-coronaviruses isolated from malayan pangolins. This evidence then suggests a possible evolutionary pathway for SARS-CoV-2, beginning in bats and involving recombination in intermediate mammalian hosts, such as pangolins, prior to human transmission (5).

1.2 SARS-CoV-2 structure and infection mechanism

The SARS-CoV-2 genome comprises a positive-sense, single-stranded RNA (ssRNA+) molecule of 26–32 kilobases (kb) and it is surrounded by the nucleocapsid, which is itself encased in a lipidic envelope. This genome encodes for several proteins including the major structural ones, such as, the membrane protein (M), envelope protein (E), nucleocapsid protein (N) and the spike protein (S) (1,6).

The M protein is the most abundant structural protein and gives shape to the virus, it also plays a key part in the assembly process after infection, and it is also an important immunogenic agent (7). The E protein is highly expressed during viral replication in the interior of the cell. It is located in crucial protein trafficking points, such as the endoplasmic reticulum or the Golgi apparatus. While only a minor portion of the production is ultimately

incorporated into the viral envelope, a significant aspect of the E protein's impact stems from its ability to form ion channels or viroporines, a process that can trigger cell death and cytokine storm (7,8). The N protein interacts with the virus' RNA, forming the nucleocapsid. This binding creates a ribonucleoprotein complex, vital for maintaining the genome organized as needed for replication and transcription (7). Lastly, the S protein, a transmembrane protein, is essential for mediating viral entry and its arrangement in the envelope gives the virus its distinctive crown-like appearance (Figure 1A). It is comprised of 2 subunits: S1 and S2, critical for membrane fusion after cleavage (6,7).

The S protein of SARS-CoV-2 interacts with the host's cell membrane receptor, angiotensin-converting enzyme 2 (ACE2) (9). ACE2 is an enzyme, and a receptor present in most tissues and is especially abundant in the lower respiratory tract, lung, heart, ileum, oesophagus, kidney and bladder, which could explain the variety of symptoms (10).

This mechanism is not only dependent on the ACE2 receptor, but also on the subsequent cleavage of the S2 subunit, which determines the two primary pathways for viral entry: I) endocytosis and II) membrane fusion. The endocytosis pathway requires virus internalization, which occurs after the S protein binds to ACE-2. Once the virus is endocytosed, the cleavage process of the S protein mediated by cathepsin-L, can occur in the acidic endolysosomes (Figure 1B) (9). Alternatively, the membrane fusion pathway, doesn't involve cellular ingestion of the virus. The transmembrane protease serine-2 (TMPRSS2) primes the virus splicing the S2 subunit and enabling the fusion of the viral envelope with the host cell membrane (Figure 1C) (9). Furthermore, studies suggest a broader distribution and expression of TMPRSS2 compared to ACE2, suggesting that the abundance of the ACE2 receptor might be the limiting factor for SARS-CoV-2 entry, particularly during the initial stages of infection (5).

In the end, both pathways expose the fusion peptide (FP), crucial for membrane fusion and viral RNA release (9).

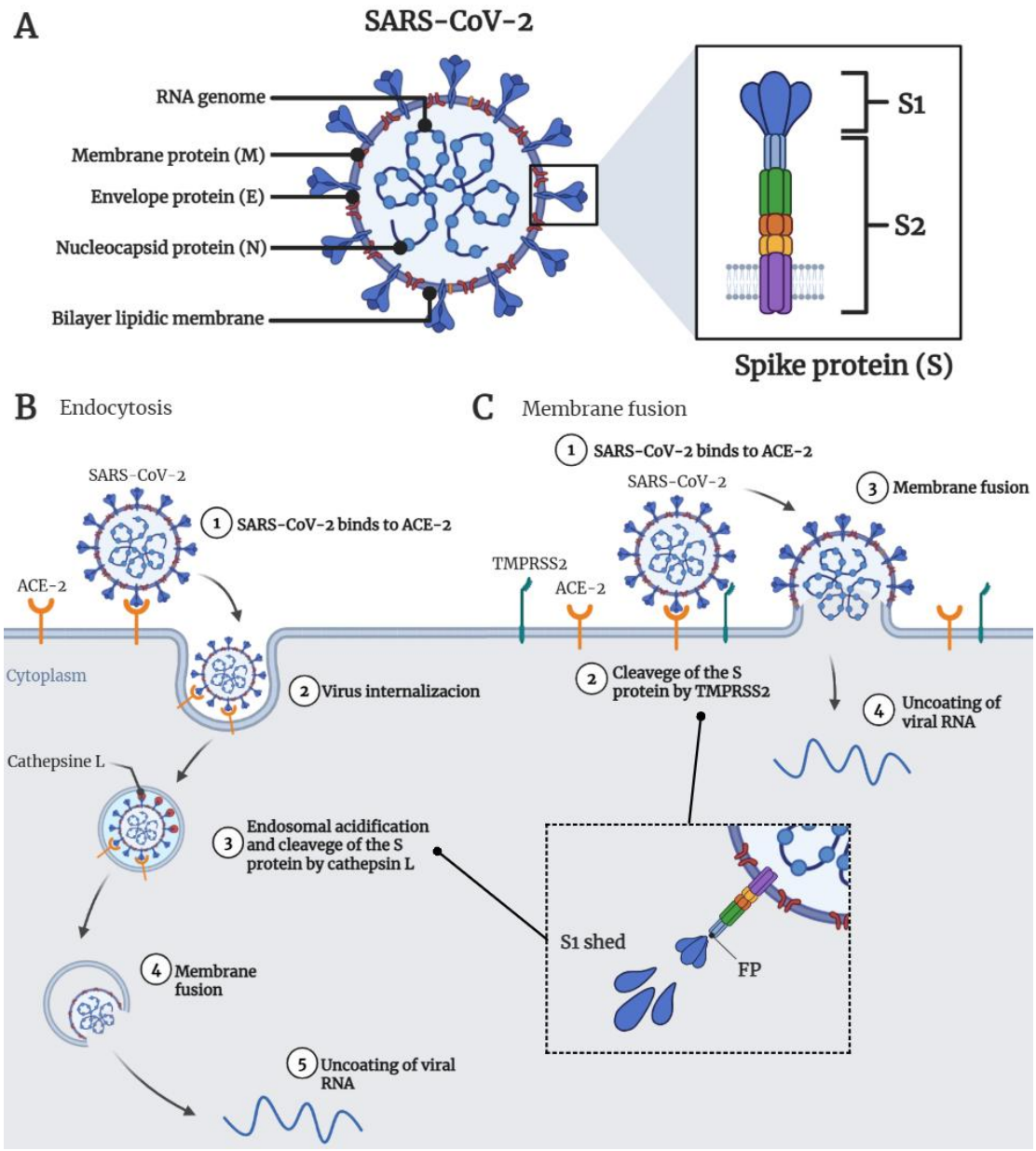

Figure 1: **Structure and mechanisms of infection of SARS-CoV-2. A)** Illustrates the structure of the SARS-CoV-2 virus. The RNA genome enclosed within the nucleocapsid protein (N), surrounded by the membrane protein (M), envelope protein (E), and embedded within the bilayer lipidic membrane. On the left there is a magnified view of the Spike protein (S), which protrudes from the viral surface, giving it a crown-like appearence. The Spike protein is further divided into two subunits, S1 and S2. **B)** SARS-CoV-2 entry via endocytosis: it occurs intracellularly and is mediated by cathepsin-L in an acidic environment. **C)** SARS-CoV-2 entry via membrane fusion: TMPRSS2 at the surface of the cell cleaves the S protein's S2' site, enabling fusion. Both pathways have the same outcome, exposing the FP and triggering membrane fusion. *Adapted from (9) in BioRender.*

1.3 SARS-CoV-2 transmission

Viral transmission from human to human mostly occurs via contact with contaminated surfaces and inhalation of aerosol particles that contain the virus. Day to day activities like talking, breathing, coughing or singing release these liquid suspensions of the virus from the epithelial lining fluid. Healthy individuals may become infected by breathing these droplets or by touching contaminated surfaces (clothes, doorknobs etc.) and then contact with their oral, eye or other mucous membranes. Less common routes of virus spread include saliva, urine or faces (11–13). In severe ill patients, viral load and longevity of the virus in these drops are higher. Furthermore, several variants, such as Alpha, exhibit increased efficiency in aerosol transmission (11,13).

1.4 Symptomatology

COVID-19's wide-ranging symptoms, from mild to severe, vary significantly between individuals and regions (14). The most common clinical manifestations include fever, dyspnea, fatigue, headache, and muscle pain. While mild patients may present with a dry cough, critically ill individuals can also develop severe complications such as severe pneumonia that can lead to acute respiratory distress syndrome (ARDS) development (15). Less common manifestations that have been reported include nausea or vomiting, dizziness, rhinorrhea, chills, diarrhea, and liver or kidney damage (15,16)Accurate understanding of this diverse symptomatology is essential for early detection and effective care. Several demographic factors have a detrimental effect on the severity of COVID-19, such as age, sex or pre-existing comorbidities (17). Advanced age poses a significant risk due to the progressive decline of the immune response, a process known as immunosenescence (18). This decline impairs the body's ability to inhibit viral replication and infection, weakening antigen-specific immunity, and consequently increasing mortality (18). Furthermore, it has also been described that viruses tend to replicate more effectively in older, worn-out or senescent cells that have weak and low-capacity mitochondria, suggesting that aging is a key factor for severity in COVID infection (18). The higher prevalence of comorbidities in older populations, heightens their risk of developing more severe health outcomes (19).

