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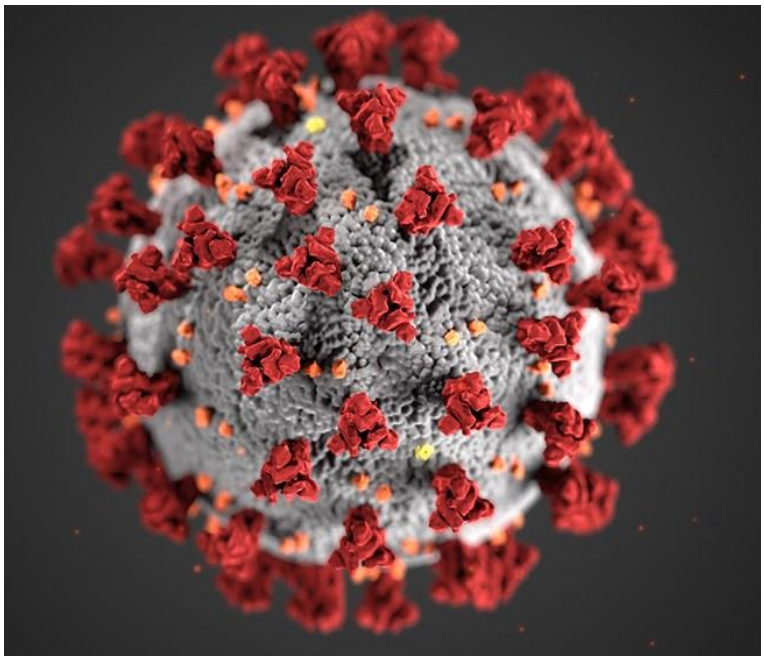

Development of Predictive Models for COVID-19 Prognosis based on Patients' Demographic and Clinical Data

Cristiana da Palma Von Rekowski

Thesis to obtain the Master's Degree in **Biomedical Engineering**

Definitive Version

Supervisors:

Dr. Cecília Ribeiro da Cruz Calado (ISEL)
Dr. Iola Pinto (ISEL)
Dr. Luís Bento (CHULC)

July 2022

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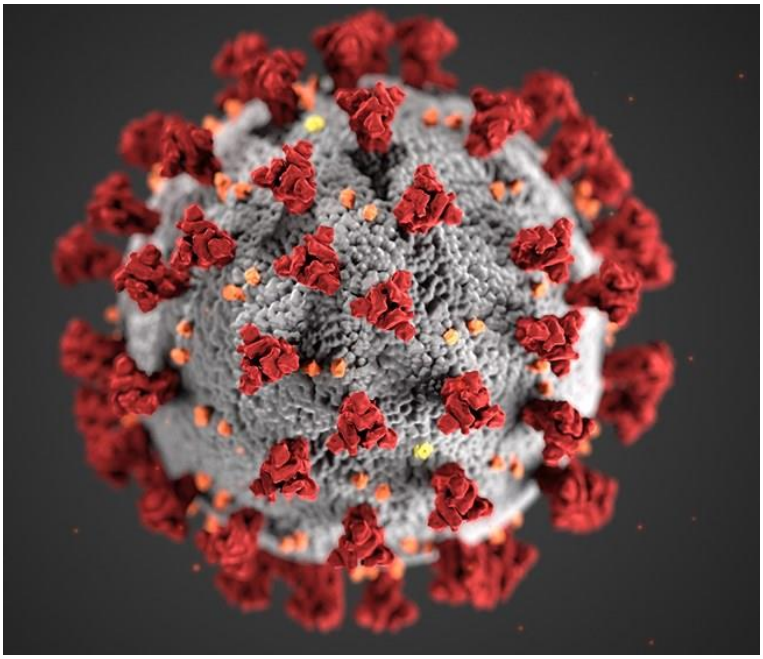

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Abstract

Background – Cases of infection by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) were first reported in late December 2019. Due to the large spectrum of clinical presentations and outcomes, the disease was named Coronavirus Disease 2019 (COVID-19) and characterized as a pandemic due to the elevated number of cases worldwide, the high transmission rate and the lack of action measures. Since then, a lot of progress has been made, but the study of demographic and clinical information and the determination of possible laboratory biomarkers for COVID-19 prognosis is crucial.

Purpose – Determine predictive biomarkers for COVID-19's outcome (death or survival), in critically ill patients, using clinical, demographic and laboratory data from the intensive care unit (ICU).

Methods – Demographic, clinical and laboratory data from 337 COVID-19 patients admitted to the ICU of *Centro Hospitalar Universitário Lisboa Central,* Portugal, between March 2020 and March 2021, was extracted from the hospital's electronic medical record system, pre-processed, and analyzed. Comparisons were made regarding death, the need of invasive mechanic ventilation (IMV), the first three COVID-19 waves and age groups. Longitudinal data was gathered over the course of the patients stay in the ICU. To infer about the evolution of the patients' condition in the first week of ICU admission, a comparative analysis was carried out between the data from the 2nd (335 patients) and 7th days (216 patients). Comparisons of laboratory parameters between discharged and deceased patients, at these time points were performed. The associations between the several biomarkers and death were tested by means of Univariate Generalized Estimating Equations (GEEs) models. Additionally, to analyze the impact of some biomarkers in mortality, crude odds ratios were estimated and interpreted, with the corresponding 95% confidence intervals (CIs). Death event-free survival rates were obtained by the Kaplan-Meier estimator. All *P* values were considered statistically significant at $P<0.05$.

Results – Deceased patients were considerably older, had more comorbidities, required more IMV, and spent less time in the hospital than discharged patients. Death rates did not differ significantly between COVID-19 waves. Patients from the 1st wave were significantly older and relied more on IMV and extracorporeal membrane oxygenation (ECMO). Most of the detected differences regarding laboratory biomarkers were found between discharged and deceased patients from the 2nd and 3rd waves, being that the deceased ones had almost always worse results. In general, worse results were obtained in the 1st wave and in the 7th day of ICU admission. In 2nd day of ICU admission, 2nd wave, higher mortality rates were observed for patients with lymphocyte (LYM) levels under normality ranges. In the 3rd wave, mortality rates were higher for patients with high sensitivity troponin I (hs-cTn I) levels above normality ranges in the 2nd day of ICU admission, with LYM levels under normality ranges in the 7th day of ICU admission, and with platelet (PLT) levels below normality ranges, either in the 2nd or 7th days of ICU admission. Through the univariate logistic regression's results in 2nd day of ICU admission, 2nd wave, hs-cTn I, red blood cell (RBC) counts, platelet-lymphocyte ratio (PLR) and neutrophil-lymphocyte ratio (NLR) showed significant association with the risk of death. In 7th day of ICU admission, C-reactive protein (CRP), RBC counts, hematocrit (HCT), hemoglobin (HGB), white blood cell (WBC) and

neutrophil (NEU) counts, eosinophil (EO) counts and NLR, revealed significant association with the risk of death. In the 2nd day of ICU admission, 3rd wave, hs-cTn I, PLT counts, lactate dehydrogenase (LDH) and CRP showed significant association with the risk of death. For the 7th day, PCT, CRP, WBC and NEU counts, LYM counts, NLR and PLT counts results were also associated with higher risks of death. Univariate GEEs models results demonstrated that, in the 1st wave, hs-cTn I, myoglobin, EO counts, results were associated with higher risks of death. In the 2nd wave, the risk of death was significantly associated with hs-cTn I, myoglobin levels, EO counts, WBC and NEU counts, LYM counts, and INR. Finally, in the 3rd wave, hs-cTn I, CK, EO counts, WBC and NEU counts, LYM counts, NLR and PLT counts, were also associated with the risk of death.

Conclusion - This study provides useful information for prognostic evaluation that can be used to guide treatment and monitoring. Most importantly, it consists of valuable data that can be employed as the foundation of a variety of future research. Aside from the positive results, more research is needed to develop reliable and robust biomarkers for COVID-19's outcomes.

Keywords – COVID-19 ● SARS-CoV-2 ● Multi-Organ Involvement ● Predictive Biomarkers ● ICU Outcomes ● Hematologic indexes

Resumo

Introdução – Casos de infeção pelo vírus *severe acute respiratory syndrome coronavirus* 2 (SARS-CoV-2) foram relatados pela primeira vez no final de dezembro de 2019. Devido ao grande espetro de apresentações e *outcomes* clínicos, a doença foi denominada *Coronavirus Disease 2019* (COVID-19) e considerada uma pandemia devido ao elevado número de casos em todo o mundo, à alta taxa de transmissão e à falta de medidas de ação. Apesar desta patologia estar a ser aprofundadamente investigada, o estudo de informação demográfica e clínica e a determinação de possíveis biomarcadores laboratoriais para o prognóstico da COVID-19 continua a ser crucial.

Objetivos – Determinar biomarcadores preditivos para o *outcome* da COVID-19 (morte ou vida), em pacientes críticos, usando dados clínicos, demográficos e laboratoriais da unidade de cuidados intensivos (UCI).

Métodos – Dados demográficos, clínicos e laboratoriais de 337 pacientes com COVID-19 internados na UCI do Centro Hospitalar Universitário Lisboa Central, em Portugal, entre março de 2020 e março de 2021, foram extraídos das bases de dados eletrónicas do hospital, pré-processados e analisados. Foram feitas comparações em relação ao óbito na UCI, necessidade de ventilação mecânica invasiva (VMI), três vagas de COVID-19 e faixas etárias. Dados longitudinais foram obtidos ao longo da permanência dos pacientes na UCI. Para inferir sobre a evolução do quadro dos pacientes na primeira semana de internamento na UCI, foi realizada uma análise comparativa entre os dados do 2º (335 pacientes) e 7º dias (216 pacientes). Foram realizadas comparações de parâmetros laboratoriais entre pacientes que receberam alta e pacientes falecidos, nestes momentos. As associações entre os diversos biomarcadores e a morte foram testadas por meio de modelos, do inglês, *Generalized Estimating Equation* (GEEs) univariados. Adicionalmente, para analisar o impacto de alguns biomarcadores na mortalidade, foram estimados e interpretados os *odds ratios*, com os correspondentes intervalos de confiança de 95%. As taxas de sobrevivência, em relação a cada biomarcador, foram obtidas pelo estimador Kaplan-Meier. Todos os valores de *P* foram considerados estatisticamente significantes para $P<0,05$.

Resultados – Os pacientes que faleceram eram consideravelmente mais velhos, tinham mais comorbidades, necessitavam mais de VMI e passavam menos tempo no hospital do que os pacientes que receberam alta. As taxas de mortalidade não diferiram significativamente entre as vagas de COVID-19. Os pacientes da 1ª vaga eram significativamente mais velhos e dependiam mais da VMI e da ECMO. A maioria das diferenças detetadas quanto aos biomarcadores laboratoriais foi entre pacientes que receberam alta e os que faleceram na 2ª e 3ª ondas, sendo que os falecidos demonstraram quase sempre piores resultados. A nível de biomarcadores, os piores resultados foram obtidos na 1ª vaga e no 7º dia de internamento UCI. Na 2ª vaga, as maiores taxas de mortalidade foram observadas para pacientes com níveis de linfócitos abaixo da normalidade no 2º dia de internamento na UCI. Na 3ª vaga, as taxas de mortalidade foram maiores para pacientes com níveis de troponina de alta sensibilidade acima da normalidade no 2º dia de internamento na UCI, com níveis de linfócitos abaixo da normalidade no 7º dia de internamento na UCI e com níveis de plaquetas abaixo da normalidade, no 2º e 7º dias de

internamento na UCI. Por meio de regressão logística univariada, determinou-se que, para a 2ª vaga, os resultados das troponinas de alta sensibilidade, eritrócitos, rácios entre plaquetas e linfócitos e dos rácios entre neutrófilos e linfócitos poderiam prever o risco de morte no 2º dia de internamento na UCI. O mesmo foi observado para a proteína C-reativa, hemácias, hematócrito, hemoglobina, leucócitos, neutrófilos, eosinófilos e rácio entre neutrófilos e linfócitos, no 7º dia de internamento. Na 3ª vaga, os resultados das troponinas de alta sensibilidade, plaquetas, lactato desidrogenase e proteína C-reativa também demonstraram capacidade para prever o risco de morte no 2º dia de internamento na UCI. Para o 7º dia, os resultados da procalcitonina, proteína C-reativa, leucócitos, linfócitos, neutrófilos e dos rácios entre neutrófilos e linfócitos e plaquetas e linfócitos também demonstraram capacidade preditiva para riscos de morte superiores. Através dos modelos *Generalized Estimating Equation* (GEEs) univariados, na 1ª vaga os resultados das troponinas de elevada sensibilidade, mioglobina e eosinófilos foram associados a maiores riscos de morte. Na 2ª vaga, o mesmo foi novamente verificado para as troponinas de elevada sensibilidade, a mioglobina e os eosinófilos, e também para os leucócitos, neutrófilos, linfócitos e INR. Por fim, na 3ª vaga, as troponinas de elevada sensibilidade, a creatinina cinase, eosinófilos, leucócitos, neutrófilos, linfócitos, rácio entre neutrófilos e linfócitos e as plaquetas também foram associados ao risco de morte.

Conclusão - Este estudo fornece informações úteis para uma avaliação prognóstica e que podem ser usadas para orientar o tratamento e a monitorização de pacientes com COVID-19. É ainda composto por dados que podem vir a ser empregados numa grande variedade de estudos futuros. Além dos resultados positivos, é necessária mais investigação nesta área de maneira a desenvolver biomarcadores confiáveis e robustos para os *outcomes* da COVID-19.

Palavras-Chave – COVID-19 ● SARS-CoV-2 ● Envolvimento Multi-Orgão ● Biomarcadores preditivos ● *Outcomes* da UCI ● Índices hematológicos

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Table of Contents

List of figures

List of tables

List of abbreviations

ACE2	Angiotensin-Converting Enzyme 2
ACTIV	Accelerating COVID-19 Therapeutic Interventions and Vaccines
AKI	Acute Kidney Disease
ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
Ang	Angiotensin
aPTT	Activated Partial Thromboplastin Time
ARDS	Acute Respiratory Distress Syndrome
AST	Aspartate Aminotransferase
AT1R	Ang II Type 1 Receptor
AT2R	Ang II Type 2 Receptor
AXL	Tyrosine-Protein Kinase Receptor UFO
BE	Base Excess/deficit
BMI	Body Mass Index
BP	Blood Pressure
BPH	Benign Prostate Hyperplasia
BUN	Blood Urea Nitrogen
CEPI	Coalition for Epidemic Preparedness Innovations
CI	Confidence Interval
CLpro	Chymotrypsin-Like Protease
CNS	Central Nervous System
COPD	Chronic Obstructive Pulmonary Disease
COVID-19	Coronavirus Disease 2019
CoVs	Coronaviruses
CRP	C-reactive protein
CSG	*Coronaviridae* Study Group
CT	Computed Tomography
DC	Dendritic Cells
DD	D-Dimer
DIC	Disseminated Intravascular Coagulation
DILI	Drug-Induced Liver Damage (DILI),
ECMO	Extracorporeal Membrane Oxygenation
eGFR	Estimated Glomerular Filtration Rate
EO	Eosinophil
EUL	Emergency Use Listing
FDP	Fibrin Degradation Product
FGF	Fibroblast Growth Factor
FiO_2	Fraction of Inspired Oxygen
G-CSF	Granulocyte Colony-Stimulating Factor
GEE	Generalized Estimating Equation
GGT	Gamma-Glutamyl Transferase
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
gRNA	Genome RNA
HCoV	Human Coronavirus
HCT	Hematocrit

HDL	High-Density Lipoprotein
HFO	High Flow Oxygen
HGB	Hemoglobin
HHb	Deoxyhemoglobin
hs-cTn	High-Sensitivity Cardiac Troponin
ICTV	International Committee on Taxonomy of Viruses
ICU	Intensive Care Unit
IL	Interleukin
IL-1RA	Interleukin 1 Receptor Antagonist
IL-2R	Interleukin 2 Receptor
IMV	Invasive Mechanic Ventilation
INF	Interferon
INR	International Normalized Ratio
IQR	Interquartile Range
JAK's-STAT's	Janus Kinase Signal Transducer and Activator of Transcription Proteins
Lac	Lactate
LDH	Lactate Dehydrogenase
LDL	Low-Density Lipoprotein
LYM	Lymphocyte
MasR	Mas Receptor
MCH	Mean Corpuscular Hemoglobin
MCHC	Mean Corpuscular Hemoglobin Concentration
MCP	Monocyte Chemoattractant Protein
MCV	Mean Corpuscular Volume
MERS-CoV	Middle East Respiratory Syndrome Coronavirus
MI	Myocardial Infarction
MIP	Macrophage Inflammatory Protein
MON	Monocyte
Mpro	Main Protease
mRNA	Messenger RNA
NEU	Neutrophil
NK	Natural Killer
NLR	Neutrophil-Lymphocyte Ratio
NSP	Non-Structural Protein
NT-proBNP	Natriuretic Peptide Testes
O2Hb	Oxyhemoglobin
ORF	Open Reading Frame
OSA	Obstructive Sleep Apnea
PaO_2	Partial Pressure of Oxygen
PAMP	Pathogen-Associated Molecular Pattern
PCT	Procalcitonin
PDGF	Platelet-Derived Growth Factor
PDW	Platelet Distribution Width
PHEIC	Public Health Emergency of International Concern
PLpro	Papain-Like Proteases
PLR	Platelet-Lymphocyte Ratio

PLT	Platelet
PNS	Peripheral Nervous System
pp	Polypeptides
PPR	Pattern-Recognition Receptor
PREMO	Predictive Models of COVID-19 Outcomes for Higher Risk Patients Towards a Precision Medicine
PT	Prothrombin Time
PT-act	Prothrombin Time Activity
q-PCR	Quantitative Polymerase Chain Reaction
RAS	Renin-Angiotensin System
RBC	Red Blood Cell
RBD	Receptor Binding Domain
RDW	Red Cell Distribution Width
RDW-SD	Red Cell Distribution Width
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcriptase – Polymerase Chain Reaction
SAGE	Strategic Advisory Group of Experts on Immunization
SARS-CoV	Severe Acute Respiratory Syndrome Coronavirus
SCr	Serum Creatinine
ssRNA	Single-Stranded Positive-Sense RNA
So2	Arterial oxygen saturation
TGF	Transforming Growth Factor
TLR	Toll-Like Receptors
TMPRSS2	Transmembrane Protease Serine 2
TNF	Tumor Necrosis Factor
UTR	Untranslated Region
uWBC	Uncorrected White Blood Cell count
VEGF	Vascular Endothelial Growth Factor
VOC	Variant Of Concern
VOI	Variant Of Interest
WBC	White Blood Cell
WHO	World Health Organization

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Chapter 1: Objectives and work structure

Given the significant impact of the COVID-19 pandemic from late December 2019 to the present, research into demographic and clinical data, as well as the identification of potential laboratory biomarkers for COVID-19 prognosis is critical. Therefore, this thesis' major purpose is to identify predictive biomarkers for this disease's outcome in critically ill patients. This project is inserted in a larger one, namely Predictive Models of COVID-19 Outcomes for Higher Risk Patients Towards a Precision Medicine (PREMO), which uses a variety of methodologies (for example Fourier-transform infrared spectroscopy, metabolomics, cytokine and vital signal analysis) to reach similar goals. For that reason, its intended that the results and conclusions from this thesis can be used not only as an individual source of information, but also as a supplement to future research.

In order to achieve the above-mentioned main goal, the following were also aimed:

- Gather and organize patients' clinical and demographic data into a single transversal database;
- Analyze the key clinical and demographic characteristics of the study sample through statistical descriptive measures, and compare data between discharged and deceased patients, patients who required invasive mechanical ventilation against those who did not, COVID-19 waves, and age groups;
- Organize and compile all of the patients' laboratory results into a single longitudinal database, then integrate them with the clinical and demographic information;
- Select specific sets of biomarkers (representative of several organs) and analyze them at the time of ICU admission and one week later, while comparing patients who were discharged from the ICU to those who died in the ICU, for each COVID-19 wave;
- Compare the behavior of the selected biomarkers between COVID-19 waves;
- Determine which biomarkers are predictive of a higher risk of death in the ICU;
- Analyze the associations between the several biomarkers and death, using the longitudinal data corresponding to all length of ICU stay.

This thesis was organized into five main chapters, each with its own sub-sections, the most essential of which are represented in **Figure 1.1**. Initially, an extensive literature review was conducted in order to learn more about the new virus, how it works, and how it affects the human body. The organs most affected by this virus, how it infects them, and the repercussions of this infection were also provided in this part as a foundation for the analysis of the sample study's laboratory results. The methodology was further broken down into sub-sections to clarify how clinical, demographic, and laboratory variables were chosen and organized, as well as to define the study sample. Results and discussion were merged into a single chapter, containing two main sub-sections. One was dedicated to the analysis of all patients' clinical and demographic features, as well as comparisons between those who were discharged from the ICU and those who died, those who required IMV and those who did not, and between COVID-19

waves and age groups. The other subsection was directed for the analysis of laboratory biomarkers in the ICU, more specifically cardiac-related biomarker, inflammatory and other hematologic biomarkers, and coagulation-related biomarkers. For each of these sets of biomarkers a comparative analysis was carried out between discharged and deceased patients, and COVID-19 waves, for specific days of ICU admission, in order to determine biomarkers related to the patients' outcome.

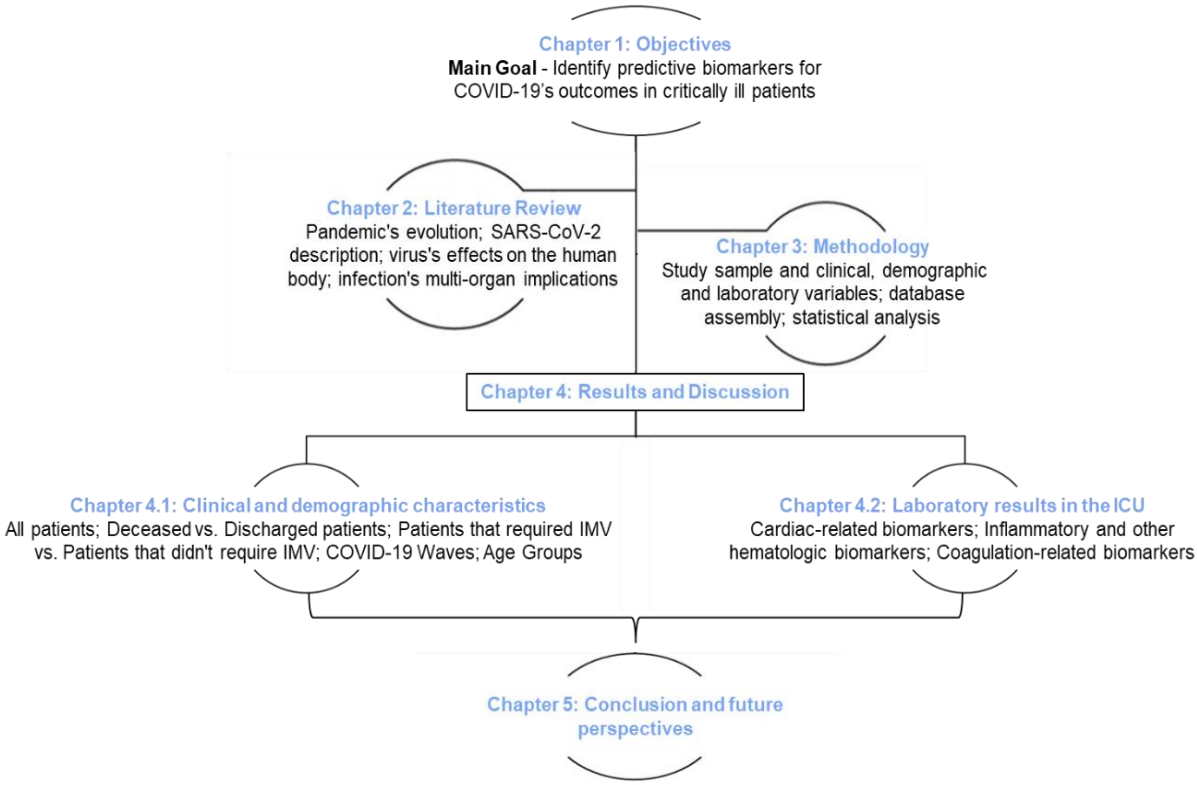

Figure 1.1. Representative scheme of the present thesis structure

Chapter 2: Literature review

2.1. General evolution of the SARS-CoV-2 pandemic

In late December 2019 several cases of patients with pneumonia of unknown cause were reported to health facilities in Wuhan, the capital of Hubei province in central China (1). After analyzing the first 27 documented cases it was concluded that the outbreak had an epidemiological link to the Huanan Seafood Wholesale Market, that was selling seafood and live animals. Due to the growing number of infected people, on December 31 the Wuhan Municipal Health Commission notified the public, as well as the World Health Organization (WHO) about the situation (1,2). On January 7, analysis from samples of bronchoalveolar lavage fluid indicated a novel coronavirus, abbreviated as 2019-nCoV by the WHO. Later in the same month it was renamed severe acute respiratory syndrome coronavirus (SARS-CoV-2) by the *Coronaviridae* Study Group (CSG), a working group of the International Committee on Taxonomy of Viruses (ICTV). Due to the large spectrum of clinical presentations and outcomes of the infection caused by this virus, the disease was named Coronavirus Disease 2019 (COVID-19) by the WHO (3). By the end of January, human-to-human transmission of the new coronavirus had been confirmed and the city of Wuhan locked down. The virus had also spread to all of the provinces of China, causing thousands of new cases to emerge daily (1). On January 30 the WHO declared a Public Health Emergency of International Concern (PHEIC), considering that only in China had been confirmed 7736 cases and 170 deaths and 12,167 cases were suspected (4,5).

Since February 2020, the situation became even more serious with the emergence of multiple COVID-19 cases from a variety of other countries mainly due to the high transmission rate of the virus and to international travel. Alarmed by the elevated levels of spread and severity of the SARS-CoV-2 and the lack of action measures, the WHO characterized COVID-19 as a pandemic on March 11. By this time 118,319 cases were confirmed globally, as well as 4,292 deaths (6). While the situation in China was more controlled, outside of the country more and more cases were reported from all continents and health-care resources became overwhelmed (1).

Due to the lack of specific treatment and prevention options against the SARS-CoV-2, like vaccines and antivirals, the WHO focused on public health measures and guidance tools aiming to slow down the transmission rate of the virus, to protect health systems and to accurately diagnose, isolate and care for all cases of COVID-19 (7,8). Thus, countries applied their own strategies to avoid the formation of new outbreaks, with included measures such as testing isolation, quarantine, and social distancing. Another focal point was the collaborative research and data sharing in order to find new and more effective solutions for the global crisis being faced, with included for example de foundation of groups and forums for presentation of candidate diagnostic methods, therapeutics, and vaccines (7). As a result, the first RT-PCR laboratory diagnostic kits were shipped to WHO Regional Offices still in February 2020 and two months later a draft of COVID-19 candidate vaccines was published by the WHO. To develop an effective and safe vaccine, the cooperation between international organizations such as the WHO, Accelerating COVID-19 Therapeutic Interventions and Vaccines (ACTIV), Epidemic Preparedness Innovations (CEPI), amongst others, was key to ensure funding (8).

The rate of vaccine candidates for SARS-CoV-2 increased rapidly as the pandemic advanced and some excellent progress was made in such small amount of time. For example, some candidate vaccines began their phase I clinical trials within 2 months and showed some promising results (9)**.** By the end of December 2020, with 79 231 893 cumulative cases and 1 754 574 deaths globally (10), 52 candidate vaccines were in clinical evaluation and 162 in preclinical evaluation (11)**.** These included DNA, RNA, non-replicating viral vectors and inactivated vaccines. On December 31, 2020, the Pfizer/BioNtech Comirnaty vaccine was listed for WHO Emergency Use Listing (EUL), followed by two AstraZeneca/Oxford vaccines (SII/Covishield and AstraZeneca/AZD1222) on February 15, 2021, and the Janssen/Ad26.COV2.S vaccine on March 12, 2021(12). The EUL and the Strategic Advisory Group of Experts on Immunization (SAGE) have worked with these and other vaccines, to determine if they are quality-assured, safe, effective and to develop policy recommendations for their development, delivery, and others matters related to health interventions (12).

As of August 2021, date until the data for the present thesis was collected, there were 215,714,824 cumulative cases and 4,490,753 cumulative deaths globally. The highest percentages of both cases and deaths were in the Americas (39%; 47%) and Europe (30%; 28%) (**Figure 2.1.1**) (13).

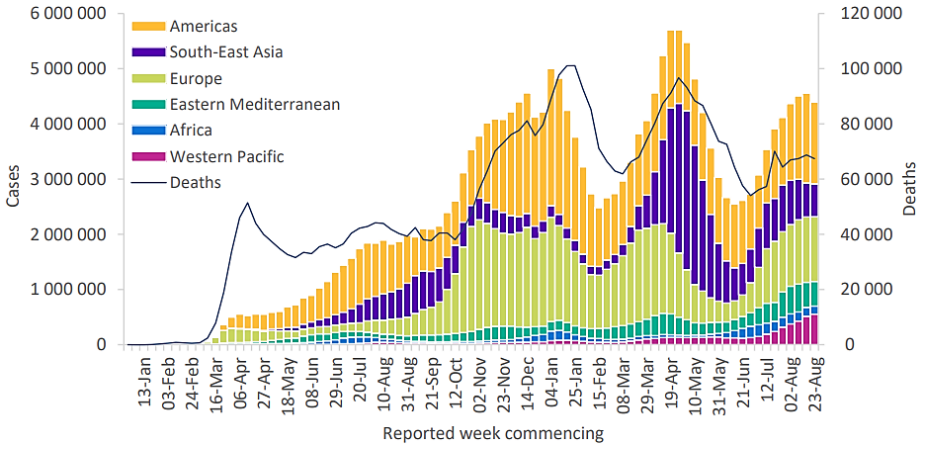

Figure 2.1.1. COVID-19 weakly reports of cases and global deaths as of August 29, 2021 (adapted from (13)).

Since the end of the year 2020, different variants that posed risks to the population appeared, leading to the development of concepts like Variants of Interest (VOI'S) and Variants of concern (VOC's) to prioritize monitoring and research. VOC's, labeled as Alpha, Beta, Gamma, and Delta by the WHO, were designated from December 18, 2020, to May 11, 2021, and reported in numerous sites (**Table 2.1.1**) (13,14). At the same time, in Portugal, 44087 cases were still active, contributing to a total of 1037927 cumulative cases, and 17743 cumulative deaths were estimated until August 2021 (15).

Table 2.1.1. SARS-CoV's-2 variants of concern and interest under monitoring as of August 2021, according to the WHO (adapted from (13,14)).

WHO labels and Lineages	Type of Variant	Date and Location of First Documentation	Date of Designation	Number of Countries with Reports of the VOC's
Alpha (B.1.1.7)	VOC	United Kingdom, September 2020	December 18, 2020	193
Beta (B.1.351)	VOC	South Africa, May 2020	December 18, 2020	141
Gamma (P.1)	VOC	Brazil, November 2020	January 11, 2021	91
Delta (B.1.617.2)	VOC	India, October 2020	May 11, 2021	170
Lambda (C.37)	VOI	Peru, December 2020	June 14, 2021	-
Mu (B.1.621)	VOI	Colombia, January 2021	August 30, 2021	39

2.2. Brief introduction on coronaviruses

Coronaviruses (CoVs) are enveloped pathogenic and non-segmented viruses with large single-stranded positive-sense RNA (ssRNA) genome, that varies between 26 to 32kb in length (one of the largest among RNA viruses) (16). Their spherical envelope, with a diameter that ranges from 80 to 160nm, bears “club-shaped”/ “crown-shaped” projections that gave origin to virus’s name (17,18).

They belong to the *Coronaviridae* family, *Cornidovirineae* suborder, *Nidovirales* order and *Riboviriae* realm. The *Coronaviridae* family includes the *Letovirinae* subfamily and the *Orthocoronavirinae* subfamily which is subdivided, based on the virus’s protein sequence, into four genera: alphacoronaviruses, betacoronaviruses, gammacoronaviruses and deltacoronaviruses. Within betacoronaviruses there are also recognized the subgenera *Embecovirus*/ lineage A, *Sarbecovirus*/ lineage B, *Merbecovirus*/ lineage C and *Embecovirus*/ lineage D (3,19,20). Alpha and betacoronaviruses only infect mammals and usually cause respiratory illness in humans and gastrointestinal symptoms in animals. Gammacoronaviruses mostly include CoVs from avian origin and aquatic animals. Deltacoronaviruses infect birds and, in some cases, mammals (swine-derived CoVs) (19,21). As of this date there are seven CoV’s known to infect humans, the Human Coronavirus (HCoV) 229E and the HCoV-NL63 (both alphacoronaviruses) and HCoV-OC43, HCoV-HKU1, Middle East respiratory syndrome coronavirus (MERS-CoV), severe acute respiratory syndrome coronavirus (SARS-CoV) and SARS-CoV-2 (all betacoronaviruses) (22).

Human coronaviruses, such as the previously mentioned HCoV-229E and HCoV-OC43, have been known to circulate in the population since the 1960’s, causing mild respiratory tract infections. However, since the beginning of the last two decades, highly pathogenic HCoVs have been linked to major outbreaks of severe pneumonia in different places around the world (23). These were the cases of the SARS-CoV and MERS viruses in November 2002 and June 2012, respectively, which resulted from zoonotic coronaviruses and lead to high morbidity and mortality (1). Both viruses may have originated in bats, although some intermediate/ secondary hosts were suspected (like civet cats for SARS-CoV and dromedary camels for MERS) due to the genetical similarities between the hostess and human viruses (more than 99% genome sequence identity) (1,23). The novel SARS-CoV-2 is another public health concern that rapidly surpassed SARS-CoV and MERS in terms of spread and infectivity. Several reports demonstrated, true phylogenetic analysis, that this virus clusters with SARS-CoVs in trees of the species *severe acute respiratory syndrome-related coronavirus,* placing it in the subgenus *Sarbecovirus* of the genus Betacoronavirus (**Figure 2.2.1**) (1,3).

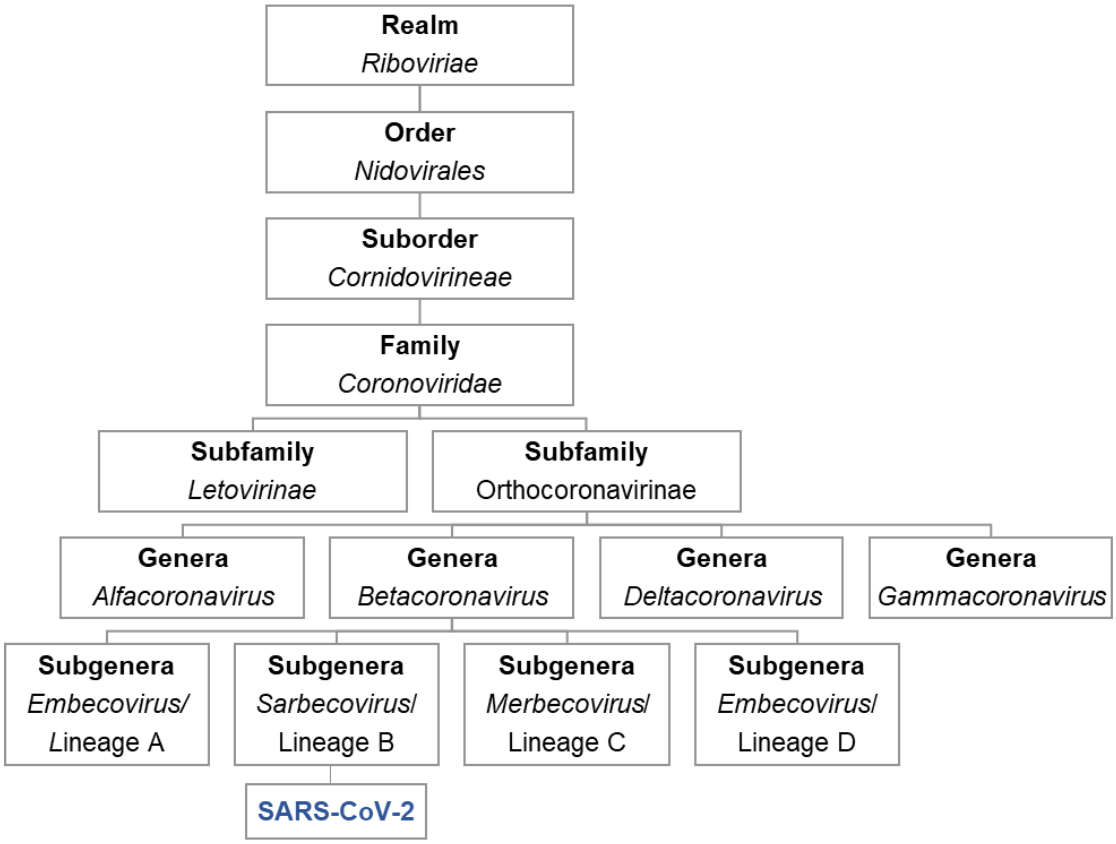

Figure 2.2.1. Taxonomy of the SARS-CoV-2.

Being a betacoronavirus, SARS-CoV-2 shares some genome sequence identity with SARS-CoV (79%) and MERS (50%). In the other hand, it is closely related (88% similarity) to bat-SL-CoVZXC21 and bat-SL-CoVZC45, two bat-derived SARS-like CoV strains, which is consistent with the fact that bats hold a reservoir for different kinds of CoVs. Nevertheless, just like for SARS-CoV and MERS, its highly likely that SARS-CoV-2 was transmitted to humans trough intermediate hostess, considering that the sequencing identity to the previously mentioned bat CoVs is less than 90% (24). Some reports indicate that pangolins might be the intermediate host for SARS-CoV-2 since, as result of homologous recombination, there is a 90,55% similarity between the S genes of pangolins and bat CoVs (25). These animals showed clinical manifestations of infection, which included interstitial pneumonia and inflammatory cell infiltration, opposed to bats that remained healthy while carrying these types of viruses (1,26).

2.3. Genome and structure of SARS-CoV-2

SARS-CoV-2 genome was recently sequenced and its length is approximately 29,9kb with a composition of four structural proteins and sixteen non-structural proteins (NSPs) (27). Besides, the virus's genome also consists of a 5'cap, a 3'-poly-A tail and two untranslated regions (UTRs), responsible for inter and intramolecular interactions, like those between the RNA of viral and cellular proteins (16,28,29). Just like most HCoVs, this new virus's genome is arranged in a specific order, that being: a 5'-UTR-replicase followed by structural proteins (S, E, M and N) and finally the UTR-3' (**Figure 2.3.1**) (30).

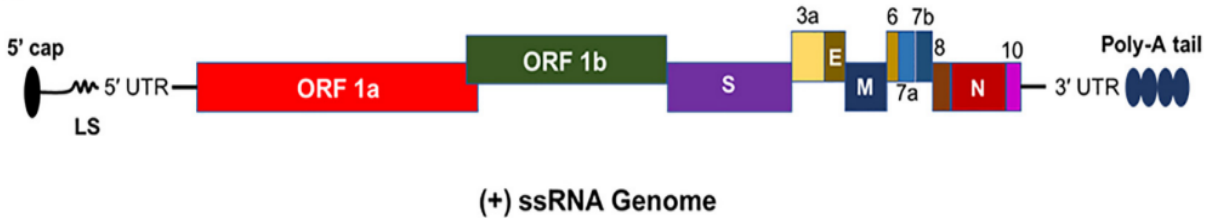

Figure 2.3.1. SARS-CoV's-2 genome representation (adapted from (31)).

It contains multiple open reading frames (ORFs), with ORF1a/b being the longest one, occupying about two-thirds of the virus's genome (29). ORF1a and ORF1b contain a frameshift mutation with produces the polypeptides 1a (pp1a) and 1ab (pp1ab). These will be processed into 16 NSPs by the action of chymotrypsin-like protease (3CLpro)/ main protease (Mpro) and papain-like proteases (PLpro). PLpro cleavage action results in NSP1, NSP2 and NSP3 that are needed for viral replication and Mpro's action in NSP4-NSP16, needed in the virus's life cycle. The rest of the genome is responsible for encoding the four structural proteins (**Figure 2.3.2**) previously mentioned: spike (S), membrane (M), envelop (E) and nucleocapsid (N) proteins, common among all CoV's (**Table 2.3.1**) (17,23,30).