Common comorbidities like diabetes and obesity, have been found to impact both innate and adaptive immune responses (20). The chronic inflammation inherent in these conditions creates a basal inflammatory state, leading to a cytokine imbalance that worsens during infection (20). Consequently, these patients are more likely to develop grave consequences like ARDS or sepsis (21). Several mechanisms associated with these comorbidities could explain the severe symptomatology that most of these patients suffer when infected with

SARS-CoV-2. For instance, ACE2 levels in adipocytes correlate with nutritional status and high lipid accumulation, which facilitates raft formation, promoting viral entry and providing essential building blocks for viral capsid assembly (22). Furthermore, clinical data suggests a close association between pre-existing cardiovascular diseases (CVD) and both increased susceptibility to SARS-CoV-2 infection and adverse COVID-19 outcomes. The prevalence of pre-existing CVD is significantly higher in severe cases and fatalities, highlighting its potential as a predictor of poor prognosis (15,23). Specifically, in hypertensive patients, several metabolic pathways are compromised. These include, stress responses and blood coagulation, as well as the metabolic pathways of ascorbate and aldarate and linoleic acid metabolism. Identifying plasma biomarkers, like myo-inositol or gelsolin, is crucial for anticipating worse clinical outcomes in these patients and understanding the shared underlying mechanisms between hypertension and COVID-19 (24).

One way to classify COVID-19 symptomatology is in pulmonary and extrapulmonary manifestations.

1.4.1 Pulmonary manifestations

The most common pulmonary manifestation in severe COVID-19 is pneumonia, an inflammatory reaction to infection targeting the alveoli and distal bronchial tree. This COVID-19 pneumonia can subsequently lead to hypoxia and, in some cases, progress into acute respiratory distress syndrome (ARDS), the most common and critical complication in severe patients (16,25,26).

ARDS in COVID-19 patients is frequently caused by altered surfactant levels and the cytokine storm. Surfactant, a mixture of lipid and proteins (27), is synthesized by type II pneumocytes in the alveoli with the aid of ACE2 (28). This substance plays a critical role in reducing air-liquid surface tension in the lungs, and infiltration of SARS-CoV-2 via the ACE2 receptors decreases this production and increases the risk of suffering ARDS (28). The elevated expression of ACE2 in the lungs of older individuals is associated with a greater risk of ARDS which could explain the higher COVID-19 mortality rate in this demographic (15).

1.4.2 Extrapulmonary manifestations

In addition to respiratory symptoms, there are also extrapulmonary manifestations, probably due to the presence of ACE2 in different tissues, making those cells more prone to SARS-CoV-2 infection (29).

Among these, cardiovascular manifestations are notable. Some patients present symptoms such as palpitation and chest distress, indicating early cardiovascular manifestations. The spectrum of potential cardiovascular complications in COVID-19 encompasses myocardial injury (20% of hospitalized patients), palpitations (10% of patients) (30), heart failure and coagulation abnormalities (15,23).

Gastrointestinal issues also commonly arise in COVID-19 patients. According to a systematic review (31), diarrhea was the most reported with 10% (95% CI 8%-12%) of patients across 93 studies. Other symptoms included nausea (6% [95% CI 3%-10%]; 27 studies), vomiting 4% [95% CI 2%-8%]; 26 studies) and abdominal pain (4% [95% CI 2%-7%]; 19 studies). These gastrointestinal issues, can often precede respiratory symptoms, complicating diagnosis and potentially leading to increased transmission in clinical settings (32). Gastrointestinal symptoms are an effect of the infection of epithelial cells in the mucous layer. Pancreatic and liver injury may also be an issue in COVID infection due to the especially high expression of ACE-2 in these tissues (15).

Furthermore, neurological manifestations are frequently reported in SARS-CoV-2 infection A meta-analysis that included 350 studies (33) identified the prevalence of several neurological manifestations in SARS-CoV-2 infection. Some of the most common are, fatigue (32% [95% CI 30%–35%]; 169 studies), anosmia (19% [95% CI 13%–25%]; 51 studies), headache (13% [95% CI 12%–15%], 202 studies) or dizziness (7% [95% CI 5%–8%]; 46 studies). Other symptoms include delirium (11% [95% CI 7%–16%]; 19 studies), agitation (45% [95% CI 3%–93%]; 3 studies) or seizures (4% [95% CI 2%–6%]; 2 studies) (15,34).

1.4.3 Severity based on the World Health Organization (WHO) criteria

To ensure consistent and reliable organization and categorization of patient data collected during the early pandemic stages, the World Health Organization (WHO) developed a standardized measurement set. This set aimed to capture patients' evolution, and the associated resources needed throughout their care. Some of the main outcomes that were thought necessary to be included for uniform assessment were the presence of organ dysfunction, levels of biochemical parameters and the duration of intervention (e.g., ventilation, organ support) among others (35,36).

This set is divided into three main points: viral load, all-cause mortality at the time of hospital discharge or within 60 days after and the patient's clinical progression, measured with the WHO Clinical Progression Scale (WHO-CPS). The WHO-CPS assesses the impact of illness by tracking patients' outcomes across their healthcare experience (35,37).

Effectively the criteria used to classify patients is the following: the patient is mild (in green) if they have 1 or 2 symptoms, no O_2 requirement and no pneumonia, moderate (in orange) when they have pneumonia and O_2 requirement and severe (in light red) if the pneumonia is grave and needs intubation or vasopressors (Table 1)

Table 1: Classification of COVID-19 patients based on the WHO clinical progression scale (37).

PATIENT STATE	DESCRIPTOR	SCORE
Uninfected	No clinical or virological evidence of infection	0
Ambulatory: Mild	No limitation of activities	1
	Limitation of activities	2
Hospitalized: Moderate	Hospitalized, no oxygen therapy	3
	Oxygen by mask or nasal prongs	4
Hospitalized: Severe	Non-invasive ventilation or high-flow oxygen	5
	Intubation and mechanical ventilation	6
	Ventilation + additional organ support (pressors, RRT, ECMO)	7
Death	Death	8

1.5 SARS-CoV-2 and inflammation

Upon infection, the immune system is activated, leading to an inflammatory response. Inflammation is a complex biological response of the body's immune system to harmful stimuli, such as pathogens, damaged cells or irritants. Its primary role is to eliminate the initial cause of cell injury and initiate repair (38).

During the early stages of SARS-CoV-2 infection, the innate immune response, actively combats the virus. In the subsequent stages, the adaptive response begins to take over, becoming the primary force, with specialized cells like T and B cells targeting the virus (39). As part of this immune response, cytokines that are generally released include, IL-7, IL-10, IL-15 or IFN-γ (40), other cytokines like, IL-17, IL-1-β and IL-6 are specifically involved in the recruitment of pro-inflammatory cells to the site of infection (41).

If the immune response becomes dysregulated, it triggers the excessive release of pro-inflammatory cytokines and the shift to a more innate immune response (39). Particularly, severe and critical COVID-19 cases are characterized by increased levels of IL-6 and IL-10

(41), leading to grave symptoms in patients such as severe pneumonia, ARDS and multi-organ failure (39,42).

The reasons behind this imbalance are varied and include the individual's genetic susceptibility, the amount of virus present in the body or pre-existing health issues (39,43). For example, patients with a previous history of CVD show a very poor prognosis, the development of a cytokine storm significantly contributes to multi-organ damage, which frequently involves the cardiovascular system (23).

This hyperactivation of the immune system after infection leads to an overexpression and release of cytokines, mainly IL-6, TNF-α, and IL-1β, an increase in neutrophil count and a decrease in lymphocyte count (39). Understanding the mechanisms by which a normally protective immune response transforms into a detrimental one, represents a pivotal aspect in determining the severity of the condition (39,43).

A key element is the suppression of type I interferon (IFN-I) responses. Normally, IFN-I, specifically IFN- α and IFN-β, limits viral propagation, enhances antigen presentation and primes T and B cell responses. However, in severe COVID-19, this IFN-I production is compromised and IL-6 and IL-1β increase (15,18,34).

IL-6 plays a major role in COVID-19 progression. This cytokine is synthesized by a wide range of cells, including immune cells like macrophages, monocytes, T or B cells but also by non-immune cells such as fibroblasts or vascular endothelial cells (44). Its production is rapidly induced by infections and tissue injuries but, abnormally high levels of IL-16 lead to organ damage, ARDS and other severe complications, making it a significant marker for COVID-19 severity and progression (45). IL-1β is another inflammatory cytokine, with significantly higher levels in severe COVID patients. It is part of the IL-1 family and it is key for the regulation of the immune response. SARS-CoV-2 seems to be able to control the production of IL-1β, consequently triggering other cytokines. Its over-production is associated with poorer outcomes in COVID-19 patients (46,47). Another pivotal cytokine in COVID-19 is tumor necrosis factor-alpha (TNF-α) (48). It can be synthesized by macrophages and monocytes but also T cells NK cells or endothelial cells. TNF- α is an early cytokine that mediates inflammatory responses and promotes immune cell infiltration. In severe COVID-19 patients, abnormally high levels of this protein are common, especially in the lungs, contributing to the risk of ARDS (47,48).

The dysregulated secretion of cytokines is also often exacerbated by pre-existing health issues, which positively correlate with severity and mortality rate (49). For example, (49) reported that patients with a history of high blood pressure (17%), hyperglycaemia (8%)

and heart disease (5%) are more prone to severe complications compared to patients without any pre-existing medical conditions (15,23).