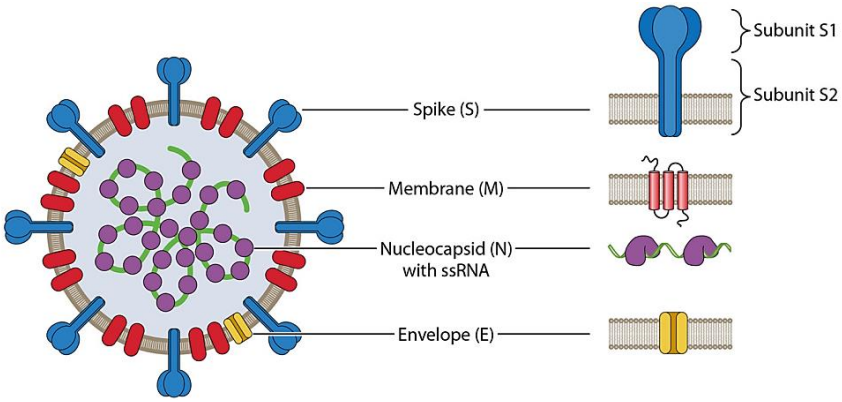

Figure 2.3.2. SARS-CoV's-2 structure representation (adapted from (32)).

Table 2.3.1. SARS-CoV's-2 structural proteins main characteristics and functions.

Structural Proteins	Main characteristics and functions	References
S	1273 aminoacid residues Trimeric protein with an extracellular N-terminus, a transmembrane region anchored in the viral membrane and an intracellular C-terminal Initiates the infection by sticking the virion to the host cell The spikes are camouflaged with polysaccharides, against the host's immune system 2 subunits in the ectodomain region: - S1 (N-terminal domain (NTD) + receptor binding domain (RBD)) for binding to the receptors on host cells - S2 (fusion peptide (FP) + heptad repeat 1 (HR1) + central helix (CH) + connector domain (CD) + heptad repeat 2 (HR2) + transmembrane domain (TM) + cytoplasmatic tail (CT)) responsible for virus and host cell membrane fusion For SARS-CoV-2, S1 recognizes the host's angiotensin-converting enzyme 2 (ACE2) via RBD	(27,31,33)
M	222 aminoacid residues Transmembrane protein tree domains: a C-terminal (inside), a N-terminal (outside) and a third transmembrane domain Transmembrane and endodomain are involved in protein-protein interaction (role in assembly of virion particles) Packaging of viral RNA Responsible for the virus's shape and assembly Generation of mature viral envelopes 3 transmembrane domains	(23,29,31)
E	75 aminoacid residues Assembly (trough viroporins) and secretion of virions from host cells Inhibition of host cell stress response	(23,31)
N	140 aminoacid residues Tree domains: N arm, central linker (CL) and C tail Packaging of viral RNA (gRNA) into a helical ribonucleocapsid (RNP) Interaction with the viral genome and M protein during viral assembly for transcription and replication	(16,23,29,31)

2.4. Host entry and SARS-CoV's-2 life cycle

The most common way of infection by SARS-CoV-2 is initiated at the host's cells surface when the angiotensin-converting enzyme 2 (ACE2) is recognized as a targeted receptor (29,30). After this, SARS-CoV-2 can use both endosomal cysteine proteases (cathepsin B/L) and transmembrane protease serine 2 (TMPRSS2) for S protein primming, contributing to its high infectivity (34,35). The cumulative effects of these proteases and furin preactivation (**Figure 2.4.1**) are beneficial for cell entry, mainly in lung epithelial and/or fibroblast cell lines. Even if host cells have low expression of TMPRSS2 or other proteases, furin preactivation boosts the virus's entry (35,36). Deletions on furin cleavage sites results in a less effective replication on respiratory cells and pathogenesis (37).

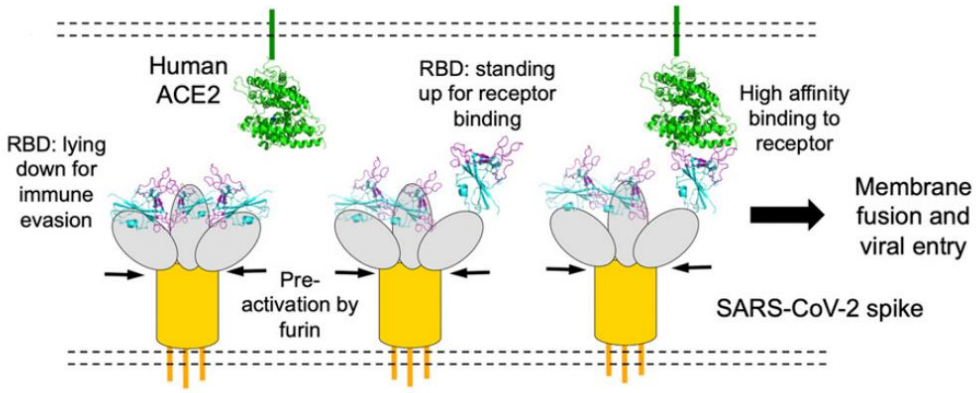

Figure 2.4.1. SARS-CoV's-2 binding to ACE2 receptor after furin pre-activation (adapted from (35)).

Fusion of the viral membrane with the host's cell membrane begins when the S1 domain binds with ACE2. Comparing to SARS-CoV, the SARS-CoV-2 RBD has a significantly stronger affinity to ACE2 receptors. In the other hand, even though the RBD can be both in a standing-up or lying-down state, SARS-CoV-2 S protein is mainly in a lying-down position causing the RBD to be less accessible, when compared to SARS-CoV (35,38). After this stage, viral entry can occur in two different ways (**Figure 2.4.2**). One of the ways involves endocytosis (which may be cathepsin-dependent) with subsequent viral envelope and endosomal wall fusion. In the other one, the virus's entry is assured through the cleavage of the S protein by TMPRSS2 which results in conformational changes in the protein, leading to the fusion of viral and host membranes (23,30,39).

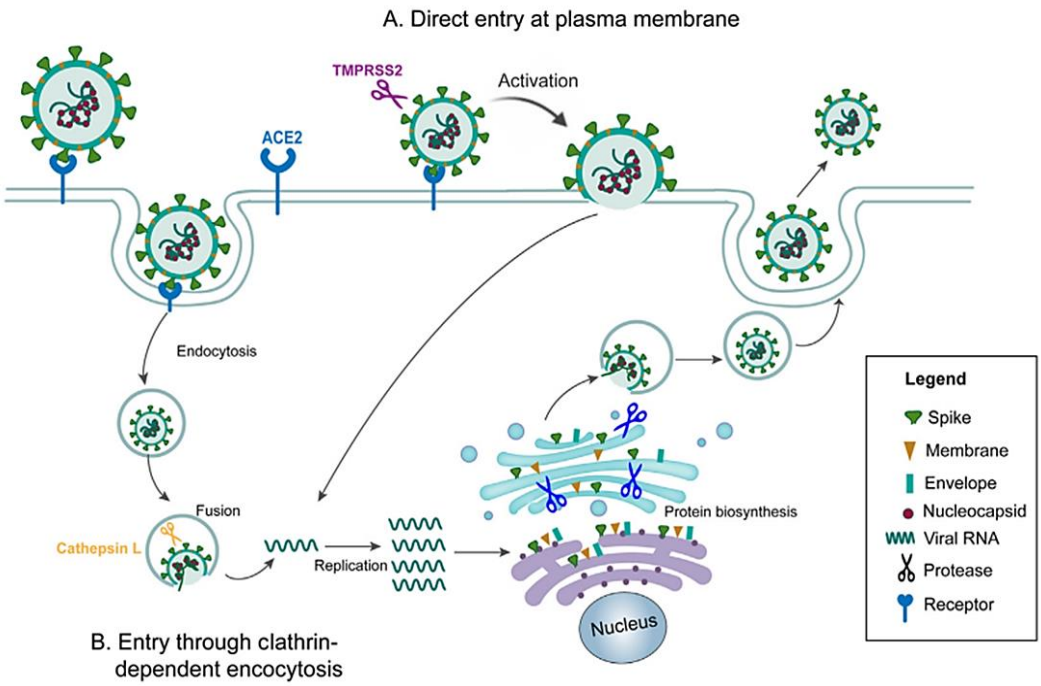

Figure 2.4.2. SARS-CoV's-2 host entry and life cycle (adapted from (39)).

Once the viral envelope and endosomal wall/ host cell membrane fusion has occurred, the virus's nucleocapsid packed genome RNA (gRNA) is released into the cellular cytoplasm. After the unpackaging of N proteins, translation of ORF1a and 1b directly from +gRNA (now serving as mRNA) into viral replicase polyproteins pp1a and pp1ab occurs in the ribosome (22). The same gRNA will also be the mold for RNA transcription. Then, interactions of some of the polyprotein-derived NSPs lead to the formation of a multi-protein replicase-transcriptase complex (RTC). This complex turns gRNA into negative-strand RNA that will be further used to create positive strands of gRNA (+gRNA's) and single guide RNA's (+sgRNA's) (**Figure 2.4.3 A, B**) (22,29). All the previous steps occur inside double membrane vesicles, that further release the sgRNA's for structural and accessory protein encoding. Note that SARS-CoV-2 also uses the host's transfer RNA for its own protein translation. The gRNA, also released from the vesicles, is encapsulated with N proteins. All translated structural and accessory proteins are then released in the endoplasmic reticulum and Golgi intermediate compartment (characteristic in CoV's). Here, the viral particles and gRNA are finally assembled into virions, then transported through secretory vesicles to the plasma membrane and secreted by exocytosis (22,23,29,40).

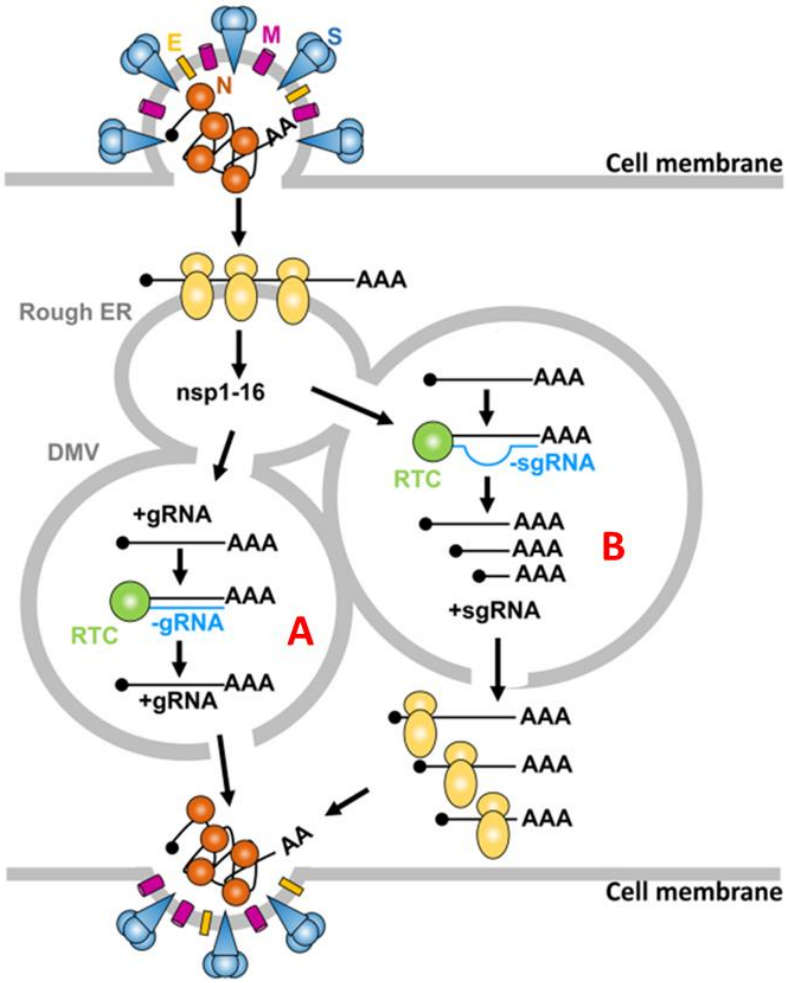

Figure 2.4.3. SARS-CoV's-2 genome replication and transcription (adapted from (22)).

Despite ACE2 being associated with vulnerability to SARS-CoV-2 infection, this enzyme alone isn't the sole factor involved in the virus infection. Ang II type 2 receptor (AT2R) per example acts as a co-receptor in the process of infection and some unidentified proteins may regulate the function of ACE2 receptor. Although ACE2 is the most commonly known receptor for SARS-CoV-2, its expression is relatively lower in tissues like the ones from the respiratory tract in comparison, per example, with the ileum (41). This led to the search of alternative receptors or co-receptors for viral entry trough the respiratory system. Thus, other receptors, such as tyrosine-protein kinase receptor UFO (AXL) were found. In this case, overexpression of AXL in HEK293T cells promotes SARS-CoV-2 entry and the blockage of this receptors significantly reduces infection (42). Other examples include CD147 and CD209l, transmembrane glycoproteins that function as receptors for SARS-CoV. Due to the similarity between this virus and SARS-CoV-2, these proteins could be other potential receptors used for cell entry in COVID-19 (41).

2.5. Angiotensin-Converting Enzyme 2 as a SARS-CoV'S-2 receptor and its influence in disease progression

ACE2 is a transmembrane protein present in cell membranes of organs like the lungs (tracheal, bronchial, and alveolar epithelial cells, type II pneumocytes and macrophages), heart (endothelium of coronary arteries, myocytes, fibroblasts) and kidneys, the gastrointestinal tract (per example in enterocytes of the small intestine) and arterial and venous endothelial cells (43,44). ACE2 protein expression can also be found in the nasal and oral mucosa, nasopharynx, skin, lymph nodes, bone marrow, brain, and liver. The fact that the lungs have a wide surface of alveolar epithelial cells might be related to their vulnerability to infection (41,45).

This protein is a negative regulator of the renin-angiotensin system (RAS), which is a key factor in blood pressure homeostasis and hydroelectrolyte balance, contributing for sodium reabsorption, and preventing the adverse effects of Angiotensin (Ang) II accumulation. Unlike its homologue ACE, that has a role in vasoconstriction and the rise of arterial blood pressure (BP), ACE2 leads to vasodilation and to the decrease of BP. This is achieved by the formation of an ACE2/Ang (1-7)/Mas receptor (MasR) axis in which Ang (1-7), a vasodilator, is mainly obtained through ACE2 (**Figure 2.5.1**). This enzyme convents angiotensin Ang I into Ang (1-9) (that will be further converted into Ang (1-7) by the action of ACE) and Ang II into (Ang 1-7). This leads to the production of Ang 1-7 that binds to the MasR, resulting in the above-mentioned axis (41).

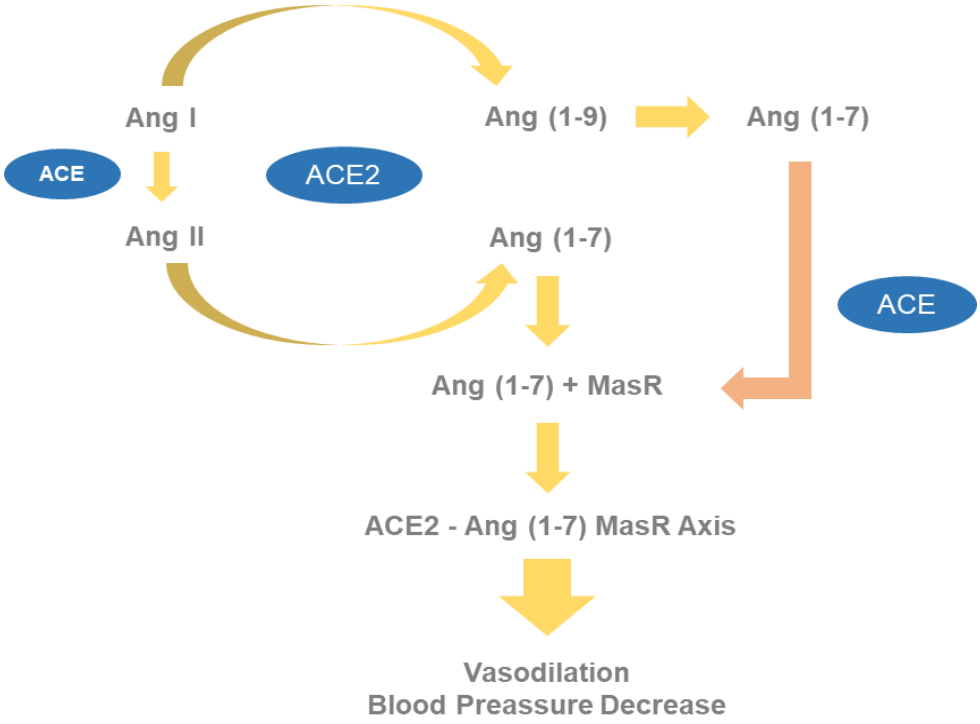

Figure 2.5.1. One of ACE2 roles in homeostasis.

The former mentioned negative regulation provided by the ACE2/Ang (1-7)/MasR axis counteracts the effects of the ACE/Ang II/Ang II type 1 receptor (AT1R) axis, like the ones that occur in hypertension and some cardiovascular diseases. Ang (1-7) alone can act on the MasR to influence different mechanisms in organs like the heart, kidneys, and brain (46). When infection by the SARS-CoV-2 occurs, ACE2 is downregulated in the lung and the ACE/Ang II/AT1R system becomes dominant, causing the accumulation of Ang II the decrease of Ang (1-7) production (41,47). Liu et al (48) demonstrated this accumulation of Ang II in SARS-CoV-2 infected patients by comparing the plasma levels of Ang II from COVID-19 patients and healthy individuals. Interestingly, the levels of Ang II were strongly associated with the viral load and lung injury in infected patients.

In cases of baseline ACE2 deficiency, for example in the presence of comorbidities like diabetes, hypertension and kidney disease, the consequences of ACE2 downregulation induced by SARS-CoV-2 infection may be much worse (44,45). Also, with age, the expression of ACE2 in the lungs decreases markedly, predominantly in men. In these situations, the protective effects of Ang (1-7), listed on the **Table 2.5.1** would be diminished, which can lead to worse prognosis. Viable therapeutic approaches could involve the administration of soluble recombinant ACE2 and Ang (1-7) (46).

Table 2.5.1. *Angiotensin (1-7) roles in physiology and disease.*

Organs	Role/ action	References
Lungs	Reduction of lung inflammation (decrease of total cell counts of eosinophils, lymphocytes, and neutrophils) Inhibition of leukocyte pro-inflammatory action by MasR activation on their surface Increase of anti-inflammatory cytokines Decrease of pro-inflammatory cytokines Attenuation of lung inflammation, airway remodeling and hyperresponsiveness in asthma cases Reduction of lung fibroblast migration and fibrosis Reduction of the expression of transforming growth factor β (TGF-β) and collagen deposition Inhibition of alveolar epithelial cell apoptosis (event that causes lung fibrosis) Reduction of pulmonary arterial hypertension Potential use in therapies for acute respiratory distress syndrome (ARDS)	(46,49)
Brain	Facilitation of the baroreflex Lowering/ increase of blood pressure Neuroprotection from brain ischemia or hemorrhage Attenuation of epileptic seizures	(46,50)
Heart	Myocardial protection Reduction of infarction area Antiarrhythmic (for example in ischemia-reperfusion arrythmias) Prevents heart disfunction, remodeling and hypertrophy Inhibition of oxidative stress	(46,51,52)
Blood Vessels	Vasodilatation Antihypertensive Anti-thrombogenic effects Antiproliferation of vascular smooth muscular cells	(46,51,52)

Muscles	Anti-fibrotic effects Reduction of apoptosis and atrophy Reduction of insulin resistance Increase of glucose uptake	(46)
Liver	Decrease in hepatic vascular resistance Inhibition of intra-hepatic vasoconstriction Decrease of hepatic fibrosis Reduction in infiltration of inflammatory cells and cytokines in cases of disease Reduces insulin resistance	(46,51)
Kidneys	Antidiuretic or natriuretic effects Small increases in glomerular filtration rate Antifibrotic and antiproliferative action Vasodilator action (per example in the afferent arterioles through local nitric oxide release)	(46,51–54)

2.6. Host's immune response to SARS-CoV-2 and cytokine storm

After SARS-CoV-2 enters the host's cells, the immune system begins mediating inflammation and antiviral activity in order to diminish/ inhibit viral replication and dissemination (4,31). The innate/ nonspecific immunity **(Figure 2.6.1, A)** is the first line of defense against any infection. It involves the expression of pattern-recognition receptors (PPR's), such as Toll-like receptors (TLR's) and retinoic acid-inducible gene I (RIG-I) - like receptors, by innate immune cells, that further recognize pathogen-associated molecular patterns (PAMP's) (55). PPR's can be localized in the cell surface or in the intracellular region, recognizing different types of PAMP's, including nucleic acids, lipoproteins, polysaccharides, and others. In the specific case of coronaviruses, recognition of viral RNA by endosomal receptors (like TLR3, 7 and 8) and cytosolic RNA sensors (like RIG-I) lead to the activation of various transcription factors that include interferon regulatory factor 3 (IRF3), nuclear factor kappa-light-chain-enhancer (NF-Kb) and activator protein 1 (AP-1) (55–57). Translocation into the nucleus occurs and the expression of pro-inflammatory cytokines and INF's (especially type I and III) is induced. The immune response is further amplified by the activation of the Janus kinase signal transducer and activator of transcription (JAK-STAT) pathway, via signaling through INF receptors, with transcription of INF-stimulated genes (in infected and non-infected cells) and subsequently expression of antiviral proteins that inhibit viral infection and pro-inflammatory cytokines. (55,58–60).

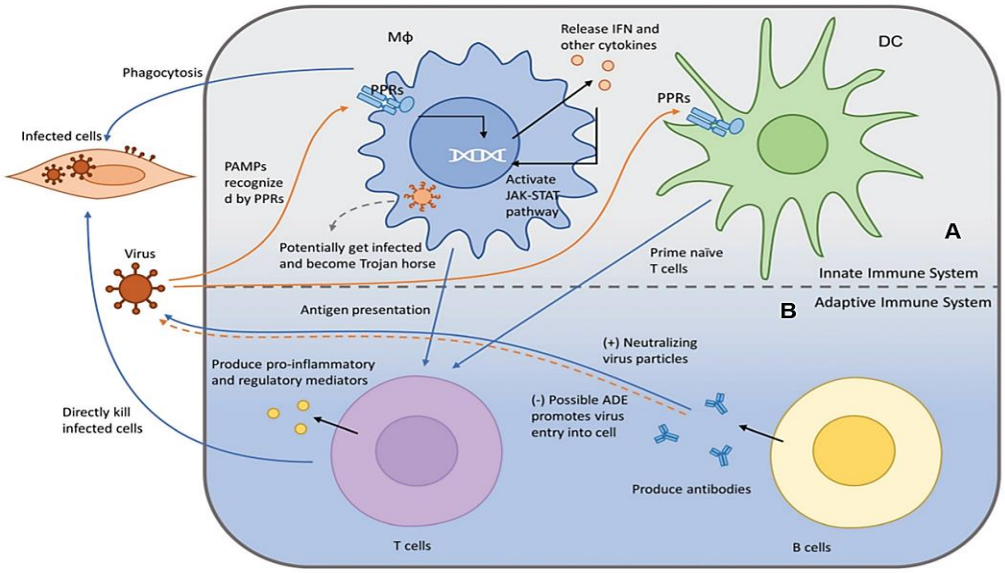

Figure 2.6.1. Immune response against viral infections. A) Innate Immune System; B) Adaptative Immune System (with both cellular and humoral immune responses) (adapted from (59)).

For the adaptative immune response **(Figure 2.6.1, B)** to begin, antigen presenting cells like DC's and macrophages present the viral antigens (like the ones released by neutrophiles (NEU) after phagocytosis) to T cells. Eventually, cytotoxic T cells (TCD8+) will destroy infected cells like type II pneumocytes, and macrophages (cellular immune response) and helper T cells (TCD4+) will induce B cells into the secretion of pathogen-specific antibodies (60,61). So, in case of the humoral immune

response, neutralizing antibodies produced by B cells are very important in interrupting the virus life cycle and preventing re-infections (58,60).

SARS-CoV-2 induces an immune response failure (per example due to high viral loads, presence of certain risk factors or genetic susceptibility) which further leads to a cytokine storm. The pathological basis may be related to damage in type II pneumocytes and capillary endothelial cells with diffuse alveolar damage followed by a two phases immune dysregulation: immunosuppression and proinflammation state (cytokine storm) (62). The lack of type I and III IFN responses in early cases of SARS-CoV-2 infection makes it harder to restrict viral infection in early stages, contributing to the virus's pathogenicity. The increase of cytokines results in an influx of macrophages, NEU, and T cells from the circulation, with multiple destructive effects in various tissues. This dysregulation of the innate immune system leads to subsequent alterations in the acquired immune response allowing the perpetuation of the imbalanced immune response (59).

The increase of multiple proinflammatory cytokines and chemokines like the ones listed on **Table 2.6.1,** which levels are proportional to the severity of COVID-19 disease**,** is manifested clinically by the development of acute respiratory distress syndrome (ARDS), damage to extrapulmonary tissues and organs, multiple-organ failure, disseminated intravascular coagulation and potentially death (all these consequences will be further explored in the next sections) (60,62,63). Another characteristic of this virus is that it can induce cell damage and death by pyroptosis releasing markers like IL-1β and lactate dehydrogenase (LDH), already reported in patients with COVID-19 and considered indicators of severity (59,62,64–66).

Table 2.6.1. *Cytokines that are elevated during COVID-19 progression.*

Cytokines/ Chemokines	**References**
Tumor Necrosis Factor (TNF)-α	(64,67–69)
Monocyte Chemoattractant Protein 1 (MCP1) and 3 (MCP3)	(64,65,67,69,70)
Fibroblast Growth Factors (FGFs)	(64)
Granulocyte Colony-Stimulating Factor (G-CSF)	(64,65,67)
Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF)	(64)
Platelet-Derived Growth Factor (PDGF)	(64)
Transforming Growth Factor (TGF)-β	(71)
Vascular Endothelial Growth Factor (VEGF)	(64)
Interferon-Gamma (IFNγ)	(64,68,71,72)
IFN-γ-induced protein 10 (IP-10)	(64,65,70)
Macrophage Inflammatory Protein 1 Alpha (MIP-1α) and Beta (MIP-1β)	(64,65)
Interleukin 1 beta (IL-1β)	(64,65)
Interleukin 1 Receptor Antagonist (IL-1RA)	(64,65,70,72)
Interleukin 2 Receptor (IL-2R)	(73)
IL-2, IL-4, IL-6, IL-7, IL-8, IL-9, IL-10, IL-17	(64,65,67–75)

Regarding the adaptative immune response to SARS-CoV-2, there are different levels of antibody responses between different patients that typically appear in the first 2 weeks after symptom onset (59). Levels of specific IgA, IgM and IgG appear to be higher and appear sooner in patients whose clinical condition is worse. Low levels of TCD4+ and TCD8+ cells have also been reported as well as promotion of TH17 cells (70,71). The low levels of the referred cells may be related to T cell exhaustion in part caused by the inflammatory cytokines. TH17 cells are involved in the activation of monocytes (MON)/ macrophages, DC's, and NEUs and in the release of more cytokines, worsening the former mentioned cytokine storm. These mediators cause damage to epithelial cells and reach the blood stream, causing further damage in other organs (58,59,62). Elevated NEU counts have been reported several times, as well as decreased eosinophil (EO), natural killer and T and B cell counts (64,70,76–78).

2.7. Baseline characteristics of COVID-19 patients

2.7.1. Age and gender

Older age is a major predictor of clinical deterioration and mortality, so as an increasing number of comorbidities (79,80). Age may determine worse outcomes, just like in other viral infections, due to the gradual decreasing innate and adaptative immune responses effectivity (81). Gender also plays an important part in disease severity since COVID-19 has a higher death rate in males. Individuals on the age group from 0 to 40 years and females under 60 have significantly less probability to die. Males with 60 years or more have a much higher risk of developing a severe disease and death (82,83). In a certain way, this can be explained by the different influences that environmental mediators, genetic and hormonal factors (like for example estrogen downregulation of Ang II and upregulation of Ang (1-7) pathways) and immune system regulation have on females and males (84,85).

2.7.2. Main comorbidities

Many of COVID-19's comorbidities are characterized by alterations in ACE2 expression/ activity and imbalances in the RAS. Based on a meta-analysis that included 125,446 patients, the most prevalent comorbidities were hypertension (32%), obesity (25%), diabetes (18%) and cardiovascular disease (16%). Others like renal disease, cerebrovascular accidents and cardiovascular diseases were more strongly associated with COVID-19 severity and higher mortality (86). Chronic lung diseases, particularly chronic obstructive pulmonary disease (COPD), and immunosuppression also predisposed patients to an unfavorable clinical outcome (70). In other reports, like one that involved 44,672 confirmed cases, chronic respiratory disease was one of the most common COVID-19 associated comorbidities (5).

2.7.3. Most common symptoms

Typically, clinical symptoms of infection by SARS-CoV-2 are reported approximately after 5,2 days of incubation (87). These mainly include fever, cough, fatigue, dyspnea, and sputum production (88). Other less common symptoms are migraines, hemoptysis, chest tightness, myalgia, shortness of breath and sore throat (32,88,89). Gastrointestinal symptoms like nausea and diarrhea with abdominal pain may also occur (64). These types of symptoms more often occur from the 7th to 14th days after the infection in conjunction with pulmonary ground-glass opacities and pneumonia.(1) In more severe cases patients tend to also develop pneumonia and respiratory failure due to ARDS, leading to their admission in ICU's (32). Acute cardiac injury and multi-organ failure are more common after de 16th day since the infection onset, in critical and deceased patients.(1)

2.7.4. Chest Computed Tomography

The diagnosis of COVID-19 is established by quantitative polymerase chain reaction (qPCR), but computed tomography (CT) has a very important role in prognosis assessment, management guidance and identification of disease related complications (90). Besides, in early stages and when nucleic acid tests show negative results, chest CT may be useful to evaluate the disease in combination

with other laboratory tests. Main findings obtained by chest CT imaging in cases of infection by SARS-CoV-2 include ground-glass opacities and bilateral consolidation (alveolar-filling process that replaces air with other fluids like blood and pus) in the lower and more peripheral parts of the lungs (70,91,92). Cases have been reported where ground-glass opacities were diagnosed in the lower and peripheral parts of the lungs and with disease progression the density of these alterations increased, their range expanded to the center of the lungs, and in the end diffused throughout the totality of the organ (48).

2.8 - Multi-organ involvement and biomarkers

2.8.1. Respiratory system

The respiratory system in an initial reservoir for viral replication and possess several characteristics that facilitate the infection. ACE2 expression was recently detected in nasal epithelial cells, a very important implication in understanding SARS-CoV-2 transmissibility, considering that the virus can be transmitted through droplets (93). Besides, the lung has a large and highly vascularized surface area that facilitates dissemination of inhaled viruses through the respiratory system and other organs (94). Like former mentioned, ACE2 is present in some pulmonary cells, but the alveolar type II epithelial cells are the ones where it's more expressed compared to other lung and bronchial tissues (43,95). These cells produce surfactant, execute immunoregulatory functions and have self-renewal capacity. Being one of the first sites of SARS-CoV-2 entry and replication, the lung's type II pneumocytes (that eventually become hyperplasic), nearby cells and the alveolo-capillary membrane become damaged with time. This generates a continuous cycle of destruction and repair that further leads to diffuse alveolar damage followed by bilateral oedema, thickening of the alveolar septa (due to interstitial fibroblast proliferation and fibrosis) and infiltration of inflammatory cells. Mainly interstitial infiltration of mononuclear cells (lymphocytes, MON, macrophages) has been reported, but also some cases of multinucleated giant cells and neutrophilic granulocytes in the alveoli (81). This process is facilitated by the increased vascular permeability caused by the augmented Ang II production. The infiltration of immune cells leads to excessive release of pro-inflammatory cytokines and chemokines, damaging the epithelial cells and reaching the blood circulation, causing damage to multiple organs (**Figure 2.8.1.1**) (96).

Like mentioned before, in COVID-19 the first symptoms are usually mild and from a respiratory nature (dry cough, shortness of breath, fever, chest pain, fatigue) although, in more severe cases, viral pneumonia and further complications like ARDS, cardiovascular pathologies and secondary infections can develop (96). There are no SARS-CoV-2 derived manifestations that allow a differentiation from other viral respiratory illness/ pneumonia.(97) ARDS (known for diffuse alveolar damage or epithelial-cell hyperplasia) is the leading cause of mortality in COVID-19 cases and is defined as an acute and diffuse inflammatory lung injury that presents with hypoxemia and bilateral pulmonary opacities (followed by lung collapse) on lung imaging, associated with increased pulmonary vascular permeability, increased physiological dead space and decreased lung compliance (96,98). This complication requires respiratory support, that ranges from high flow oxygenation to noninvasive or invasive mechanical ventilation, and the admission to an intensive care unit (ICU). Oxygenation is also improved when patients are turned from a supine to a prone position which generates enough transpulmonary pressure to exceed airway opening pressure in dorsal regions of the lung. This way, more severely collapsed areas are expanded, thereby increasing ventilation-perfusion, and improving gas exchange (99). ARDS can be divided into 3 categories of severity based on the degree of hypoxemia/ inspired oxygen ratio (PaO_2/FiO_2): mild (200 mmHg < PaO_2/FiO_2 ≤ 300 mmHg), moderate (100 mmHg < PaO_2/FiO_2 ≤ 200 mmHg) and severe (PaO_2/FiO_2 ≤ 100 mmHg) (98,100). Hypoxemia itself is has a strong association with

worse clinical outcomes and the PaO_2/FiO_2 ratio serves as an independent risk factor for predicting death in COVID-19 ICU patients (70,101). In cases of patients admitted to the ICU, complications like thrombosis and pulmonary embolism were found, despite the use of anticoagulation medication, leading the worsening of lung function and respiratory failure (94).

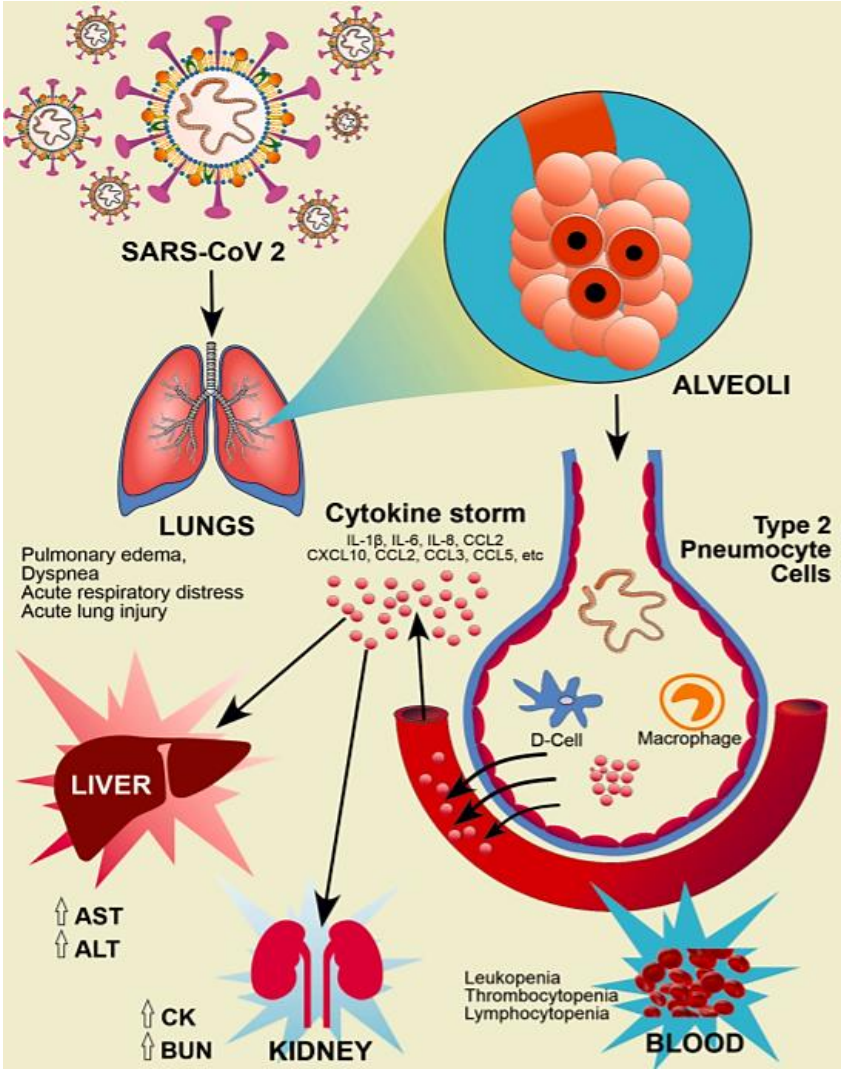

Figure 2.8.1.1. SARS-CoV-2 entry through the lungs, pathogenesis, and multiple organ damage. The lung's type II pneumocyte cells enter a continuous cycle of destruction and repair that further leads to diffuse alveolar damage, facilitated by the increased vascular permeability. This is followed by bilateral oedema, thickening of the alveolar and infiltration of inflammatory cells (like lymphocytes, monocytes, macrophages, and neutrophils). This process than leads to excessive release of pro-inflammatory cytokines and chemokines, damaging the epithelial cells and reaching the blood circulation, causing damage to multiple organs (adapted from (96)).

2.8.2. Cardiovascular system

According to a study based on ACE2 expression in human tissues, the heart is one of the organs with the most prominent expression of this protein, especially in cardiomyocytes and endothelial cells and/or pericytes (**Figure 2.8.2.1**) (95,102). In another research based on single cell RNA sequencing, more than 7.5% of myocardial cells had ACE2 expression (comparing to approximately 1% in type II pneumocytes and 2% in epithelial cells from the respiratory tract) which means SARS-CoV-2 can induce direct cardiotoxicity when entering cardiomyocytes (103). These can be some of the explanations for direct cardiac involvement in COVID-19 and the resulting cytokine storm and resulting hyperinflammation state with several consequences like, per example, myocardial disfunction. High expression of ACE2 in microvascular pericytes in capillaries all through the body may explain other clinical manifestations in the cardiovascular system, namely the high risk of thromboembolism and vascular disfunction (95).

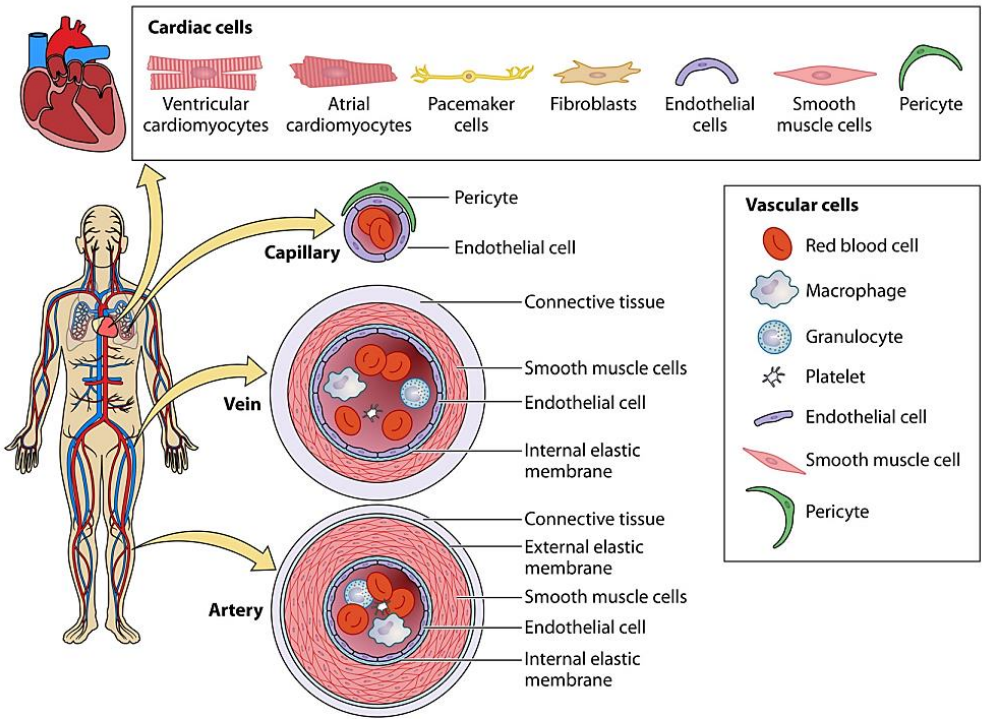

Figure 2.8.2.1. Cardiovascular system's main cells and their localization. Expression of ACE2 mainly occurs in cardiomyocytes, endothelial cells and pericytes (adapted from (32)).