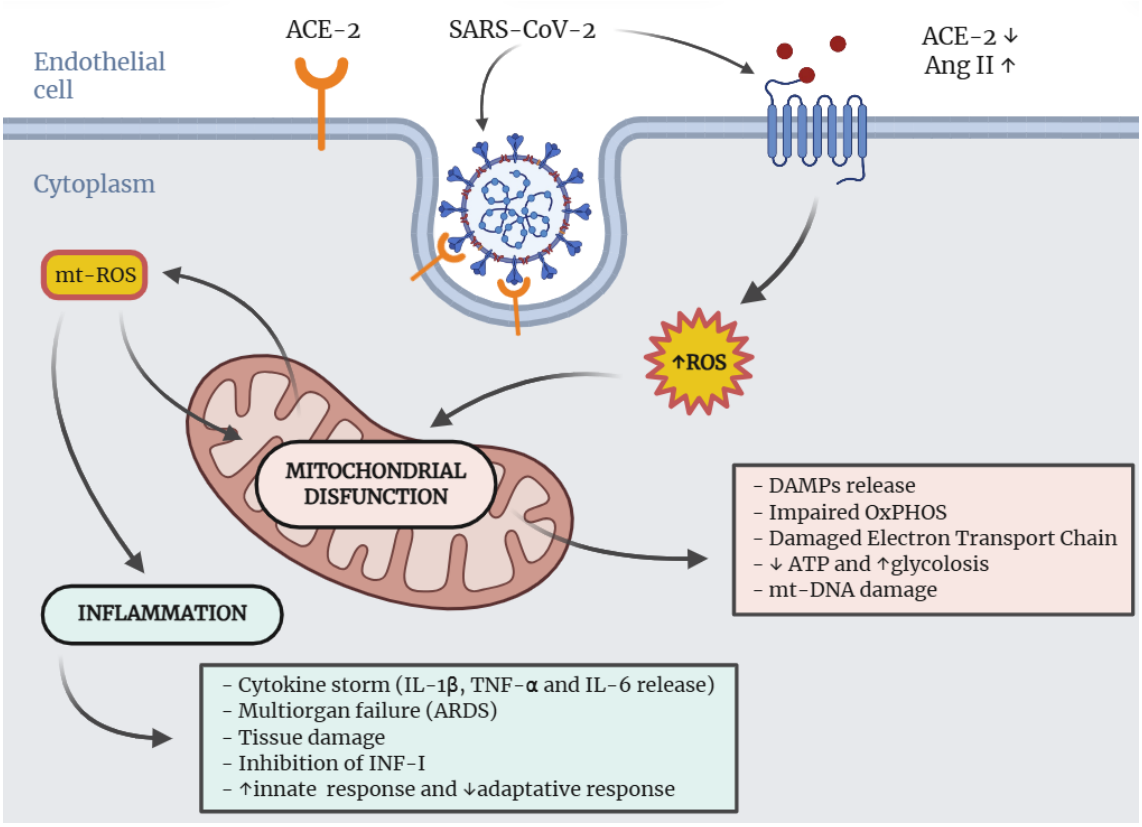

Figure 2: Schematic representation illustrating the interplay between SARS-CoV-2 infection, ACE2 receptor interaction, mitochondrial dysfunction, oxidative stress and inflammation within an endothelial cell, ultimately contributing to severe manifestations of COVID-19. *Created with Biorender*.

1.6 Immunometabolism and the inflammatory response

Mitochondria are essential organelles that maintain homeostasis by synthesizing ATP through oxidative phosphorylation and serving as a vital source of reactive oxygen species (ROS). They are fluid, changing morphology in response to physiological and pathological stimuli, affecting their function (50). The complex relation between cellular metabolism and the immune system is termed, immunometabolism and it is crucial for immune cell activation and effector functions (51).

The equilibrium of immunometabolism is significantly perturbed during SARS-CoV-2 infection (51). Mitochondrial function is also heavily affected by SARS-CoV-2 infection promoting the hyperproduction of mitochondrial ROS (mt-ROS). This mitochondrial dysfunction has been correlated with the exacerbated inflammatory response observed in severe COVID-19 individuals (50,52) (Figure 2). Understanding these changes may be key

to comprehending the different immune responses associated with severity in COVID-19 patients.

During viral infection, immune cell activation triggers significant metabolic reprogramming. T cells, for example, heavily rely on mitochondrial functionality and after T-cell receptor (TCR) engagement, a metabolic reprogramming takes place. This change is characterized by the activation of OxPHOS, generating ATP to fuel its proliferation and activation. This metabolic transformation supports the TCR signalling machinery and the nucleic acid synthesis necessary for quiescent T cells to transition into a proliferative state (18). OxPHOS activity is indispensable for the rapid reactivation of memory CD8+ T cells. These cells characterized by a high respiratory capacity and abundant mitochondrial mass, making them able to rapidly respond after antigen re-encountering. An altered OxPHOS translates as weakened immune memory and slower response in re-exposure to infection(18). In general, mitochondrial activity appears to be crucial for lymphocytes to effectively combat viral infections and damaged mitochondria in these cells weakens such defence by contributing to chronic inflammation, the cytokine storm and the inhibition of IFN-I (18,53).

Consequently, a vicious cycle is created. Inflammation provokes mitochondrial dysfunction, which in turn fuels inflammation (54). For instance, IL-1β and TNF-α can damage mitochondrial DNA (mt-DNA) and IL-6 can induce the expression of mitochondrial fission proteins and contributes to decreasing mitochondrial respiration in cells. Conversely dysfunctional mitochondria release the mtDNA, which act as damage-associated molecular patterns (DAMPs), which further activate innate immune pathways and promote inflammation (52).

1.7 Mitochondrial dysfunction and oxidative stress

ROS are chemically reactive molecules that encompass both free radical and non-radical products generated during normal metabolic processes within various organelles. While often associated with cellular damage under conditions of oxidative stress, ROS play crucial roles, including host defence, cellular signalling pathways or energy production in the mitochondria. Is in this last organelle, where most of ROS production takes place (55).

Upon viral infection, including SARS-CoV-2 infection, ROS production exerts an antiviral effect by activating immune cells and inhibiting viral replication (54). However, the equilibrium between oxidation and reduction reactions, often referred to as the "redox status," undergoes a significant disruption in the context of severe COVID-19. This imbalance is characterized by an overproduction of ROS and a diminished antioxidant capacity, with key antioxidant enzymes such as peroxiredoxin 1, superoxide dismutase 1

and glucose-6-phosphate dehydrogenase, getting significantly reduced (22,54) (Figure 2). This results in redox irregularities and is an important element to the mechanisms underlying the development of severe clinical manifestations associated with COVID-19 (54).

These metabolic shifts in immune cells caused by SARS-CoV-2 infection contribute directly to the production of ROS, establishing a link between immunometabolism and oxidative stress, adding to the severity of this dysregulation. H_2O_2 amplifies pro-inflammatory cytokines production by activating the NF-κB pathway, which primes macrophages, neutrophils and endothelial cells. The process is facilitated by NADPH, which generates even more free radicals and supporting viral replication (54,56).

Mitochondria are recognized as the main source of intracellular oxidants, also referred as mt-ROS, both during normal cell function and when redox control is disrupted. The delicate redox balance is susceptible to various factors, being alterations in normal mitochondrial dynamics a particularly significant one (54,57). Mitochondria is also a key component in the endocytic pathway that enables SARS-CoV-2 replication and survival. Given their central role in numerous interconnected pathways disrupted by COVID-19, mitochondrial functions and immunometabolism are recognized as key targets for many existing viruses. Also, +RNA viruses are known to utilize host intracellular membrane structures, including mitochondria, as platforms for RNA replication. This viral manipulation leads to metabolic disruptions, including energy deficits, dysregulation of ROS levels, and a shift towards aerobic glycolysis, ultimately benefiting viral propagation (53,58,59). Damaged mitochondria also act as pivotal sources of DAMPs, including cardiolipin, n-formyl-peptides, mitochondrial transcription factor A (TFAM), mt-ROS or mt-DNA. These molecular cues, are similar to bacterial components and therefore trigger a strong NLRP3 activation, initiating downstream inflammatory cascades (18,53). Interestingly, in non- immunological cells, mitochondria is the major producer of ROS (mt-ROS), conversely, in immune cells, the major sources of ROS are NADPH oxidase and xanthine oxidase (56).

Additionally, the mitochondrial antiviral signalling (MAVS) protein's function is intrinsically tied to an intact mitochondrial membrane potential and OXPHOS activity. Consequently, mitochondrial dysfunction directly impairs MAVS signalling, compromising the initial antiviral alarm system and increasing viral replication (18,60).

During SARS-CoV-2 infection, the redox balance status contributes to endothelial dysfunction and thrombosis, potentially causing multi-organ damage, including long-term lasting tissue damage and in critical cases, ARDS, as mentioned before. The resulting oxygen

deprivation in other tissues intensifies cellular stress, triggers inflammatory cascades, amplifies cytokine release and activates immune cells (Figure 3).

Although the role of ROS in tissue damage has been described, there is a lack of clarity regarding the specific levels of circulating ROS and how they relate to pro-inflammatory cytokines in the context of mitochondrial dysfunction across COVID-19 levels of severity.

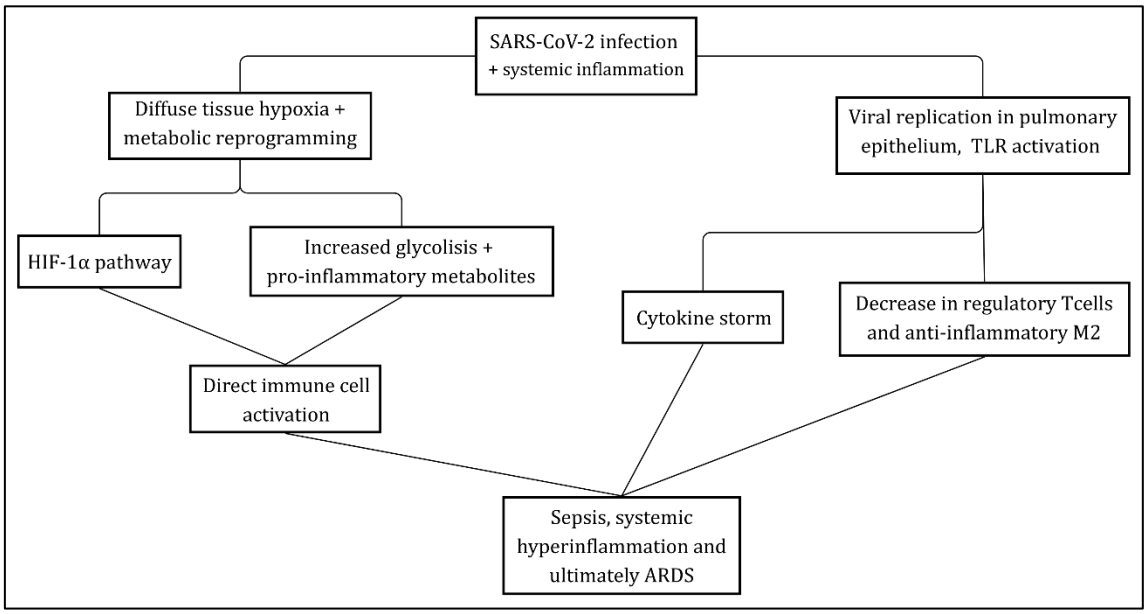

Figure 3: How SARS-CoV-2 infection and systemic inflammation can lead to sepsis, systemic hyperinflammation and ultimately ARDS. *Adapted from (21)*.