Focusing on the indirect damage in the cardiovascular system caused by SARS-CoV-2, some clinical manifestations (that may also result from direct damage) include myocardial ischemia and type 1 and 2 myocardial infarction (MI), myocarditis, arrythmias (new-onset atrial fibrillation and flutter, sinus tachycardia and bradycardia, prolongation of the QTc segment …), cardiomyopathy, cardiogenic shock, and the former mentioned thromboembolic complications (104). These are driven mainly by the hyperinflammation state caused by the cytokine storm, which further leads to vascular inflammation, hypercoagulability, and other disturbances related to RAS counter regulation (105). Early research even mentioned that myocardial injury and other manifestations are more likely to be caused by this type of systemic changes rather than direct damage resulting from the viral infection (106).

Myocardial ischemia/ injury and myocarditis itself can also be considered as some of the leading causes of death in COVID-19 patients (106). According to the 2007 guideline issued by The National Academy of Clinical Biochemistry, a concentration of cardiac troponins superior to the 99th percentile of the values for a reference control group is indicative of myocardial injury due to possible MI (107). In a retrospective cohort study, more than half of the deceased patients had increased high-sensitivity cardiac troponin (hs-cTn) I during hospitalization, which was also associated with disease severity (108). It's not only important the study of the levels of cardiac troponins in a certain moment in time, but also their behavior during disease progression. Per example, there are several cases where levels of cardiac troponins from survivors remain stable while the one's from non-survivors, that were initially higher, continued to rise until death (106,108,109). Myocarditis is an inflammatory disease of the heart's muscle, leading to inflammatory infiltrates and injury to the myocardium without an ischemic etiology, opposed to what happens in MI's (110). The prevalence among COVID-19 patients is unclear, but some results indicate up to 12.5% of abnormalities similar to myocarditis (106). Others even argued that it may be responsible for approximately 7% of COVID-19 related deaths (111).

Cardiac arrythmias are also a very common cardiovascular manifestation, which high prevalence may be related to hypoxia (resulting from consequences of viral infection in the lung), electrolyte imbalance (resulting from COVID-19's interactions with the RAS, contributing to hypokalemia), inflammatory stress, metabolic disarray and may also be a result of other manifestations (like myocardial injury and myocarditis) (111,112). It's been reported that arrhythmias lead to the admission to ICUs of 44.4% of COVID-19 patients (113). This high percentages can be the result of overestimation since the majority of causes for arrhythmias are not reported and can be secondary to other clinical manifestations, or even related to patients' preexisting conditions (110).

COVID-19 related heart failure may be caused by pre-existing cardiac disfunction and/ or by newly developed myocarditis and cardiomyopathies. Per example, the right ventricle is very vulnerable to increases in pulmonary resistance. Since SARS-CoV-2 typically affects the lungs first, the functional reduction of residual gas volume can increase vascular resistance in the lungs and therefore increase pulmonary resistance. This causes a volume overload in the right ventricle, which can possibly lead to pulmonary hypertension and pulmonary hearth disease (112).

Dysfunction in the coagulation process has also been reported in COVID-19 cases, this can be evidenced per example by elevated levels of D-dimer (DD), fibrin degradation products and prothrombin time and by other manifestations in different coagulation indexes (**Table 2.8.2.1**) (94,111). DD was found to be associated with a fatal outcome in COVID-19 when levels are above 1ug/mL, showing increased coagulation activity. One study showed that DD and fibrin degradation products levels are also more prone to be elevated in COVID-19 non-survivors and that 71,4% of this population developed disseminated intravascular coagulation (DIC). This condition can be caused in part by the expression of tissue factor and secretion of von Willebrand factor (promotors of prothrombin and thrombin and platelet (PLT) aggregation, respectively) due to cytokine release and it's related to sepsis (113–115). DIC aggravates multiorgan damage and is related to thrombosis, not only of large vessels, but also epicardial

vessels and the ones belonging to the microcirculation (being one of the causes of thrombosis of coronary arteries) (105). Venous thromboembolism is also a problem mainly since infected patients may enter a state of prolonged immobilization besides already being in risk of vascular inflammation and coagulation disfunction (111). Plaque rupture, induction of coagulation factors and PLT aggregation through local inflammation and hemodynamic changes can also lead to ischemia and thrombosis, especially in cases of history of atherosclerosis (108). Pulmonary embolism, usually diagnosed a few days after ICU admission, is also a frequent result of endothelial inflammation combined with severe hypoxemia. Hypoxemia itself may lead to vasoconstriction and reduction of blood supply to pulmonary capillaries, promoting their occlusion (114).

Table 2.8.2.1. *Biomarkers for Coagulation Dysregulation in COVID-19*

Categories	Increased Biomarkers	Decreased Biomarkers	References
Coagulation Indexes	D-Dimer (DD) Fibrinogen (FIB) Prothrombin Time (PT)/ International normalized ratio (INR) Partial Thromboplastin Time Fibrin Degradation Product (FDP)	Antithrombin Prothrombin Time Activity (PT-act)	(64,70,77,114–116)

The exact mechanism of coagulopathy is unknown but recent studies suggest a strong relationship with RAS axis dysregulation. This dysregulation may be caused by the effects of SARS-CoV-2 infection that facilitates the loss of ACE2 expression leading to accumulation of Ang II, and through this increasing the risk of thrombosis and endothelial dysfunction and decrease in Ang (1-7) levels and thereby its vasodilatory, anti-inflammatory, cardioprotective action and other effects that may play a role in the attenuation of COVID-19's clinical manifestations (32).

Considering all the mentioned clinical manifestations resulting from cardiovascular disfunction due to SARS-CoV-2 infection, monitoring cardiac function and standard biomarkers, like cardiac troponins and coagulation indexes, is recommended and crucial in severe cases of COVID-19 (94).

2.8.3. Gastrointestinal tract

The pathophysiology of gastrointestinal tract involvement is more likely multifactorial. Virus direct tissue damage through ACE2 entry is plausible due to the presence of these receptors in stomach epithelial cells, in enterocytes from the small intestine (including the duodenum, jejunum and ileum) and rectal epithelia. Higher levels of ACE2 expression were detected in the small intestine and duodenum and the lowest in the stomach and large intestine (32,95,117). ACE2 levels are higher in differentiated enterocytes when compared with the immature ones (118). TMPRSS2 levels are also highly expressed in the gastrointestinal tract (119).

According to a multicenter cohort study involving 318 confirmed COVID-19 patients, approximately 61% of them reported at least one gastrointestinal symptom, with 20,3% of the patients reporting it as their predominant complaint and 14,2% as the initial presenting symptom. The most reported manifestations were loss of appetite, diarrhea, and nausea. Among these patients, symptoms like fatigue, myalgia and sore throat, loss of smell or taste were reported in higher rates, when compared with patients with no gastrointestinal manifestations (120). Other studies also reported cases of abdominal pain, anorexia and rare situations that involved mesenteric ischemia, gastrointestinal vasculitis, and gastrointestinal bleeding (104,121).

Reports of histological analysis of COVID-19 patients demonstrated some of the consequences of SARS-CoV-2 cell entry, which included infiltrating plasma cells and lymphocytes (LYM) in the esophagus, stomach, duodenum, and rectum, with interstitial edema (117). A case also resulted in preeminent endothelial inflammation of submucosal vessels from the small intestine and accumulation of apoptotic bodies, together with evidence of mesenteric ischemia suggesting small-bowel injury (122). These manifestations may explain some of the former mentioned symptoms and, even though they haven't been associated with a higher risk of mortality, they seem to correlate with longer periods of illness (94).

2.8.4. Renal involvement

ACE2 expression in the kidneys is very high, allowing direct damage by SARS-CoV-2 infection, especially in the renal tubular epithelium and podocytes (81,95,123). Indirect damage also occurs due to imbalance of the RAS, cytokine storm (which causes the release of G-CSF, interleukins and IFN causing direct or indirect injury to the kidneys) and other immune responses, hypoxemia, coagulation disfunction and consequences of damage in other organs (124).

Histopathological findings from a postmortem analysis of 26 COVID-19 patients reported a range of abnormalities. Substantial endothelial damage was observed, which may induce acute kidney injury (AKI), lead to proteinuria and elevated serum creatinine levels(81). AKI, which was significant in these cases, is the abrupt loss of kidney function that affects 0,9% to 37% of the patients, depending on centers and countries, it occurs approximately 7 to 14 days after admission. Acute and diffuse proximal tubular injury, obstruction of peritubular and glomerular capillary loops by aggregates of erythrocytes, microvascular disfunction secondary to endothelial damage and other findings related to SARS-CoV-2 invasion of kidney tissue were also observed (104,125). Other reports show an elevated incidence of podocytopathies, kidney diseases that involve injury to podocytes (highly specialized cells in the glomerulus) including glomerulonephritis, leading to proteinuria or nephrotic syndrome (121,126). Some types of glomerulonephritis have been reported in COVID-19 patients that have poor prognosis and require dialysis in more than half of the cases (121).

Besides proteinuria, hematuria is also associated with worse clinical outcomes and higher mortality in COVID-19 patients with renal dysfunction (104). Other relevant parameters were serum creatinine (SCr), typically elevated in severe cases of COVID-19, and blood urea nitrogen (BUN) (127). One study demonstrated that a low estimated glomerular filtration rate (eGFR) (indicating renal insufficiency) on admission and development of AKI during hospitalization are independent risk factors for poor clinical outcomes (74). In general, and according to a cohort study, reports indicate that hematuria, proteinuria, elevated SCr, elevated BUN and decreased eGFR are independent risk factors for disease progression (128).

This said, obtaining urine analysis of SARS-CoV-2 infected patients may be useful for risk stratification (104). It may also be important to consider risk factors for AKI like older age, diabetes mellitus, cardiovascular disease, black race, ventilation, and hypertension. In a study that involved 5,449 COVID-19 patients, 89,7% of those that were on mechanical ventilation developed AKI. 52,2% of this group required renal replacement therapy (129). So, special attention is required for patients that are in need for mechanical ventilation.

2.8.5. Liver involvement

Liver ACE2 expression was reported to be just above cutoff (95). On the other hand, studies have reported a much higher expression of ACE2 in cholangiocytes (epithelial cells of the bile duct), leading to the conclusion that direct liver injury due to virus infection may not be a result of hepatocyte damage, but cholangiocyte dysfunction (32,95,123,130). Bile duct cells are related to initiation and regulation of immune responses and liver regeneration, thus the disruption of these functions can lead to hepatobiliary damage (131).

Despite the former mentioned facts, the mechanisms of direct injury by SARS-CoV-2 infection are still unclear. Histological findings such as mild lobular LYM infiltrations, centrilobular sinusoidal dilation and patchy necrosis on liver sections have been reported (132). In another study, biopsy samples of liver tissue from a COVID-19 patient have indicated moderate microvesicular steatosis and mild lobular and portal inflammation (133). These findings may in fact be related to direct injury caused by SARS-CoV-2 infection or to drug-induced liver damage (DILI), caused by the high doses of antiviral medications, antibiotics or steroids, cytokine storm or even underlying diseases (**Figure 2.8.5.1**) (81,132,133).

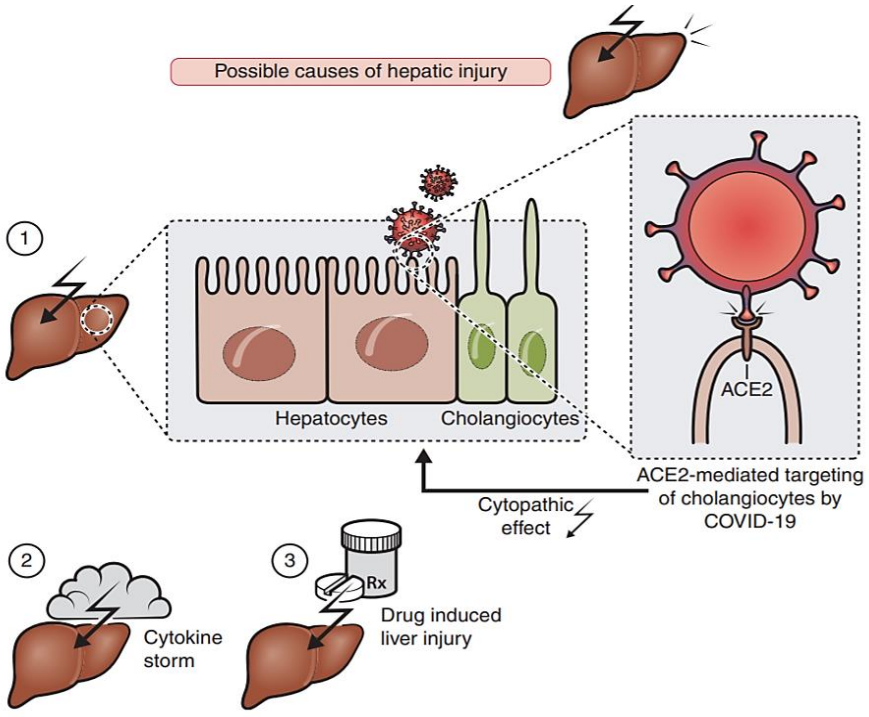

Figure 2.8.5.1. Possible causes of hepatic injury. 1) SARS-CoV-2 direct liver damage through cholangiocyte entry. 2) Immune-mediated process of liver injury through cytokine storm. 3) Drug-induced liver injury related to medication for COVID-19 management (adapted from (130)).

Besides histopathological findings and other aspects related to liver injury (such as older age and pre-existing liver disease), biochemical biomarkers are another tool that needs to be considered when searching for indicators of liver involvement in COVID-19 cases (130). According to a systematic review and meta-analysis of 20 retrospective studies regarding the association between liver injury and severe COVID-19, higher levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin and lower levels of albumin are associated to COVID-19 disease severity (**Table 2.8.5.1**) (131). Gamma-glutamyl transferase (GGT) values can be found in some COVID-19 related studies. These are cholestatic markers, meaning that GGT elevations, like the ones detected in a recent review that reported unpublished data (with GGT elevations in 30 of the 56 cases), are related to cholangiocyte injury (131,134). Although less frequently, elevated alkaline phosphatase (ALP) levels were also found in COVID-19 patients, and sometimes related to disease severity.(135,136) Regardless, in a systematic review and meta-analysis, the impact of SARS-Cov-2 infection in most liver enzymes (like AST, ALT, ALP, and others) was not significant at initial presentation. This points to the need of more studies with larger sample sizes and control groups (137).

***Table 2.8.5.1**. Biomarkers for Liver Injury in COVID-19*

Categories	Increased Biomarkers	Decreased Biomarkers	References
Liver-Related Biochemical Biomarkers	Aspartate Aminotransferase (AST) Alanine Aminotransferase (ALT) Alkaline Phosphatase (ALP) Gamma-glutamyl Transferase (GGT) Total Bilirubin	Albumin	(48,64,70,77,78,116,130,135,136,138)

2.8.6. Pancreas

ACE2 is expressed in both the exocrine and endocrine pancreas. Studies have determined low levels of ACE2 expression the interlobar ducts and endothelial cells/ pericytes (exocrine pancreas) (95). Other reports indicated that the levels of expression on these cells are positively higher when compared to the one's from the endocrine tissue (beta cells from Langerhans islets) (139). This way, SARS-CoV-2 can cause direct pancreatic injury by affecting beta cells and indirectly by targeting duct cells and endothelial cells/ pericytes (**Figure 2.8.6.1**), contributing to insulin deficiency and hyperglycemia (even in patients without preexisting diabetes), as it occurred in previous cases of SARS-CoV infection (32,104,140,141). Systemic effects may also be related to pancreatic damage since the global state of inflammation and the accumulation of prodiabetic metabolites can ultimately damage beta cells (141).

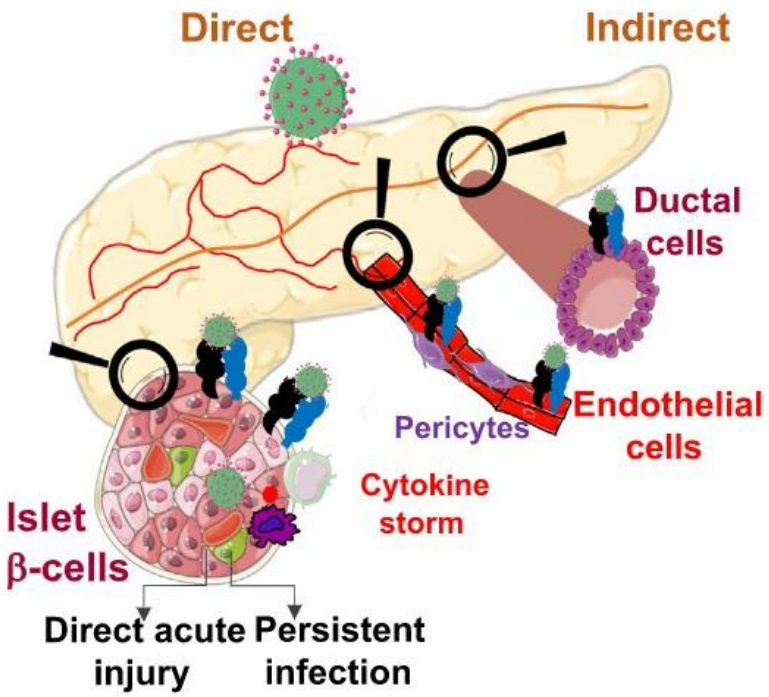

Figure 2.8.6.1. Possible causes of pancreatic injury include SARS-CoV-2 direct liver damage through beta cell entry or indirect damage by pericyte, endothelial and ductal cell targeting. Indirect damage leads to cell structural and functional transformation, causing local inflammation with cytokine and chemokine release, possibly contributing for beta cell involvement (adapted from (141)).

Although it's unclear whether or how SARS-CoV-2 infection results in injuries in the endocrine pancreas, besides direct perturbation of beta cell function, immune-mediated factors that facilitate insulin resistance and beta cell hyperstimulation (and eventually their dysfunction) are some of the factors involved in pancreatic injury (142–144). A cohort that involved 551 patients with COVID-19 reported altered glucometabolic control, with insulin resistance and abnormal cytokine profiles. Data also indicated that metabolic abnormalities persisted at least 2 months after patients had recovered from COVID-19. Finally, it was concluded that SARS-CoV-2 induces insulin resistance and disrupts beta cell function, which can lead to hyperglycemia (143). The destruction of beta cells may also trigger new-

onset/ acute diabetes.(144) Diabetes is a major risk factor for COVID-19 hospitalization and/or admission to the ICU and severe illness. In a retrospective cohort involving 1158 patients with COVID-19, 23,4% had diabetes and 27,1% pre-diabetes (141). Another cohort study with 28,095 COVID-19 patients also reached the conclusion that diabetes increased hospitalization and critical care risks for these patients (145).

As a result of exocrine pancreas damage, acute pancreatitis may occur in 32,5% of critically ill patients (more frequently in women). Symptoms include severe acute upper abdominal pain, nausea and vomiting and results in pancreatic enlargement and elevated pancreatic enzymes (amylase or lipase) in 7,5-17% of patients (121,142). Elevation of these pancreatic enzymes is related to a poor prognosis in COVID-19 patients, especially in the critically ill (121,142,146). Although, the elevation of these markers can also be related to kidney injury, degradation of the oxygenation (cases linked to mechanical ventilation and shock), other infections…(146). Hyperlipasemia was found in 11,7% of the patients infected with SARS-CoV-2 included in a systematic review and it was determined that these patients had a 3-fold higher risk of a poor clinical outcome including need for ICU admission and mechanical ventilation, and a bigger risk of death (147).

2.8.7. Neurological involvement

SARS-CoV-2 related neurological manifestations are diverse. Cases of encephalopathy, encephalitis, and cerebrovascular pathologies due to neuroinvasion or neurotropic damage have been reported, just like other manifestations related to neuroinflammatory damage (per example cases of Guillian-Barré syndrome and other inflammatory demyelinating polyneuropathies) (121). Despite all these manifestations, brain tissue showed a consistent lack of ACE2 expression in different studies (95). Possible routes of virus entry into the nervous system include the olfactory epithelium (that contains neuronal and nonneuronal cells that express both ACE2 and TMPRSS2 that eventually undergo apoptosis, causing anosmia), carriage across the blood-brain barrier and through infected leukocytes (104,148,149). The virus may also infect the peripheral (PNS) and central (CNS) nervous systems by direct infection of nerve endings in tissues and through axonal routes that reach the CNS, respectively (149). Entry through the olfactory bulb is a way to invade various parts of the brain, including the brainstem. Since this structure is responsible for respiratory regulation by, per example, detecting fluctuations in oxygen and carbon dioxide concentrations, allowing the modulation of the respiratory rate it's partially involved in the mechanisms of respiratory failure. The invasion of the brainstem may also cause dysfunction of the cardiac center, that regulates the heart rate, leading to cardiac manifestations or worsening of the preexisting ones (150).

Different factors are responsible for the mentioned clinical manifestations, being cytokine storm one of them. The purpose of this immune response is to fight the viral infection in the nervous system, but the fact that its exaggerated and dysregulated can lead to the development of meningitis, encephalitis, meningoencephalitis, death, and other types of damage to de central and even peripherical nervous system (149). Other factors include the direct effects of the virus in the nervous system and neurological complications that result from the systemic effects of COVID-19 (per example vascular effects that can result in ischemic stroke, intracerebral hemorrhage, transient ischemic attack…) (148). A report of a histopathologic examination of a patient infected with SARS-CoV showed neuron denaturation and necrosis, encephalic edema and broad gliocyte hyperplasia. Immunohistochemical staining also demonstrated that infection by this type of viruses results in elevated expression of cytokines in the brain and infiltration of MON, macrophages, and T cells (151).

Symptoms resulting from the mentioned effects of SARS-CoV-2 in the nervous system include headache, confusion, dizziness, anosmia, ageusia, myalgias, fatigue and visual impairment (94,104). In a European multicenter study approximately 86% and 88% of the 417 COVID-19 patients that were involved reported olfactory and gustatory dysfunction, respectively (152). Another study regarding the neurologic manifestations of patients with COVID-19 in Wuhan, China, of the 214 patients, 36,4% had neurologic manifestations (24,8% related to the CNS and 8,9% to the PNS). Patients in more severe conditions had significantly more nervous system-related manifestations, including acute cerebrovascular disease, ischemic stroke, cerebral hemorrhage, impaired consciousness, and skeletal muscle injury. Other symptoms included dizziness, headache, ataxia, seizures, nerve pain and taste, smell, and vision impairment (153).

Chapter 3 – Methodology

3.1. Study population and data assembly

Patients hospitalized to the ICU of *Centro Hospitalar Universitário Lisboa Central* between March 2020 and March 2021 and diagnosed with COVID-19, after a positive result on a reverse-transcriptase-polymerase-chain-reaction (RT-PCR) assay, were included in the study population. The present study is inserted in the project Predictive Models of COVID-19 Outcomes for Higher Risk Patients Towards a Precision Medicine (PREMO), approved by the former mentioned institution's ethics board and is under all legal and ethics considerations.

A large database including demographic data, information on symptom onset, RT-PCR diagnosis, dates of hospital/ ICU admission and discharge and implementation of certain therapies (defined as "Patient data") was built in the platform Microsoft Excel. All this information was gathered from the hospital's electronic medical record system. Results of blood gas analysis, hemograms, ionograms, urine tests and other daily laboratorial analysis were also extracted to individual Microsoft Excel files, for each patient, that were further condensed into another database (named "Laboratory Results").

In the development of this master thesis, only patients over 18 years of age and with sufficient information regarding the study variables were included. Patients without hospital discharge, ICU admission and discharge dates (due to lack of data or because the individuals were still admitted) were excluded, as well as without either symptom onset or RT-PCR COVID-19 diagnosis dates. In groups of patients that required invasive mechanic ventilation (IMV) and/or extracorporeal membrane oxygenation (ECMO), those without dates of beginning and/or end of the referred techniques were also excluded. From the remaining patients, those who had information regarding their laboratory results were included in the present study, leading to a sample of 337 patients.

3.2. Clinical and demographic data

Age and body mass index (BMI) were calculated with the data collected at hospital admission (date of birth, weight, and height) and the following age groups were considered: 30 years or younger; 30 through 39 years; 40 through 49 years; 50 through 59 years; 60 through 69 years; 70 through 79 years and 80 years or older. BMI was calculated based on the *Quelet* Index, which is obtained when the body weight (kilograms) is divided by the squared height (meters) of an individual (154,155). With this information the patients were also categorized according to their BMI, in order to evaluate their degree of obesity: less or equal to 29,9 Kg/m^2 (normal BMI); 30 through 34,9 Kg/m^2 (Class I Obesity); 35 through 39,9 Kg/m^2 (Class II Obesity); and equal to or greater than 40 Kg/m^2 (Class III Obesity) (154,155). Obese and non-obese sub-groups were also used as study variables.

The number and type of comorbidities from each patient, previously assessed by health professionals, were collected from medical records. The resulting lists of comorbidities were then analyzed and separated into individual nominal variables for future statistical analysis. Some individual

comorbidities were grouped (**Table 3.2.1**) due to the original low frequencies, with the validation of health professionals.

***Table 3.2.1**. Grouping of individual comorbidities*

Comorbidity Group (nominal variables**)**	**Individual Comorbidity**
Rheumatic Diseases	Arthritis
	Rheumatic Disease
	Systemic Sclerosis/ Scleroderma
Pulmonary Vascular Disease	Pulmonary Embolism
	Pulmonary Hypertension
Cardiovascular Disease	Aortic Dissection
	Aortic Stenosis
	Congestive Heart Failure
	Takotsubo Cardiomyopathy
	Unspecified Congenital Heart Disease
Heart Arrythmias	Atrioventricular Block
	Atrial Fibrillation
	Bradyarrhythmia
History of Organ Transplantation	Heart Transplant
	Lung Transplant
	Liver Transplant
	Renal Transplant
Hematologic Cancer	Leukemia
	Chronic Lymphatic Leukemia
	Multiple Myeloma
	Hodgkin Lymphoma
Tissue and/or Organ Cancer	Myeloproliferative Syndrome
	Carcinoma
	Bladder Cancer
	Colon Cancer
	Breast Cancer
	Thyroid Cancer
	Prostate Cancer
	Prostate Adenocarcinoma
	Lung Cancer
Nervous System Disease	Epilepsy
	Myasthenia Gravis
	Multiple Sclerosis
	Parkinson Disease

Patients were also grouped according to the number of comorbidities, so an ordinal variable with the following categories was created: 0 comorbidities; 1 to 2 comorbidities; 3 to 4 comorbidities; 5 or more comorbidities.

Due to the existence of groups with low frequencies, variables like “Admission Motive” had some categories condensed. Thus, the category “Central Nervous System Disorders” includes coma, convulsions, and focal neurological deficits.

COVID-19 waves were established according to the hospital's directives. The first wave was considered from March 10 to August 22, 2020, the second wave from August 23 to December 19, 2020, and the third wave from December 20, 2020 to June 5, 2021. Patients were distributed among the different COVID-19 waves according to the date of RT-PCR diagnosis or of symptom onset, whichever was earlier. This criterion, regarding these two dates, was applied throughout the study.

Respiratory support included IMV, ECMO and high flow oxygen (HFO). For IMV and ECMO, days between symptom onset/RT-PCR diagnosis and IMV onset, between symptom onset/RT-PCR diagnosis and ECMO onset, between ICU admission and IMV onset, and the number of days with IMV/ECMO were calculated.

For the variables "Days in the ICU", "Days in the Hospital" and "Days between ICU and Hospital Discharge", dates of ICU/hospital discharge, for patients who died, coincided with the date of death. A nominal variable for patients deceased in the ICU was generated, namely "Deaths in the ICU", and another one for the final outcome, with the categories "Hospital Discharge" and "Total Deaths".

3.3. Laboratory data

As formerly mentioned, results of blood gas analysis, hemograms, ionograms, urine tests and other daily laboratorial analysis were extracted to individual Microsoft Excel files, for each patient, and then condensed into the database "Laboratory Results". Contrary to the clinical and demographic data, in this case the results from all variables were longitudinal. As a result, each variable was measured multiple times for each individual, allowing the detection of changes along the time they were admitted to the hospital. As an example, a patient could be admitted to the ICU for 42 days, but have a total of 1002 results during the entire stay, or be admitted for 2 days and have a total of 100 measurements. Thus, everything depended on the length of time patients were admitted to the ICU, the severity of their illness, and the necessity for more clinical analysis to fully understand a patient's situation.

Through pre-processing techniques, the formerly mentioned database was merged with the one with the patients' clinical and demographic information, into a single longitudinal database with approximately 1000 variables (it's important to mention that these included the date and time of each patients' sample analysis). Since this part of the study aimed to analyze the laboratory results of patients that were admitted to the ICU, all the others were excluded from this database (thus it was renamed "ICU Laboratory Data"). Subsequently, a sample of 335 patients was obtained and variables of interest were selected.

All variables related to hemograms/blood tests (**Table 3.3.1**), blood gas analysis (**Table 3.3.2**), and urinalysis (**Table 3.3.3**), were analyzed and, with clinical guidance, only the ones of interest were kept. The remaining ones were saved for future work. Maximum and minimum values of the selected variables were determined, depending on the goal of the analysis, in order to obtain a single measure per patient, per day. Since some continuous variables had doctor's notes and symbols as components, individual corrections were made, and these errors were considered "missing values".

***Table 3.3.1**. Selected variables of interest obtained from hemograms/blood tests and their daily value of interest*

Parameter	Value of interest	Parameter	Value of interest
Activated Partial Thromboplastin Time (aPTT) Ratio	Maximum	LDH (U/L)	Maximum
Albumin (g/L)	Minimum	LDL cholesterol (mg/dL)	Maximum
ALP (U/L)	Maximum	Lipase (U/L)	Maximum
ALT (U/L)	Maximum	LYM (x 10^9/L) / (%)	Minimum
Ammonia (µg/dL)	Maximum	Macrocytic PLTs (%)	Minimum
AST (U/L)	Maximum	Magnesium (mEq/L)	Maximum
Atypical LYM	Minimum	Mean corpuscular hemoglobin (MCH) (pg)	Minimum
Basophiles (x 10^9/L) / (%)	Maximum	Mean Corpuscular Hemoglobin Concentration (MCHC) (x 10g/L)	Minimum
Blood Urea (mg/dL)	Maximum	Mean corpuscular volume (MCV) (fL)	Minimum
Calcium (mEq/L)	Minimum	Mean platelet volume (MPV) (fL)	Minimum
Chlorine (mEq/L)	Maximum	MONs (x 10^9/L) / (%)	Maximum and minimum
Cholinesterase (U/L)	Maximum	Myoglobin (ng/mL)	Maximum
C-Reactive Protein (CRP) (mg/L)	Maximum	Natriuretic Peptide Testes (NT-proBNP) (pg/mL)	Maximum
Creatine kinase (CK) (U/L)	Maximum	NEUs (x 10^9/L) / (%)	Maximum and minimum
Creatinine (mg/dL)	Maximum	Pancreatic amylase (U/L)	Maximum
Cystatin C (mg/L)	Maximum	Plaquetocrit (%)	Minimum
DDs (µg/L)	Maximum	Platelet Distribution Width (PDW) (%)	Minimum
EOs (x 10^9/L) / (%)	Maximum	PLTs (x 10^9/L) / (%)	Minimum
Erythroblasts	Maximum	Potassium (mEq/L)	Maximum
Erythrocytes	Minimum	Procalcitonin (PCT) (ng/mL)	Maximum
Estimated Glomerular Filtration Rate (eGFR) (mL/min./1.73 m2)	Minimum	Red Cell Distribution Width (RDW) (%)	Maximum and minimum
Factor V	Minimum	Red Cell Distribution Width (RDW-SD) (fL)	Maximum and minimum
Ferritin (FER) (ng/mL)	Maximum	Sedimentation rate (mm/hr)	Maximum
FIB (g/L)	Minimum	Serum Folic Acid (ng/mL)	Minimum
GGT (U/L)	Maximum	Sodium (mEq/L)	Maximum and minimum
Glucose (mg/dL)	Maximum and minimum	Thyroid Stimulating Hormone (mIU/L)	Maximum and minimum
HDL cholesterol (mg/dL)	Maximum	Total Bilirubin (mg/dL)	Maximum
Hematocrit (HCT)	Minimum	Total cholesterol (mg/dL)	Maximum
Hemoglobin	Minimum	Total proteins (g/L)	Minimum
High-sensitivity cardiac troponin (hs-cTn) I (pg/mL)	Maximum	Triglycerides (mg/dL)	Maximum
Homocysteine (µmol/L)	Maximum	Uncorrected White Blood Cell count (uWBC)	Maximum and minimum
Immature Granulocytes	Maximum and minimum	Uric acid (mg/dL)	Maximum
Interleucin-6 (IL-6) (pg/mL)	Maximum	Vitamin B12 (pg/mL)	Minimum
INR	Maximum	Vitamin D25 (ng/mL)	Minimum
Iron (mEq/L)	Minimum	WBC's (x 10^9/L)	Maximum and minimum

***Table 3.3.2**. Selected variables of interest from blood gas analysis and their daily value of interest*

Parameter	Value of interest	Parameter	Value of interest
Acid-base balance (pH)	Maximum and minimum	Deoxyhemoglobin (HHb)	Maximum
Arterial oxygen saturation (So2)	Minimum	Lactate (Lac)	Maximum
Base excess/deficit (BE (ecf))	Minimum	Oxyhemoglobin (O2Hb)	Minimum
Bicarbonate ($HCO3^-$(act))	Minimum	Partial pressure of carbon dioxide (Pco2)	Maximum and minimum
Calcium (Ca^{++})	Minimum	Partial pressure of oxygen (Po2)	Minimum

***Table 3.3.3**. Selected variables of interest from urinalysis and their daily value of interest*

Parameter	Value of interest	Parameter	Value of interest
Bilirubin (mg/dL)	Positive	Proteins (mg/dL)	Positive
Erythrocytes (/µL)	Maximum	Urobilinogen (mg/dL)	Positive

3.4. Statistical analysis

Initially, an exploratory analysis of all the variables recorded was carried out, according to its typology and the type of data: transversal or longitudinal data.

Categorical variables were represented by their absolute frequencies and percentages. Continuous variables were represented by their medians and interquartile range (25th percentile-75th percentile), since they were presented with an asymmetric distribution and deviations from normality. To assess the normality of the continuous variable's, Kolmogorov-Smirnov and Shapiro-Wilk tests were used, as appropriate. Additionally, given the objectives and according to clinical criteria, some variables were categorized, and others were recoded.

Concerning qualitative variables, to make comparisons between two independent groups, specifically, to compare patients who were discharged from the hospital and the ones who died, as well, between patients who required IMV and the ones who didn't, non-parametric chi-square (χ^2) or fisher's exact test (if the applicability conditions of the first test were not verified) were used. For ordinal and continuous variables, in general, Mann-Whitney U test was applied, given the normality wasn't verified. For comparisons between three or more independent groups, for example, in the case where it is intended to compare the data referring to the three COVID-19 waves, or to comparisons between age groups data, Kruskal-Wallis One-Way ANOVA test was applied (with significance values adjusted by the Bonferroni correction, for pairwise comparisons). For comparisons between two related samples, Wilcoxon matched pair signed rank test was used for continuous variables.

The symbology used to represent each statistical test can be found in **Table 3.4.1.**

Table 3.4.1*. Symbology used to represent statistical tests*

Test	Symbol
Chi-square	●
Fisher's exact test	■
Mann-Whitney U	*
Kruskal-Wallis one-way ANOVA	†

In terms of laboratory results, longitudinal data refers to information collected throughout patients ICU stay. To infer about the evolution of the patients' condition in the first week of ICU admission, a comparative analysis was carried out between the data from the 2nd and the 7th days. Comparisons of laboratory parameters between discharged and deceased patients, at these time points were performed. When relevant, Pearson's correlation coefficient (*r*) was used to study linear associations between quantitative variables.

To visualize data pattern over time, panel data line plots were used, being the panel the patient. To visualize the relationship between the covariates and time, median band lines plots were used. It provides a convenient but crude way to show the tendency in the relationship between time and each

one of the main biomarkers of interest in ICU context. Median band lines for all biomarkers were obtained by dividing the x axis into equal-width intervals (bands obtained by a specific mathematical formula) and then calculating the median of the x and y axis for each interval. Then a line plot is created with the crossed medians.

Due to the decreasing number of patients since the 7th day of ICU admission, the median line charts were redone to represent those days only, ending in the 10th day for representative reasons only.

Regarding the entire period of ICU stay, graphical evolution of all patients' results was accessed, for the global sample, by each COVID-19 wave, and considering the groups defined by the outcomes of interest. Considering the total longitudinal data corresponding to the UCI stay, the associations between the several biomarkers and death were tested by means of Univariate Generalized Estimating Equations models (GEEs), family binomial and logit link function, with robust standard errors and an exchangeable working correlation structure (156,157).

Additionally, to analyze the impact of some biomarkers in mortality, crude odds ratios were estimated and interpreted, with the corresponding 95% confidence intervals (CIs).

To study the death outcome, death event-free survival rates were obtained using the Kaplan-Meier estimator. To compare the survival curves, obtained considering by stratifying the data according to the cut-off point that separates the normal and non-normal reference values of a biomarker, the Log Rank (Mantel-Cox), Breslow (Generalized Wilcoxon) and Tarone-Ware test, were used, as appropriate.

Descriptive and inferential statistics were obtained by SPSS software (version 26.0), and so were the population pyramids. All remaining graphical representations and the GEE models results were obtained using STATA software (version 12.0).

Chapter 4 – Results and discussion

4.1. Clinical and demographic characteristics

4.1.1. All patients and comparisons relative to their outcomes

Clinical characteristics and demographics of all the enrolled patients were detailed on **Table 4.1.1.1.** In total, from March 2020 to March 2021, 337 patients with COVID-19 were admitted to the hospital's ICU. Their median age was 64 (P_{25}= 53; P_{75}=73), ranging from 19 to 92 years old. More than half of the patients were aged 60 years and older, since 27% of them had ages between 60 and 69 years old, and 23% between 70 and 79 years old. Only 3% of the patients were under 30 years old. Concerning gender, 227 (67,4%) patients were male and 110 (32,6%) were female. BMI was calculated for 85,8% of the population, due to missing values in the variable "Weight", resulting in a BMI median of 27,7 (P_{25}= 24,7; P_{75}=31,1), Kg/m^2. Most of the patients (71,3%) had a BMI of 29,9 Kg/m^2 or lower, resulting in 28,7% of them being considered obese.

Approximately 85% of the patients had at least one comorbidity, being that the majority belonged to the group of 1 to 2 comorbidities (53,6%). The most prevalent comorbidities were arterial hypertension (57%), diabetes mellitus (37,1%), obesity (28,7%) and dyslipidemia (26,1%), which correspond to the most common comorbidities reported in the Portuguese population (158). Respiratory diseases like asthma (3,3%), COPD (5%), obstructive sleep apnea (OSA) (3,3%) and pulmonary emphysema (1,2%), were less frequent. Regarding vascular disease, myocardial ischemia was the most common (6,8%). Stroke (3%), pulmonary vascular disease (0,9%) and other types of cardiovascular diseases (1,2%) weren't as common. History of heart arrhythmias was detected in 5,3% of the patients. Tissue and organ cancer (5,9%) were more common than hematologic cancers (1,5%). Only 6,2% of the patients had history of chronic renal disease. Other comorbidities were less frequent, involving less than 5% of the patients.

At admission, most of the patients belonged to the third (41,2%) and second (40,4%) COVID-19 waves. This can be transposed to the general situation of the country in the different waves (**Figure 4.1.1.1**). As of May 2021, the Alpha variant (B.1.1.7) was still dominant in the country. Until June 9, 2021, 118 cases of the Beta (B.1.351), 145 of the Gamma (P.1) and 101 of the Delta (B.1.617) variants were reported. It was expected that the cases caused by the variant Alpha would decrease due to the increase of the Delta variant in other countries (159).

Almost all patients were admitted for medical causes (95,3%), being the main admission motive "COVID-19 induced ARDS" (81%). Other motives included "Acute Respiratory Failure or ARDS" (5%), "Monitoring" (3,3%), "Central Nervous System Disorders" (2,4%) and "Septic Shock or Sepsis" (2,1%). This can be related to the fact that the hospital's ICU, at a certain time, was only meant for COVID-19 patients. The most frequent method of respiratory support was IMV (75,4%), followed by HFO (31,2%) and ECMO (11%). The median of days patients spent with IMV, 8 (P_{25}= 4; P_{75}=14) days, was lower than the median of days they spent with ECMO, 9 (P_{25}= 4; P_{75}=22) days. However, the maximum time a patient spent on IMV was 58 days and on ECMO 42 days. The median number of days between

symptom onset/ a confirmed RT-PCR diagnosis, and IMV onset was of 9 (P_{25}= 5; P_{75}=12) days, and for ECMO onset 13 (P_{25}= 9,5; P_{75}=17) days.

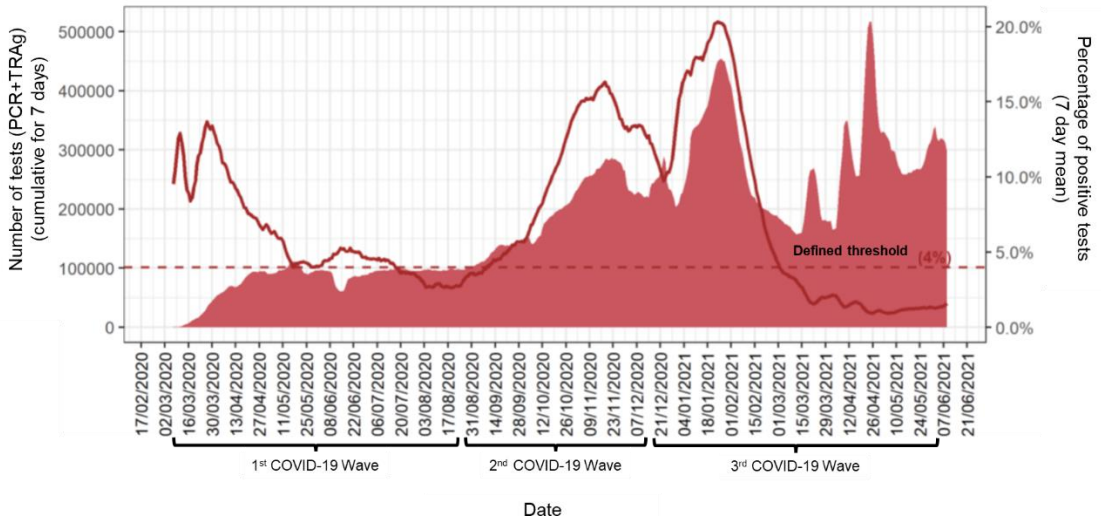

Figure 4.1.1.1. Number of laboratory tests for detection of SARS-CoV-2 (area in red) and percentage of positive results (red line) per week in Portugal. The defined threshold (4%) is according to European directives. Indication for COVID-19 waves added according to *Centro Hospitalar Universitário Lisboa Central* (adapted from (159)).

Periods of time between symptom onset/ RT-PCR diagnosis and hospital or ICU admission, days between hospital and ICU admission, days spent in the hospital or in the ICU and total of days between ICU and hospital discharge are displayed on **Figure 4.1.1.2**. It's important to mention that 21 of all patients only began having symptoms or had a RT-PCR confirmed diagnosis after being admitted to the hospital. Data from these patients wasn't considered when calculating the median number of days between symptom onset or RT-PCR diagnosis and hospital admission. Also, data from patients whose ICU admission dates were earlier than those of hospital admission weren't considered when calculating the number of days between hospital and ICU admission.

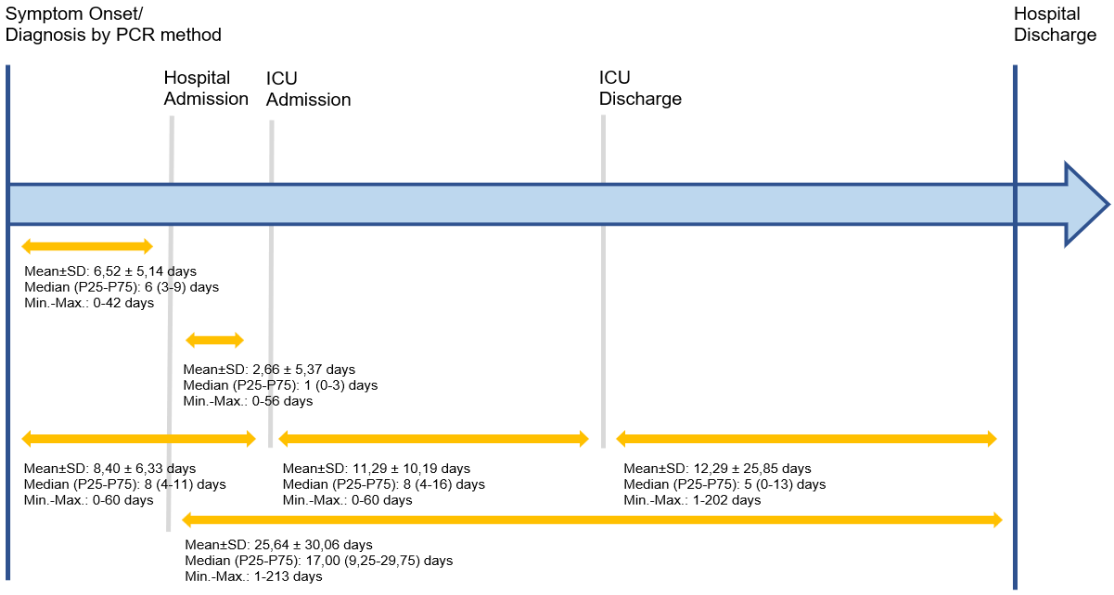

Figure 4.1.1.2. COVID-19 patients' timeline since symptom onset or confirmed diagnosis by RT-PCR (whichever was earlier) until hospital discharge.

***Table 4.1.1.1**. Demographic data from all patients and comparisons between hospital discharged and deceased patients*

Variables	Total Missing's n (%) / N	All Patients (N=337)	Discharged from hospital (n=197)	Deceased (n=140)	P Value
Age					
Age, years	-	64,0 (53,0-73,0)	58,0 (49,0-68,0)	71,0 (63,0-78,0)	<0,001*
Age groups					
<30 years	-	10 (3,0)	8 (4,1)	2 (1,4)	<0,001*
30-39 years	-	16 (4,7)	14 (7,1)	2 (1,4)	
40-49 years	-	34 (10,19	30 (15,2)	4 (2,9)	
50-59 years	-	72 (21,4)	55 (27,9)	17 (12,1)	
60-69 years	-	91 (27,0)	50 (25,4)	41 (29,3)	
70-79 years	-	78 (23,1)	30 (15,2)	48 (34,3)	
≥80 years	-	36 (10,7)	10 (5,1)	26 (18,6)	
Gender					
Female	-	110 (32,6)	61 (31,0)	49 (35,0)	0,436•
Male	-	227 (67,4)	136 (69,0)	91 (65,0)	
BMI					
BMI, Kg/m^2	48 (14,2%) / 289	27,7 (24,7-31,1)	27,7 (24,7-31,3)	27,1 (24,7-31,1)	0,439*
BMI Categories					
≤29,9 Kg/m^2	48 (14,2%) / 289	206 (71,3)	122 (61,9)	84 (60,0)	0,585*
30-34,9 Kg/m^2		44 (15,2)	28 (14,2)	16 (11,4)	
35-39,9 Kg/m^2		25 (8,7)	14 (7,1)	11 (7,9)	
≥40 Kg/m^2		14 (4,8)	10 (5,1)	4 (2,9)	
Presence of Comorbidities					
Yes	-	285 (84,6)	158 (80,2)	127 (90,7)	0,008•
No	-	52 (15,4)	39 (19,8)	13 (9,3)	
Comorbidities					
Arterial Hypertension	-	192 (57,0)	93 (47,2)	99 (70,7)	<0,001•
Diabetes Mellitus	-	125 (37,1)	68 (34,5)	57 (40,7)	0,246•
Dyslipidemia	-	88 (26,1)	48 (24,4)	40 (28,6)	0,386•
Obesity	48 (14,2%) / 289	83 (28,7)	52 (26,4)	31 (22,1)	0,590•
Asthma	-	11 (3,3)	9 (4,6)	2 (1,4)	0,131▪
COPD	-	17 (5,0)	6 (3,0)	11 (7,9)	0,047•
OSA	-	11 (3,3)	4 (2,0)	7 (5,0)	0,212▪
Bronchiectasis	-	3 (0,9)	1 (0,5)	2 (1,4)	-
Extrinsic Allergic Alveolitis	-	1 (0,3)	0 (0,0)	1 (0,7)	-

Pulmonary Emphysema	-	4 (1,2)	1 (0,5)	3 (2,1)	-
Pulmonary Vascular Disease	-	3 (0,9)	1 (0,5)	2 (1,4)	-
Myocardial Ischemia	-	23 (6,8)	13 (6,6)	10 (7,1)	0,845•
Cardiovascular Disease	-	4 (1,2)	1 (0,5)	4 (2,9)	-
Heart Arrythmias	-	18 (5,3)	6 (3,0)	12 (8,6)	0,026•
Stroke	-	10 (3,0)	3 (1,5)	7 (5,0)	0,100▪
Thalassemia	-	1 (0,3)	0 (0,0)	1 (0,7)	-
Hypothyroidism	-	14 (4,2)	7 (3,6)	7 (5,0)	0,512•
Chronic Renal Disease	-	21 (6,2)	8 (4,1)	13 (9,3)	0,051•
Chronic Liver Disease	-	1 (0,3)	1 (0,5)	0 (0)	-
Hematologic Cancer	-	5 (1,5)	4 (2,0)	1 (0,7)	-
Tissue and/or Organ Cancer	-	20 (5,9)	8 (4,1)	12 (8,6)	0,084•
Rheumatic Diseases	-	4 (1,2)	2 (1,0)	2 (1,4)	-
Nervous System Disease	-	9 (2,7)	7 (3,6)	2 (1,4)	0,315▪
Hyperuricemia	-	6 (1,8)	3 (1,5)	3 (2,1)	-
HIV Positive	-	7 (2,1)	4 (2,0)	3 (2,1)	-
BPH	-	11 (3,3)	4 (2,0)	7 (5,0)	0,212▪
History of Organ Transplantation	-	6 (1,8)	3 (1,5)	3 (2,1)	-
Number of Comorbidities					
0 comorbidities	-	52 (15,4)	39 (19,8)	13 (9,3)	<0,001*
1-2 comorbidities	-	153 (45,4)	96 (48,7)	57 (40,7)	
3-4 comorbidities	-	119 (35,3)	59 (29,9)	60 (42,9)	
≥5 comorbidities	-	13 (3,9)	3 (1,5)	10 (7,1)	
COVID-19 Wave at Admission					
First wave	-	62 (18,4)	37 (18,8)	25 (17,9)	0,729•
Second wave	-	136 (40,4)	76 (38,6)	60 (42,9)	
Third wave	-	139 (41,2)	84 (42,6)	55 (39,3)	
Type of Patient at Admission					
Medical	-	321 (95,3)	184 (93,4)	137 (97,9)	-
Surgical (Urgency) and Trauma	-	11 (3,3)	8 (4,1)	3 (2,1)	
Surgical (Elective)	-	1 (0,3)	1 (0,5)	0 (0,0)	
Neurocritical	-	4 (1,2)	4 (2,0)	0 (0,0)	
Admission Motive					
Acute Respiratory Failure or ARDS	-	17 (5,0)	13 (6,6)	4 (2,9)	-
COVID-19 induced ARDS	-	273 (81,0)	153 (77,7)	120 (85,7)	
Cardiogenic Shock or poorly defined shock	-	5 (1,5)	3 (1,5)	2 (1,4)	
Septic Shock or Sepsis	-	7 (2,1)	4 (2,0)	3 (2,1)	
Cardiac Arrest	-	5 (1,5)	2 (1,0)	3 (2,1)	

Heart Failure	-	1 (0,3)	1 (0,5)	0 (0,0)	
Renal Failure	-	2 (0,6)	2 (1,0)	0 (0,0)	
Acid-Base Disorders	-	3 (0,9)	1 (0,5)	2 (1,4)	
Acute Abdomen	-	3 (0,9)	2 (1,0)	1 (0,7)	
Acute Pancreatitis	-	1 (0,3)	0 (0,0)	1 (0,7)	
Polytrauma	-	1 (0,3)	1 (0,5)	0 (0,0)	
Central Nervous System Disorders	-	8 (2,4)	6 (3,0)	2 (1,4)	
Monitoring	-	11 (3,3)	9 (4,6)	2 (1,4)	
Respiratory Support					
IMV	-	254 (75,4)	128 (65,0)	126 (90,0)	<0,001•
Days between symptom onset/RT-PCR diagnosis and IMV onset	-	9,0 (5,0-12,0)	9,0 (6,0-12,8)	9,0 (4,0-12,0)	0,164*
Days between ICU admission and IMV onset	-	0,0 (0,0-1,0)	0,0 (0,0-1,0)	0,0 (0,0-1,0)	0,459*
Days with IMV	-	8,0 (4,0-14,0)	7,0 (4,0-15,0)	8,0 (3,0-13,3)	0,577*
ECMO	-	37 (11,0)	20 (10,2)	17 (12,1)	0,565•
Days between symptom onset/RT-PCR diagnosis and ECMO onset	-	13,0 (9,5-17,0)	12,0 (9,0-16,0)	14,0 (11,0-17,0)	0,497*
Days with ECMO	-	9,0 (4,0-22,0)	10,0 (4,3-18,5)	9,0 (3,5-28,0)	0,821*
HFO	-	105 (31,2)	82 (41,6)	23 (16,4)	<0,001•
Outcomes					
Days between symptom onset/ RT-PCR diagnosis and Hospital Admission	34 (10,1%) / 303	6,0 (3,0-9,0)	7,0 (3,0-9,0)	5,0 (2,0-8,0)	0,005*
Days between symptom onset/ RT-PCR diagnosis and ICU Admission	-	8,0 (4,0-11,0)	8,0 (5,0-11,0)	7,0 (3,25-10,0)	0,099*
Days between Hospital and ICU Admission	15 (4,5) / 322	1,0 (0,0-3,0)	1,0 (0,0-3,0)	1,0 (0,0-3,0)	0,717*
Days in the ICU	-	8,0 (4,0-16,0)	8,0 (4,0-18,0)	8,0 (4,0-14,0)	0,463*
Days in the Hospital	13 (3,9) / 324	17,0 (9,3-29,8)	22,0 (14,5-39,0)	12,0 (7,0-18,0)	<0,001*
Days between ICU and Hospital Discharge	-	5,0 (0,0-13,0)	11,0 (5,5-21,0)	0,0 (0,0-0,0)	<0,001*
Deaths in the ICU	-	124 (36,8)	-	-	-
Hospital Discharge	-	197 (58,5)	-	-	-
Total Deaths	-	140 (41,5)	-	-	

P values weren't calculated if the variable's frequencies were too small/ null. P values weren't also calculated for the variables "Type of Patient at Admission" and "Admission Motive" because it wasn't clinically relevant.

In total, 140 (41,5%) of the 337 patients died and 197 (58,5%) were discharged from the hospital. From the total 337 patients, 124 (36,8%) died in the ICU. This means that 88,6% of the deaths occurred in the ICU.

Comparisons between hospital discharged (n=197) and deceased patients (n=140) (**Table 4.1.1.1**) reveled differences in age, since deceased patients were significantly older (71 (P_{25}= 63; P_{75}=78), vs. 58 (P_{25}= 49; P_{75}=68), years; *P*<0,001), and between age groups (*P*<0,001)). More than half of the discharged patients had ages between 50 and 69 years old, and more than 60% of the deceased patients were aged between 60 and 79 years old (**Figure 4.1.1.3A**). In both groups, the percentage of males was higher. Deceased patients were significantly more likely to have comorbidities (90,7% vs. 80,2%; *P*=0,008), hypertension (70,7% vs. 47,2%; *P*<0,001), COPD (7,9% vs. 3,0%; *P*=0,047) and hearth arrhythmias (8,6% vs. 3,0%; *P*=0,026). They were also more prone to a larger number of comorbidities (*P*<0,001), thus 42,9% of these patients had 3 to 4 comorbidities. On the other hand, 48,7% of those who were discharged from the hospital had between 1 and 2 comorbidities (**Figure 4.1.1.3B**).

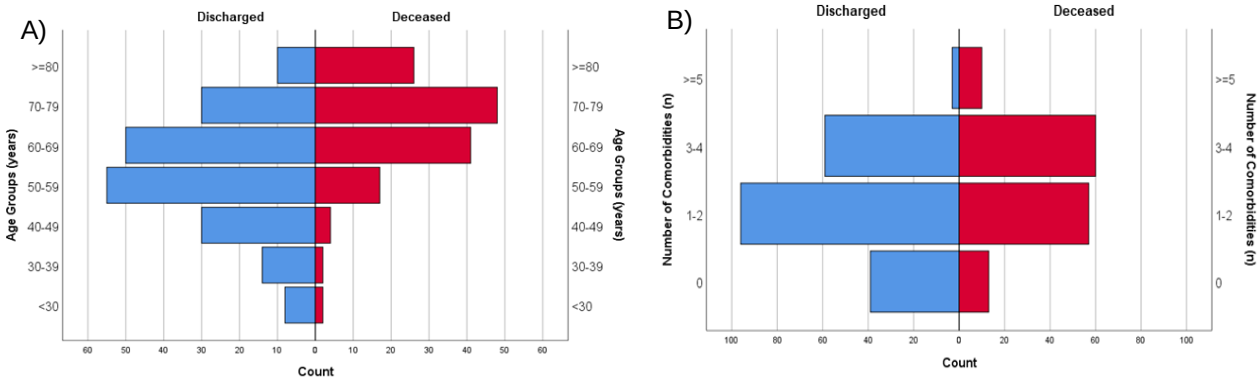

Figure 4.1.1.3. Population Pyramid of patients discharged from the hospital and patients who died in each age group (A) and in each number of comorbidities (B).

Most of the discharged patients were admitted during the third COVID-19 wave (42,6%), whether the deceased were during the second one (42,9%). At admission, in both groups, the most frequent type of patients was “Medical” (97,9% in the deceased group and 93,4% in the discharged group) and the most common reason for admission was “COVID-19 induced ARDS” (85,7% in the deceased group and 77,7% in the discharged group). Deceased patients were significantly more likely to need IMV (90% vs. 65%; *P*<0,001), whether patients discharged from the hospital required more HFO than the others (41,6% vs. 16,4%; *P*<0,001). Days between symptom onset or RT-PCR diagnosis and hospital admission were significantly less in the deceased group (5 (P_{25}= 2; P_{75}=8) vs. 7 (P_{25}= 3; P_{75}=9), days; *P*=0,005) and so were the number of days at the hospital (12 (P_{25}= 7; P_{75}=18), vs. 22 (P_{25}= 14,5; P_{75}=39,0), days; *P*<0,001) and between ICU and hospital discharge (0 (P_{25}= 0; P_{75}=0), vs. 11 (P_{25}= 5,5; P_{75}=21,0), days; *P*<0,001).

4.1.2. Comparisons relative to IMV

The median age for mechanically ventilated (n=254) patients was 64 (P_{25}= 54; P_{75}=73) years and for non-mechanically ventilated (n=83) patients 59 (P_{25}= 50; P_{75}=73) years (**Table 4.1.2.1**). No significant associations were detected between the need for ventilation and the defined age groups (*P*=0,164) (**Figure 4.1.2.1A**). The age group with the highest number of mechanically ventilated patients was the one from 60 to 69 years old (29,9%) and for non-mechanically ventilated from 50 to 59 years old (26,5%). In both groups the frequency of males was superior. The median BMI of mechanically ventilated patients was significantly higher (27,7 (P_{25}= 25,4; P_{75}=31,3), Kg/m^2 vs. 26,0 (P_{25}= 23,9; P_{75}=30,5) Kg/m^2; *P*=0,041) and, in both groups, more than 70% of the patients had a BMI less or equal to 29,9 Kg/m^2. The need for mechanical ventilation was associated with a greater number of comorbidities (87% vs. 77,1%; *P*=0,030). Also, most patients had 1 to 2 comorbidities (46,5% vs. 42,4%), followed by 3 to 4 comorbidities (35,8% vs. 33,7%).

The need for mechanical ventilation was also associated to COVID-19 waves at admission (*P*=0,023). Mechanically ventilated patients were more frequently admitted in the third wave (41,7%), whether non- mechanically ventilated ones in the second wave (50,6%) (**Figure 4.1.2.1B**).

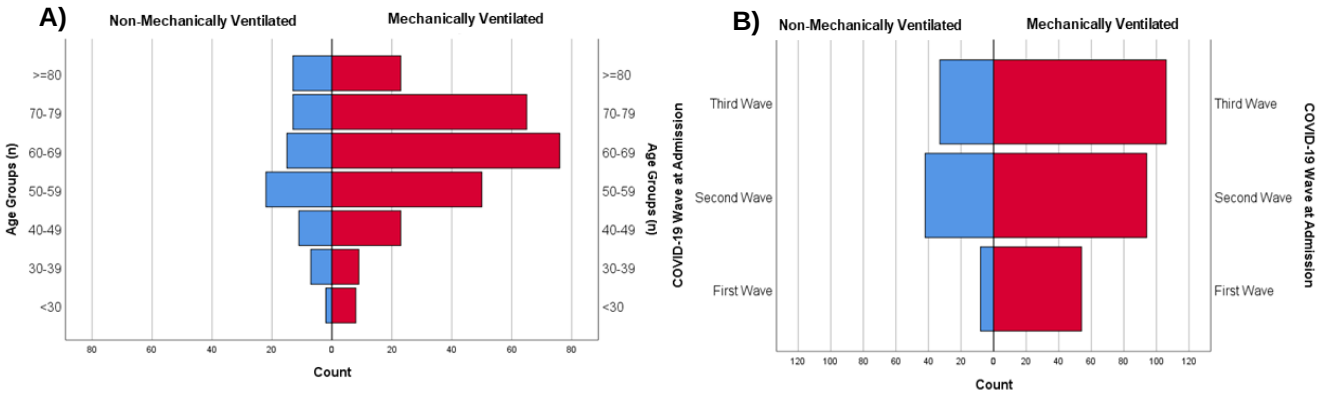

Figure 4.1.2.1. Population Pyramid of non-mechanically ventilated patients and mechanically ventilated patients in each age group (A) and in each COVID-19 wave at admission (B).

Only mechanically ventilated patients received ECMO (14,6%), and all non- mechanically ventilated patients required HFO. The internment was significantly longer for mechanically ventilated in comparison to non-mechanically ventilated patients, both in the ICU (10,5 (P_{25}= 6; P_{75}=19) vs. 4 (P_{25}= 2; P_{75}=6) days; *P*<0,001) and in the hospital (20 (P_{25}= 12; P_{75}=34) days vs. 11 (P_{25}= 6; P_{75}=18) days; *P*=<0,001). Hospital mortality was also significantly higher in mechanically ventilated patients (49,6% vs. 16,9%; *P*=<0,001), including in the ICU (43,7% vs. 15,7%; *P*=<0,001). Mechanically ventilated patients also had a lower percentage of hospital discharges (50,4% vs. 83,1%; *P*=<0,001).

Table 4.1.2.1. Comparisons between mechanically ventilated and non-mechanically ventilated patients

Variables	Mechanically Ventilated (n=254)	Non-Mechanically Ventilated (n=83)	P Value
Age			
Age, years	64,0 (54,0-73,0)	59,0 (50,0-73,0)	0,212*
Age groups			
<30 years	8 (3,1)	2 (2,4)	
30-39 years	9 (3,5)	7 (8,4)	
40-49 years	23 (9,1)	11 (13,3)	
50-59 years	50 (19,7)	22 (26,5)	0,164*
60-69 years	76 (29,9)	15 (18,1)	
70-79 years	65 (25,6)	13 (15,7)	
≥80 years	23 (9,1)	13 (15,7)	
Gender			
Female	77 (30,3)	33 (39,8)	0,111•
Male	177 (69,7)	50 (60,2)	
BMI			
BMI, Kg/m^2	27,7 (25,4-31,3)	26,0 (23,9-30,5)	0,041
BMI Categories			
≤29,9 Kg/m^2	155 (70,1)	51 (75,0)	
30-34,9 Kg/m^2	38 (17,2)	6 (8,8)	0,589
35-39,9 Kg/m^2	17 (7,7)	8 (11,8)	
≥40 Kg/m^2	11 (5,0)	3 (4,4)	
Presence of Comorbidities			
Yes	221 (87,0)	64 (77,1)	0,030•
No	33 (13,0)	19 (22,9)	
Comorbidities			
Arterial Hypertension	152 (59,8)	40 (48,2)	0,063•
Diabetes Mellitus	96 (37,8)	29 (34,9)	0,640•
Dyslipidemia	68 (26,8)	20 (24,1)	0,630•
Obesity	66 (29,9)	17 (25,0)	0,438•
Asthma	9 (3,5)	2 (2,4)	1,000▪
COPD	14 (5,5)	3 (3,6)	0,773▪
OSA	11 (4,3)	0 (0,0)	-
Bronchiectasis	2 (0,8)	1 (1,2)	-
Extrinsic Allergic Alveolitis	0 (0,0)	1 (1,2)	-
Pulmonary Emphysema	4 (1,6)	0 (0,0)	-
Pulmonary Vascular Disease	1 (0,4)	2 (2,4)	-
Myocardial Ischemia	17 (6,7)	6 (7,2)	0,866•
Cardiovascular Disease	2 (0,8)	3 (3,6)	0,098▪
Heart Arrythmias	13 (5,1)	5 (6,0)	0,780▪
Stroke	7 (2,8)	3 (3,6)	0,712▪
Chronic Renal Disease	19 (7,5)	2 (2,4)	0,097•
Tissue and/or Organ Cancer	18 (7,1)	2 (2,4)	0,179▪
Number of Comorbidities			
0 comorbidities	33 (13,0)	19 (22,9)	
1-2 comorbidities	118 (46,5)	35 (42,2)	0,078*
3-4 comorbidities	91 (35,8)	28 (33,7)	
≥5 comorbidities	12 (4,7)	1 (1,2)	
COVID-19 Wave at Admission			
First wave	54 (21,3)	8 (9,6)	
Second wave	94 (37,0)	42 (50,6)	0,023•
Third wave	106 (41,7)	33 (39,8)	
Type of Patient at Admission			
Medical	244 (96,1)	77 (92,8)	
Surgical (Urgency) and Trauma	8 (3,1)	3 (3,6)	-
Surgical (Elective)	0 (0)	1 (1,2)	
Neurocritical	2 (0,8)	2 (2,4)	

Admission Motive			
Acute Respiratory Failure or ARDS	15 (5,9)	2 (2,4)	
COVID-19 induced ARDS	213 (83,9)	60 (72,3)	
Cardiogenic Shock or poorly defined shock	4 (1,6)	1 (1,2)	
Septic Shock or Sepsis	6 (2,4)	1 (1,2)	
Cardiac Arrest	5 (2,0)	0 (0,0)	
Heart Failure	0 (0,0)	1 (1,2)	
Renal Failure	1 (0,4)	1 (1,2)	-
Acid-Base Disorders	1 (0,4)	2 (2,4)	
Acute Abdomen	2 (0,8)	1 (1,2)	
Acute Pancreatitis	1 (0,4)	0 (0,0)	
Polytrauma	1 (0,4)	0 (0,0)	
Central Nervous System Disorders	4 (1,6)	4 (4,8)	
Monitoring	1 (0,4)	10 (12,0)	
Respiratory Support			
ECMO	37 (14,6)	0 (0,0)	-
Days between symptom onset/RT-PCR diagnosis and ECMO onset	13,0 (9,5-17,0)	-	-
Days with ECMO	9,0 (4,0-22,0)	-	-
HFO	22 (8,7)	83 (100,0)	<0,001•
Outcomes			
Days between symptom onset/ RT-PCR diagnosis and Hospital Admission	6,0 (3,0-8,0)	7,0 (4,0-9,0)	0,060*
Days between symptom onset/ RT-PCR diagnosis and ICU Admission	8,0 (4,0-11,0)	8,0 (5,0-10,0)	0,752*
Days between Hospital and ICU Admission	1,0 (0,0-4,0)	1,0 (0,0-1,8)	0,037*
Days in the ICU	10,5 (6,0-19,0)	4,0 (2,0-6,0)	<0,001*
Days in the Hospital	20,0 (12,0-34,0)	11,0 (6,0-18,0)	<0,001*
Days between ICU and Hospital Discharge	0,0 (0,0-13,0)	6,0 (0,0-11,0)	0,253*
Deaths in the ICU	111 (43,7)	13 (15,7)	<0,001•
Hospital Discharge	128 (50,4)	69 (83,1)	<0,001•
Total Deaths	126 (49,6)	14 (16,9)	

P values weren't calculated if the variable's frequencies were too small/ null. P values weren't also calculated for the variables "Type of Patient at Admission" and "Admission Motive" because it wasn't clinically relevant.

4.1.3. Comparisons relative to COVID-19 waves

Regarding comparisons made between COVID-19 waves (**Table 4.1.3.1**), the distribution of age (*P*=0,021) and the distribution of patients by age groups (*P*=0,048) were significantly different, more specifically between the third (n=139) and the first (n=62) waves (*P*=0,022 and 0,048 respectively) (**Figure 4.1.3.1**). For all waves, the percentage of males was higher (72,6%; 70,6%; 61,9%). The BMI distribution was statistically different between the second and third waves (*P*=0,010), and the same was verified for BMI categories (*P*=0,022). Concerning the presence of comorbidities, no significant differences were found but, in all waves, 80 to 90% of patients had one or more comorbidities. Only obesity was significantly different among COVID-19 waves (*P*=0,008), probably due to the former identified differences in BMI. Between COVID-19 waves, the need for IMV, ECM, and HFO was significantly different (*P*=0,023, *P*=<0,001 and *P*=0,009, respectively). Focusing on IMV, it was more frequent in the first COVID-19 wave, which is in accordance with national statics (160).

***Table 4.1.3.1**. Comparisons between COVID-19 first, second and third waves*

Variables	First Wave (n=62)	Second Wave (n=136)	Third Wave (n=139)	P Value
Age				
Age, years	69,0 (57,8-76,0)	65,5 (52,0-74,0)	60,0 (53,0-71,0)	0,021†
Age groups				
<30 years	2 (3,2)	6 (4,4)	2 (1,4)	
30-39 years	1 (1,6)	6 (4,4)	9 (6,5)	
40-49 years	7 (11,3)	15 (11,0)	12 (8,6)	
50-59 years	7 (11,3)	24 (17,6)	41 (29,5)	0,048†
60-69 years	17 (27,4)	37 (27,2)	37 (26,6)	
70-79 years	20 (32,3)	28 (20,6)	30 (21,6)	
≥80 years	8 (12,9)	20 (14,7)	8 (5,8)	
Gender				
Female	17 (27,4)	40 (29,4)	53 (38,1)	0,190•
Male	45 (72,6)	96 (70,6)	86 (61,9)	
BMI				
BMI, Kg/m^2	27,7 (24,9-31,3)	26,1 (24,5-29,3)	28,1 (25,9-31,5)	0,013†
BMI Categories				
≤29,9 Kg/m^2	38 (61,3)	92 (67,6)	76 (54,7)	
30-34,9 Kg/m^2	10 (16,1)	7 (5,1)	27 (19,4)	0,026†
35-39,9 Kg/m^2	4 (6,5)	7 (5,1)	14 (10,1)	
≥40 Kg/m^2	1 (1,6)	8 (5,9)	5 (3,6)	
Presence of Comorbidities				
Yes	55 (88,7)	113 (83,1)	117 (84,2)	0,589•
No	7 (11,3)	23 (16,9)	22 (15,8)	
Comorbidities				
Arterial Hypertension	34 (54,8)	79 (58,1)	79 (56,8)	0,912•
Diabetes Mellitus	22 (35,5)	53 (39,0)	50 (36,0)	0,840•
Dyslipidemia	15 (24,2)	38 (27,9)	35 (25,2)	0,812•
Obesity	15 (28,3)	22 (19,3)	46 (37,7)	0,008•
COPD	5 (8,1)	8 (5,9)	4 (2,9)	0,254•
Myocardial Ischemia	3 (4,8)	6 (4,4)	14 (10,1)	0,140•
Heart Arrythmias	4 (6,5)	8 (5,9)	6 (4,3)	0,772•
Chronic Renal Disease	5 (8,1)	8 (5,9)	8 (5,8)	0,803•

Tissue and/or Organ Cancer	6 (9,7)	4 (2,9)	10 (7,2)	0,127•
Number of Comorbidities				
0 comorbidities	7 (11,3)	23 (16,9)	22 (15,8)	0,359†
1-2 comorbidities	34 (54,8)	65 (47,8)	56 (38,8)	
3-4 comorbidities	19 (30,6)	43 (31,6)	57 (41,0)	
≥5 comorbidities	2 (3,2)	5 (3,7)	6 (4,3)	
Respiratory Support				
IMV	54 (87,1)	94 (69,1)	106 (76,3)	0,023•
Days between symptom onset/RT-PCR diagnosis and IMV onset	9,0 (4,8-14,5)	9,0 (5,0-12,3)	9,0 (4,0-11,0)	0,291†
Days between ICU admission and IMV onset	0,0 (0,0-1,0)	0,0 (0,0-1,0)	0,0 (0,0-1,0)	0,601†
Days with IMV	6,0 (1,8-17,5)	7,0 (2,8-12,5)	8,0 (5,0-15,3)	0,090†
ECMO	14 (22,6)	18 (13,2)	5 (3,6)	<0,001•
Days between symptom onset/RT-PCR diagnosis and ECMO onset	12,5 (10,0-17,0)	13,0 (8,8-16,3)	15,0 (9,0-24,0)	0,740†
Days with ECMO	8,5 (3,0-19,0)	12,5 (4,0-30,3)	9,0 (4,5-15,0)	0,782†
HFO	17 (27,4)	55 (40,4)	33 (23,7)	0,009•
Outcomes				
Days between symptom onset/ RT-PCR diagnosis and Hospital Admission	6,0 (3,0-9,0)	6,0 (3,0-9,0)	6,0 (3,0-8,8)	0,680†
Days between symptom onset/ RT-PCR diagnosis and ICU Admission	8,0 (5,0-13,0)	8,0 (5,0-11,0)	7,0 (4,0-10,0)	0,066†
Days between Hospital and ICU Admission	1,0 (0,0-5,0)	1,0 (0,0-3,0)	1,0 (0,0-2,0)	0,243†
Days in the ICU	9,0 (3,8-20,0)	8,0 (4,0-14,0)	9,0 (4,0-17,0)	0,544†
Days in the Hospital	21,0 (12,3-43,8)	17,0 (9,0-25,8)	17,0 (9,0-29,8)	0,062†
Days between ICU and Hospital Discharge	4,0 (0,0-13,3)	1,5 (0,0-11,8)	5,0 (0,0-14,0)	0,491†
Death in the ICU	20 (32,3)	53 (39,0)	51 (36,7)	0,662•
Total Deaths	25 (40,3)	60 (44,1)	55 (39,6)	0,729•
Hospital Discharge	37 (59,7)	76 (55,9)	84 (60,4)	

P values are relative to comparisons between patients who were admitted to the hospital at the first, second and third waves of COVID-19 considered in Portugal. *P* values weren't calculated if the variable's frequencies were too small/ null.

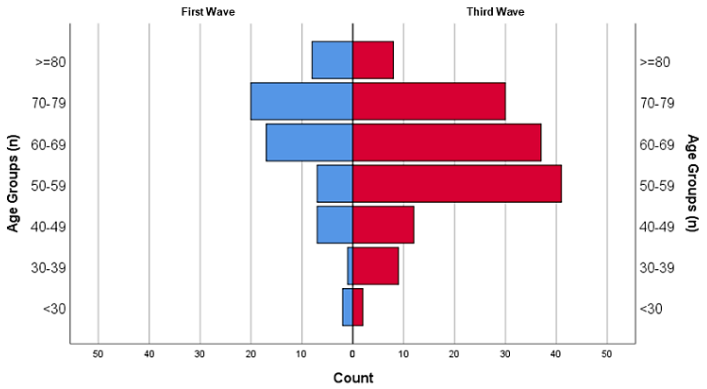

Figure 4.1.3.1. Population Pyramid of patients admitted during the first and second COVID-19 waves in each age group.

Outcome variables didn't show any significant differences between the analyzed groups. The percentage of total deaths (40,3%; 44,1%; 39,6%) and deaths in the ICU (32,3%; 39,0%; 36,7%) had no significant differences between COVID-19 waves (*P*=0,729 and 0,662, respectively). Since further along a detailed analysis on laboratory biomarkers during ICU permanence will be made, a Kaplan-Meier survival curve was obtained for patients admitted to the ICU for each COVID-19 wave (**Figure 4.1.3.2**). Comparisons between COVID-19 waves were performed using the Tarone-Ware test since the Kaplan-Meier survival curves cross at early time points. Thus, no significant differences were detected between waves (*P*=0,367), as presented before.

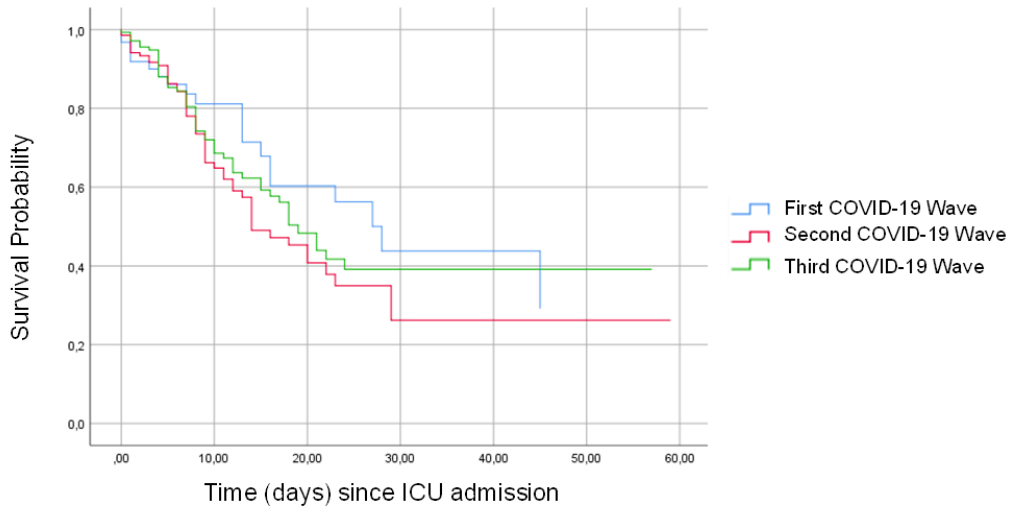

Figure 4.1.3.2. Kaplan-Meier survival curves since ICU admission until ICU discharge for each COVID-19 Wave.

4.1.4. Comparisons relative to age groups

Regarding comparisons between age groups (**Table 4.1.4.1**), it was observed that men were more prevalent in each one of them, except for 80 years and over (47,2%). Thus, it was possible to conclude that the distribution of gender was significantly different between age groups (*P*=0,019). More than half of the patients had a BMI of 29,9 Kg/m^2 or less in every age group. The presence of comorbidities was significantly different between groups (*P*<0,001). It was also possible to observe that the percentage of patients with comorbidities increased with age. The distribution of arterial hypertension, diabetes mellitus and dyslipidemia were also significantly different between the age groups (*P*<0,001 for all mentioned variables). Again, the higher the age, the higher the percentage of patients with arterial hypertension. The remaining comorbidities had no significant differences between groups or couldn't be compared due to the small sample's sizes of some age groups. Although, significant differences were found between age groups regarding the number of comorbidities (*P*<0,001). All patients aged up to 59 years old were more likely to have a maximum of 2 comorbidities, whether those older than 59 years old were more likely to have 3 to 4 comorbidities.

There was significant association between age and the need of IMV (*P*=0,028), as well as between age and the number of days they spent on IMV (*P*=0,003). The number of days on IMV showed significant differences specifically between the age groups of 80 years old or above and 50 to 59 years old (*P*=0,001). The three age groups with a higher frequency of patients that needed IMV were between 60 and 69 years old, followed by 70 to 79 years old and finally 50 to 59 years old. The maximum median number of days spent on IMV was of 12,5 (P_{25}= 6,0; P_{75}=17,8) days, in the ages between 50 and 59 years old. Due to small sample sizes of certain age groups in the population that required ECMO, comparisons between groups couldn't be obtained. HFO was adopted in a higher percentage of patients with ages between 30 and 39 years old.

Both the number of days spent in the hospital and in the ICU were significantly different between age groups (*P*=0,002 and *P*=0,011 respectively). In both cases the highest median number of days spent at the hospital (21 days) and at the ICU (11 days) were from the patients belonging to the age group from 50 to 59 years old. The number of days between ICU and hospital discharge also showed significant differences between age groups (*P*=0,003), more specifically between 80 years or older and 50 to 59 years old (*P*=0,004) and 70 to 79 and 50 to 59 years old (*P*=0,011).