2. HYPOTHESYS AND OBJECTIVES

COVID-19 patients may suffer from a broad spectrum of clinical manifestations, resulting in varying degrees of COVID-19 severity. Preliminary results from the INIM group indicate that immune system cells from COVID-19 patients, exhibit different mitochondrial activity across severity levels. These results show mitochondrial dysfunction in the immune cells of severe patients, inducing high levels of inflammation and oxidative stress.

Based on these findings, we hypothesized that critically ill patients, characterized by complex clinical symptoms, may experience mitochondrial stress and dysfunction, which could result in elevated levels of ROS and pro-inflammatory cytokines in plasma, whereas in mild patients, these molecules are balanced, causing a controlled immune response with fewer symptoms.

The main objective is to characterize plasma ROS levels and correlate them with different levels of inflammation based on COVID-19 severity.

The secondary objectives are:

I) To characterize the COVID-19 patients' cohort, based on clinical data analysis.

II) To assess the inflammatory status by measuring proinflammatory cytokine levels (IL-6, TNF-α, and IL-1β) in plasma

III) To evaluate the levels of systemic oxidative stress by measuring Total Oxidative Status in plasma

IV) To evaluate the possible relationship of ROS and pro-inflammatory cytokine levels with clinical and demographic characteristics

3. MATERIALS AND METHODS

3.1 Characterization of participants

The preliminary study's cohort was comprised of 42 patients infected by SARS-CoV-2 confirmed by reverse transcriptase-polymerase chain reaction technique. The participants were recruited during the first to third waves (March 2020 to February 2021) at "Hospital universitari Joan XXIII, Tarragona". Individuals were not vaccinated against SARS-CoV-2 at the time of blood sampling. These patients were classified into three groups according to severity following the WHO's clinical progression scale (37) (Table 1). Mild patients had some symptoms but did not suffer from pneumonia or respiratory-related manifestations (WHO 1-2; n=12), moderate patients had moderate pneumonia and might have required O_2 (WHO 3-4; n=17) and severe patients had pneumonia and more grave clinical complications and needed intubation or additional organ support (WHO 5-7; n=13) (Figure 4). To determine the presence of pneumonia, pulmonary radiographic imaging was used. Relevant clinical data on the disease progression was collected in a specially designed database that included demographic information, comorbidities and risk factors, previous treatments and treatment during admission and symptoms.

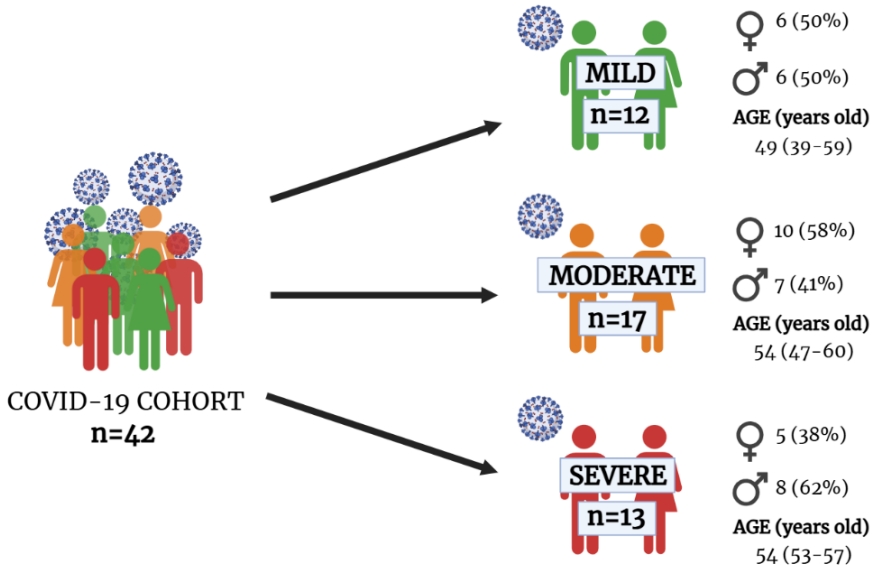

Figure 4. Study cohort and groups: preliminary study cohort is comprised by42 COVID-19 patients divided into 3 groups based on severity following the WHO's clinical progression scale: mild (n=12, in green), moderate (n=17, in orange) and severe (n=13, in red). Each group is further characterized by number and percentage (%) of participants by sex, as well as the age (years old) with the median (25^{th}-75^{th} interquartile range). *Created with Biorender*.

3.2 Sample collection

Clinical evaluation, blood cell counts and biochemical parameters were assessed at the time of admission. Serum samples were then stored at –80 Cº at the facilities of the "BioBank - Institut de'Investigació Sanitària Pere Virgili (IISPV)" for future use.

3.3 Ethics

Ethical and scientific oversight for the protocols was provided by the respective institutional committees. Following the Declaration of Helsinki, this study's approval was granted by the committee for ethical clinical research, operating under the principles of Goo clinical practice at the IISPV (079/2020, CEIm IISPV). This independent committee, ensures methodological, ethical and legal compliance for all clinical trials and research endeavours. Written informed consent was secured from patients or their relatives, in alignment with the declaration of Helsinki.

3.4 Measure of plasma cytokines

Enzyme- linked immunosorbent assay (ELISA) kits from Thermo Fisher Scientific were used to analyse plasma samples from COVID-19 patients for the concentration of IL-1β (#BMS224HS), TNF-α (#BMS223-2HS), and IL-6 (#EH2IL6). Contrary to the standard protocol, the samples were not diluted in any of the three assays. Prior to main analysis, preliminary assays, conducted to determine optimal detection range, revealed that the undiluted samples were the most optimal concentration for reliable results.

3.5 Measure of plasma Total Oxidative Status

Total Oxidant Status (TOS) Colorimetric Assay Kit (EEA027) from Thermo Fisher Scientific was used for TOS quantification in plasma. This kit measures the total oxidative state of a sample, which is relevant because individual oxidant molecules are difficult and expensive to measure. Under acidic conditions, oxidizing agents in the sample convert Fe^{2+} to Fe^{3+}, which then forms a blue-purple complex with xylenol orange. The intensity of this colour is measured at 590 nm and is directly proportional to the concentration of oxidizing substances. The results are expressed in μmol/L Equally to the previously mentioned ELISA kits, samples were not diluted, based on preliminary testing.

3.6 Data Processing and statistical analysis

Non-parametric methods were used in this study. Qualitative variables such as sex, COVID-19 symptoms, risk factors and comorbidities were assessed using Chi-squared ($\chi 2$) test, while quantitative variables such as age, haematological and coagulation parameters and

inflammatory and biochemical markers, were analysed via Kruskal-Wallis test. Data was presented as “number (%)” and “median (25^{th}-75^{th} interquartile range)” respectively.

For statistical analysis of ELISAS, raw data underwent a $\log_{10}$ transformation to ensure normality and ANOVA with post-hoc Tukey was applied. For TOS data, statistical differences between severity groups were determined using the Kruskal-Wallis test. In instances where a significant difference was observed, Mann-Whitney’s test was applied.

Correlation analysis between levels of cytokines and ROS and several characteristics of the cohort was assessed. Correlation between quantitative variables and inflammatory results was performed by Point-biserial correlation analysis. For correlations between quantitative variables, Spearman’s correlation test was used. Significant correlations were represented with scatterplots for a visual distribution of each group’s data. P-values <0.05 were considered significant. All statistical analysis and data processing were performed using SPSS version 15.0 for windows and Microsoft excel. Graphical plots were performed with GraphPad prism 9 and SPSS version 24.0.

4. RESULTS

4.1 Description of the cohort

The cohort comprised 42 patients with COVID-19, categorized into three severity groups based on the WHO clinical progression scale: mild (n = 12), moderate (n = 17) and severe (n = 13). Although the median ages of the groups were comparable, the mild group showed the lowest median age at 49.5 years while the moderate and severe groups had similar median ages, 54 and 53.5 years, respectively. Regarding sex distribution, women were more prevalent in the moderate group (58.8%), followed by the mild group (50%). Men comprised most of the moderated group, with 61.5%, but these differences in sex distribution were not significant (Table 2).

Weight was significantly different between groups (p = 0.031) increasing from mild (76 kg) to severe (107 kg) patients. Consequently, a similar trend can be observed with the BMI (p = 0.013). Regarding previous comorbidities, hypertension prevalence was significantly higher in the severe group, reaching 61.5% (p = 0.042) (Table 2). Notably, exercise, a risk factor in its absence, had a prevalence of 20% in the severe group compared to 66,7% and 80% in the moderate and mild groups (p = 0.009) (Table 2).

Table 2: Demographic data and previous characteristics of the cohort classified by COVID-19 severity. Data are presented as "n (%)" or "median (25th -75th IQR)". Statistical: Kruskal-Wallis test and $\chi 2$ in SPSS. P-values < 0.05 were considered significant and are indicated in bold.