Death in the ICU was significantly different between groups (*P*=<0,001), being the highest frequency for patients of 80 years or older (63,9%) and with ages between 70 to 79 years old (55,1%). These age groups were also the only ones where the number of patients that died was higher than the number of patients that was discharged from the hospital. 72,2% of the patients aged 80 years or older and 61,5 of patients aged between 70 and 79 years old died in the ICU or in the hospital. All patients aged between 40 and 49 years old died solely in the ICU. The final outcome variable (death vs. hospital discharge) also showed significant differences between the age groups (*P*=<0,001). It was also verified than the higher the age group, the smaller the percentage of hospital discharges (varying from 80% in patients aged 30 years old or below to 27,8% in patients aged 80 years old or above).

***Table 4.1.4.1**. Comparisons between age groups*

Variables	<30 (n=10)	30-39 (n=16)	40-49 (n=34)	50-59 (n=72)	60-69 (n=91)	70-79 (n=78)	≥80 (n=36)	P Value
Sex								
Female	1 (10)	3 (18,8)	9 (26,5)	22 (30,6)	24 (26,4)	32 (41,0)	19 (52,8)	0,019•
Male	9 (90)	13 (81,3)	25 (73,5)	50 (69,4)	67 (73,6)	46 (59,0)	17 (47,2)	
BMI								
BMI, Kg/m^2	24,2 (21,9-27,7)	27,7 (23,2-30,9)	27,8 (25,4-35,2)	28,4 (24,8-33,1)	27,7 (25,3-31,3)	27,1 (24,5-29,7)	26,4 (24,2-27,7)	0,096†
BMI Categories								
≤29,9 Kg/m^2	7 (87,5)	11 (73,7)	21 (67,7)	44 (63,8)	52 (66,7)	46 (78,0)	25 (86,2)	0,178†
30-34,9 Kg/m^2	0 (0,0)	2 (13,3)	2 (6,5)	12 (17,4)	17 (21,8))	7 (11,9)	4 (13,8)	
35-39,9 Kg/m^2	0 (0,0)	0 (0,0)	5 (16,1)	8 (11,6)	8 (10,3)	4 (6,8)	0 (0,0)	
≥40 Kg/m^2	1 (12,5)	2 (13,3)	3 (9,7)	5 (7,2)	1 (1,3)	2 (3,4)	0 (0,0)	
Presence of Comorbidities								
Yes	3 (30,0)	8 (50,0)	26 (76,5)	59 (81,9)	79 (86,8)	75 (96,2)	35 (97,2)	<0,001•
No	7 (70,0)	8 (50,0)	8 (23,5)	13 (18,1)	12 (13,2)	3 (3,8)	1 (2,8)	
Comorbidities								
Arterial Hypertension	1 (10,0)	1 (6,3)	3 (26,5)	30 (41,7)	59 (64,8)	60 (76,9)	32 (88,9)	<0,001•
Diabetes Mellitus	1 (10,0)	0 (0,0)	5 (14,7)	21 (29,2)	45 (49,5)	40 (51,3)	13 (36,1)	<0,001•
Dyslipidemia	0 (0,0)	0 (0,0)	3 (8,8)	12 (16,7)	32 (35,2)	30 (38,5)	11 (30,6)	<0,001•
Obesity	1 (12,5)	4 (26,7)	10 (32,3)	25 (36,2)	26 (33,3)	13 (22,0)	4 (13,8)	0,210•
COPD	0 (0,0)	0 (0,0)	0 (0,0)	2 (2,8)	7 (7,7)	5 (6,4)	3 (8,3)	-
Myocardial Ischemia	0 (0,0)	0 (0,0)	1 (2,9)	5 (6,9)	9 (9,9)	5 (6,4)	3 (8,3)	-
Heart Arrythmias	0 (0,0)	0 (0,0)	0 (0,0)	1 (1,4)	2 (2,2)	9 (11,5)	6 (16,7)	-
Chronic Renal Disease	0 (0,0)	0 (0,0)	2 (5,9)	1 (1,4)	7 (7,7)	7 (9,0)	4 (11,1)	-
Tissue and/or Organ Cancer	0 (0,0)	0 (0,0)	0 (0,0)	3 (4,2)	5 (5,5)	8 (10,3)	4 (11,1)	-
Number of Comorbidities								
0 comorbidities	7 (70,0)	8 (50,0)	8 (23,5)	13 (18,1)	12 (13,2)	3 (3,8)	1 (2,8)	<0,001†
1-2 comorbidities	3 (30,0)	8 (50,0)	23 (67,6)	36 (50,0)	35 (38,5)	33 (42,3)	15 (41,7)	
3-4 comorbidities	0 (0,0)	0 (0,0)	3 (8,8)	21 (29,2)	38 (41,8)	38 (48,7)	19 (52,8)	
≥5 comorbidities	0 (0,0)	0 (0,0)	0 (0,0)	2 (2,8)	6 (6,6)	4 (5,1)	1 (2,8)	

Respiratory Support								
IMV	8 (80,0)	9 (56,3)	23 (67,6)	50 (69,4)	76 (83,5)	65 (83,3)	23 (63,9)	0,028•
Days between symptom onset/ RT-PCR diagnosis and IMV onset	4,5 (4,0-13,5)	11,0 (8,0-12,5)	6,0 (3,0-14,0)	9,0 (5,0-13,0)	9,0 (6,0-11,0)	8,0 (4,5-12,0)	9,0 (4,0-10,0)	0,722†
Days between ICU admission and IMV onset	0,0 (0,0-0,0)	0,0 (0,0-4,5)	0,0 (0,0-1,0)	0,0 (0,0-1,0)	0,0 (0,0-1,0)	0,0 (0,0-0,0)	0,0 (0,0-1,0)	0,496†
Days with IMV	9,0 (3,0-23,5)	6,0 (3,0-7,5)	6,0 (3,0-11,0)	12,5 (6,0-17,8)	6,5 (3,0-16,0)	8,0 (5,0-13,5)	4,0 (1,0-10,0)	0,003†
ECMO	4 (40,0)	2 (12,5)	6 (17,6)	12 (16,7)	10 (11,0)	3 (3,8)	0 (0,0)	-
Days between symptom onset/RT-PCR diagnosis and ECMO onset	16,5 (5,5-26,8)	16,5 (8,00-.)	9,5 (8,5-13,3)	13,0 (8,5-13,0)	14,5 (11,0-16,0)	17,0 (13,0-.)	-	0,625†
Days with ECMO	17,5 (5,0-31,5)	11,5 (4,00-.)	13,5 (7,8-20,5)	9,5 (3,8-23,5)	9,5 (2,8-27,5)	4,0 (0,0-.)	-	0,616†
HFO	3 (30,0)	8 (50,0)	13 (38,2)	26 (36,1)	21 (23,1)	19 (24,4)	15 (41,7)	0,102•
Outcomes								
Days between symptom onset/ RT-PCR diagnosis and Hospital Admission	3,0 (3,0-6,5)	5,0 (2,0-6,5)	6,0 (2,0-8,0)	6,0 (3,0-9,0)	7,0 (5,0-9,0)	5,0 (2,5-8,5)	6,0 (2,0-9,0)	0,252†
Days between symptom onset/ RT-PCR diagnosis and ICU Admission	4,5 (4,0-10,3)	8,0 (4,3-11,5)	6,5 (3,0-10,0)	8,0 (5,0-11,8)	9,0 (6,0-11,0)	7,0 (4,0-12,0)	8,0 (3,3-10,0)	0,724†
Days between Hospital and ICU Admission	1,0 (0,5-1,5)	3,0 (1,0-7,0)	1,0 (0,0-3,0)	1,0 (0,0-3,0)	1,0 (0,0-3,0)	1,0 (0,0-4,0)	1,0 (0,0-1,0)	0,165†
Days in the ICU	9,5 (5,0-25,0)	7,0 (3,3-11,0)	7,0 (3,0-13,5)	11,0 (5,0-20,8)	8,0 (5,0-20,0)	8,0 (4,8-16,3)	5,5 (2,3-9,0)	0,011†
Days in the Hospital	17,0 (11,5-36,5)	17,0 (14,0-22,0)	18,0 (8,0-24,3)	21,0 (13,0-35,0)	18,0 (10,0-31,5)	17,0 (9,0-29,0)	10,0 (5,0-15,0)	0,002†
Days between ICU and Hospital Discharge	6,5 (2,3-17,8)	7,0 (1,0-8,0)	6,5 (0,0-12,3)	9,5 (0,0-15,0)	2,0 (0,0-14,0)	0,0 (0,0-10,3)	0,0 (0,0-8,0)	0,003†
Death in the ICU	0 (0,0)	1 (6,3)	4 (11,8)	14 (19,4)	39 (42,9)	43 (55,1)	23 (63,9)	<0,001•
Total Deaths	2 (20,0)	2 (12,5)	4 (11,8)	17 (23,6)	41 (45,1)	48 (61,5)	26 (72,2)	<0,001•
Hospital Discharge	8 (80,0)	14 (87,5)	30 (88,2)	55 (76,4)	50 (54,9)	30 (38,5)	10 (27,8)	

P values weren't calculated if the variable's frequencies were too small/ null.

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4.2. Laboratory results in the ICU

The database "ICU Laboratory Data" was used in this subchapter. The first goal for each set of biomarkers was to characterize the sample on the 1st day of ICU admission. Since most variables had several missing values on that specific day, with clinical guidance it was agreed that the 2nd day of admission was the best for this first analysis. Thus, for the 2nd day of ICU admission, comparisons between deceased and discharged patients' biomarkers were obtained for each COVID-19 wave. Additionally, comparisons of biomarker's distribution across COVID-19 waves for each group of patients were also obtained. In order to evaluate the patients' evolution after one week of ICU admission, the same analysis as for the 2nd day of ICU admission was made for the 7th day (selected with clinical guidance and because evaluation of the biomarkers was not possible after the 7th day of ICU admission due to the high number of missing values). For both the 2nd and the 7th days of ICU admission, survival analysis for nominal variables and univariate logistic regression for all variables were used. Finally, in each COVID-19 wave, comparisons between the results of the 2nd and 7th days, as well as median time course graphic representations of each biomarker between deceased and discharged patients, were utilized to assess each biomarker's evolution. For longitudinal data regarding the entire ICU admission time, GEEs were applied.

4.2.1. Cardiac biomarkers

Regarding the cardiac biomarkers, 325 patients' cardiac-related laboratory results were analyzed in the 2nd day of ICU admission (**Table 4.2.1.1**). It was observed that 40% of them had elevated hs-cTn I, 33,9% had elevated CK, 62,8% had elevated myoglobin and 97,6% had elevated LDH results. The percentage of patients with indications of a probable coronary syndrome (hs-cTn I ≥ 64pg/mL) was of 27,9%. Regarding NTproBNP, median results were highly above the normality range, but weren't considered since the sample consisted only of 6 patients. For this reason, NTproBNP results weren't analyzed any further.

Table 4.2.1.1. *Cardiac-related biomarkers in the 2nd day of ICU admission*

Biomarkers	**Normality Ranges**	**Missing Values**	**All Patients** (n=325)
hs-cTn I (pg/mL)	< 34,2	60 (18,5%)	20,20 (6,60-80,75)
Increased hs-cTn I			106 (40%)
In risk of CS			74 (27,9%)
CK (U/L)	30-200	36 (11,1%)	103,00 (47,00-313,00)
Increased CK			98 (33,9%)
Myoglobin (ng/mL)	< 85	247 (76,0%)	110,30 (62,63-464,88)
Increased Myoglobin			49 (62,8%)
NTproBNP (pg/mL)	<400	319 (98,2%)	4506,50 (327,25-16490,25)
Increased NTproBNP			4 (66,7%)
LDH (U/L)	125-220	37 (11,4%)	490 (391,25-625,75)
Increased LDH			281 (97,6%)

Risk of coronary syndrome (CS) was considered for hs-cTn I ≥ 64pg/mL, according to hospital directives. Remaining reference values according to the hospital's laboratory directives.

Comparisons between discharged and deceased patients in the ICU (**Table 4.2.1.2**) showed significant differences regarding hs-cTn I in the second (*P*=0,042) and third (*P*=0,003) COVID-19 waves. Deceased patients had significantly higher concentrations of hs-cTn I, and the number of patients with elevated results or in risk of CS in the 2nd (*P*=0,026) and 3rd (*P*=0,018) waves, was higher when compared to the ones discharged from the ICU.

Table 4.2.1.2. *Cardiac-related biomarkers of all patients and comparisons between the ones discharged from the ICU and those who died, for all COVID-19 waves in the 2nd day of ICU admission*

	Biomarkers	**All patients** (n=59)	**Discharged** (n=41)	**Deceased** (n=18)	***P* Value**
First COVID-19 Wave	**hs-cTn I (pg/mL)**	45,15 (14,50; 219,85)	40,10 (10,80; 248,00)	60,10 (26,20-213,00)	0,374*
	Increased hs-cTn I	31 (57,4%)	21 (53,8%)	10 (66,7%)	0,393•
	In risk of CS	19 (35,2%)	12 (30,8%)	7 (46,7%)	0,273•
	CK (U/L)	100,00 (45,00-356,25)	113,50 (56,00; 388,50)	82,50 (31,75-209,00)	0,260
	Increased CK	20 (34,5%)	16 (40%)	4 (22,2%)	0,188•
	Myoglobin (ng/mL)	119,60 (37,20-919,35)	98,30 (32,30; 846,15)	143,40 (103,90-1150,15)	0,445*
	Increased Myoglobin	15 (71,4%)	10 (62,5%)	5 (100%)	0,262▪
	LDH (U/L)	466,00 (358,50-586,75)	457 (357-583)	499,00 (389,50-590,50)	0,593*
	Increased LDH	55 (98,2%)	38 (97,4%)	17 (100%)	1,000▪
Second COVID-19 Wave	**Biomarkers**	**All patients** (n=133)	**Discharged** (n=83)	**Deceased** (n=50)	***P* Value**
	hs-cTn I (pg/mL)	17,00 (5,40-80,80)	14,00 (4,45-45,25)	21,20 (8,23-275,68)	**0,042***
	Increased hs-cTn I	41 (35,7%)	19 (27,5%)	22 (47,8%)	**0,026•**
	In risk of CS	30 (26,1)	14 (20,3%)	16 (34,8%)	0,083•
	CK (U/L)	91,00 (39,00-201,50)	80,00 (34,00-170,00)	101,50 (53,75-275,75)	0,172*
	Increased CK	30 (24,8%)	17 (22,7%)	13 (28,3%)	0,489•
	Myoglobin (ng/mL)	78,40 (50,90-519,60)	76,70 (42,75-398,70)	234,90 (58,85-721,75)	0,491*
	Increased Myoglobin	12 (46,2%)	7 (41,2%)	5 (55,6%)	0,683▪
	LDH (U/L)	454,00 (368,25-566,00)	432,00 (352,25-523,75)	508,00 (407,50-627,25)	**0,010***
	Increased LDH	116 (96,7%)	73 (96,1%)	43 (97,7%)	1,000▪
Third COVID-19 Wave	**Biomarkers**	**All patients** (n=133)	**Discharged** (n=85)	**Deceased** (n=48)	***P* Value**
	hs-cTn I (pg/mL)	18,25 (6,35-73,25)	9,50 (4,80-39,20)	33,10 (15,10-147,65)	**0,003***
	Increased hs-cTn I	34 (35,4%)	14 (25,5%)	20 (48,8%)	**0,018•**
	In risk of CS	25 (26%)	12 (21,8%)	13 (31,7%)	0,275•
	CK (U/L)	132,50 (53,25-362,00)	115,00 (42,00-342,00)	221,00 (60,00-393,00)	0,083*
	Increased CK	48 (43,6%)	25 (37,3%)	23 (53,5%)	0,095•
	Myoglobin (ng/mL)	173,30 (73,10-255,80)	101,00 (68,30-226,10)	207,00 (74,83-260,53)	0,520*
	Increased Myoglobin	22 (71,0%)	11 (73,3%)	11 (68,8%)	1,000▪
	LDH (U/L)	568,50 (428,00-705,25)	475,00 (379,50-611,75)	650,50 (534,00-826,25)	**<0,001***
	Increased LDH	110 (98,2%)	66 (97,1%)	44 (100%)	0,519▪

All calculated percentages do not include missing values.

Elevation of hs-cTn I is typically related to cardiac myocyte injury/ necrosis. It's been described several times that this type of cardiac manifestation in patients with COVID-19 leads to a higher risk of mortality (161–165). A cohort study including 416 patients demonstrated through a Cox regression model that patients with cardiac injury are at a higher risk of death (hazard ratio 3,41 (95% CI; 1,62-7,16)) (166). In another multihospital retrospective cohort study involving 3000 patients, myocardial injury was common, but mostly associated with a low elevation of hs-cTn I at admission (167). The same

was verified for the present study sample. Although the frequency of patients at risk of CS was higher than the one of patients with mildly elevated cardiac troponin levels (34,2 <hs-cTn I ≤ 64), the median levels of hs-cTn I at admission were only mildly elevated in the 1st COVID-19 wave. So, in general, besides 40% of the patients having showed elevated troponin levels, the median was within the normality range (20,20 pg/mL) (**Table 4.2.1.1**).

Even though a concentration of cardiac troponins above the 99th percentile of the values corresponding to a reference control group is indicative of a possible MI, other clinical factors must be considered to establish a diagnosis (107). Cardiac troponins are "organ-specific, not disease-specific" thereby meaning their elevation can result from other factors besides MI, such as myocarditis, increased ventricular tension, excess catecholamines, diminished renal clearance and other causes for myocyte injury and/or necrosis. Chronic diseases like chronic kidney disease, hearth failure and pulmonary arterial hypertension are other examples (168). In the present study sample, besides not documented in this master thesis, several cases of acute myocarditis were observed by health professionals. In these cases, troponin elevations were a result of non-ischemic myocardial injury, thus not resulting in a type I infarction, but presenting as a pseudoinfarct/ non-obstructive myocardial infarct (169).

In all COVID-19 waves, a higher percentage of deceased patients had an elevation of cardiac troponins (26,7% vs. 25,6%; 34,8% vs. 15,9%; and 26,8% vs. 20%). Taking this fact into account, Kaplan-Meier survival curves for each COVID-19 wave were obtained for the groups: "Increased hs-cTn I" and "non-increased hs-cTn I" (**Figure 4.2.1.1**).

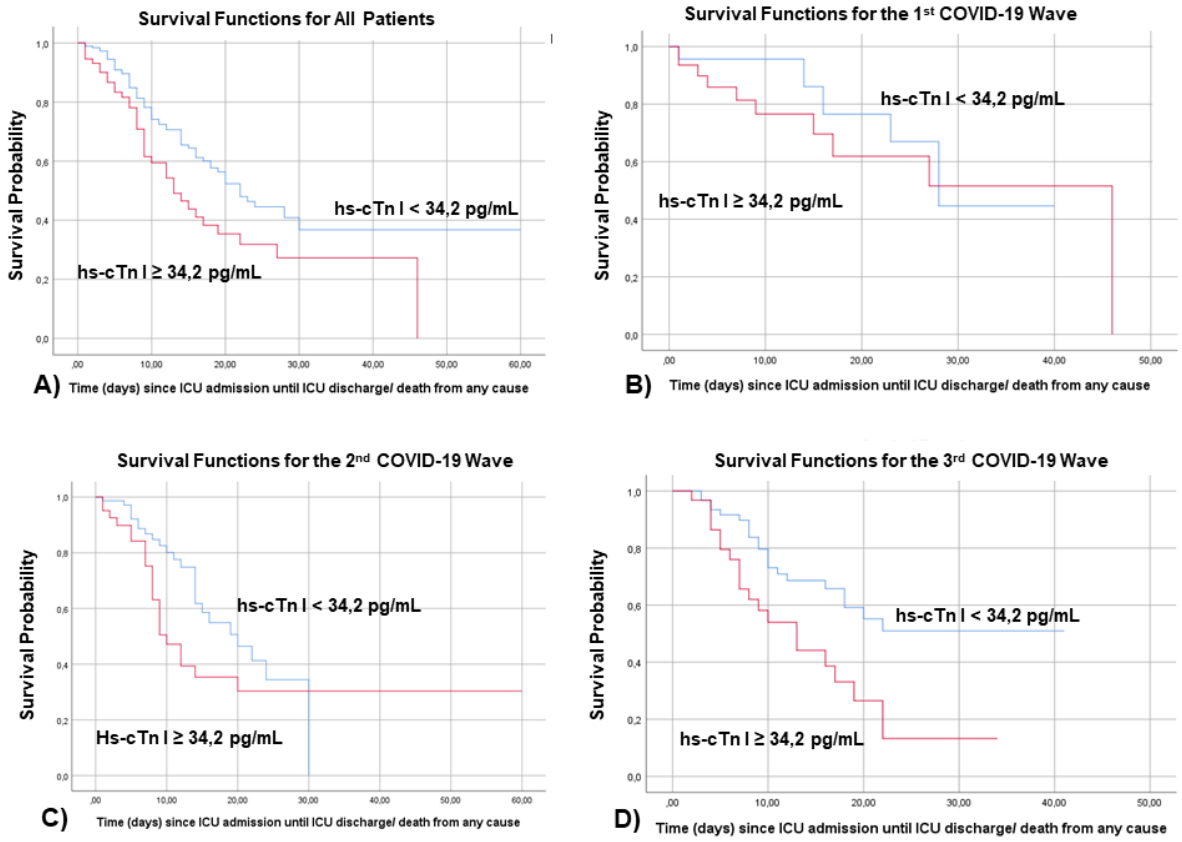


Figure 4.2.1.1. Survival curves for patients with COVID-19 according to the hs-cTn I threshold of 34,2 pg/mL in the 2nd day of ICU admission (**A**) and separate survival curves for each COVID-19 wave (**B, C** and **D**).

The survival functions were significantly different considering the hs-cTn I threshold of 34,2 pg/mL (**Figure 4.2.1.1A**). Analyzing the survival data for each wave, the survival curves were significantly different in the 2nd and 3rd COVID-19 waves (**Figures 4.2.1.1C** and **D**), results which are further supplemented by the tests reflected on **Table 4.2.1.3** (*P*=0,013; 0,013 and 0,006 respectively).

Table 4.2.1.3. *Comparisons between survival functions for all patients with and without increased hs-cTn I levels, and separate analysis for all COVID-19 waves, in the 2nd day of ICU admission*

	All patients	**1st Covid-19 Wave**	**2nd Covid-19 Wave**	**3rd Covid-19 Wave**
	***P* Value**	***P* Value**	***P* Value**	***P* Value**
Log Rank (Mantel-Cox)	**0,013**	0,505	0,065	0,003
Breslow (Generalized Wilcoxon)	0,007	0,265	0,008	0,010
Tarone-Ware	0,008	0,329	**0,013**	**0,006**

Regarding CK and myoglobin, deceased patients had almost always higher median concentrations (except CK that was higher for the discharged patients of the 1st COVID-19 wave) and higher frequency of patients with values out of normality ranges (except for myoglobin in the 3rd wave). Kaplan Meier survival curves stratified according to reference normality cut-off points for these biomarkers didn't show significant differences for any of the COVID-19 waves. Although, elevated CK has been associated with increased mortality and disease severity in COVID-19, as concluded in a systematic review and meta-analysis involving 2471 patients (170). It is still unclear whether this elevation is caused by the virus itself (since it may invade muscles and other nervous system cell's via ACE2 receptors causing direct viral myositis) or by other factors such as renal impairment, toxic effects of cytokines, preexisting myopathies and muscle dysfunction acquired during hospital stay/ disease course (170–172). Myoglobin exists in skeletal and cardiac muscle and increases in circulation after myocyte damage/ necrosis. It was shown, that when combined with CK, it has a better prediction of worse outcomes and death in COVID-19 patients (AUC of 0,883 (95%CI: 0,813-0,952; *P*<0,001)) (173).

In all COVID-19 waves LDH median levels were above normality ranges and, in average, for both groups (discharged and deceased) more than 97% of the patients had elevated LDH values. This was expected since, as already described, almost all COVID-19 patients demonstrate elevated LDH results (174). Also, the median levels of this biomarker were significantly different between discharged and deceased patients for the 2nd (*P*=0,010) and 3rd (*P*<0,001) waves. Besides being described that LDH levels could be an independent predictor of myocardial injury, other studies also determined that they are significantly associated with disease severity. This can be related to the typical multiorgan dysfunction of COVID-19 patients, with systemic inflammation, tissue damage and necrosis (including in the myocardium) (73,174,175). For each wave, Kaplan Meier survival curves stratified according to the reference normality cut-off points for this biomarker didn't show any significant differences.

To finalize the cardiac biomarker's analysis in the 2nd day of ICU admission, univariate logistic regression for all patients in each COVID-19 wave was applied to determine which biomarkers were related to the disease's outcome (ICU discharge or death) (**Table 4.2.1.4**). The univariate logistic regression analysis demonstrated that increased hs-cTn I levels, in the 2nd and 3rd waves, and that LDH

levels, in the 3rd wave, were significantly associated with a higher risk of death. Thus, mortality risk is increased 2,4-fold in the 2nd wave and 2,8-fold in the 3rd wave for patients with increased hs-cTn I results. In the case of LDH, every unit increase (U/L) results in a 0,3% increase in mortality risk.

Table 4.2.1.4. *Statistically significant results from univariate logistic regression for cardiac-related biomarkers in the 2nd day of ICU admission*

		Univariate logistic regression for patients in the 2nd day of ICU admission		
		Crude OR	**95% CI**	**P Value**
2nd COVID-19 Wave	**Increased hs-cTn I**	2,412	1,102-5,280	**0,028**
3rd COVID-19 Wave	**Increased hs-cTn I**	2,789	1,178-6,604	**0,020**
	LDH (U/L)	1,003	1,001-1,005	**0,001**

Separate comparisons between COVID-19 waves for the groups of discharged and deceased patients (**Table 4.2.1.5**) lead to the conclusion that, in the 2nd day of ICU admission, the levels of hs-cTn I were significantly different between waves for the discharged patients (*P*=0,009) and that the levels of LDH were also significantly different between waves for the deceased patients (*P*<0,001).

Table 4.2.1.5. *Cardiac-related biomarkers' distributions with significant differences across COVID-19 waves, for discharged and deceased patients in the 2nd day of ICU admission*

	Hs-cTn I levels comparison between:	***P* Value**
Discharged patients	**All COVID-19 Waves (**n=163**)**	**0,009†**
	1st and 2nd COVID-19 Waves	**0,019†**
	1st and 3rd COVID-19 Waves	**0,015†**
	2nd and 3rd COVID-19 Waves	1,000†
	LDH levels comparison between:	***P* Value**
Deceased patients	**All COVID-19 Waves (**n=105**)**	**<0,001†**
	1st and 2nd COVID-19 Waves	1,000†
	1st and 3rd COVID-19 Waves	**0,004†**
	2nd and 3rd COVID-19 Waves	**0,001†**

hs-cTn I median levels were much higher in the 1st wave (**Figure 4.2.1.2**), leading to significant differences between the 1st and 2nd (P=0,019) and 1st and 3rd (P=0,015) COVID-19 waves. Significant elevation of cardiac biomarkers in the 1st wave was also described in other studies. For example, one study in the United Kingdom observed a possible correlation between prolonged "symptom-to-call" time, higher peak hs-cTn I levels and decreased six months survival after myocardial infarction (176). The same was verified in other countries like Spain and China (177,178). Possible triggers were changes on patients' behavior due to the pandemic, like hospital/ medical care avoidance to prevent contracting the disease (176,178). Hence, this type of information can be very valuable for health professionals in future pandemics or other SARS-CoV-2 variants. In the other hand, LDH median levels were higher in the 3rd COVID-19 wave (**Figure 4.2.1.3**) for the 2nd day of ICU admission, leading to significant differences between the 1st and 3rd (P=0,004) and 2nd and 3rd (P=0,001) COVID-19 waves. Despite that, all COVID-19 waves showed elevated median levels in both discharged and deceased patients' groups and almost all patients had elevated LDH results. The remaining cardiac biomarkers didn't show significant differences across the three waves and therefore the results of their statistical analysis were not presented.

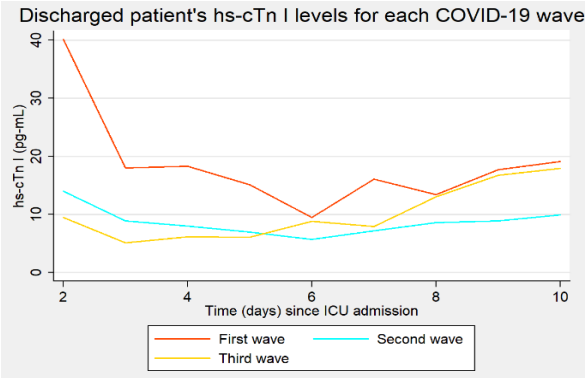


Figure 4.2.1.2. hs-cTn I (pg/mL) median time course by COVID-19 wave, for discharged patients, between the 2nd and 10th days of ICU admission.

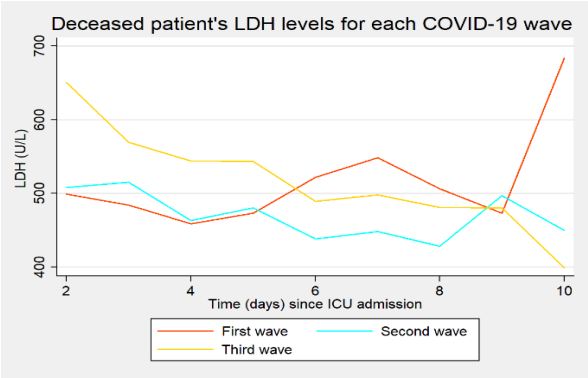


Figure 4.2.1.3. LDH (U/L) median time course by COVID-19 wave, for deceased patients, between the 2nd and 10th days of ICU admission.

To evaluate the biomarkers after one week in the ICU, 216 patients were included (**Table 4.2.1.6**). It was concluded that 47,2% had elevated CK, 87,5% had elevated myoglobin and 98,4% had elevated LDH results, all higher percentages than in the 2nd day of ICU admission. On the other hand, 26,9% of the patients had elevated hs-cTn I levels, which is less than in the 2nd day of ICU admission (26,9% vs.40%), and the percentage of patients with indications of a probable CS was also smaller (17,2% Vs. 27,9%).

Table 4.2.1.6. *Cardiac-related biomarkers in the 7th day of ICU admission*

Biomarkers	**Missing Values**	**All Patients** (n=216)
hs-cTn I (pg/mL)	71 (32,9%)	12,40 (5,35-39,10)
Increased hs-cTn I		39 (26,9%)
In risk of CS		25 (17,2%)
CK (U/L)	53 (24,5%)	185,00 (65,00-481,00)
Increased CK		77 (47,2%)
Myoglobin (ng/mL)	192 (88,9%)	256,15 (172,48-591,03)
Increased Myoglobin		21 (87,5%)
LDH (U/L)	23 (10,6%)	429,00 (338,00-525,00)
Increased LDH		190 (98,4%)

Comparisons between discharged and deceased patients (**Table 4.2.1.7**) showed significant differences regarding the proportion of patients in risk of CS (*P*=0,037) and the median levels of LDH (*P*=0,013) for the 1st COVID-19 wave. The same was verified for LDH in the 3rd wave (*P*=0,005). hs-cTn I levels were significantly different between discharged and deceased patients from the 2nd and 3rd waves (*P*=0,038 and 0,045 respectively). Finally, in the 3rd wave, CK median levels were different between the same groups (*P*=0,011) and so was the proportion of patients with elevated CK values (*P*=0,006).

In all COVID-19 waves, once again, a higher percentage of deceased patients had an elevation of cardiac troponins (23,8% vs. 45,5%; 18,9% vs. 25,0%; and 20,7% vs. 47,4%). This time around, Kaplan Meier survival curves stratified according to the reference normality cut-off points for this biomarker didn't show any significant differences in each wave.

Table 4.2.1.7. *Cardiac-related biomarkers of all patients and comparisons between the ones discharged from the ICU and those who died, for all COVID-19 waves in the 7th day of ICU admission*

	Biomarkers	**All patients** (n=39)	**Discharged** (n=27)	**Deceased** (n=12)	***P* Value**
First COVID-19 Wave	**hs-cTn I (pg/mL)**	20,35 (10,33-41,63)	16,10 (8,85-33,00)	27,00 (12,50-118,50)	0,133*
	Increased hs-cTn I	10 (31,3%)	5 (23,8%)	5 (45,5%)	0,252▪
	In risk of CS	5 (15,6%)	1 (4,8%)	4 (36,4%)	**0,037▪**
	CK (U/L)	212,50 (66,75-410,50)	220,00 (55,00-479,50)	195,00 (69,00-373,00)	0,919*
	Increased CK	19 (52,8%)	14 (56%)	5 (45,5%)	0,559•
	Myoglobin (ng/mL)	277,40 (188,70-642,50)	236,25 (174,00-616,10)	733,00 (733,00-733,00)	0,364*
	Increased Myoglobin	10 (90,9%)	9 (90%)	1 (100%)	1,000▪
	LDH (U/L)	445,00 (339,00-521,00)	413,00 (336,00-470,50)	548,50 (392,00-631,75)	**0,013***
	Increased LDH	37 (100%)	25 (100%)	12 (100%)	-
Second COVID-19 Wave	**Biomarkers**	**All patients** (n=87)	**Discharged** (n=52)	**Deceased** (n=35)	***P* Value**
	hs-cTn I (pg/mL)	9,10 (4,25-29,00)	7,20 (2,95-23,55)	13,25 (7,23-37,35)	**0,038***
	Increased hs-cTn I	14 (21,5%)	7 (18,9%)	7 (25,0%)	0,555•
	In risk of CS	9 (13,8%)	4 (10,8%)	5 (17,9%)	0,483▪
	CK (U/L)	105,50 (32,25-290,25)	103,50 (20,75-331,50)	105,50 (50,25-243,50)	0,371*
	Increased CK	22 (34,4%)	13 (34,2%)	9 (34,6%)	0,973•
	Myoglobin (ng/mL)	359,00 (58,90-456,90)	306,45 (90,05-1366,98)	359,00 (30,80-.)	0,857*
	Increased Myoglobin	5 (71,4%)	3 (75%)	2 (66,7%)	1,000▪
	LDH (U/L)	400,50 (332,75-532,50)	377,50 (323,00-515,00)	448,00 (333,75-564,50)	0,194*
	Increased LDH	75 (96,2%)	46 (95,8%)	29(96,7%)	1,000▪
Third COVID-19 Wave	**Biomarkers**	**All patients** (n=90)	**Discharged** (n=58)	**Deceased** (n=32)	***P* Value**
	hs-cTn I (pg/mL)	12,20 (5,40-50,90)	7,90 (3,60-22,75)	30,80 (11,70-145,60)	**0,045***
	Increased hs-cTn I	15 (31,3%)	6 (20,7%)	9 (47,4%)	0,051•
	In risk of CS	11 (22,9%)	5 (17,2%)	6 (31,6%)	0,304▪
	CK (U/L)	235,00 (107,00-636,00)	186,00 (71,00-483,00)	459,50 (206,00-849,75)	**0,011***
	Increased CK	36 (57,1%)	17 (43,6%)	19 (79,2%)	**0,006•**
	Myoglobin (ng/mL)	220,50 (153,78-734,90)	234,90 (168,80-.)	206,20 (108,70-.)	1,000*
	Increased Myoglobin	6 (100%)	3 (100%)	3 (100%)	-
	LDH (U/L)	434,00 (345,50-516,75)	374,50 (312,50-501,50)	498,00 (410,25-561,00)	**0,005***
	Increased LDH	78 (100%)	48 (100%)	30 (100%)	-

All calculated percentages do not include missing values.

In the other hand, just like in the 2nd day of ICU admission, only in the 2nd and 3rd COVID-19 waves was there a significant difference between discharged and deceased patients regarding hs-cTn I median levels (*P*=0,038 and 0,045 respectively). LDH median levels were significantly different between the same groups in the 3rd wave (*P*=0,005), which also happened in the 2nd day of ICU admission.

Moving on to comparisons between COVID-19 waves for discharged and deceased patients' cardiac biomarkers in the 7th day of ICU admission (**Table 4.2.1.8**), it was determined that CK median levels were significantly different between waves for deceased patients (*P*=0,003). The referred biomarker's levels were much higher in the 3rd wave, for deceased patients, leading to significant differences between the 2nd and 3rd (P=0,003) waves (**Figure 4.2.1.4**).

Table 4.2.1.8. *Cardiac-related biomarkers' distributions with significant differences across COVID-19 waves, for discharged and deceased patients in the 7th day of ICU admission*

	CK levels comparison between:	*P* Value
Deceased patients	**All COVID-19 Waves (n=61)**	**0,003†**
	1st and 2nd COVID-19 Waves	1,000†
	1st and 3rd COVID-19 Waves	0,097†
	2nd and 3rd COVID-19 Waves	**0,003†**

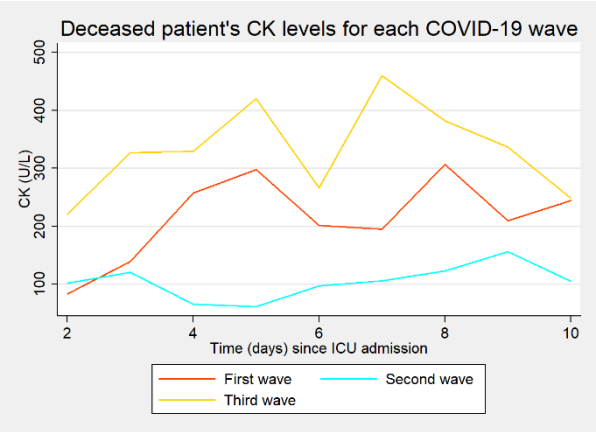


Figure 4.2.1.4. CK (U/L) median time course by COVID-19 wave, for deceased patients, between the 2nd and 10th days of ICU admission.

To assess the patients' progress throughout their ICU stay, comparisons were made between the 2nd and 7th days of ICU admission (**Table 4.2.1.9**). Due to the large number of missing values, comparisons between myoglobin levels weren't obtained.

Table 4.2.1.9. *Comparisons regarding median cardiac-related biomarker's levels of all patients, the ones discharged from the ICU and those who died between the 2nd and 7th days of ICU admission for each COVID-19 wave*

		hs-cTn I (pg/mL)		CK (U/L)		LDH (U/L)	
		n	**P Value**	**n**	**P Value**	**n**	**P Value**
1st COVID-19 Wave	**Discharged patients**	20	**0,001**	24	0,511	23	**0,007**
	Deceased patients	9	0,374	11	0,594	11	0,722
	All patients	29	**0,001**	35	0,351	34	**0,032**
2nd COVID-19 Wave	**Discharged patients**	33	**0,001**	35	0,980	42	**<0,001**
	Deceased patients	28	**0,007**	25	0,389	27	**0,022**
	All patients	61	**<0,001**	60	0,546	69	**<0,001**
3rd COVID-19 Wave	**Discharged patients**	24	0,081	34	0,365	40	**<0,001**
	Deceased patients	17	0,076	21	0,259	27	**<0,001**
	All patients	41	**0,016**	55	0,146	67	**<0,001**

P-values represent the comparison of the median of a given biomarker on the 2nd day with that of the 7th day of ICU admission. All *P* values obtained through related-samples Wilcoxon matched-pair signed rank test.

In all three COVID-19 waves all patients showed significant differences regarding their hs-cTn I results between the 2nd and 7th days of ICU admission (*P*=0,001; <0,001 and 0,016 in each wave, respectively) (**Figure 4.2.1.5A**). Median hs-cTn I values were always higher in the 2nd day, for both groups of discharged and deceased patients, especially in the 1st wave. This can be related to the fact that the patients that belong to this wave were significantly older. For all waves hs-cTn I levels started to decrease in the beginning and then increased between the 5th and 6th days of ICU admission (**Figures 4.2.1.5B-D**). The 2nd wave was the only one where the differences between the two days were significant between all groups of patients (*P* value of 0,001 for discharged patients, of 0,007 for deceased patients and inferior to 0,001 for all patients) since there was a marked decrease of hs-cTn I levels (**Figure 4.2.1.5C**). Other studies had contrary results were hs-cTn I levels rose until the 3rd day and then declined from the 4th to the 7th days for survivors, while for deceased patients an abrupted increase occurred until death (179).