	Mild (n = 12)	Moderate (n = 17)	Severe (n = 13)	p-value
	DEMOGRAPHIC DATA			
Sex (woman)	6 (50.00%)	10 (58.80%)	5 (38.50%)	0.438
Age (years)	49.5 (39-59.5)	54 (47-60)	53.5 (53-57)	0.484
Weight (kg)	76 (57-88)	79.5 (72-98)	107 (80-118)	**0.031**
Height (m)	1.70 (1.57-1.78)	1.67 (1.64-1.69)	1.71 (1.59-1.8)	**0.009**
BMI (kg/m²)	24.22 (22.48-28.48)	29.64 (26.92-32.27)	35.20 (26.22-41.5)	**0.012**
	COMORBIDITIES			
Dyslipemia	2 (16.70%)	3 (17.60%)	5 (38.50%)	0.404
Hypertension	2 (16.70%)	6 (35.30%)	8 (61.50%)	**0.041**

Cardiovascular Problems	1 (8.30%)	2 (11.80%)	1 (7.70%)	0.899
Chronic Obstuctive Pulmonary Disease (COPD)	1 (8.30%)	1 (5.90%)	1 (7.70%)	0.967
Cancer	1 (8.30%)	0 (0.00%)	1 (7.70%)	0.498
Obesity				
Obese	2 (18.20%)	7 (46.70%)	6 (60.00%)	0.092
BMI <25	7 (63.60%)	2 (13.30%)	2 (20.00%)	
BMI 25-35 (overweight)	4 (36.40%)	8 (53.30%)	3 (30.00%)	
BMI >35 (obesity)	0 (0.00%)	5 (33.30%)	5 (50.00%)	
		HABITS AND RISK FACTORS		
Alcohol	3 (27.30%)	3 (18.80%)	3 (30.00%)	0.830
Exercise	8 (80.00%)	10 (66.70%)	2 (20.00%)	**0.009**
Smoker				
Yes	0 (0.00%)	2 (11.80%)	0 (0.00%)	0.404
Ex-smoker	2 (18.20%)	4 (23.50%)	1 (10.00%)	
		PREVIOUS TREATMENTS		
Antihypertensives	2 (16.70%)	6 (35.30%)	6 (46.20%)	0.287
Lipid-lowering drugs (statins)	3 (25.00%)	4 (23.50%)	2 (15.40%)	0.811
Lipid-lowering drugs (fibrates)	1 (8.30%)	0 (0.00%)	0 (0.00%)	0.277
Diabetes Treatment (insulina)	0 (0.00%)	0 (0.00%)	1 (7.70%)	0.318
Diabetes Treatment (oral)	1 (8.30%)	1 (5.90%)	1 (7.70%)	0.964
Analgesics	1 (8.30%)	2 (11.80%)	4 (30.80%)	0.252
Opioid Analgesics	0 (0.00%)	1 (5.90%)	1 (7.70%)	0.639
Antacids	1 (8.30%)	0 (0.00%)	0 (0.00%)	0.277

Antibiotics	0 (0.00%)	3 (17.60%)	1 (7.70%)	0.270
Antibiotics and hydroxychloroquine	0 (0.00%)	3 (17.60%)	0 (0.00%)	0.092
Antiaggregate	0 (0.00%)	2 (11.80%)	0 (0.00%)	0.213
Antiarrhythmics	0 (0.00%)	0 (0.00%)	1 (7.70%)	0.318
Oral Anticoagulants	0 (0.00%)	1 (5.90%)	2 (15.40%)	0.317
Antidepressants	1 (8.30%)	2 (11.80%)	2 (15.40%)	0.862
Antiemetic	0 (0.00%)	1 (5.90%)	0 (0.00%)	0.470
Antihistamines	1 (8.30%)	0 (0.00%)	1 (7.70%)	0.488
Anti-inflammatory	0 (0.00%)	1 (5.90%)	0 (0.00%)	0.470
Benzodiacepine	3 (25.00%)	2 (11.80%)	2 (15.40%)	0.634
Bisphosphonate	0 (0.00%)	0 (0.00%)	1 (7.70%)	0.634
Adrenergic blockers	0 (0.00%)	1 (5.90%)	0 (0.00%)	0.470
Anticholinergic bronchodilators	0 (0.00%)	1 (5.90%)	0 (0.00%)	0.470
Beta 2 bronchodilators	0 (0.00%)	1 (5.90%)	1 (7.70%)	0.639
Corticosteroids bronchodilators	0 (0.00%)	1 (5.90%)	1 (7.70%)	0.639
Corticosteroids and beta 2 bronchodilators	0 (0.00%)	0 (0.00%)	1 (7.70%)	0.639
Corticosteroids	0 (0.00%)	0 (0.00%)	1 (7.70%)	0.318
Diuretic	0 (0.00%)	1 (5.90%)	1 (7.70%)	0.318
Hypouricemic agent	0 (0.00%)	1 (5.90%)	1 (7.70%)	0.639
Thyroid hormone	1 (8.30%)	2 (11.80%)	1 (7.70%)	0.639
Proton Pump Inhibitors	1 (8.30%)	3 (17.60%)	1 (7.70%)	0.637

BMI: Body Mass Index, ICU: Intensive Care Unit, COPD: Chronic Obstructive Pulmonary Disease

Regarding hospital admissions and COVID-19 symptomatology, hospitalization was required for 100% of severe and moderate patients ($p < 0.001$), with 84.6% of severe cases also admitted to the Intensive Care Unit (ICU) ($p < 0.001$). O_2 saturation was significantly

lower with increasing severity, from 98% in mild patients to 91% in severe ($p < 0.01$). Several symptoms also showed significant differences across severity groups, including dyspnea ($p = 0.005$), respiratory failure ($p < 0.001$) and pneumonia ($p < 0.001$) being more prevalent in the severe group. Conversely, headache ($p = 0.019$) was more frequent in mild patients (41.7%) compared to moderate (5.90%) and severe (7.70%) groups. The most frequently administered treatments for these COVID-19 patients, were hydroxychloroquine ($p = 0.004$), kaletra ($p = 0.010$) and antibiotics, all of which were used more frequently in moderate patients (Table 3).

Table 3: Symptomatology and COVID-19 treatments of the cohort classified by COVID-19 severity. Data are presented as "n (%)" or "median (25th -75th IQR)". Statistical: Kruskal-Wallis test and $\chi 2$ in SPSS. P-values < 0.05 were considered significant and are indicated in bold.

	Mild (n = 12)	**Moderate (n = 17)**	**Severe (n = 13)**	**p-value**
		HOSPITAL ADMISSION		
Hospital Admission	4 (33.30%)	17 (100.00%)	13 (100.00%)	**<0.001**
ICU	0 (0.00%)	0 (0.00%)	11 (84.60%)	**<0.001**
		SYMPTOMS		
Death	0 (0.00%)	0 (0.00%)	2 (15.40%)	0.113
Systolic Blood Pressure (mmHg)	125 (120-131)	132 (114-149.50)	133.5 (122-149)	0.475
Diastolic Blood Pressure (mmHg)	82 (79-89)	86 (72-90.50)	80 (74-80)	**0.049**
O2 Saturation (Admission) (%)	98 (96.50-98.50)	95 (93-97)	91 (88-93)	**<0.001**
Fever	9 (75.00%)	12 (70.60%)	12 (92.30%)	0.291
Cough	5 (41.70%)	13 (76.50%)	8 (61.50%)	0.160
Dyspea	4 (33.30%)	9 (52.90%)	12 (92.30%)	**0.005**
Arthromyalgia	1 (8.30%)	3 (17.60%)	3 (23.10%)	0.653
Asthenia	3 (25.00%)	4 (23.50%)	4 (30.80%)	0.727

Thoracic Pain		2 (16.70%)	5 (29.40%)	0 (0.00%)	0.087
Headache		5 (41.70%)	1 (5.90%)	1 (7.70%)	**0.019**
Anosmia		2 (16.70%)	2 (11.80%)	1 (7.70%)	0.931
Ageusia		2 (16.70%)	1 (5.90%)	2 (15.40%)	0.625
Dysgeusia		0 (0.00%)	2 (11.80%)	0 (0.00%)	0.201
Odynophagia		0 (0.00%)	2 (11.80%)	0 (0.00%)	0.201
Nausea/Vomiting		0 (0.00%)	3 (17.60%)	2 (15.40%)	0.247
Diarrhea		5 (41.70%)	4 (23.50%)	7 (53.80%)	0.160
Respiratory Failure		0 (0.00%)	7 (41.20%)	13 (100.00%)	**<0.001**
Pulmonary Thromboembolism (PTE)		0 (0.00%)	0 (0.00%)	2 (15.40%)	0.113
Pneumonia					
	Yes	0 (0.00%)	16 (94.1%)	13 (100%)	**<0.001**
	Mild Pneumonia	1 (8.30%)	1 (5.90%)	0 (0.00%)	
	Moderate Pneumonia	1 (8.30%)	15 (88.20%)	0 (0.00%)	
	Severe Pneumonia	0 (0.00%)	0 (0.00%)	13 (100.00%)	
			COVID-19 TREATMENT		
Antibiotics		6 (50.00%)	13 (76.50%)	9 (69.20%)	0.320
Hydroxychloroquine		0 (0.00%)	10 (58.80%)	5 (38.50%)	**0.004**
Azithromycin		1 (8.30%)	7 (41.20%)	3 (23.10%)	0.134
Kaletra		0 (0.00%)	9 (52.90%)	5 (38.50%)	**0.010**
Tocilizumab		0 (0.00%)	1 (5.90%)	0 (0.00%)	0.470

Corticosteroids	3 (25.00%)	4 (23.50%)	6 (46.20%)	0.360
Remdesivir	1 (8.30%)	3 (17.60%)	1 (7.70%)	0.637

Regarding immune cell population, lymphocyte counts decreased from mild (26.10%) to severe (17%) groups, conversely, neutrophil levels showed a clear increase trend from 67.30% in mild patients to 74% in severe (table 4). Notably, the levels of D-dimer display a clear peak in the severe groups (795 mg/L) compared to the levels in both mild (433.5 mg/L) and moderate (405 mg/L) groups (Table 4). Gamma-glutamyl transferase, total protein and lactate dehydrogenase significantly increased with the severity of COVID-19 ($p = 0.027$, $p = 0.005$, $p = 0.01$). Moreover, although not statistically significant, troponin levels tended to increase in moderate and severe groups (mild: 2 ng/L; moderate: 4 ng/L; severe: 10 ng/L) (Table 4) Finally, trends in inflammatory markers in this cohort displayed clear changes across severities. The median erythrocyte sedimentation rate (ESR) decreased with increasing severity (mild: 114 mm/h; moderate: 56 mm/h; severe: 41 mm/h). In contrast, ferritin levels increased in the severe group (786 ng/ml) compared to the mild (423 ng/ml) and moderate (358 ng/ml) groups. A similar pattern was observed for C-reactive protein (CRP), with higher levels in the severe group (6.1 mg/ml) compared to the mild (4.3 mg/ml) and moderate (4.7 mg/ml) groups (Table 4).