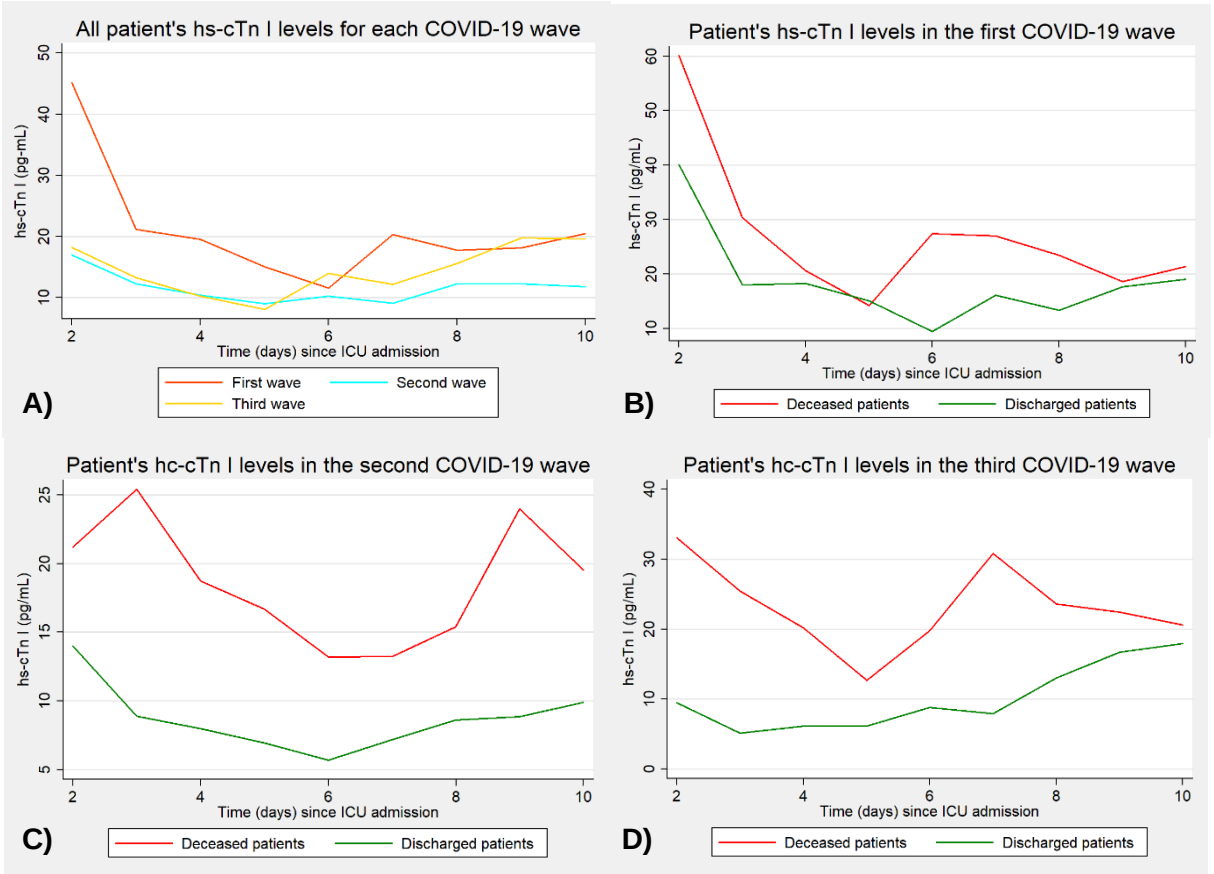


Figure 4.2.1.5. Median time course for all patients' hs-cTn I levels in each COVID-19 wave (**A**) and for the groups of discharged and deceased patients in the 1st (**B**), 2nd (**C**) and 3rd (**D**) COVID-19 waves, between the 2nd and 10th days of ICU admission.

Median LDH levels were also significantly different for all patients between the 2nd and 7th days of ICU admission, except in the 1st COVID-19 wave (*P*<0,001 for the 2nd and 3rd waves) (**Figure 4.2.1.6A**). Between the 2nd and 7th days, LDH levels peaked approximately in the first two days of ICU admission for all patients and then started to decrease (except for the deceased patients of the 1st wave) (**Figures 4.2.1.6B-C**). Thus, in the 1st wave there were only significant differences between the two analyzed days for discharged patients (*P*=0,007). On the other hand, in the 2nd wave significant differences were found for both groups (*P*<0,001 for discharged patients and *P*=0,022 for deceased

ones) and the same was verified in the 3rd wave (*P*<0,001 for both groups). The high increases of this biomarker in early stages of the decease observed in this study has been documented by others, which classified LDH as one of the most premature biomarkers related to inflammation and cell death (180).

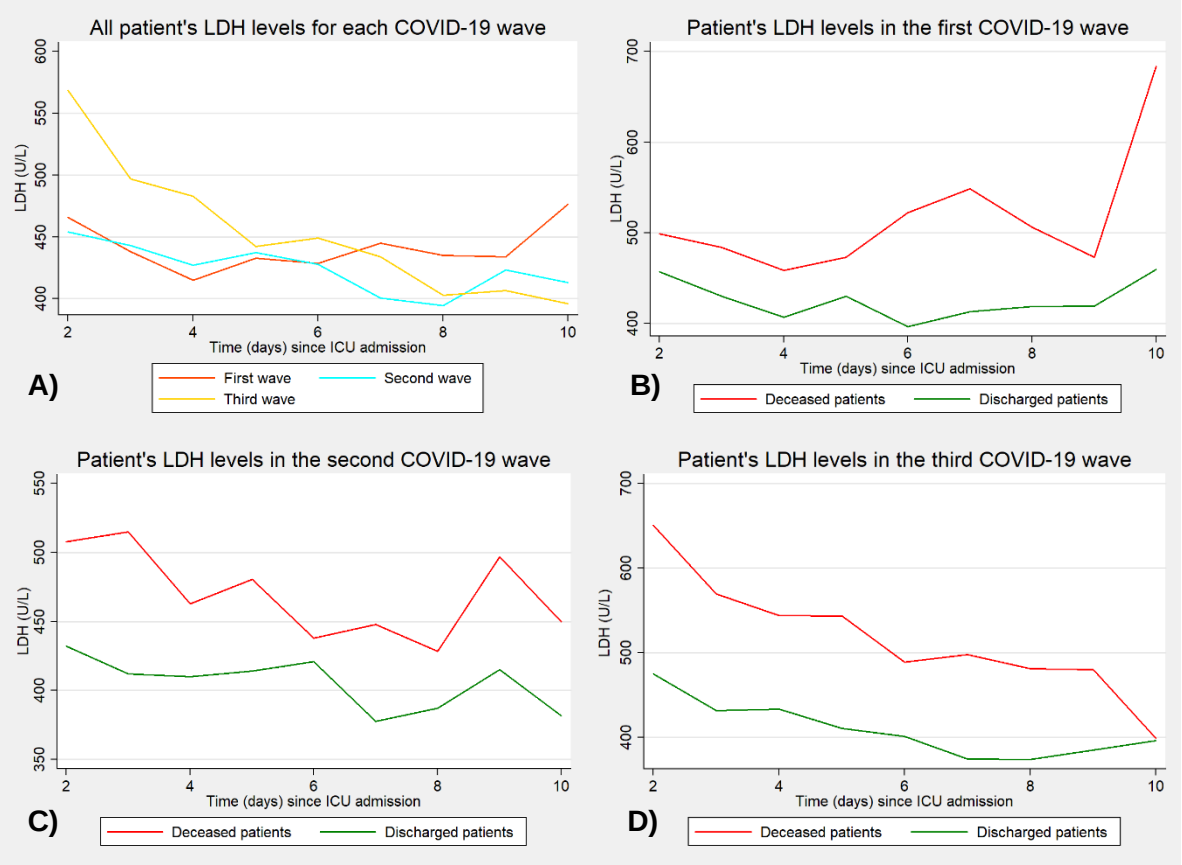


Figure 4.2.1.6. Median time course for all patients' LDH levels in each COVID-19 wave (**A**) and for the groups of discharged and deceased patients in the 1st (**B**), 2nd (**C**) and 3rd (**D**) COVID-19 waves, between the 2nd and 10th days of ICU admission.

Considering the total longitudinal data corresponding to the UCI stay, the associations between cardiac-related biomarkers and death were tested by means of univariate GEEs models (**Table 4.2.1.10**). Similarly to the previously obtained results, patients with increased hs-cTn I levels had a higher risk of death. This time, not only in the 2nd and 3rd waves, but in all three of them (*P*=0,019; 0,002 and 0,003 respectively). Thus, patients with increased hs-cTn I results had an increased risk of death of 0,4% in the 1st wave, 1,7% in the 2nd wave, and 2,1% in the 3rd wave. Also, variables that for the 2nd and 7th days of ICU admission weren't associated with the patients' outcome, like increased risk of CS, myoglobin, and CK, were associated with higher risks of death when GEEs models were applied. Patients in risk of CS had a 0,4% increased chance of death in the 1st wave, and of 2,1% in the 2nd wave. For patients with increased myoglobin, the risk of death was 2,4% higher in the 1st wave and 18,9% in the 2nd wave. Increased CK levels resulted in a 2,3% greater risk of death in the 3rd wave. These results can be very useful in future research when the entire period of ICU admission is thoroughly analyzed, and multivariate models are applied.

Table 4.2.1.10. *Statistically significant results from univariate GEEs models for cardiac-related biomarkers in each COVID-19 wave*

		OR	95% CI	*P* Value
1st COVID-19 Wave	**Increased hs-cTn I**	1,004	1,001-1,007	0,019
	In risk of CS	1,004	1,001-1,008	0,017
	Increased Myoglobin	1,024	1,009-1,038	0,002
2nd COVID-19 Wave	**Increased hs-cTn I**	1,017	1,006-1,027	0,002
	In risk of CS	1,021	1,006-1,035	0,006
	Increased Myoglobin	1,189	1,034-1,367	0,015
3rd COVID-19 Wave	**Increased hs-cTn I**	1,021	1,007-1,035	0,003
	Increased CK	1,023	1,012-1,034	<0,001

4.2.2. Inflammatory and other hematological biomarkers

Regarding inflammatory and other hematological biomarkers, to characterize the sample in the 2nd day of ICU admission 325 patients' laboratory results were analyzed (**Table 4.2.1.1**).

Table 4.2.2.1. *Inflammatory and other hematological biomarkers in the 2nd day of ICU admission*

Biomarkers	Normality Ranges	Missing Values	All Patients (n=325)
Max. PCT (ng/mL)	<0,06	68 (20,9%)	0,26 (0,11-1,04)
Increased PCT			231 (89,9%)
Max. CRP (mg/L)	<5,0	4 (1,2%)	162,30 (92,20-243,95)
Increased CRP			321 (100%)
Max. IL-6 (pg/mL)	<7,0	299 (92%)	83,37 (25,53-178,10)
Increased IL-6			26 (100%)
Min. RBC x 10^{12}/L	4,4-5,9	5 (1,5%)	4,14 (3,60-4,56)
Decreased RBC			212 (66,3%)
Min. HCT (%)	40,0-50,0	5 (1,5%)	35,90 (31,75-39,58)
Decreased HCT			248 (77,5%)
Min. HGB x 10g/L	13,0-16,0	5 (1,5%)	11,90 (10,53-13,20)
Decreased HGB			222 (69,4%)
Min. WBC x 10^9/L	4,0-10,0	5 (1,5%)	9,01 (6,48-12,15)
Decreased WBC			22 (6,9%)
Max. WBC x 10^9/L	4,0-10,0	5 (1,5%)	10,02 (7,59-13,70)
Increased WBC			161 (50,3%)
Min. LYM x 10^9/L	0,8-5,0	5 (1,5%)	0,74 (0,51-1,09)
Decreased LYM			178 (55,6%)
Max. NEU x 10^9/L	1,6-7,0	5 (1,5%)	8,58 (6,29-11,92)
Increased NEU			212 (66,3%)
Min. EO x 10^9/L	0,03-0,6	5 (1,5%)	0,00 (0,00-0,00)
Decreased EO			275 (85,9%)
Min. MON (x 10^9/L)	0,1-1,0	5 (1,5%)	0,40 (0,25-0,68)
Decreased MON			11 (3,4%)
Max. MON (x 10^9/L)	0,1-1,0	5 (1,5%)	0,51 (0,34-0,79)
Increased MON			48 (15%)
Max. Platelet-lymphocyte ratio (PLR)	<192	5 (1,5%)	334,73 (226,54-509,85)
Increased PLR			261 (81,6%)
Max. Neutrophil-lymphocyte ratio (NLR)	0,78-3,53	5 (1,5%)	10,89 (6,72-17,22)
Increased NLR			305 (95,3%)

Reference values according to the hospital's laboratory directives and adapted from (116).
PLR reference values according to (181).

According to the results, 89,9% of the patients had increased PCT values. PCT is normally produced by the parafollicular cells of the thyroid gland. In case of bacterial sepsis, PCT levels rise due to the increase of its production in all parenchymal tissues (stimulated by TNF-α and IL-6, per example), being very useful for antibiotic therapy monitoring. In the other hand, in viral infections its production is downregulated (by mediators like IFNγ). Despite this statement, PCT is increased in COVID-19 patients, suggesting that these patients may have secondary bacterial infections that lead to these increases (182,183). Thus, accordingly to a meta-analysis, PCT levels were associated with approximately a fivefold higher risk of severe infection by SARS-Cov-2 (OR, 4,76; 95% CI; 2,74-8,29) (184). CRP is a

nonspecific biomarker produced in the liver in case of inflammation and is triggered by a variety of inflammatory mediators such as IL-6 (183). In this study, median CRP results were far above reference values (162,30 mg/L) and elevated in all involved patients. For levels greater than or equal to 40mg/L, other authors have suggested this biomarker as a reliable indicator of COVID-19 severity and risk of death (185). IL-6, reported as the most secreted cytokine by macrophages, is strongly elevated in severe COVID-19 patients (68,186). In the present study population IL-6 results were increased for all patients (n=26), however the number of missing values for this variable was very high.

Median levels of red blood cells (RBCs) and HCT were below normality ranges and more than half the patients, in both cases, had decreased results (66,3% and 77,5% respectively). Decreases in erythrocyte levels have been linked to the infection by SARS-CoV-2. After the virus binds to RBCs, a series of metabolic changes cause an increase in reactive oxygen species, resulting in cell damage, lysis, and the inability to transport oxygen. SARS-Cov-2 also binds to hemoglobin (HGB), causing its denaturation and thus the inhibition of viral replication. This defense mechanism interferes in oxygen transport and results in hypoxia, a common symptom in COVID-19 patients (187). In this study median levels of HGB were below normality ranges and 69,4% of the patients had decreased results.

It has been reported that WBCs levels increase (leukocytosis) during SARS-CoV-2 infection, more commonly in severe cases (186). WBC count at admission was even significantly correlated with death in another research (188). In this study, maximum WBCs median levels were slightly above normality ranges and 50,3% of the patients had leukocytosis. As formerly described, the increase in WBCs could be driven by the increases verified in NEU counts (73). As a matter of fact, NEU counts were elevated in the present analysis and strongly associated with WBCs counts (Φ=0,809; P<0,001). These cells increase in circulation as the disease progresses due to the presence of infectious agents and tissue damage (189). Supported by neutrophilia, leukocytosis seems to correlate with more severe outcomes (190).

Like in any other viral infection, COVID-19 patients are presented with lymphopenia. With data from a longitudinal cohort of 5628 Korean COVID-19 patients, it was demonstrated that early lymphopenia is associated with poor prognosis, just like in various other reports (78,116,191,192). Possible underlying triggers are LYM depletion in the spleen and lymph nodes through cell apoptosis and viral antigen stimulation (193). In the present case, median LYM levels were decreased and 64,1% of the patients had lymphopenia. Eosinopenia is another response of infection with unclear pathophysiology. It can be a result of the migration of EOs to inflammation sites or, on contrary, the resistance of their mobilization from the bone marrow, or even direct cell apoptosis (190). Decreases in EO counts have been reported several times and considered a severity biomarker (116,194). Finally, MONs are also activated during viral infections, being recruited by inflammatory mediators. When they arrive to the target tissues, their phenotype changes, and they become macrophages or DCs, which fight inflammation. In COVID-19, cases of hyperactivation of these cells are common, resulting in pro-inflammatory cytokine release with cytotoxic effector cells recruitment, causing more tissue damage (195). Studies like the one from Rajamanickam *et al.* (196), demonstrated that COVID-19 severity is associated with elevated MON counts. In the other hand, Kilercik *et al*. (197) determined that decreased

MON percentages are significantly correlated with disease severity. The median of minimum MON count in this study was between normality ranges, and only 3,4% of the patients had decreased results. The median of maximum MON count was similarly normal, and 15% of the patients had increased values.

PLR is a relatively new and inexpensive marker of systemic inflammation. It has been used in cases of cardiovascular and autoimmune diseases as a predictor of inflammation and mortality and related to tumor size and metastasis (198,199). Recently, PLR has been used as a severity biomarker in COVID-19 cases and associated with higher morbidity and mortality. According to Hashem *et al*., in patients requiring ICU internment, PLR results at admission could be used as predictors of severe infection by SARS-CoV-2 (181). However, more evidence in this matter is needed (some authors describe PLR results as confounding) and so is a reliable cut-off value (200). For the present study, median maximum PLR results were above the normality range, and an elevated percentage of patients had increased results (81,6%). NLR is another easily calculated inflammatory biomarker. As opposed to PLR, NLR has showed more reliable results and has a high prognostic value (201). COVID-19 patients' NLR can be used as an independent risk factor for hospital mortality, according to *Liu et al* (202). Regarding this biomarker, almost all patients had increased levels (95,3%) and the median of maximum NLR results were also elevated.

For comparisons between discharged and deceased patients (**Table 4.2.2.2**), IL-6 results weren't considered due to the high number of missing values. Minimum values of WBCs weren't also analyzed since the results were within normality ranges. The same rule was also applied for both minimum and maximum MON counts. No significant differences were observed in the 2nd day of ICU admission between discharged and deceased patients from the 1st COVID-19 wave, for any of the analyzed variables. Although, for both groups, all median results were outside normality ranges. Regarding the 2nd wave, RBC counts were significantly different between discharged and deceased patients, being that median levels were lower for the deceased ones since 80,9% of them had decreased results. Other reports have also described decreased RBCs and HGB results in Portuguese ICUs and that these low RBC counts are independently associated with mortality (OR=9,021; *P*<0,001) (203). In the present case, HGB median results were slightly below normality ranges for all COVID-19 waves. In the 2nd wave, they were even significantly different between discharged and deceased patients (*P*=0,045), taking into account the fact that 80,9% of the deceased ones had decreased HGB results.

In both the 2nd and 3rd waves, median LYM counts were significantly different between discharged and deceased patients (*P*=0,014 and 0,019 respectively). It was also verified that the deceased ones always revealed a worse degree of lymphocytopenia. Formerly, other authors have obtained similar results, namely the patients who died had lower LYM counts (191). Taking the former results into account, Kaplan-Meier survival curves for each COVID-19 wave were obtained for the nominal variable "Decreased LYM", versus "non-decreased LYM" results (**Figure 4.2.2.1**).

Table 4.2.2.2. *Inflammatory and other hematological biomarkers of all patients and comparisons between the ones discharged from the ICU and those who died, for all COVID-19 waves in the 2nd day of ICU admission*

Biomarkers	First COVID-19 Wave				Second COVID-19 Wave				Third COVID-19 Wave			
	All patients (n=59)	**Discharged** (n=41)	**Deceased** (n=18)	**P Value**	**All patients** (n=133)	**Discharged** (n=83)	**Deceased** (n=50)	**P Value**	**All patients** (n=133)	**Discharged** (n=85)	**Deceased** (n=48)	**P Value**
Max. PCT (ng/mL)	0,45 (0,17-1,32)	0,35 (0,14-1,15)	0,66 (0,22-1,44)	0,299*	0,18 (0,08-0,85)	0,13 (0,06-0,97)	0,31 (0,10-0,68)	0,238*	0,27 (0,13-0,89)	0,21 (0,11-0,46)	0,60 (0,16-1,68)	**0,002***
Increased PCT	48 (100%)	32 (100%)	16 (100%)	-	82 (82,8%)	46 (79,3%)	36 (87,8%)	0,270•	101 (91,8%)	60 (89,6%)	41 (95,3%)	0,478▪
Max. CRP (mg/L)	223,30 (127,80-261,40)	208,30 (117,45-254,30)	242,25 (176,05-283,10)	0,122*	133,95 (76,98-209,88)	117,70 (63,50-206,30)	148,10 (94,50-225,30)	0,223*	166,05 (93,00-248,68)	151,90 (67,43-232,93)	199,90 (129,00-256,28)	**0,024***
Increased CRP	59 (100%)	41 (100%)	18 (100%)	-	130 (100%)	83 (100%)	47 (100%)	-	132 (100%)	84 (100%)	48 (100%)	-
Min. RBC x 10^{12}/L	4,10 (3,50-4,42)	4,16 (3,65-4,42)	4,01 (3,19-4,41)	0,391*	4,05 (3,57-4,50)	4,28 (3,59-4,63)	3,86 (3,41-4,26)	**0,030***	4,21 (3,72-4,64)	4,23 (3,75-4,64)	4,21 (3,59-4,64)	0,542*
Decreased RBC	41 (70,7%)	28 (70%)	13 (72,2%)	0,863•	91 (70%)	53 (63,9%)	38 (80,9%)	**0,042•**	80 (60,6%)	49 (58,3%)	31 (64,6%)	0,480•
Min. HCT	34,60 (30,25-38,40)	34,65 (30,58-38,18)	33,70 (28,10-39,08)	0,680*	35,65 (31,80-39,10)	36,30 (32,60-39,80)	34,80 (30,30-37,50)	0,093*	36,65 (32,60-40,33)	36,65 (33,35-40,48)	36,30 (31,48-40,30)	0,569*
Decreased HCT	47 (81%)	33 (82,5%)	14 (77,8%)	0,724▪	104 (80%)	64 (77,1%)	40 (85,1%)	0,273•	97 (73,5%)	62 (73,8%)	35 (72,9%)	0,911•
Min. HGB	11,55 (9,98-13,05)	11,60 (10,15-12,98)	10,85 (9,33-13,20)	0,545*	11,85 (10,48-13,20)	12,10 (10,80-13,30)	11,50 (9,80-12,60)	**0,045***	12,25 (10,90-13,38)	12,40 (10,98-13,28)	11,95 (10,40-13,68)	0,380*
Decreased HGB	42 (72,4%)	30 (75%)	12 (66,7%)	0,538▪	93 (71,5%)	55 (66,3%)	38 (80,9%)	0,077•	87 (65,9%)	54 (64,3%)	33 (68,8%)	0,603•
Max. WBC	10,73 (8,78-13,41)	10,66 (8,59-13,12)	10,84 (9,26-17,34)	0,290*	10,23 (7,28-13,88)	9,56 (7,16-13,92)	10,77 (7,76-13,60)	0,453*	9,42 (7,36-13,66)	9,03 (7,34-13,57)	10,00 (7,38-13,74)	0,488*
Increased WBC	35 (60,3%)	18 (45%)	9 (50%)	0,724•	66 (50,8%)	32 (38,6%)	23 (48,9%)	0,250•	60 (45,5%)	32 (38,1%)	23 (47,9%)	0,271•
Min. LYM x 10^9/L	0,67 (0,50-1,10)	0,78 (0,51-1,18)	0,61 (0,47-1,10)	0,501*	0,74 (0,51-1,15)	0,83 (0,56-1,20)	0,63 (0,42-0,96)	**0,014***	0,75 (0,54-1,01)	0,77 (0,63-1,10)	0,64 (0,45-0,93)	**0,019***
Decreased LYM	31 (53,4%)	25 (62,5%)	12 (66,7%)	0,760•	69 (53,1%)	47 (56,6%)	33 (70,2%)	0,126•	78 (59,1%)	55 (65,5%)	33 (68,8%)	0,701•
Max. NEU x 10^9/L	8,80 (7,24-11,48)	8,57 (6,68-11,25)	9,14 (7,94-14,08)	0,243*	8,62 (6,02-12,45)	7,93 (5,32-12,53)	9,30 (6,85-12,43)	0,323*	8,05 (6,08-11,86)	7,80 (5,83-11,58)	9,11 (6,35-12,54)	0,289*
Increased NEU	45 (77,6%)	20 (50%)	12 (66,7%)	0,238•	85 (65,4%)	39 (47%)	27 (57,4%)	0,252•	82 (62,1%)	38 (45,2%)	25 (52,1%)	0,449•
Min. EO x 10^9/L	0,00 (0,00-0,01)	0,00 (0,00-0,01)	0,00 (0,00-0,0,4)	0,915*	0,00 (0,00-0,00)	0,00 (0,00-0,01)	0,00 (0,00-0,00)	**0,022***	0,00 (0,00-0,00)	0,00 (0,00-0,01)	0,00 (0,00-0,00)	**0,011***
Decreased EO	45 (77,6%)	32 (80%)	13 (72,2%)	0,516▪	117 (90%)	73 (88%)	44 (93,6%)	0,374▪	113 (85,6%)	68 (81%)	45 (93,5%)	**0,044•**
Max. PLR	294,79 (196,06-544,19)	256,11 (193,85-414,59)	435,27 (250,07-681,48)	0,064*	358,53 (247,53-519,85)	330,56 (235,63-429,07)	431,37 (269,84-576,47)	**0,008***	334,73 (225,61-505,10)	324,70 (236,08-461,08)	365,66 (203,25-581,29)	0,601*
Increased PLR	45 (77,6%)	31 (77,5%)	14 (77,8%)	1,000▪	110 (84,6%)	67 (80,7%)	43 (91,5%)	0,102•	106 (80,3%)	68 (81,0%)	38 (79,2%)	0,804•
Max. NLR	12,01 (6,17-22,06)	10,73 (5,52-15,75)	14,67 (9,23-29,01)	0,122*	10,47 (6,36-18,87)	8,86 (5,61-15,50)	15,88 (8,87-23,50)	**0,003***	10,92 (7,40-15,78)	9,90 (6,74-15,25)	12,70 (8,48-17,78)	**0,032***
Increased NLR	53 (91,4%)	37 (92,5%)	16 (88,9%)	0,641▪	124 (95,4%)	79 (95,2%)	45 (95,7%)	1,000▪	128 (97,0%)	81 (96,4%)	47 (97,9%)	1,000▪

All calculated percentages do not include missing values.

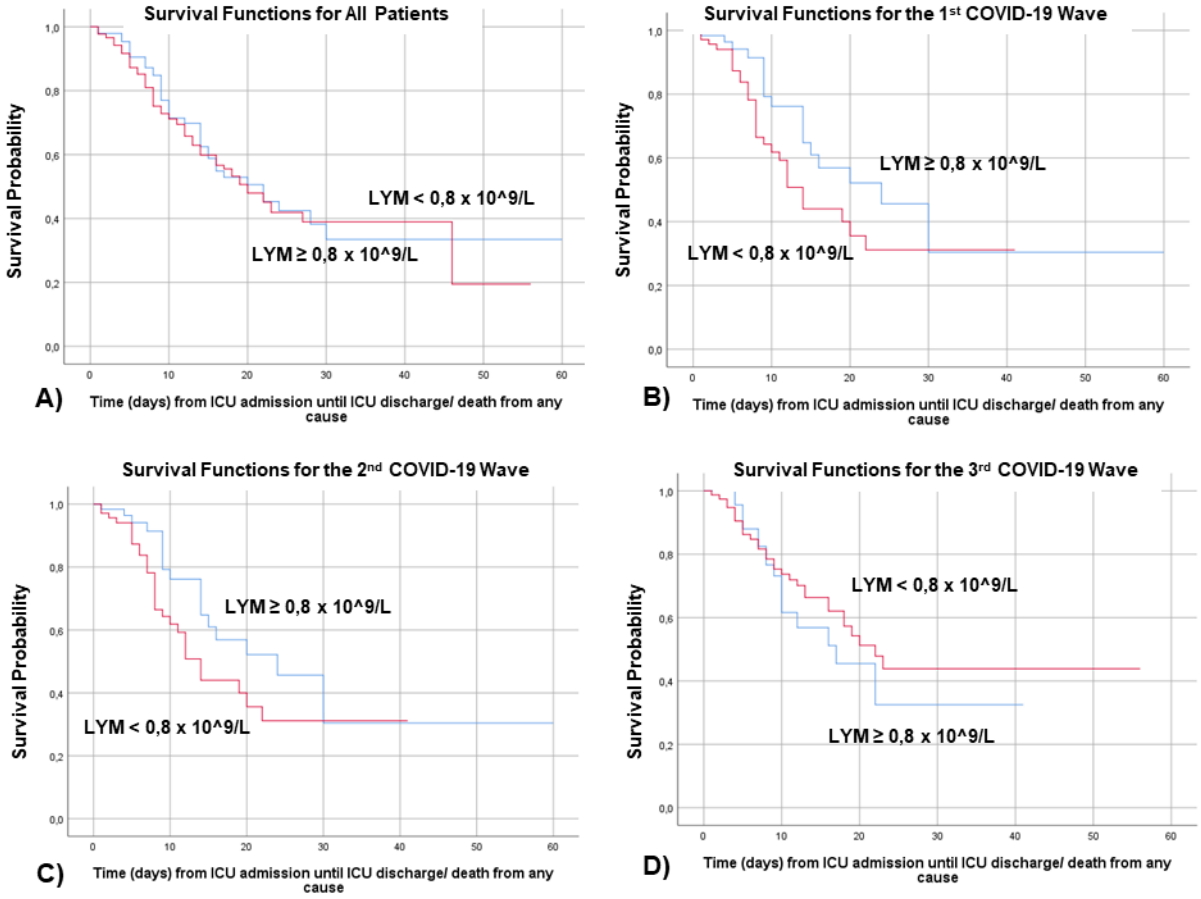


Figure 4.2.2.1. Survival curves from all patients with COVID-19 according to the LYM threshold of 0,8 X10^9/L in the 2nd day of ICU admission (**A**) and separate survival curves for each COVID-19 wave (**B, C** and **D**).

The survival rate of patients with LYM counts lower than 0,8x10^9/L was significantly lower than that of the patients with LYM counts equal or superior to 0,8x10^9/L, in the 2nd COVID-19 wave (**Figure 4.2.1.1C**). These results are further supplemented by the tests reflected on **Table 4.2.1.3** (*P*=0,033).

Table 4.2.1.3. *Comparisons between survival functions for all patients with and without decreased LYM counts, and separate analysis for all COVID-19 waves in the 2nd day of ICU admission*

	All patients	**1st Covid-19 Wave**	**2nd Covid-19 Wave**	**3rd Covid-19 Wave**
	***P* Value**	***P* Value**	***P* Value**	***P* Value**
Log Rank (Mantel-Cox)	0,583	0,898	0,060	0,470
Breslow (Generalized Wilcoxon)	0,295	0,813	**0,031**	0,781
Tarone-Ware	0,401	0,855	**0,033**	0,602

Median EO counts were below normality ranges for discharged and deceased patients, in all COVID-19 waves. Significant differences between both groups regarding median EO counts were verified in the 2nd and 3rd waves (*P*=0,022 and 0,011 respectively). The frequency of patients with decreased results was higher in the group of deceased patients for both waves (93,6% and 93,5%). Other reports described similar results. For example, Cortés-Vieyra *et al.* (204) determined that

eosinopenia was more frequent in deceased patients rather than in recovered ones, and that the survival rate of patients without eosinopenia was greater.

PLR was significantly different between discharged and deceased patients in the 2nd COVID-19 wave (*P*=0,008), and the same was observed for NLR in the 2nd and 3rd waves (*P*=0,003 and 0,032 respectively). Both ratios were highly elevated in all the waves, for both groups. Higher PLR and NLR values in deceased patients on admission have been reported by other authors (200,201). In one case, ROC curves were obtained to analyze the diagnostic values of PLR and NLR. The AUCs obtained for each ratio were 0,535 (95% CI; 0,46-0,60) and 0,703 (95% CI; 0,64-0,76), respectively (201).

In all COVID-19 waves PCT and CRP values were highly increased, but only in the 3rd wave were there significant differences between the two groups of patients (*P*=0,002 and 0,024 respectively). Higher PCT levels in deceased COVID-19 patients have been reported, especially as the disease worsened (205,206). Other authors also showed that CRP values were much higher in patients who died, in comparisons with those who recovered (186).

To finalize this set biomarkers' analysis in the 2nd day of ICU admission, univariate logistic regression for each COVID-19 wave was applied to determine which biomarkers were related to the patients' outcome (discharge from the ICU or death) (**Table 4.2.1.4**). The univariate logistic regression analysis demonstrated that decreased RBC counts, PLRs and NLRs were associated with an increased risk of death in the 2nd wave. Thus, in this wave, the mortality risk was increased 2,4-fold for patients with decreased RBC counts. Also, for every unit increase in the PLR, the risk of mortality increased 0,2% and for every unit increase in the NLR the same risk increased by 5,7%. In the 3rd wave, for every unit increase in the levels of CRP, the risk of mortality increased by 0,4%. These results are in conformity with the former mentioned outcomes from other authors and with the differences found between groups in the 2nd and 3rd COVID-19 waves.

Table 4.2.1.4. *Statistically significant results from univariate logistic regression for Inflammatory and other hematological biomarkers in the 2nd day of ICU admission*

		Univariate logistic regression for patients in the 2nd day of ICU admission		
		Crude OR	**95% CI**	**P Value**
2nd COVID-19 Wave	**Decreased RBCs**	2,390	1,018-5,611	0,045
	Max. PLR	1,002	1,001-1,004	0,007
	Max. NLR	1,057	1,018-1,097	0,004
3rd COVID-19 Wave	**Max. CRP (mg/L)**	1,004	1,000-1,008	0,029

After assessing the differences between groups of patients in each COVID-19 wave, it was also investigated if these groups' laboratory results showed differences between waves. Separate comparisons between COVID-19 waves for discharged and deceased patients (**Table 4.2.2.5**) led to the conclusion that, in the 2nd day of ICU admission, the median levels of PCT were significantly different between waves for discharged patients (*P*=0,035) and that the median levels of EO were significantly different between waves for deceased patients (*P*=0,026). Median CRP levels were significantly different between waves for both discharged and deceased patients (*P*=0,013 and 0,009 respectively).

Table 4.2.2.5. *Inflammatory and other hematological biomarkers' distributions with significant differences across COVID-19 waves, for discharged and deceased patients in the 2nd day of ICU admission*

Discharged patients	**PCT median levels comparison between:**	***P* Value**
	All COVID-19 Waves (n=157**)**	**0,035†**
	2nd and 1st COVID-19 Waves	**0,029†**
	3rd and 1st COVID-19 Waves	0,228†
	2nd and 3rd COVID-19 Waves	0,879†
	CRP median levels comparison between:	***P* Value**
	All COVID-19 Waves (n=208**)**	**0,013†**
	2nd and 1st COVID-19 Waves	**0,010†**
	3rd and 1st COVID-19 Waves	0,094†
	2nd and 3rd COVID-19 Waves	0,986†
Deceased patients	**CRP median levels comparison between:**	***P* Value**
	All COVID-19 Waves (n=113**)**	**0,009†**
	2nd and 1st COVID-19 Waves	**0,012†**
	3rd and 1st COVID-19 Waves	0,549†
	2nd and 3rd COVID-19 Waves	0,108†
	EO median levels comparison between:	***P* Value**
	All COVID-19 Waves (n=113**)**	**0,026†**
	2nd and 1st COVID-19 Waves	**0,025†**
	3rd and 1st COVID-19 Waves	0,063†
	2nd and 3rd COVID-19 Waves	1,000†

CRP median levels were much higher in the 1st wave, for both groups, leading to significant differences between the 1st and 2nd COVID-19 waves for both the discharged (P=0,010) and deceased (P=0,012) patients (**Figure 4.2.2.2A, B**). PCT median results were also much higher in the 1st wave, for the discharged patients, resulting in significant differences between the 1st and 2nd COVID-19 waves (P=0,029) (**Figure 4.2.2.3C**). The same happened for deceased patients regarding EOs median results, thus significant differences between the 1st and 2nd COVID-19 waves were observed (P=0,025) (**Figure 4.2.2.2D**). Buttenshon *et al*.(207) obtained similar results. When compared to patients from the 1st COVID-19 wave, patients from the 2nd wave appeared to have a less severe disease course. Additionally, a smaller number of patients required IMV and the time from symptom onset until hospital admission was shorter. In the present study, the same was verified relatively to the need for IMV but the time from symptom onset until hospital admission was the same for all COVID-19 waves. The significant differences detected between COVID-19 waves may result from different factors. Examples are the different restrictions that were in effect during the three waves; the fact that the initial treatment of COVID-19 was merely symptomatic; that the disease management evolved during the pandemic; and that different variants have been associated with the mortality rate of the disease but influence its course very differently (207).

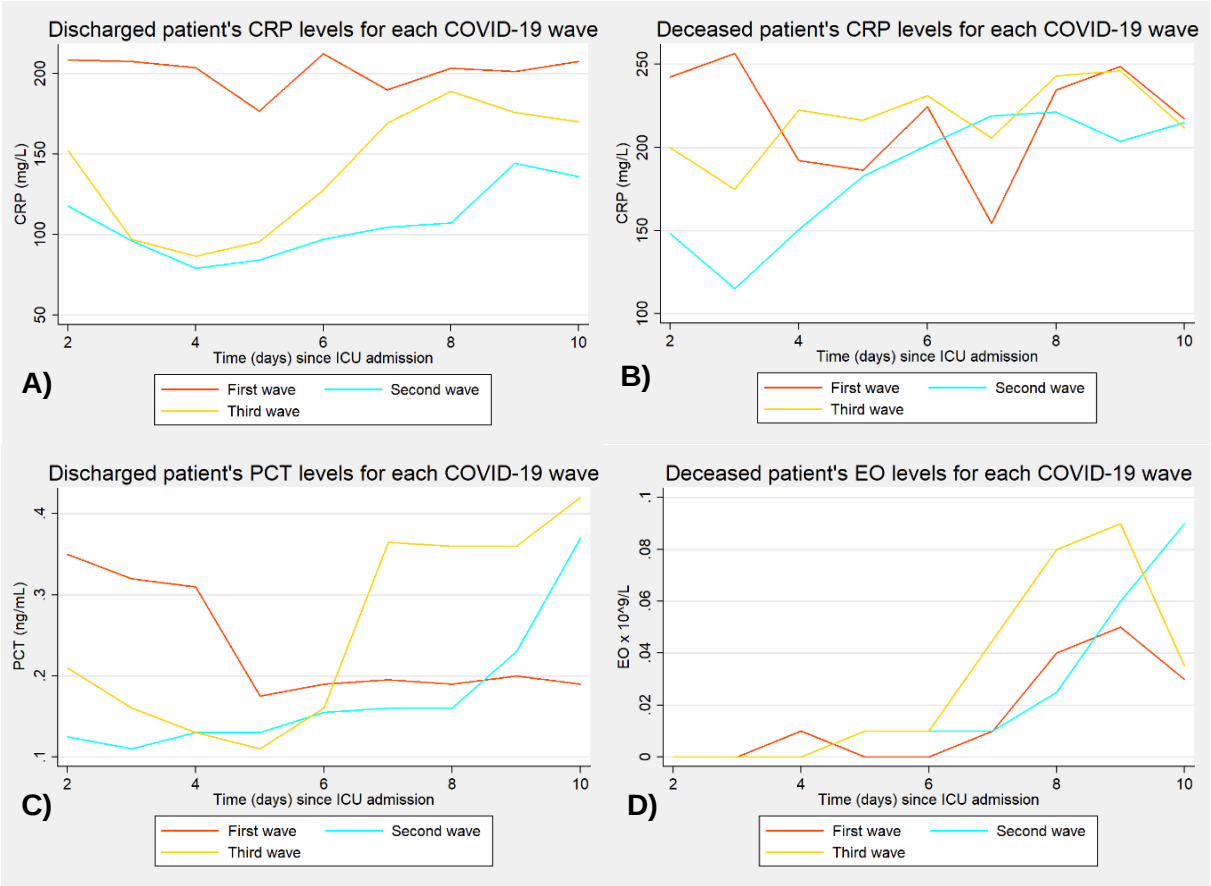


Figure 4.2.2.2. Median time course, in each COVID-19 wave, for discharged (A) and deceased (B) patients' CRP levels, discharged patients' PCT levels (C) and deceased patients' EO levels (D), between the 2nd and 10th days of ICU admission.

To evaluate the patients' evolution after one week in the ICU, the same analysis made for the 2nd day was applied to the 7th day of ICU admission (**Table 4.2.2.6**). Thus 216 patients were included. In comparison to the 2nd day of ICU admission, PCT and CRP median results increased, but the percentage of patients with elevated values was smaller in both cases (89,9% vs. 66,2% and 100% vs. 99,1%). This could indicate that patients whose results did not return to normal had a worsening of their condition. RBC's, HCT and HGB medians decreased, with a rise in the percentages of patients with values under normality ranges (66,3% vs. 86%; 77,5% vs. 88,8%; and 69,4% vs. 88,3% respectively). WBC's and NEU's median results increased, elevating the percentage of patients with values above normality ranges (50,3% vs. 64,5% and 66,3% and 77,6%). LYM and EO medians also increased, diminishing the number of patients with results below normality ranges (55,6% vs. 48,1% and 85,9% vs. 36,4%). In general, the patients' condition between the two analyzed days worsened despite the implemented therapies in the ICU, which weren't considered in the present study.

Table 4.2.2.6. *Inflammatory and other hematological biomarkers in the 7th day of ICU admission*

Biomarkers	**Missing Values**	**All Patients** (n=216)
Max. PCT (ng/mL)	59 (27,3%)	0,32 (0,12-1,04)
Increased PCT		143 (66,2%)
Max. CRP (mg/L)	2 (0,9%)	180,65 (72,10-260,03)
Increased CRP		212 (99,1%)
Min. RBC x 10^{12}/L	2 (0,9%)	3,70 (3,20-4,08)
Decreased RBC		184 (86,0%)
Min. HCT (%)	2 (0,9%)	33,20 (28,78-36,75)
Decreased HCT		190 (88,8%)
Min. HGB x 10g/L	2 (0,9%)	10,70 (9,30-11,90)
Decreased HGB		189 (88,3%)
Max. WBC x 10^9/L	2 (0,9%)	11,82 (9,18-15,34)
Increased WBC		138 (64,5%)
Min. LYM x 10^9/L	2 (0,9%)	0,81 (0,57-1,22)
Decreased LYM		103 (48,1%)
Max. Neu x 10^9/L	2 (0,9%)	9,65 (7,21-13,46)
Increased NEU		166 (77,6%)
Min. EO x 10^9/L	2 (0,9%)	0,05 (0,01-0,13)
Decreased EO		78 (36,4%)
Max. PLR	2 (0,9%)	368,14 (218,58-518,34)
Increased PLR		173 (80,8%)
Max. NLR	2 (0,9%)	11,40 (7,03-18,95)
Increased NLR		208 (97,2%)

Comparisons between discharged and deceased patients for the 7th day of ICU admission (**Table 4.2.2.7**) once again didn't show significant differences between groups for the 1st COVID-19 wave. In the 2nd wave, all biomarker's medians were significantly different between groups, except for PCT, LYM's and PLR. In all cases, the group of deceased patients had worse results comparing to the discharged patients. Finally, in the 3rd wave, significant differences between groups were verified for PCT, CRP, WBCs, LYMs and NLR. Once again, the group of deceased patients remained the one with worse findings. This rise in the number of biomarkers with significant differences between groups on the 7th day of ICU admission is probably since the deceased patients' condition worsened more rapidly over time than that of discharged patients. In the 1st wave, patients' condition also deteriorated from the 2nd to the 7th days of ICU admission, but with no significant differences between groups.

All the analyzed biomarkers had an unfavorable evolution in the studied period of time, except for EO and LYM counts, which adapted increasing tendencies. These observations will be analyzed in detail further along.

Kaplan-Meier survival curves stratified according to the reference normality cut-off points were obtained for the present set of biomarkers, in the 7th day of ICU admission, for every COVID-19 wave. Survival functions were only significantly different considering the LYM threshold of 0,8 X10^9/L (**Figure 4.2.2.3**).

Table 4.2.2.7. *Inflammatory and other hematological biomarkers of all patients and comparisons between the ones discharged from the ICU and those who died, for all COVID-19 waves in the 7th day of ICU admission*

	First COVID-19 Wave				Second COVID-19 Wave				Third COVID-19 Wave			
Biomarkers	**All patients** (n=39)	**Discharged** (n=27)	**Deceased** (n=12)	**P Value**	**All patients** (n=87)	**Discharged** (n=52)	**Deceased** (n=35)	**P Value**	**All patients** (n=90)	**Discharged** (n=58)	**Deceased** (n=32)	**P Value**
Max. PCT (ng/mL)	0,31 (0,15-1,04)	0,20 (0,14-1,10)	0,36 (0,24-1,92)	0,313*	0,18 (0,09-0,70)	0,16 (0,05-0,54)	0,25 (0,10-0,79)	0,126*	0,44 (0,17-1,59)	0,37 (0,12-0,76)	1,16 (0,26-5,88)	**0,008***
Increased PCT	31 (100%)	22 (100%)	9 (100%)	-	45 (78,9%)	21 (67,7%)	24 (92,3%)	**0,023•**	67 (97,1%)	40 (95,2%)	27 (100%)	0,517▪
Max. CRP (mg/L)	189,30 (81,50-259,70)	189,80 (83,60-258,10)	154,35 (52,30-260,68)	0,499*	156,35 (53,05-245,15)	104,55 (35,90-226,00)	219,00 (119,58-267,88)	**0,003***	183,10 (89,80-273,55)	169,20 (74,45-260,70)	205,55 (154,45-283,53)	**0,019***
Increased CRP	39 (100%)	27 (100%)	12 (100%)	-	85 (98,8%)	51 (98,1%)	34 (100%)	1,000▪	88 (98,9%)	56 (98,2%)	32 (100%)	1,000▪
Min. RBC x 10^{12}/L	3,67 (3,17-4,01)	3,74 (3,41-3,88)	3,38 (3,07-4,01)	0,685*	3,71 (3,22-4,17)	3,86 (3,37-4,40)	3,30 (2,92-3,88)	**0,013***	3,70 (3,24-4,08)	3,72 (3,36-4,06)	3,69 (3,00-4,26)	0,611*
Decreased RBC	36 (92,3%)	25 (92,6%)	11 (91,7%)	1,000▪	71 (82,6%)	39 (75%)	32 (94,1%)	**0,022•**	77 (85,6%)	51 (89,5%)	26 (81,3%)	0,337▪
Min. HCT	32,30 (28,40-36,60)	32,90 (30,70-36,50)	30,30 (26,98-37,50)	0,518*	33,10 (28,65-37,53)	34,30 (29,15-39,10)	30,95 (27,38-34,23)	**0,015***	33,80 (29,05-36,45)	33,80 (29,90-35,85)	32,50 (27,08-38,95)	0,794*
Decreased HCT	37 (94,9%)	26 (96,3%)	11 (91,7%)	0,526▪	74 (86%)	41 (78,8%)	33 (97,1%)	**0,024▪**	79 (88,8%)	52 (91,2%)	27 (84,4%)	0,485
Min. HGB	10,40 (9,50-11,50)	10,60 (9,80-11,50)	9,90 (8,73-12,10)	0,558*	10,70 (9,28-12,03)	11,30 (9,50-12,88)	9,95 (8,85-11,20)	**0,008***	10,80 (9,30-11,80)	10,80 (9,60-11,65)	10,20 (8,65-12,48)	0,596*
Decreased HGB	36 (92,3%)	25 (92,6%)	11 (91,7%)	1,000▪	75 (87,2%)	42 (80,8%)	33 (97,1%)	**0,044▪**	78 (87,6%)	51 (89,5%)	27 (84,4%)	0,515▪
Max. WBC	12,83 (9,53-14,73)	12,83 (8,55-14,20)	13,47 (10,70-19,72)	0,159*	11,85 (9,27-16,38)	10,83 (7,94-14,33)	14,19 (10,59-18,38)	**0,014***	11,05 (9,15-14,86)	10,29 (8,87-13,17)	13,45 (9,53-18,83)	**0,043***
Increased WBC	27 (69,2%)	17 (63%)	10 (83,3%)	0,276▪	58 (67,4%)	32 (61,5%)	26 (76,5)	0,149•	53 (59,6%)	31 (54,4%)	22 (68,8%)	**0,185•**
Min. LYM x 10^9/L	0,82 (0,63-1,34)	0,86 (0,73-1,35)	0,68 (0,53-1,26)	0,298*	0,86 (0,56-1,10)	0,94 (0,57-1,33)	0,78 (0,44-0,99)	0,090*	0,76 (0,55-1,27)	0,90 (0,60-1,37)	0,61 (0,42-0,80)	**0,002***
Decreased LYM	18 (46,2%)	10 (37%)	8 (66,7%)	0,087•	38 (44,2%)	20 (38,5%)	18 (52,9%)	0,186•	47 (52,8%)	23 (40,4%)	24 (75%)	**0,002•**
Max. NEU x 10^9/L	11,10 (7,70-13,03)	9,52 (6,70-12,55)	12,25 (9,30-16,61)	0,104*	9,86 (7,17-13,63)	9,28 (6,22-12,31)	12,20 (8,96-16,10)	**0,003***	9,32 (7,25-13,27)	8,71 (7,07-11,00)	12,21 (8,33-17,20)	**0,010***
Increased NEU	30 (76,9%)	19 (70,4%)	11 (91,7%)	0,228▪	66 (76,7%)	35 (67,3%)	31 (91,2%)	**0,010•**	70 (78,7%)	44 (77,2%)	26 (81,3%)	0,654•
Min. EO x 10^9/L	0,07 (0,00-0,14)	0,09 (0,01-0,15)	0,01 (0,00-0,11)	0,118*	0,04 (0,00-0,10)	0,06 (0,01-0,13)	0,01 (0,00-0,05)	**0,004***	0,07 (0,02-0,16)	0,08 (0,02-0,19)	0,05 (0,01-0,13)	0,229*
Decreased EO	14 (35,9%)	7 (25,9%)	7 (58,3%)	0,075▪	36 (41,9%)	16 (30,8%)	20 (58,8%)	**0,010•**	28 (31,5%)	15 (26,3%)	13 (40,6%)	0,163•
Max. PLR	353,19 (190,85-500,00)	352,99 (190,85-500,00)	374,27 (200,19-508,83)	0,869*	361,94 (218,36-522,26)	346,71 (201,15-527,78)	386,26 (248,86-526,32)	0,318*	393,83 (218,45-559,93)	341,67 (200,03-512,87)	424,03 (304,20-590,37)	0,207*
Increased PLR	29 (74,4%)	20 (74,1%)	9 (75%)	1,000▪	70 (81,4%)	40 (76,9%)	30 (88,2%)	0,187•	74 (83,1%)	46 (80,7%)	28 (87,5%)	0,411•
Max. NLR	12,96 (7,70-18,49)	8,82 (5,83-15,98)	17,80 (12,77-18,75)	0,056*	11,28 (6,34-20,33)	9,26 (4,89-18,42)	14,05 (8,36-32,17)	**0,002***	11,18 (7,49-18,68)	9,61 (6,10-15,66)	15,35 (10,08-32,38)	**<0,001***
Increased NLR	37 (94,9%)	26 (96,3%)	11 (91,7%)	0,526▪	83 (96,5%)	49 (94,2%)	34 (100%)	0,274▪	88 (98,9%)	56 (98,2%)	32 (100%)	1,000▪

All calculated percentages do not include missing values.

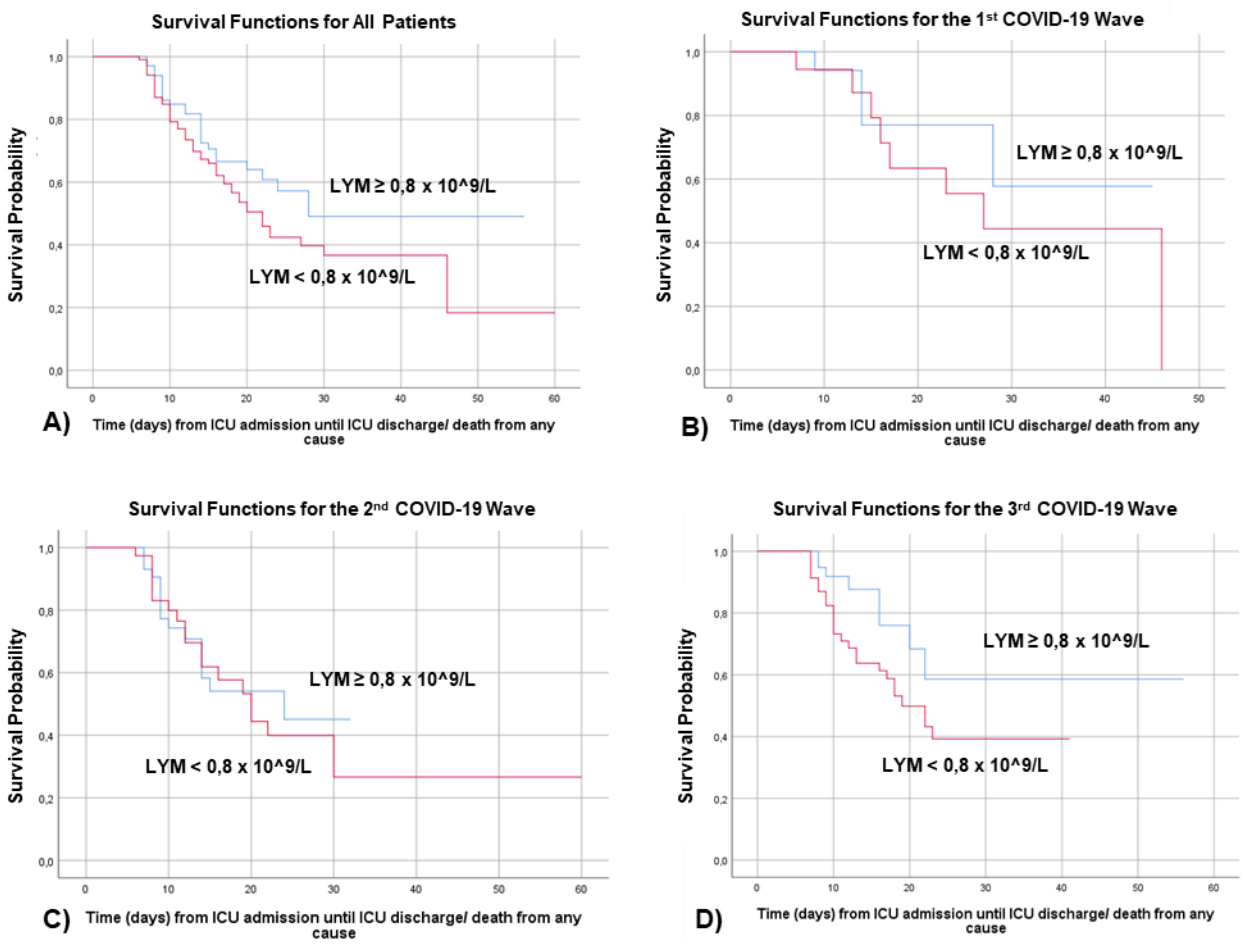


Figure 4.2.2.3. Survival curves from all patients with COVID-19 according to the LYM threshold of 0,8 X10^9/L in the 7th day of ICU interment (**A**) and separate survival curves for each COVID-19 wave (**B, C** and **D**).

The survival rate of patients with LYM counts inferior to 0,8x10^9/L was significantly lower than that of the patients with LYM counts equal or superior to 0,8x10^9/L, in the 3rd COVID-19 wave (**Figure 4.2.1.3D**). These results are further supplemented by the tests reflected on **Table 4.2.1.8** (*P*=0,048). Similarly to Zhang *et al*. (208), median LYMs counts remained low for the group of deceased patients in several time points. This is a very important finding, since decreases in LYMs counts are related to immune injury and poor prognosis. On the other hand, the groups of discharged patients had slightly decreased/ normal median LYMs counts that increased from the 2nd to the 7th days of ICU admission.

Table 4.2.2.8. *Comparisons between survival functions for all patients with and without decreased LYM counts, and separate analysis for all COVID-19 waves in the 7th day of ICU admission*

	All patients	**1st Covid-19 Wave**	**2nd Covid-19 Wave**	**3rd Covid-19 Wave**
	***P* Value**	***P* Value**	***P* Value**	***P* Value**
Log Rank (Mantel-Cox)	0,081	0,415	0,772	**0,048**
Breslow (Generalized Wilcoxon)	0,113	0,473	0,958	**0,029**
Tarone-Ware	0,096	0,431	0,895	**0,033**

To complete the biomarker' analysis on the 7th day of ICU admission, univariate logistic regression was used to establish which biomarkers were related to the disease's outcome for all patients, in each COVID-19 wave (**Table 4.2.1.9**). The results were statistically significant for several variables in the 2nd and 3rd COVID-19 waves. This was probably due to the worsening of the patients' condition since the 2nd day of ICU admission and the lack of differences between groups in the 1st wave, for all biomarkers. Both in the 2nd and 3rd waves, for every unit increase in CRP levels (mg/L), the risk of mortality increased 0,5% (*P*=0,014 and 0,022 respectively). While in the 2nd wave, for every unit increase in WBC counts, the risk of mortality increased 10,5%, in the 3rd one it increased 11,8%. Regarding NEU counts, for every unit increase, the risk of mortality increased by 13,1% in the 2nd wave and 16,6% in the 3rd. Increased NEU counts also led to a 5-fold increased risk of mortality in the 2nd wave. Still regarding the same wave, the risk of mortality is increased by 5,3-fold in patients with decreased RBC counts and 8,9-fold in patients with decreased HCT results. Decreased EO counts also led to a 3,2-fold higher risk of death. Once again, in both the 2nd and 3rd waves, for every unit increase in NLR, the risk of death increased by 7,6%. Finally, in the 3rd wave, according to former obtained results, decreased LYM counts resulted in a 4,4-fold higher risk of mortality. These results led to the conclusion that patients with worse results in the 7th day of ICU admission were in higher risk of death that those in the 2nd day of ICU admission, depending on the evaluated biomarkers.

Table 4.2.2.9. *Statistically significant results from univariate logistic regression for Inflammatory and other hematological biomarkers in the 7th day of ICU admission*

		Univariate logistic regression for patients in the 7th day of ICU admission		
		Crude OR	**95% CI**	**P Value**
2nd COVID-19 Wave	**Max. CRP (mg/L)**	1,005	1,001-1,010	0,014
	Decreased RBC	5,333	1,120-25,390	0,035
	Decreased HCT	8,854	1,087-72,142	0,042
	Min. HGB	0,734	0,576-0,935	0,012
	Max. WBC	1,105	1,018-1,199	0,017
	Max. NEU x 10^9/L	1,131	1,034-1,236	0,007
	Increased NEU	5,019	1,342-18,772	0,017
	Decreased EO	3,214	1,304-7,920	0,011
	Max. NLR	1,076	1,027-1,128	0,002
3rd COVID-19 Wave	**Max. PCT (ng/mL)**	1,326	1,026-1,714	0,031
	Max. CRP (mg/L)	1,005	1,001-1,009	0,022
	Max. WBC	1,118	1,020-1,225	0,018
	Decreased LYM	4,435	1,699-11,574	0,002
	Max. NEU x 10^9/L	1,166	1,051-1,294	0,004
	Max. NLR	1,076	1,028-1,127	0,002

Moving on to the separate comparisons between COVID-19 waves for discharged and deceased patients, this time for the 7th day after ICU admission (**Table 4.2.2.10**), significant differences for CRP levels in the group of discharged patients (*P*=0,026) and for PCT levels in the group of deceased patients (*P*=0,036) were obtained. PCT median levels were much higher in the 1st wave, leading to significant differences between the 1st and 2nd COVID-19 waves the group of deceased patients (P=0,030) (**Figure 4.2.2.4**). Besides not showing significant differences, for the group of discharged patients, differences between COVID-19 waves for CRP levels were most likely since these levels were also higher in the 1st wave (**Figure 4.2.2.4A**), in comparison with the 2nd wave.

Table 4.2.2.10. *Inflammatory and other hematological biomarkers' distributions with significant differences across COVID-19 waves, for discharged and deceased patients in the 7th day of ICU admission*

Discharged patients	**CRP levels comparison between:**	***P* Value**
	All COVID-19 Waves (n=136)	**0,026†**
	2nd and 1st COVID-19 Waves	0,057†
	3rd and 1st COVID-19 Waves	1,000†
	2nd and 3rd COVID-19 Waves	0,080†
Deceased patients	**PCT levels comparison between:**	***P* Value**
	All COVID-19 Waves (n=62)	**0,036†**
	2nd and 1st COVID-19 Waves	1,000†
	2nd and 3rd COVID-19 Waves	**0,030†**
	1st and 3rd COVID-19 Waves	0,987†

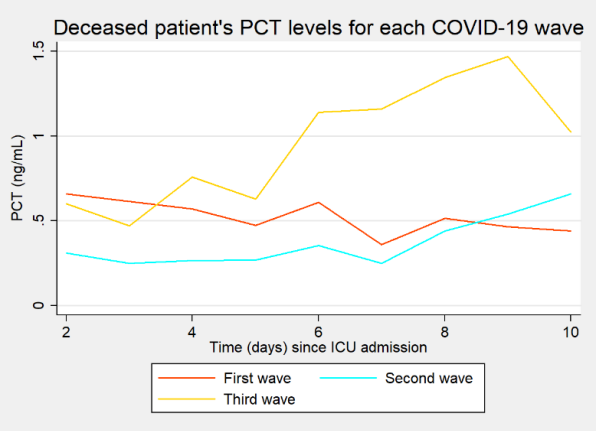


Figure 4.2.2.4. PCT (ng/mL) median time course by COVID-19 wave, for deceased patients, between the 2nd and 10th days of ICU admission.

To assess the patients' progress throughout their ICU stay, comparisons were made between the 2nd and 7th days of ICU admission (**Table 4.2.1.11**). Variables with no significant differences between the two days weren't displayed.

Table 4.2.2.11. *Comparisons of Inflammatory and other hematological biomarkers of all patients, the ones discharged from the ICU and those who died between the 2nd and 7th days of ICU admission for each COVID-19 wave*

		RBC x 10^{12}/L	HCT (%)	HGB x 10g/L	WBC x 10^9/L	LYM x 10^9/L	NEU x 10^9/L	EO x 10^9/L	NLR
1st COVID-19 Wave	**Discharged patients** (n=26)	**<0,001**	**0,004**	**<0,001**	0,091	**0,005**	0,269	**0,001**	0,469
	Deceased patients (n=12)	**0,028**	**0,041**	**0,028**	0,136	0,367	0,117	0,123	0,308
	All patients (n=38)	**<0,001**	**<0,001**	**<0,001**	**0,032**	**0,005**	0,072	**<0,001**	0,255
2nd COVID-19 Wave	**Discharged patients** (n=52)	**<0,001**	**<0,001**	**<0,001**	0,672	**0,021**	0,816	**<0,001**	0,489
	Deceased patients (n=34)	**<0,001**	**<0,001**	**<0,001**	**<0,001**	0,874	**<0,001**	**0,001**	**0,023**
	All patients (n=86)	**<0,001**	**<0,001**	**<0,001**	**0,010**	0,061	**0,039**	**<0,001**	0,294
3rd COVID-19 Wave	**Discharged patients** (n=57)	**<0,001**	**<0,001**	**<0,001**	0,221	**0,001**	0,636	**<0,001**	**0,040**
	Deceased patients (n=32)	**<0,001**	**<0,001**	**<0,001**	**0,024**	0,594	**0,047**	**<0,001**	0,112
	All patients (n=89)	**<0,001**	**<0,001**	**<0,001**	**0,018**	**0,010**	0,088	**<0,001**	0,613

P-values represent the comparison of the median of a given biomarker on the 2nd day with that of the 7th day of ICU admission. All *P* values obtained through related-samples Wilcoxon matched-pair signed rank test.

RBC's, HCT and HMG median results were significantly different between the 2nd and 7th days of ICU admission. For both the discharged and deceased patients, median HGB levels dropped in the course of the 7 considered days, along with the decrease of HCT (**Figure 4.2.2.5A, B**) and RBC counts (**Figure 4.2.2.5C, D**). In this study the discussed changes were observed, in different degrees, for all tree COVID-19 waves (despite only being displayed, as an example, the results of the 1st COVID-19 wave). These changes were already verified by other authors, who correlated the decreases in HGB with pulmonary involvement and subsequent oxygen demand (209). HGB levels were always significantly lower in the group of deceased patients, results which were consistent with the ones reported by Kilercik *et al.* (197).

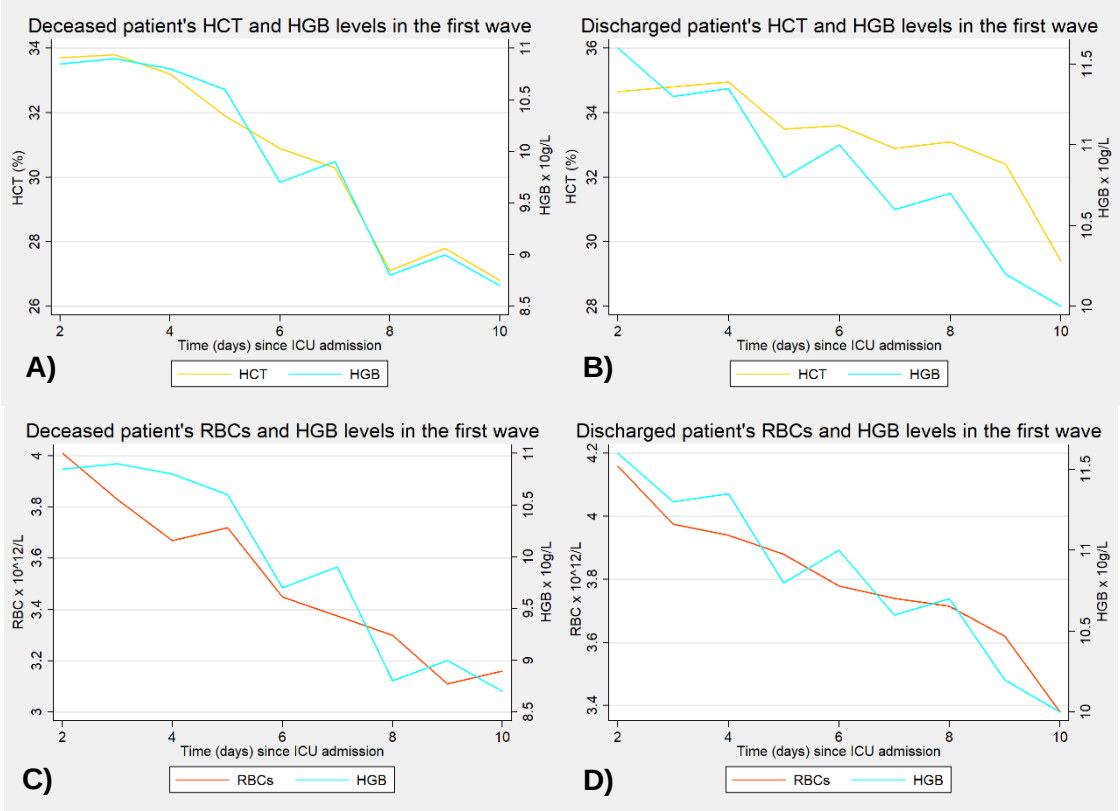


Figure 4.2.2.5. Median time course for deceased and discharged patients HGB levels drop between the 2nd and 10th days of ICU admission, along with HCT (**A** and **B**) and RBCs (**C** and **D**).

Regarding median WBC counts, significant differences were found between the 2nd and 7th days of admission for all patients, in every COVID-19 wave (*P*=0,032; 0,010 and 0,018 respectively) (**Figure 4.2.2.6A**). A significant increase in these biomarkers can be seen along the 7 days of ICU admission. Significant increases in WBCs median levels between the two days were also verified for the 2nd (*P*<0,001) and 3rd (*P*=0,024) COVID-19 waves, in the group of deceased patients (**Figure 4.2.2.6B, C**). In the group of discharged patients, a small increase in WBC median levels also occurred, but wasn't significative or as severe as for the deceased patients.

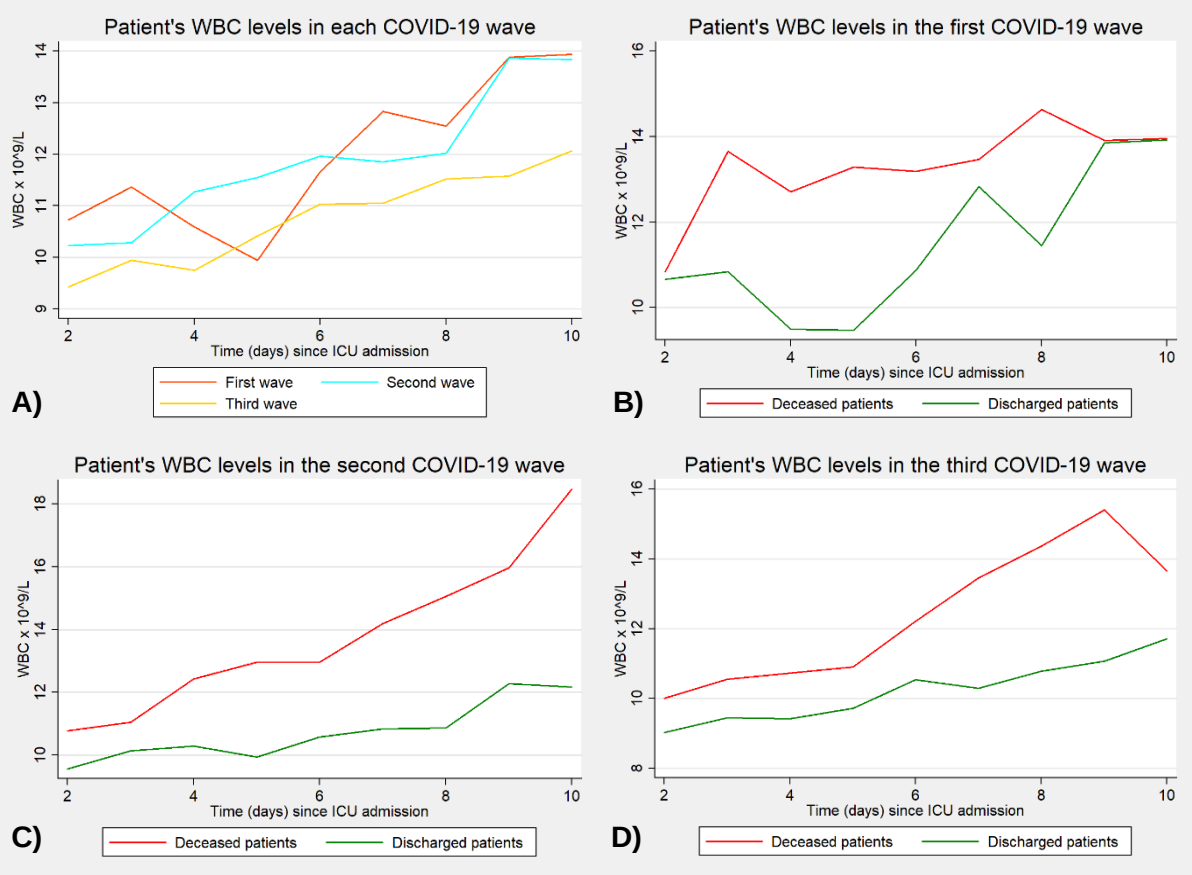


Figure 4.2.2.6. Median time course for all patients' WBC levels in each COVID-19 wave (**A**) and for the groups of discharged and deceased patients in the 1st (**B**), 2nd (**C**) and 3rd (**D**) COVID-19 waves, between the 2nd and 10th days of ICU admission.

Increases in NEUs median levels between the two considered days were also significant for the 2nd (*P*<0,001) and 3rd (*P*=0,047) COVID-19 waves, in the group of deceased patients (**Figure 4.2.2.7B, C**). This type of increases in the first days of ICU admission, which persist throughout the patients ICU stay, were already described by Chen *et al.* (210). In their report, these changes were correlated with a fatal outcome. In the group of discharged patients, a small increase in NEU median levels also occurred, but wasn't significative or as severe as for the deceased patients. In the 1st wave (**Figure 4.2.2.7A**), the group of discharged patients had a marked increase of NEU median levels, but it was most likely caused by outliers or even hospital acquired infections, as blood NEU levels typically increase in the presence of infectious agents. This could be a plausible explanation since in cases of COVID-19, patients' NEU counts are mostly increased in the first days and start to decrease after treatment (189).

By examining the graphic representation of median band lines both WBC and NEU counts, it was once again demonstrated that the current leukocytosis is supported by neutrophilia as the graphic representations are quite similar, even when there is no separation between COVID-19 waves or patient groups (**Figure 4.2.2.7D**).

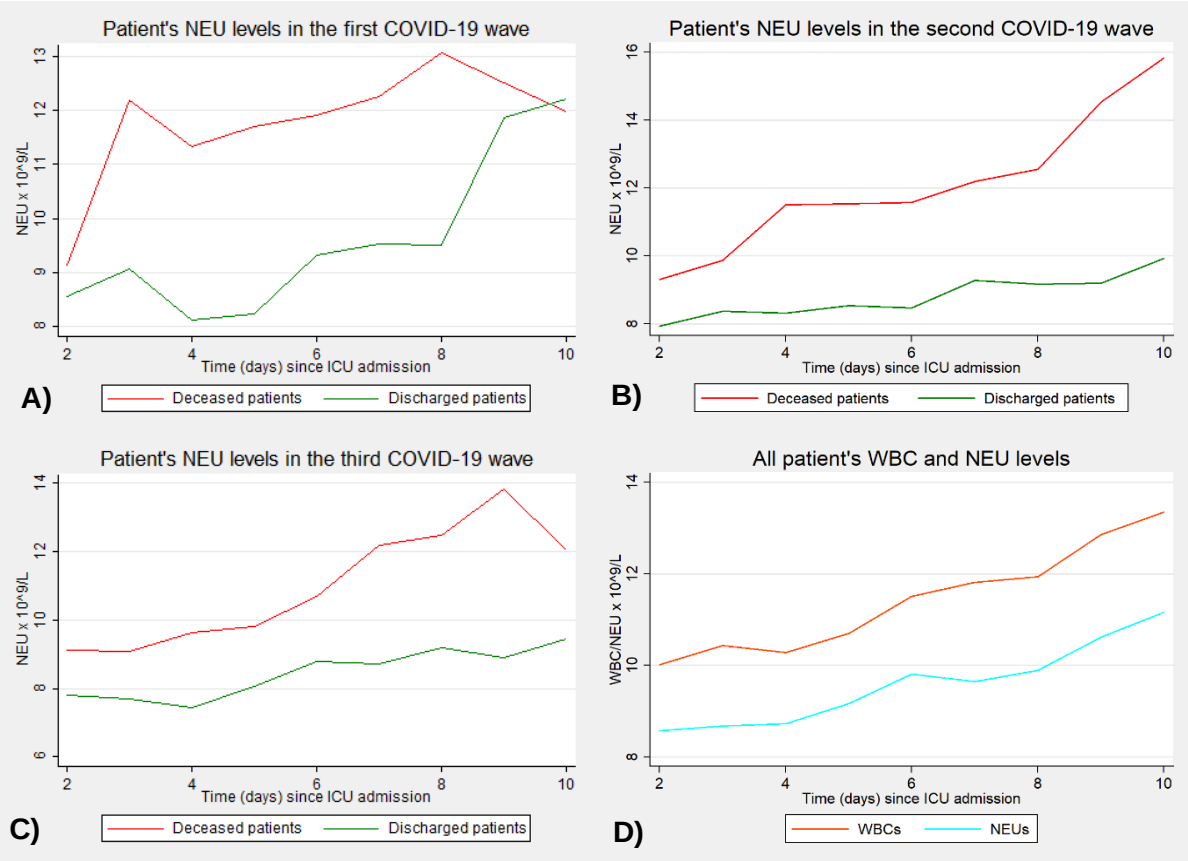
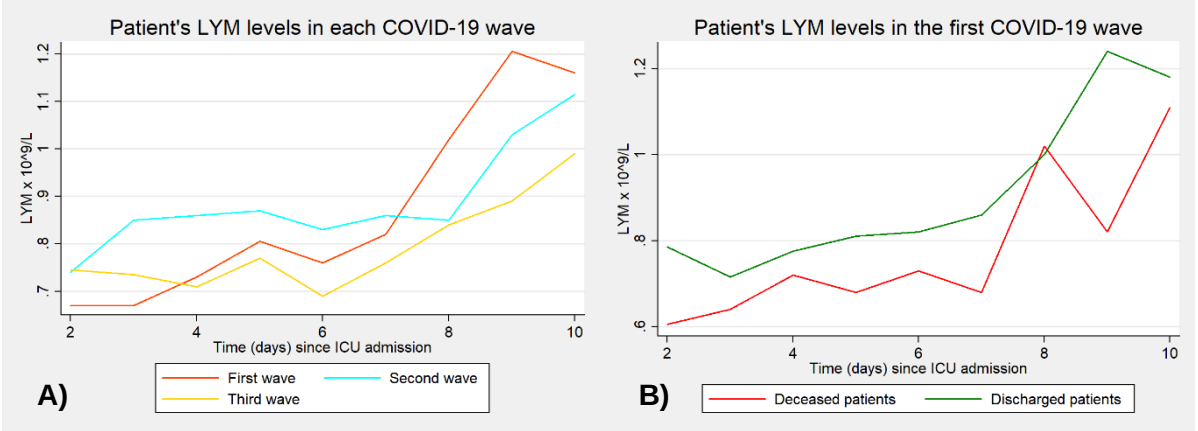


Figure 4.2.2.7. Discharged and deceased patients' NEU median time course between the 2nd and 10th days of ICU admission, for the 1st (**A**), 2nd (**B**) and 3rd (**C**) COVID-19 waves and comparison between WBC and NEU levels (**D**) from all patients.

Median LYM counts were significantly higher in the 7th day of ICU admission, in comparison with the 2nd day, for all patients in the 1st (*P*=0,005) and 3rd (*P*=0,010) COVID-19 waves (**Figure 4.2.2.8A**). In the 2nd wave, LYM levels were relatively stable and between normality ranges until the 8th day of ICU admission. Despite being in the lower range of normality in the first days of ICU admission, LYM levels of discharged patients showed an increasing tendency in each COVID-19 wave (*P*=0,005; 0,021 and 0,001 respectively) (**Figure 4.2.2.8B-D**).



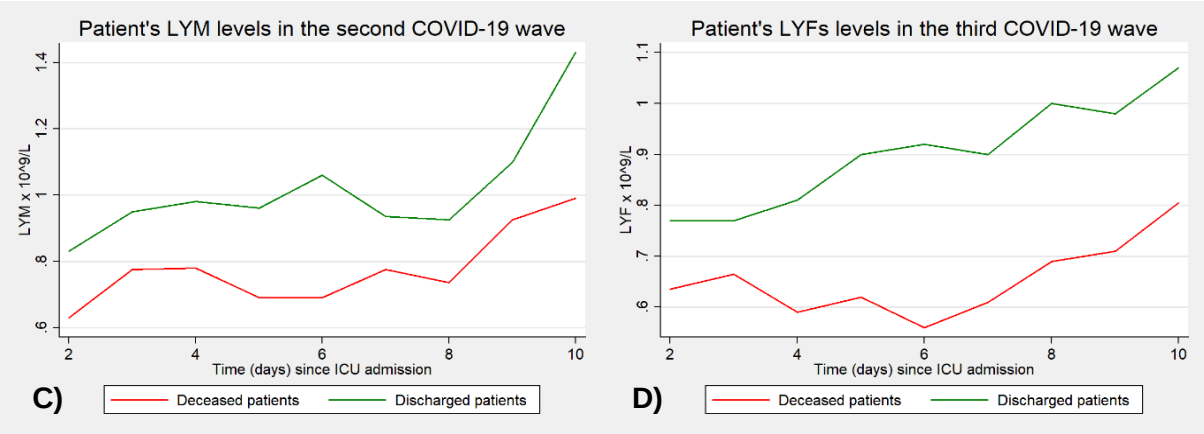


Figure 4.2.2.8. Median time course for all patients' LYM levels in each COVID-19 wave (**A**) and for the groups of discharged and deceased patients in the 1st (**B**), 2nd (**C**) and 3rd (**D**) COVID-19 waves, between the 2nd and 10th days of ICU admission.

Deceased patients, on the other hand, maintained lower levels of LYM in the first week of ICU internment, or those even declined, as seen in the 3rd wave. Findings like these were already reported in other large longitudinal studies (179,210). After day six of ICU internment, the LYM levels of deceased patients appeared to start increasing (finding not wet described in other reports). This growing trend contrasts with certain authors' studies, which show persistent declines in LYM levels during the course of the hospitalization (211).