Table 4: Biochemistry characteristics of the cohort classified by COVID-19 severity. Data are presented as "n (%)" or "median (25th -75th IQR)". Statistical: Kruskal-Wallis test and $\chi 2$ in SPSS. P-values < 0.05 were considered significant and are indicated in bold.

	Mild (n = 12)	**Moderate (n = 17)**	**Severe (n = 13)**	**p-value**
		HAEMOGRAM		
White Blood Cells Count ($x10^9$/L)	4.90 (3.78-6.74)	7.14 (5.46-8.19)	7.48 (4.54-8.74)	0.288
Hematies	4.48 (4.05-4.90)	4.42 (3.96-4.80)	4.56 (4.05-5.12)	0.602
Hemoglobine (g/dL)	13 (10.65-14.30)	12.90 (12.00-14.00)	12.50 (11.10-13.90)	0.943
Hematocritics (%)	39.45 (32.75-42.85)	38.90 (36.60-42.10)	39.60 (35.90-42.10)	0.939
Platelet Count ($x10^9$/L)	220 (184-247.50)	219 (168-311)	235 (176-280)	0.896

Neutrophils (%)	67.30 (56.85-76.55)	73 (64.90-80.00)	74 (62-83.80)	0.576
Lymphocytes (%)	26.10 (15.95-30.15)	18.70 (10.40-24.00)	17 (10.40-23.10)	0.396
Total Lymphocytes (%)	1005 (785-1370)	1420 (980-1930)	1030 (750-1390)	0.132
Monocytes (%)	7.05 (5.45-8.65)	7.50 (6.00-10.60)	7.10 (5.30-8.40)	0.333
		COAGULATION ANALYSIS		
Activated Thromboplastin Time (sec)	27.70 (26.40-30.80)	28.20 (27.00-32.00)	30.15 (29.00-30.65)	0.322
Prothrombin Time (sec)	12.95 (11.85-13.40)	12.85 (11.75-14.25)	12.75 (12.10-13.60)	0.960
D-Dimer (mg/L)	433.50 (276-982)	405 (297-758)	795.50 (573.50-1075)	0.213
Fibrinogen (g/L)	675 (503.50-786.50)	676 (615-825)	660 (544.50-709)	0.572
		BIOCHEMICAL ANALYSIS		
Glucose (mg/dL)	103 (86-118)	110 (103-130)	104 (91-126)	0.647
Total Cholesterol (mg/dL)	133 (129-137)	160 (144-176)	150 (119-163)	0.164
Urea (mg/dL)	28.50 (21.50-34.00)	33 (29-35)	39 (30-43)	0.115
Creatinine (mg/dL)	0.75 (0.66-0.81)	0.74 (0.68-0.94)	0.84 (0.79-1.05)	0.366
GOT/AST (U/L)	30.50 (25.00-39.50)	30 (21-48)	44 (34-81)	0.146
GPT/ALT (U/L)	41 (27-56.50)	33 (22-58)	61.50 (33-85.50)	0.185
Gamma-glutamyl transferase (U/L)	83.50 (58.50-312.50)	60.50 (39-82)	126 (70-227)	**0.027**
Alkaline Phosphatase (IU/L)	73 (69-439)	74 (57-95)	66.50 (50.50-92.50)	0.517
Total Protein (g/dL)	5.59 (4.8-5.67)	6.54 (6.4-6.9)	6.1 (5.85-6.56)	**0.005**

Albumin (g/dL)	3.38 (3.21-4.37)	3.91 (3.70-4.30)	3.97 (3.60-4.06)	0.728
LDH (U/L)	245 (188.50-307)	276 (235-328)	378.50 (272-467)	**0.010**
Troponin (ng/L)	2 (1-3.50)	4 (2-12)	10 (3-20)	0.061
	INFLAMMATORY MARKERS			
Erythrocyte sedimentation rate (mm)	114 (58-141)	56 (46-68)	41 (22-67)	0.075
Ferritin (ng/mL)	423 (151-637)	358 (160-639)	786 (471-1428)	0.06
C-reactive protein (mg/mL)	4.35 (1.25-7.40)	4.70 (3.10-11.00)	6.10 (2.20-12.10)	07.16

GOT/AST: Aspartate Aminotransferase, GPT/ALT: Alanine Aminotransferase, LDH: Lactate Deshidrogenase

4.2 Increased levels of inflammation and oxidative stress based on the severity of COVID-19

Firstly, the inflammatory status was assessed by measuring IL-6, IL-1β and TNF-α, as these cytokines are related to mitochondrial dysfunction and stress. IL-6 showed a significant increase with severity of COVID-19 ($p = 0.030$). Post-hoc analysis using Tukey test, indicated a significant increase specially between the mild and severe groups ($p = 0.023$) (Figure 5A). For IL-1β, levels showed the same trend although it was not significant (Figure 5B). TNF- α levels were similar between groups showing the same trend independently of COVID-19 severity (Figure 5C).

Regarding Total Oxidant Status levels, it was significantly higher in severe patients compared with the moderate group ($p = 0.034$), but not between the mild and moderate (Figure 5D). To elucidate the relation between oxidative stress and inflammation correlation analysis between IL-6, IL-1β and TNF-α and TOS, was performed. No significant correlation was found between these cytokines and TOS levels (Figures 5 E, F and G).

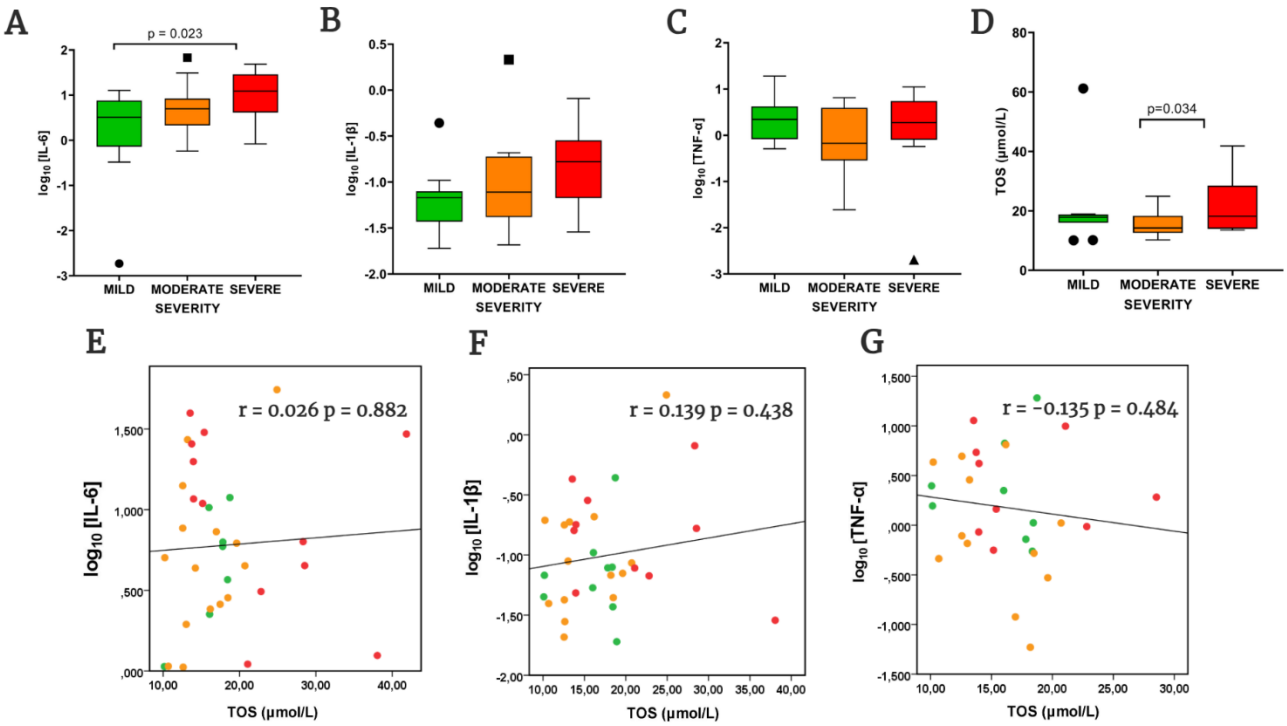

Figure 5: IL-6, IL-1β, TNF-α, and TOS levels in relation to COVID-19 severity. A) Box plots illustrating the distribution of $\log_{10}$[IL-6], **B)** $\log_{10}$[IL-1β], **C)** $\log_{10}$[TNF-α], and **D)** TOS levels based on different COVID-19 severity groups. Scatter plot of spearman correlation of TOS and **E)** $\log_{10}$[IL-6], **F)** $\log_{10}$[IL-1β], **G)** $\log_{10}$[TNF-α] (Mild in green, Moderate in orange, Severe in red). P-value < 0.05 was considered significant.

4.3 Sex, age and weight as factors influencing cytokine and oxidative stress levels.

Demographic factors such as gender and age have been reported to influence the inflammatory and immune response. In this study, in order to see how these factors may affect, several correlations have been performed. A significant positive correlation was observed between age and the levels of both IL-6 ($r = 0.441$; $p = 0.006$) and TOS ($r = 0.326$; $p = 0.04$) indicating that IL-6 levels and TOS increase with age (Figure 6A) although independent of COVID-19 severity, as the groups did not show significant differences in age (Figure 6B and 6C respectively). Regarding sex, the point-biserial correlation revealed a significant association sex and IL-6 levels ($\rho b = 0.356$; $p = 0.030$). Il-6 concentrations were higher in male patients compared to women ($p = 0.027$) (Figure 6E). This trend persisted when taking COVID-19 severity into account, but it did not reach statistical significance (Figure 6F) Weight was also analysed and correlation significant correlations with IL-6 and TOS were observed (Figure 6G). Il-6 levels increased with weight ($r = 0.428$; $p = 0.018$) (Figure 6H), while TOS showed a significant negative correlation with weight ($r = -0.38$; $p = 0.031$) (Figure 6I).

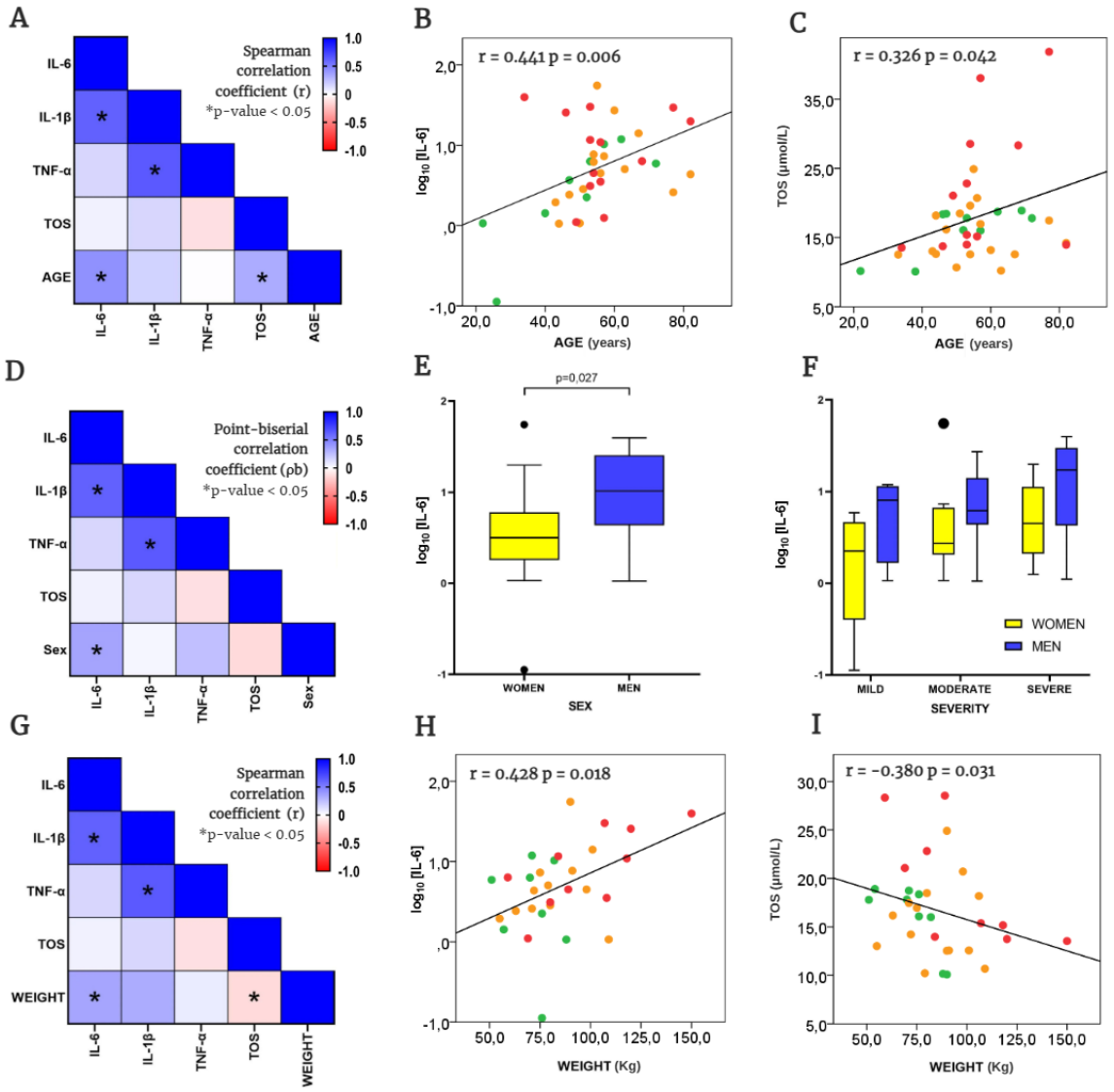

Figure 6: **Sex, age and weight influence in cytokine and TOS levels. A)** Spearman correlation coefficient (r) heatmap between age (years), cytokine levels and TOS. **B)** Scatter plot with trendline representing the correlation between age and log10 of IL-6 levels and **C)** TOS (µmol/L) in plasma. **D)** Point-biserial correlation coefficient (ρb) heatmap between sex, cytokine levels and TOS. **E)** Tukey box plot representing the log10 of IL-6 levels according to sex. **F)** Tukey box plot representing the log10 of IL-6 levels according to sex and COVID-19 severity group. **G)** Spearman correlation coefficient (r) heatmap between weight (Kg), cytokine levels and TOS. **H)** Scatter plot with trendline representing the correlation between weight and log10 of IL-6 levels and **I)** TOS (µmol/L) in plasma. Statistically significant associations were considered when *p-value < 0.05. Green dots represent mild patients, orange moderate and red severe. Yellow boxes represent women and blue represent men.

4.4 The relationship between circulating biochemical and immunological parameters with inflammation and oxidative stress levels.

To further understand the relationship between inflammation, oxidative stress and the broader clinical status, additional correlation analyses were performed between

inflammatory cytokines, TOS and clinical parameters, including hemogram (Figure 7A), biochemical analysis (Figure 7B) and inflammatory and coagulation markers (Figure 7C). IL-6 showed significant negative correlations with total lymphocyte count ($r = -0.343$; $p = 0.038$), lymphocytes ($r = -0.401$; $p = 0.014$) and platelet count ($r = -0.358$; $p = 0.29$) while exhibiting a significant positive correlation to the neutrophile count ($r = 0.350$; $p = 0.034$). TOS negatively correlated with hematies ($r = -0.391$; $p = 0.014$), haemoglobin ($r = -0.436$; $p = 0.006$) and haematocrits ($r = -0.378$; $p = 0.018$). Additionally, IL-1β also displayed a significant positive correlation with white blood cell count ($r = 0.371$; $p = 0.028$) (Figure X3A). Biochemical data revealed several relevant correlations, primarily involving IL-6 (Figure 7B). This cytokine was positively correlated with aspartate aminotransferase (AST/GOT; $r = 0.434$; $p = 0.008$), gamma-glutamyl transferase (GGT; $r = 0.447$; $p = 0.015$), lactate dehydrogenase (LDH; $r = 0.579$; $p < 0.001$), and troponin ($r = 0.434$; $p = 0.015$). Furthermore, TNF-α displayed a significant negative correlation with total cholesterol ($r = -0.555$; $p = 0.049$). Notably, ferritin ($r = 0.465$; $p = 0.017$) and CRP ($r = 0.579$; $p < 0.001$) were also found to be positively and significantly correlated with IL-6 (Figure 7C).

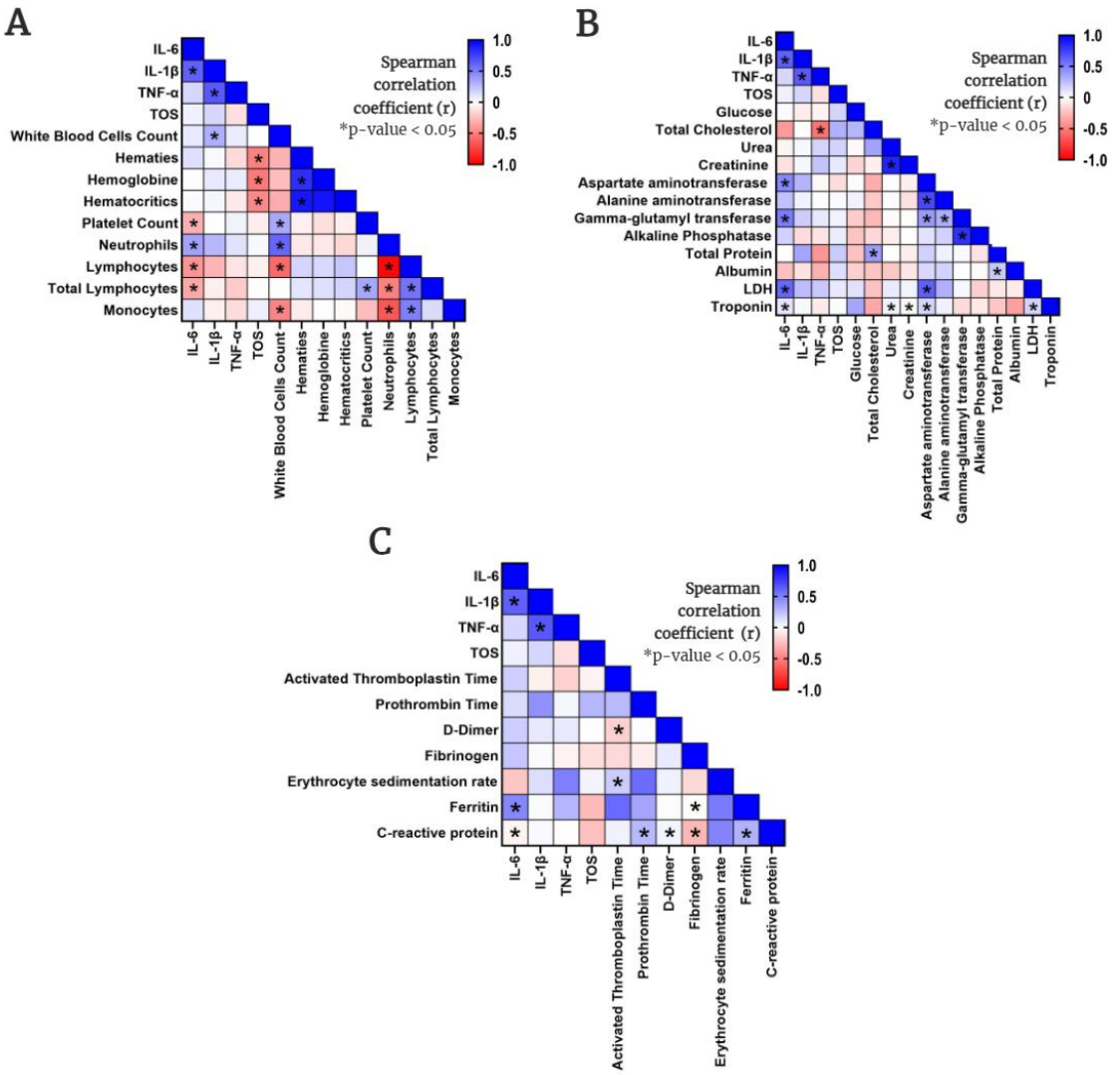

Figure 7: Relations between IL-6, IL-1β, TNF-α, TOS and clinical parameters of the study cohort. A) Spearman correlation coefficient (r) heatmap between IL-6, IL-1β, TNF-α, TOS and haemogram data of the cohort. **B)** Spearman correlation coefficient (r) heatmap between IL-6, IL-1β, TNF-α, TOS and biochemical analysis data of the cohort. **C)** Spearman correlation coefficient (r) heatmap between IL-6, IL-1β, TNF-α, TOS, inflammatory markers and coagulation markers data of the cohort. Statistically significant associations were considered when *p-value < 0.05.

5. DISCUSSION

SARS-CoV-2 infection causes a great variability of clinical manifestations resulting in different degrees of COVID-19 severity (14). Multiple studies have shown that COVID-19 infection could be associated with alterations in metabolism and mitochondrial function, affecting the inflammatory and stress state of these patients and consequently increasing the circulating levels of ROS (61). During COVID-19 infection, ROS acts both as a cause and a consequence of the infectious process. Excessive production of ROS is key to understanding the inappropriate immune response associated with COVID-19 severity, including tissue damage and increased inflammation (62). Although the levels of pro-inflammatory cytokines across severity have already been described in different studies (63,64), the levels of circulating ROS and their relationship to inflammation and COVID-19 severity, remain to be explored. In this preliminary study, using a cohort of 42 patients stratified according to COVID-19 severity, the measured inflammation markers together with TOS were higher in patients with more severe symptomatology. Interestingly, no correlation was observed between cytokine levels and TOS, although variables such as age and weight did correlate with TOS.

Inflammation is a fundamental biological response to harmful stimuli, and it plays a critical role in the severity of COVID-19 (39,65). Elevated levels of IL-6, a cytokine generally known to induce the synthesis of acute phase proteins and stimulate antibody production (65), are associated with higher severity in COVID-19 (66)which is consistent with the results of this study. IL-6 levels also increase with mortality, higher ICU admissions and a greater need for mechanical ventilation (66). Our results further support this, showing that severe COVID-19 patients present lower O_2 saturation, explaining the higher ventilation requirements. Furthermore, COVID-19 patients are also characterized by lymphopenia (low lymphocyte count) and neutrophilia (high neutrophile count), which have been previously recognized and established as key indicators of COVID-19 progression and poor prognosis (67). The negative correlation found between lymphocyte count and IL-6 levels, suggests that systemic inflammation may directly contribute to this immune cell dysregulation, while IL-6 typically plays a role in activating the immune cells, an excessive amount can shut down the formation of lymphocytes (68). On the other hand, the neutrophilia aligns with the heightened inflammatory state. Neutrophils' positive correlation with IL-6 levels is likely due to the cytokine's ability to promote the formation, recruitment and longevity of these cells (69).

Along with IL-6, other inflammatory markers such as CRP, ferritin or D-dimer, increased together with COVID-19 severity. Each of these markers contributes distinctly to the

disease's progression. For instance, CRP actively induces the production of pro-inflammatory cytokines and apoptosis (70). Similarly, high ferritin plays a critical role in COVID-19 severity by dysregulating iron metabolism and increasing iron levels (71). Meanwhile, elevated D-dimer is strongly related to severe coagulation dysfunction and extended and less favourable recovery periods (39,72,73). The positive correlation between CRP and ferritin and IL-6 levels contributes to the inflammatory cascade (74) and could be explained by the hepatic synthesis and release of these acute-phase proteins, induced by IL-6 (73,75). Regarding the biochemical state of severe patients both gamma-glutamyl transferase and LDH correlate positively with severity. Interestingly, LDH, gamma-glutamyl transferase and troponin also correlated positively with IL-6.

Increased gamma-glutamyl transferase shows evidence of bile duct injury, possibly caused by the body's overactive immune response (76). Likewise, higher LDH, an established marker for tissue damage, is likely a consequence of the widespread cellular damage prompted by the cytokine storm that contributes to the severity of the inflammatory response in COVID-19 patients (77). Finally, troponin, a cardiac injury marker, also presented a positive correlation with IL-6, further highlighting the multi-organ damage caused by the overproduction of proinflammatory cytokines (78).

Another key pro-inflammatory cytokine is IL-1β, which is key in initiating the immune response, recruiting immune cells and modulating adaptive immune responses among other functions (79). IL-1β plays an essential role in the pathogenesis of COVID-19, contributing to systemic inflammation and organ dysfunction (80). Similarly to IL-6, it was found to increase with severity, a finding consistent with previous studies (81,82). On the other hand, TNF-α leads immune cells to the infection site and stimulates cytokine production (83). In COVID-19, this cytokine is controversial, some studies consider TNF- α as one of the major pro-inflammatory cytokines upregulated and that it is positively associated with severity and viral load (84), while others found that TNF- α does not play a significant role in COVID-19 severity (73). Our results align with the second option, as we did not find any significant differences in TNF-a levels between groups, although this might be caused by the size and heterogeneous nature of the cohort.

Together with inflammation, increased systemic oxidative stress is associated with moderate and severe COVID-19 patients (55). Consistent with this, our preliminary study, which assessed oxidative stress through total oxidant status, found that TOS was significantly higher in severe patients compared to moderate and mild individuals. Excessive ROS exacerbates the storm, contributing to uncontrolled characteristic tissue damage found in severe patients with complications like ARDS (54,55). This significant increase in both

TOS and cytokines like IL-6, likely originates from mitochondrial stress, creating a vicious cycle that drives disease severity (55,85). Conversely, TOS and red blood cell count (hematies), hemoglobine and haematocrit showed a negative correlation, likely due to damage and dysfunction of red blood cells caused by oxidative stress, making these more susceptible to lysis, reducing their ability to carry oxygen and explaining the need for mechanical ventilation and the low O_2 saturation of these patients (86). Moreover, ROS has been previously associated with inflammation (85), although our results do not show a direct correlation between TOS and the analysed proinflammatory cytokines, IL-6, IL-1β and TNF-α. This lack of correlation may be because oxidative stress is the result of an unbalance between ROS and anti-oxidant molecules (87). During acute infection, anti-oxidant usage increases dramatically to neutralize ROS (62). This means that, while ROS production may be elevated due to inflammation, it might not be detectable because of the extensive scavenging by anti-oxidant molecules (62).

Risk factors such as age, sex or pre-existing comorbidities can strongly influence the development of severe COVID-19 (17). Previous studies have found a higher probability of suffering severe COVID-19 in older patients (17,88), primarily because of their weak immunity (17). Although the cohort did not find significant differences between groups regarding age, higher levels of IL-6 were found in older individuals, a finding consistent with other studies that linked IL-6 levels with COVID-19 severity. Specifically, (89) concluded that advanced age (over 65 years) and elevated IL-6 levels, pose higher vulnerability to poor outcomes. Regarding oxidative stress, infection can trigger especially high levels of ROS in compromised patients, which may lead to adverse clinical outcomes (90). This aligns with the higher levels of TOS in older patients found in our study and is consistent with previous literature (18).

Previous studies have shown that men are generally more prone to suffer from more severe COVID-19 (17). Although no statistically significant differences were found between groups in our study, it was observed that elevated IL-6 levels positively correlated with sex, with higher IL-6 levels in male patients. Of note, this finding is independent of severity as this study did not differ in sex distribution across groups. Higher IL-6 concentrations in male COVID-19 patients, may be partly explained by testosterone levels, which typically decrease in male patients during COVID-19 infection (66). Lower levels of this hormone have been previously associated with elevated IL-6, CRP, and D-dimer, as well as with lymphopenia, neutrophilia and prolonged hospital stays (66). Another important risk factor positively correlated with IL-6 is weight, which is concordant with previous studies and reflects the intense systemic inflammation common in chronic inflammatory diseases such as obesity

(91) as well as the pre-existing oxidative stress characteristic of this demographic, making them more susceptible to more severe outcomes (90).

5.1 Limitations of the study

In this study several limitations should be considered. Firstly, although our findings align with previously published data, the small sample size limited the results. Secondly, the inherent inter-individual variability observed in COVID-19 patients, which added more factors that affected the levels of ROS and inflammation. Additionally, this study was performed only during the acute phase of infection. Nevertheless, as a preliminary study, it provided an initial insight into how ROS levels are related to inflammation based on COVID-19 severity.

6. CONCLUSIONS

To conclude, this study found that inflammation and oxidative stress are associated with COVID-19 severity, causing these patients to have higher levels of IL-6, IL-1β and TOS. Older, male and overweight patients exhibited elevated levels of inflammation and oxidative stress, indicating a higher susceptibility to severe COVID-19 in these demographics. These results represent the complex relationship between inflammation and oxidative stress, potentially driven by mitochondrial dysfunction. Future research could focus on the validation of these results in a larger cohort, to allow for a more robust generalization of the results. Implementing a longitudinal study design would also provide valuable insight about the progression of COVID-19 and would help to understand the evolution of the infection, tracking markers from the acute phase to the recovery.

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