EO median levels also shown significant differences between the 2nd and 7th days of admission for all patients, in every COVID-19 wave (*P*<0,001 for all cases) (**Figure 4.2.2.9A**). The group of deceased patients showed null median EO counts in the first four days of ICU admission for the 2nd and 3rd COVID-19 waves (**Figure 4.2.2.9C, D**) and in the first 2 days for the 1st wave (**Figure 4.2.2.9B**). In the other hand, this only happened in the first two days of ICU admission for the group of discharged patients, also in the 2nd and 3rd COVID-19 waves. These results are consistent with recent research, which demonstrated that in recovered patients, EO counts of zero occurred in a lower percentage (49,2%) than in deceased patients (78,3%) (204). Right after these low results, EO counts of the present study sample tended to increase (except for the group of deceased patients from the 1st COVID-19 wave). After the 4th/5th days of ICU admission, discharged patients started to have EO counts between normality ranges (0,03-0,6x10^9/L) in all waves, since for deceased patients that only happened between the 7th/8th days. Besides not being observed in the present study, eosinophilia may play a protective role in COVID-19 patients due to the better outcomes of patients with this clinical manifestation (204). According to Nair *et al*.(212) eosinophilia is associated with milder disease courses and better outcomes. Thus, patients with elevated EO counts required less time with IMV and oxygen supplementation, spent less time in the ICU, and had lower CRP levels. Because EO's have beneficial functions such as controlling the inflammation generated by NEUs (204), it is reasonable to deduce that the fact that EO counts of dying patients took longer to start increasing led to worse outcomes.

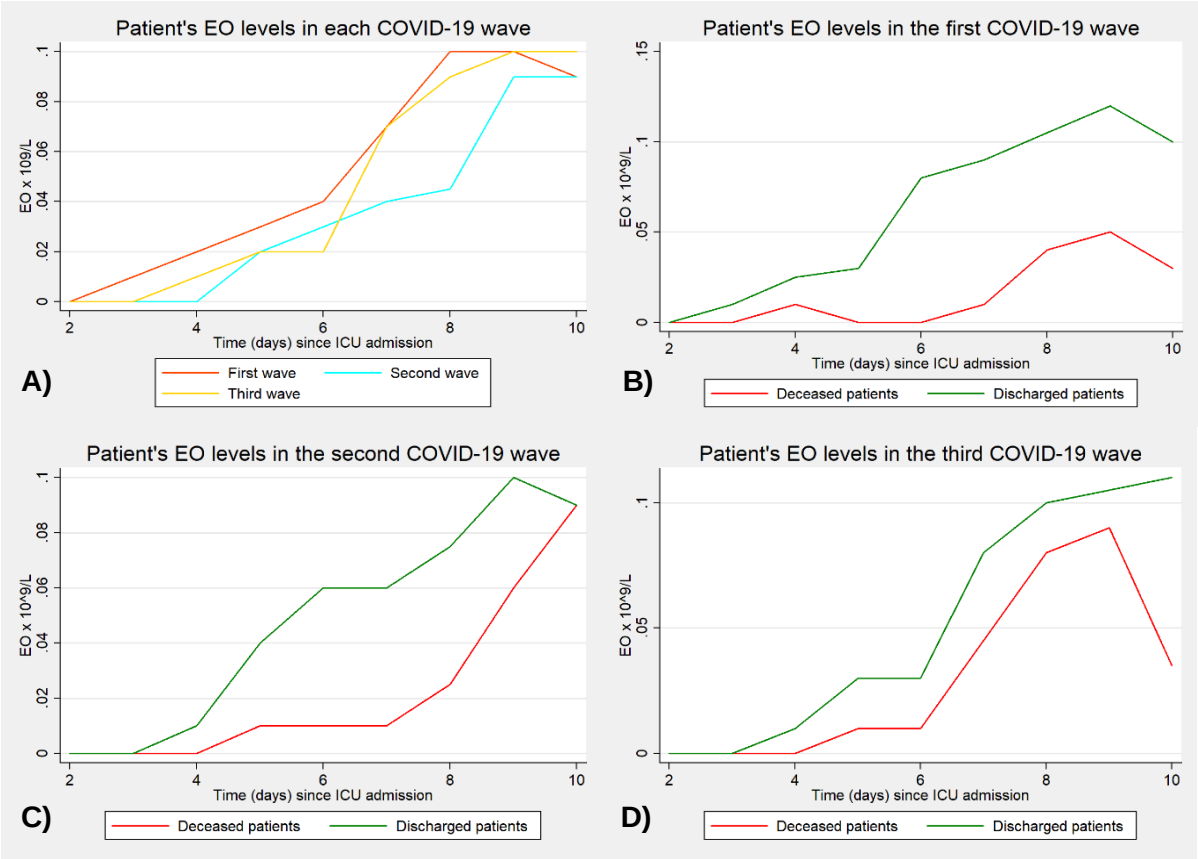
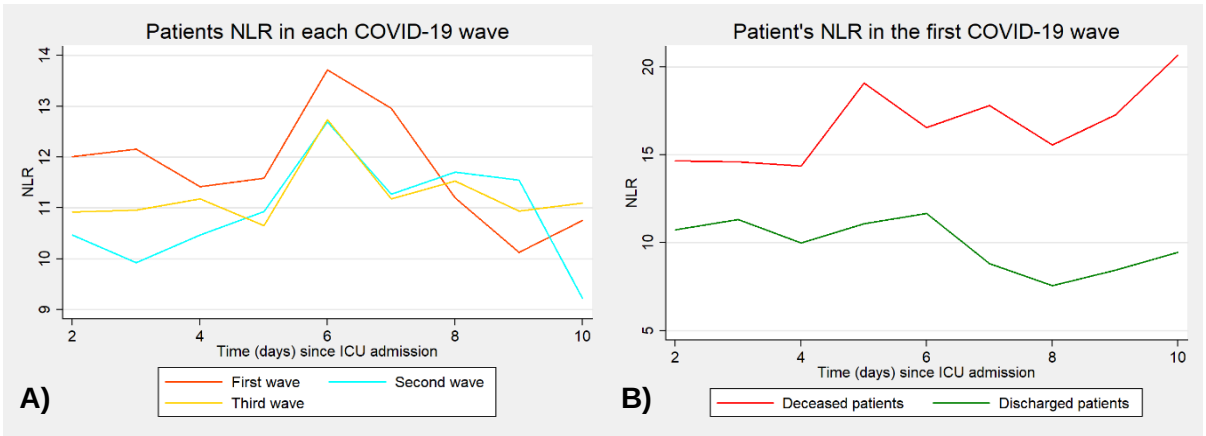


Figure 4.2.2.9. Median time course for all patients' EO levels in each COVID-19 wave (**A**) and for the groups of discharged and deceased patients in the 1st (**B**), 2nd (**C**) and 3rd (**D**) COVID-19 waves, between the 2nd and 10th days of ICU admission.

Significant differences between the NLR results of the 2nd and 7th days of ICU admission were only verified for the group of deceased patients in the 2nd COVID-19 wave (*P*=0,023) and for the group of discharged patients in the 3rd wave (*P*=0,040). Despite this observation, all patients' NLR had an increasing tendency, in all waves, until the 6th day of ICU admission (**Figure 4.2.2.10A**). Ye *et al.* (213) verified that the initial value of NLR and its peak were significantly higher in the group of deceased patients (*P*<0,001). In this study, NLR values and peak values were always greater in the group of deceased patients, in all COVID-19 waves (**Figure 4.2.2.10B-D**). The increasing values of NLR means either that NEU counts are increasing or that the LYM counts are decreasing (190). In this case, NLR increased probably due to the increases in NEU counts.



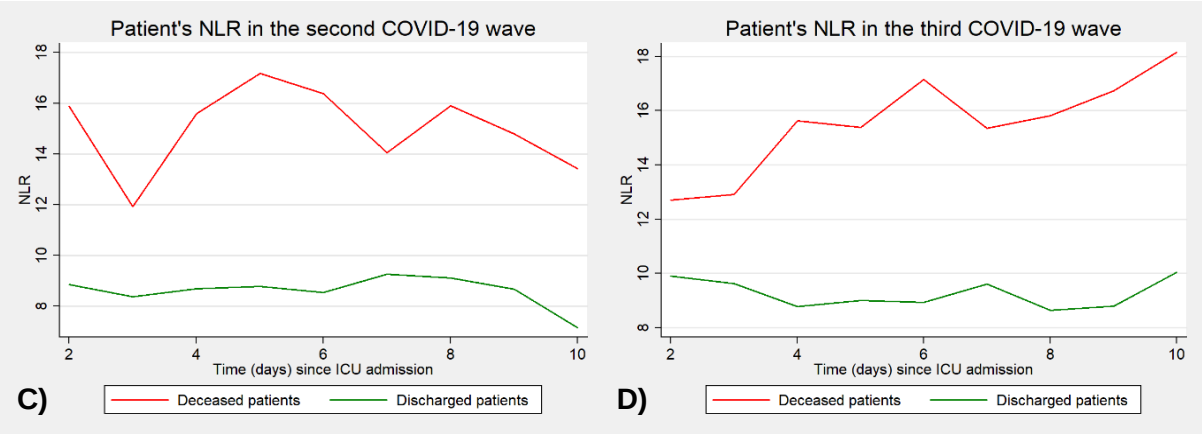


Figure 4.2.2.10. Median time course for all patients' NLR in each COVID-19 wave (**A**) and for the discharged and deceased patients in the 1st (**B**), 2nd (**C**) and 3rd (**D**) COVID-19 waves, between the 2nd and 10th days of ICU admission.

Considering the longitudinal data corresponding to the UCI stay, associations between inflammatory and other hematological biomarkers and death were tested by means of univariate GEEs models (**Table 4.2.1.12**). For the entire period of ICU admission, in the 1st wave, for every unit increase in EO counts, the risk of death decreased by 0,3%. Also, patients with decreased EO counts had an increased risk of death of 0,9% in the 2nd wave, and of 1,4% in the 3rd wave. For every unit increase of WBCs and NEUs, the risk of death increased 0,1% and 0,2% respectively, both in the 2nd and 3rd waves. In the 2nd wave, patients with increased WBCs and NEU, had an increased risk of death of 1,5% and 1,8% respectively. Regarding LYMs, for every unit increase the risk of death decreased 1,5% in the 2nd wave and 1,6% in the 3rd wave. Also, for patients with decreased LYM counts, the risk of death increased 1,4% in the 2nd wave and 2,7% in the 3rd wave. When compared to univariate logistic regression, variables that were not related to the risk of mortality in the 2nd and 7th days of ICU admission were shown to be in fact associated with an elevated risk of death when the longitudinal data was analyzed through univariate GEEs models. These findings demonstrate the importance of studying data using various approaches in order to reach diverse conclusions, such as the fact that some biomarkers predict higher risks of death when analyzed on a single day, and others only detected that risk when analyzed over a longer period of time.

Table 4.2.2.12. *Statistically significant results from univariate GEEs models for inflammatory and other hematologic biomarkers in each COVID-19 wave*

		OR	95% CI	*P* Value
1st COVID-19 Wave	**Min. EO x 10^9/L**	0,997	0,993-1,000	0,043
2nd COVID-19 Wave	**Min. LYM x 10^9/L**	0,985	0,977-0,993	<0,001
	Decreased LYM	1,014	1,005-1,023	0,002
	Decreased EO	1,009	1,004-1,015	<0,001
	Max. WBC x 10^9/L	1,001	1,001-1,002	0,001
	Increased WBC	1,015	1,007-1,024	<0,001
	Max. Neu x 10^9/L	1,002	1,000-1,002	<0,001
	Increased NEU	1,018	1,009-1,027	<0,001
3rd COVID-19 Wave	**Min. LYM x 10^9/L**	0,984	0,973-0,995	0,004
	Decreased LYM	1,027	1,017-1,038	<0,001
	Min. EO x 10^9/L	0,968	0,954-0,982	<0,001
	Decreased EO	1,014	1,006-1,022	0,001
	Max. WBC x 10^9/L	1,001	1,000-1,002	0,032
	Max. Neu x 10^9/L	1,002	1,000-1,003	0,006
	Increased NEU	1,013	1,001-1,024	0,021

4.2.3. Coagulation biomarkers

Regarding coagulation-related biomarkers, to characterize the sample in the 2nd day of ICU admission 325 patients' laboratory results were analyzed (**Table 4.2.3.1**). Other coagulation biomarkers like activated thromboplastin time, factor V and homocysteine weren't included in the following analysis due to high number of missing values. In the studied sample, 37,9% of the patients had increased INR, 89,9% had increased DDs, 81,8% had increased FIB and 88% had increased FER. Minimum and maximum values for PLT counts were obtained, being that 14,1% of the patients had decreased results (thrombocytopenia) and 4,7% of them had increased results (thrombocytosis).

Table 4.2.3.1. *Coagulation-related biomarkers in the 2nd day of ICU admission*

Biomarkers	**Normality Ranges**	**Missing Values**	**All Patients** (n=325)
Max. INR	0,80-1,20	11 (3,4%)	1,17 (1,09-1,27)
Increased INR			119 (37,9%)
Min. PLTs (x10^9/L)	150-450	5 (1,5%)	239,50 (183,25-301,00)
Decreased PLTs			45 (14,1%)
Max. PLTs (x10^9/L)	150-450	5 (1,5%)	258,50 (201,00-323,50)
Increased PLTs			15 (4,7%)
Max. DDs (µg/L)	< 230	11 (3,4%)	758,00 (362,50-2412,00)
Increased DDs			267 (89,9%)
Max. FIB (g/L)	2-4	111 (34,2%)	5,40 (4,50-6,70)
Increased FIB			175 (81,8%)
Max. FER (ng/mL)	30-340	108 (33,2%)	1032,60 (573,10-2285,05)
Increased FER			191 (88,0%)

Comparisons between patients discharged from the ICU and those who died (**Table 4.2.3.2**) reveled no significant differences regarding median INR, FIB and FER levels in all COVID-19 waves. Median INR results were only above normality ranges for deceased patients, in the 2nd wave. Although, in the same wave, 50% of the patients had elevated results, meaning that the increases above normality ranges for INR weren't very significative. In a systematic review and meta-analysis, patients that didn't survive during follow-up had significantly elevated INR values, especially in the first 24 to 48 hours after admission, in comparison to those who were discharged (214). INR increasing trends are related to endothelial cell damage or activation caused by the infection by SARS-CoV-2. When compromised, endothelial cells express high levels of tissue factor, the main trigger of the coagulation cascade (in COVID-19 cases the extrinsic cascade is the most affected, thereby influencing INR results). This activation leads to the formation of thrombin, which further converts FIB into fibrin. This protein is involved in the coagulation of blood, together with PLTs that get entangled in the fibrin clot. In order to maintain homeostasis, a set of cofactors and inhibitors regulated a process called fibrinolysis were plasmin breaks down the fibrin clot. During this process fibrin and degradation products like DDs are released (215,216). Thus, one can understand why there could be increases in the INR, FIB and DDs results and decreases in PLTs counts (PLTs are less available in the blood stream since they are being used to form clots), and why these biomarkers results could be correlated.

***Table 4.2.3.2**. Coagulation-related biomarkers of all patients and comparisons between the ones discharged from the ICU and those who died, for all COVID-19 waves in the 2nd day of ICU admission*

	Biomarkers	**All patients** (n=59)	**Discharged** (n=41)	**Deceased** (n=18)	***P* value**
First COVID-19 Wave	**Max. INR**	1,18 (1,12-1,30)	1,18 (1,12-1,29)	1,17 (1,11-1,36)	0,857*
	Increased INR	21 (36,8%)	14 (35,9%)	7 (38,9%)	0,828•
	Min. PLTs x10^9/L	228,50 (162,25-280,25)	213,50 (166-270)	260,00 (156,75-337,50)	0,187*
	Decreased PLTs	11 (19%)	8 (20%)	3 (16,7%)	1,000▪
	Max. DDs (µg/L)	1227,00 (502,00-3949,00)	1127 (549-3679)	1305 (493,00-5997,75)	0,747*
	Increased DDs	54 (98,2%)	36 (97,3%)	18 (100%)	1,000▪
	Max. FIB (g/L)	5,80 (4,13-7,53)	5,80 (3,78-7,53)	5,90 (4,88-7,53)	0,608*
	Increased FIB	34 (77,3%)	20 (71,4%)	14 (87,5%)	0,283▪
	Max. FER (ng/mL)	1138,80 (692,20-2583,30)	1191,20 (731,70-2421,80)	947,60 (557,50-3173,30)	0,924*
	Increased FER	44 (88%)	32 (91,4%)	14 (93,3%)	0,654▪
Second COVID-19 Wave	**Biomarkers**	**All patients** (n=133)	**Discharged** (n=83)	**Deceased** (n=50)	***P* value**
	Max. INR	1,21 (1,13-1,31)	1,19 (1,14-1,31)	1,22 (1,12-1,34)	0,455*
	Increased INR	63 (50%)	39 (49,4%)	24 (51,1%)	0,854•
	Min. PLTs x10^9/L	251,00 (193,00-304,75)	257 (196-308)	239 (191-297)	0,541*
	Decreased PLTs	16 (12,3%)	11 (13,3%)	5 (10,6%)	0,663•
	Max. DDs (µg/L)	699,50 (358,50-2743,50)	629 (330-2660)	853 (390-3652)	0,243*
	Increased DDs	111 (89,5%)	68 (86,1%)	43 (95,6%)	0,131▪
	Max. FIB (g/L)	5,20 (4,10-6,23)	5,20 (4,40-6,48)	5,10 (3,83-6,08)	0,321*
	Increased FIB	75 (76,5%)	49 (79%)	26 (72,2%)	0,443•
	Max. FER (ng/mL)	906,50 (501,20-1805,95)	928,80 (584,50-1901,50)	770,30 (348,90-1797,98)	0,180*
	Increased FER	80 (86%)	53 (89,8%)	29 (85,3%)	0,216▪
Third COVID-19 Wave	**Biomarkers**	**All patients** (n=133)	**Discharged** (n=85)	**Deceased** (n=48)	***P* value**
	Max. INR	1,11 (1,04-1,21)	1,11 (1,04-1,22)	1,12 (1,04-1,20)	0,429*
	Increased INR	35 (26,7%)	26 (31,3%)	9 (18,8%)	0,117•
	Min. PLTs x10^9/L	227,00 (182,00-310,25)	246,50 (198,75-326,50)	210,50 (147,00-266,75)	**0,006***
	Decreased PLTs	18 (13,6%)	6 (7,1%)	12 (25%)	**0,004•**
	Max. DDs (µg/L)	660,50 (323,75-1517,00)	479,00 (268,50-1024,50)	795,00 (420,50-1922,50)	**0,024***
	Increased DDs	102 (86,4%)	60 (82,2%)	42 (93,3%)	0,086•
	Max. FIB (g/L)	5,75 (4,73-7,00)	6,10 (5,00-7,00)	5,60 (4,70-6,75)	0,349*
	Increased FIB	66 (91,7%)	39 (90,7%)	27 (93,1%)	1,000▪
	Max. FER (ng/mL)	1265,55 (685,68-2580,35)	1265,55 (692,15-2508,20)	1270,95 (662,00-3639,83)	0,634*
	Increased FER	67 (90,5%)	45 (93,8%)	23 (88,5%)	0,691▪

All calculated percentages do not include missing values.

On average, INR median results were higher for deceased patients. Other authors have obtained similar results where, at admission, non-survivors developed not only significant higher PT, but also higher aPTT in comparison with survivors (217, 218). There was also a higher frequency of patients with increased results in this group, except in the 3rd COVID-19 wave. In this case, besides 31,3% of the discharged patients having increased results (in comparison with the 18,8% of the deceased patients), median INR was still lower than the one from deceased patients.

Regarding FIB median results, all groups of patients from every COVID-19 wave demonstrated values above normality ranges. No significant differences were found between groups regarding FIB results. In the first two waves, the percentage of patients with increased results varied from 70% to 88%. In the 3rd wave, this percentage increased, reaching percentages above 90% for both groups. Li *et al.*

(219) found that ΔFIB was one of the co-variates associated with in-hospital death of patients with COVID-19 (OR=6,45 (1,31; 31,69); *P*=0,022). High FIB in early stages of the disease has been classified as a risk factor for severe COVID-19 and correlated with excessive inflammation and ICU admission (220–222).

DDs median results were higher in the group of deceased patients for all COVID-19 waves, but significant differences between groups were only verified in the 3rd wave (*P*=0,024). Independently of the wave, deceased patients also had higher percentages of increased results but, in general, this percentages were always above 85% for all groups. DDs can help diagnose thrombosis and can be used as a biomarker for poor prognosis in the early stages of infection (186). The high results seen in this study can be related to the fact that, as formerly reported, DDs are higher in COVID-19 patients that need intensive care. According to Zhang e*t al.* (223) DDs levels above 2mg/L can be used as mortality indicators with 92% sensitivity and 83% of specificity.

Median PLT counts didn't decrease below normality ranges, but 7-20% of the discharged and 10-25% of the deceased patients had decreased values. Deceased patients had, more commonly, lower mean PLT counts, but only in the 3rd COVID-19 wave were there significant differences between discharged and deceased patients regarding their PLR counts (*P*=0,006) and the proportion of cases with decreased results (*P*=0,004). Just like in the present study, thrombocytopenia (more frequently) and thrombocytosis have been seen among COVID-19 patients, but most studies have correlated thrombocytopenia with unfavorable outcomes (186). Low PLT counts have been associated with disease severity and mortality in COVID-19 patients (218).

Finally, FER, an indirect marker of the quantity of iron stored in the body, was highly above normality ranges in all COVID-19 waves. This results from the attack to HGB by SARS-CoV-2, that leads to the release of iron into the circulation. Besides reducing the rate of oxygen binding, the excess of iron can cause oxidative damage to organs like the lungs and worsen the inflammatory process. The excess iron is stored in proteins like FER, further increasing the blood's viscosity and thus contributing to thrombotic mechanisms (224). According to Feld *et al.* (225), although elevated FER levels are associated with mortality, prediction of important outcomes like death using solely this biomarker aren't reliable. Controversially, according to another study, FER can be used as a predictive biomarker for severe disease and/or worse outcomes in COVID-19. Yet, FER results must be interpreted cautiously due to effect of other comorbidities and diseases. The same authors also detected higher FER serum levels in groups of non-survivors, when compared to survivors (226). In the present study, higher median FER results were verified in the group of discharged patients for the 1st and 2nd COVID-19 waves. In the 3rd wave, the opposite was observed, although a higher percentage of discharged patients had increased results (93,8% Vs. 88,5%). In the presence of this results, study of patients' underling comorbidities and diseases would be useful.

Next, Kaplan-Meier survival curves from each COVID-19 wave were obtained for the groups of patients with and without "Decreased PLTs" (**Figure 4.2.3.1**).

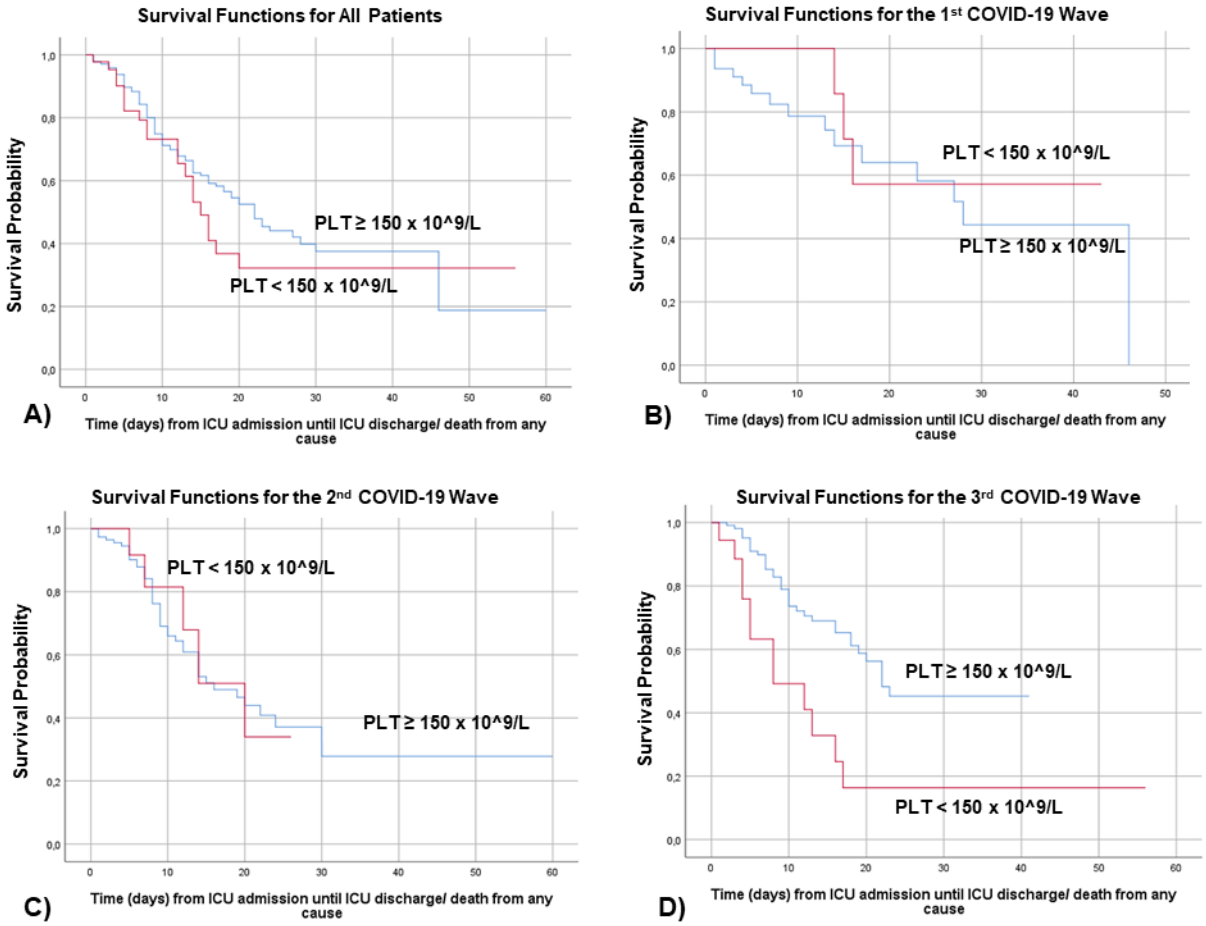


Figure 4.2.3.1. Survival curves from all patients with COVID-19 according to the PLT threshold of 150 x10^9/L in the 2nd day of ICU interment (**A**) and separate survival curves for each COVID-19 wave (**B, C** and **D**).

The survival rate of patients with PLT counts inferior to 150x10^9/L was significantly lower (*P*<0,001) than that of patients with PLT counts equal or superior to 150x10^9/L, in the 3rd COVID-19 wave (**Table 4.2.3.3**). He *et al.* (227) obtained similar results, stating that patients with higher PLT levels at admission were associated with preferred survival.

Table 4.2.3.3. *Comparisons between survival functions for all patients with and without decreased PLT counts, and separate analysis for all COVID-19 waves in the 2nd day of ICU admission*

	All patients	**1st Covid-19 Wave**	**2nd Covid-19 Wave**	**3rd Covid-19 Wave**
	***P* Value**	***P* Value**	***P* Value**	***P* Value**
Log Rank (Mantel-Cox)	0,278	0,537	0,775	**<0,001**
Breslow (Generalized Wilcoxon)	0,292	0,275	0,566	**<0,001**
Tarone-Ware	0,252	0,377	0,647	**<0,001**

Towards finishing the biomarkers' analysis in the 2nd day of ICU admission, univariate logistic regression for all patients in each COVID-19 wave was applied to determine which biomarkers were related to the disease's outcome (**Table 4.2.3.4**). Statistically significant results were only obtained for PLTs in the 3rd wave. Thus, for every unit increase in PLT counts, the risk of death decreases by 0,6% and for patients with decreased PLT counts the mortality risk in increased 4,3-fold. These results are consistent with the former survival analysis for the 3rd COVID-19 wave.

Table 4.2.3.4. *Statistically significant results from univariate logistic regression for coagulation-related biomarkers in the 2nd day of ICU admission*

		Univariate logistic regression for patients in the 2nd day of ICU admission		
		Crude OR	**95% CI**	**P Value**
3rd COVID-19 Wave	**Min. PLTs x10⁹/L**	0,994	0,989-0,998	0,006
	Decreased PLTs	4,333	1,506-12,465	0,007

Following the analysis of patients' coagulation biomarkers in the 2nd day of ICU admission, in each COVID-19 wave, it was also determined whether the laboratory results of discharged and deceased patients differed between waves. Separate comparisons between COVID-19 waves for discharged and deceased patients (**Table 4.2.3.5**) lead to the conclusion that, in the 2nd day of ICU admission, the median INR was significantly different between waves for discharged and deceased patients (*P*<0,001 in both cases). The median levels of DDs and PLT were also significantly different between waves for the group of discharged patients (*P*=0,003 and 0,041 respectively).

Table 4.2.3.5. *Coagulation-related biomarkers' distributions with significant differences across COVID-19 waves, for discharged and deceased patients in the 2nd day of ICU admission*

Discharged patients	**DDs levels comparison between:**	***P* Value**
	All COVID-19 Waves (n=189**)**	**0,003†**
	2nd and 1st COVID-19 Waves	0,070†
	3rd and 1st COVID-19 Waves	**0,002†**
	3rd and 2nd COVID-19 Waves	0,410†
	INR comparison between:	***P* Value**
	All COVID-19 Waves (n=201**)**	**<0,001†**
	1st and 2nd COVID-19 Waves	1,000†
	3rd and 1st COVID-19 Waves	**0,012†**
	3rd and 2nd COVID-19 Waves	**<0,001†**
	PLT levels comparison between:	***P* Value**
	All COVID-19 Waves (n=207**)**	**0,041†**
	1st and 2nd COVID-19 Waves	0,067†
	1st and 3rd COVID-19 Waves	0,057†
	2nd and 3rd COVID-19 Waves	1,000†
Deceased patients	**INR levels comparison between:**	***P* Value**
	All COVID-19 Waves (n=113**)**	**<0,001†**
	1st and 2nd COVID-19 Waves	1,000†
	3rd and 1st COVID-19 Waves	**0,041†**
	3rd and 2nd COVID-19 Waves	**<0,001†**

The fact that median DDs levels were significantly higher in the 2nd day of ICU admission for the 1st wave in contrast to the 3rd wave (*P=0,*002) could explain the considerable disparities identified between discharged patients' DDs median levels between waves (**Figure 4.2.3.2**). Median PLTs levels also displayed significant differences between waves for the discharged patients, possibly because PLTs counts were significantly lower in the 1st wave, in comparison with the 3rd one (*P=0,*041) (**Figure 4.2.3.3**).

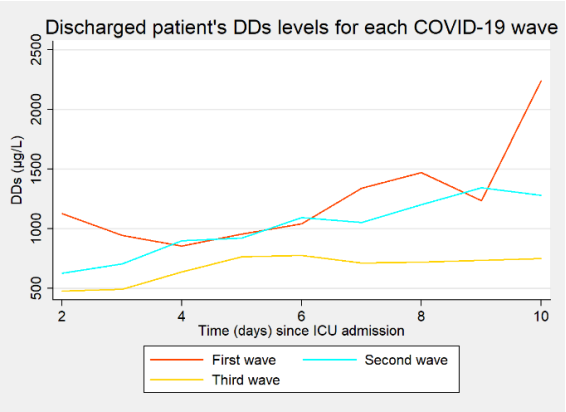


Figure 4.2.3.2. DDs (µg/L) median time course by COVID-19 wave, for discharged patients, between the 2nd and 10th days of ICU admission.

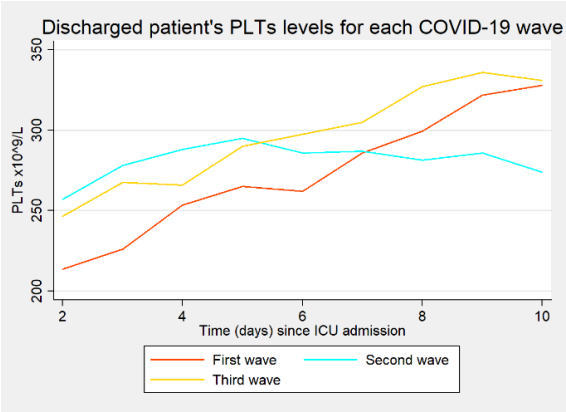


Figure 4.2.3.3. PLTs x10^9/L median time course by COVID-19 wave, for discharged patients, between the 2nd and 10th days of ICU admission.

INR median levels disparities between waves, for both the discharged and deceased groups, were due to significant differences between the results of the 1st and 3rd (*P*=0,012 and 0,041 respectively) and 2nd and 3rd (*P*<0,001 for both groups) COVID-19 waves. This means that both the patients of the discharged and the decease groups had lower median INR results in the 3rd wave (**Figures 4.2.3.4A, B**).

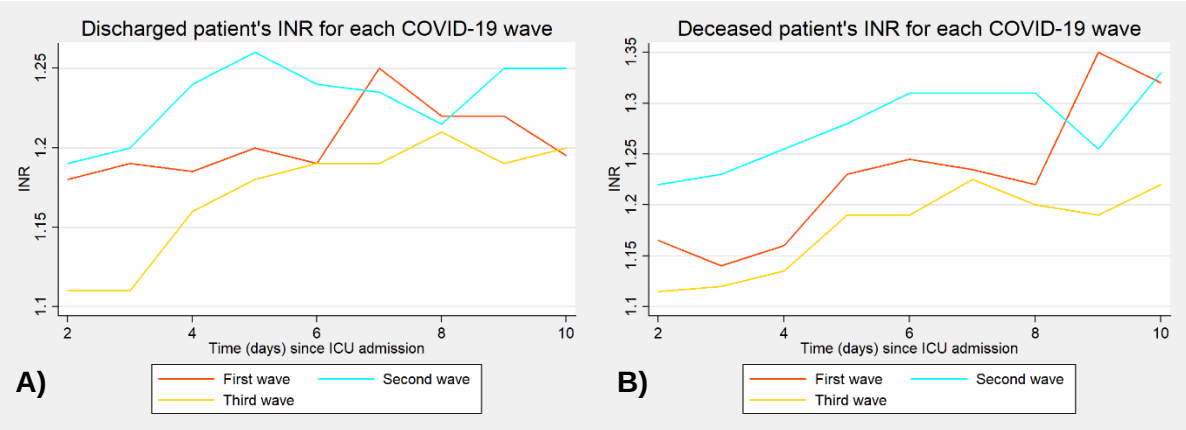


Figure 4.2.3.4. Discharged (**A**) and deceased (**B**) patients' median time course by COVID-19 wave, between the 2nd and 10th days of ICU admission.

Based on this data it can be inferred that discharged patients of the 3rd wave had better INR, PLT counts and DDs results (**Figures 4.2.3.2/ 3/ 4A**). On the other hand, in the 1st wave DDs were higher for both discharged and deceased patients, PLT counts were lower, and FIB and FER levels were elevated, possibly meaning that because patients were advised to stay at home in the 1st wave, the time until hospital admission was greater (180). This could also mean that these patients arrived at the hospital at different stages of the disease and thus with different levels of severity. Because only the IQR of the number of days between symptom onset/RT-PCR diagnosis and ICU admission was somewhat higher in the 1st wave of this study, other factors such as changes in SARS-CoV-2 variants and treatment courses as the pandemic progressed could be at play.

To assess the patients' evolution after one week in the ICU, 216 patients were included (**Table 4.2.3.6**). It was concluded that the frequency of patients with results outside of normality ranges

increased for all biomarkers (excepted FER results which were increased for 88% of the study sample in the two evaluated days). The median results of the biomarkers also increased, indicating that the remaining patients' coagulation state deteriorated with time.

Table 4.2.3.6. *Coagulation-related biomarkers in the 7th day of ICU admission*

Biomarkers	**Missing Values**	**All Patients** (n=216)
Max. INR	3 (1,4%)	1,23 (1,14-1,33)
Increased INR		125 (58,7%)
Min. PLTs (x10^9/L)	2 (0,9%)	284,00 (216,50-359,00)
Decreased PLTs		19 (8,9%)
Max. DDs (µg/L)	30 (9,3%)	1176,00 (564,00-2675,50)
Increased DDs		190 (96,9%)
Max. FIB (g/L)	114 (52,8%)	6,95 (5,10-8,70)
Increased FIB		93 (91,2%)
Max. FER (ng/mL)	96 (44,4%)	1349,65 (587,25-2362,23)
Increased FER		105 (87,5%)

The next step involved analyzing the patients' condition in the 7th day of ICU admission, for each wave, between the groups of discharged and deceased patients (**Table 4.2.3.7**). This time, due to more missing values for some variables, their analysis wasn't preformed.

Table 4.2.3.7*. Coagulation-related biomarkers of all patients and comparisons between the ones discharged from the ICU and those who died, for all COVID-19 waves in the 7th day of ICU admission*

	Biomarkers	**All patients** (n=39)	**Discharged** (n=27)	**Deceased** (n=12)	***P* value**
First COVID-19 Wave	**Max. INR**	1,25 (1,15-1,36)	1,25 (1,15-1,37)	1,24 (1,09-1,34)	0,505*
	Increased INR	22 (57,9%)	16 (61,5%)	6 (50%)	0,503•
	Min. PLTs x10^9/L	267 (203-356)	286 (222-407)	234 (178,25-296,25)	0,168*
	Decreased PLTs	5 (12,8%)	3 (11,1%)	2 (16,7%)	-
	Max. DDs (µg/L)	2090 (773-4148)	1340 (435-2812)	3888 (1542,25-8718,75)	**0,018***
	Increased DDs	39 (100%)	27 (100%)	12 (100%)	-
	Max. FIB (g/L)	7,30 (6,40-8,95)	7,30 (6,50-9,65)	6,80 (3,68-8,40)	0,210*
	Increased FIB	18 (85,7%)	12 (92,3%)	6 (75%)	0,531▪
	Max. FER (ng/mL)	1589,70 (933,00-2021,00)	1592,25 (837,83-2053,80)	1450,60 (1134,50-2325,70)	0,685*
	Increased FER	30 (96,8%)	21 (95,5%)	9 (100%)	1,000▪
Second COVID-19 Wave	**Biomarkers**	**All patients** (n=87)	**Discharged** (n=52)	**Deceased** (n=35)	***P* value**
	Max. INR	1,26 (1,18-1,36)	1,24 (1,17-1,33)	1,31 (1,21-1,36)	0,105*
	Increased INR	57 (66,3%)	31 (59,6%)	26 (76,5%)	0,106•
	Min. PLTs x10^9/L	283,50 (221,75-361,75)	287,00 (223,00-358,75)	283,50 (212,25-373,50)	0,856*
	Decreased PLTs	7 (8,1%)	4 (7,7%)	3 (8,8%)	-
	Max. DDs (µg/L)	1070,50 (574,00-2652,75)	1052 (481-2473)	1693 (706-3175)	0,465*
	Increased DDs	75 (96,2%)	45 (95,7%)	30 (96,8%)	1,000▪
	Max. FIB (g/L)	5,70 (4,60-7,30)	5,45 (4,75-7,20)	6,00 (4,35-8,20)	0,593*
	Increased FIB	38 (88,4%)	23 (88,5%)	15 (88,2%)	1,000▪
	Max. FER (ng/mL)	829,70 (332,30-2090,60)	776,90 (359,75-1993,48)	935,20 (249,50-2722,55)	0,949*
	Increased FER	35 (74,5%)	20 (76,9%)	15 (71,4%)	0,668•

	Biomarkers	All patients (n=90)	Discharged (n=58)	Deceased (n=32)	*P* value
Third COVID-19 Wave	**Max. INR**	1,21 (1,12-1,28)	1,90 (1,12-1,28)	1,23 (1,14-1,28)	0,216*
	Increased INR	46 (51,7%)	25 (43,9%)	21 (65,6%)	0,049•
	Min. PLTs x10^9/L	287,00 (214,50-359,50)	305,00 (237,50-385,50)	237,00 (184,50-316,50)	**0,005***
	Decreased PLTs	7 (7,9%)	2 (3,5%)	5 (15,6%)	-
	Max. DDs (µg/L)	942 (547-2145)	715,00 (479,50-1300,50)	1817,50 (941,00-2977,50)	**<0,001***
	Increased DDs	76 (96,2%)	46 (93,9%)	30 (100%)	0,284▪
	Max. FIB (g/L)	7,60 (5,45-9,33)	7,40 (5,20-9,40)	7,70 (5,80-9,30)	0,729*
	Increased	37 (97,4%)	19 (100%)	18 (94,7%)	1,000▪
	Max. FER (ng/mL)	1776,70 (900,35-2816,05)	1330,20 (640,18-2431,15)	2072,95 (1283,23-3609,95)	0,057*
	Increased FER	40 (95,2%)	23 (95,8%)	17 (94,4%)	1,000▪

All calculated percentages do not include missing values.

In the 1st wave, significant differences between groups were found regarding median DDs results (*P=0,*018), and all patients had values above normality ranges. In the group of deceased patients, these results almost tripled since the 2nd day of ICU admission. These kinds of increases have been found in other studies. Non-survivors revealed median DD values of 3,4 µg/L in a longitudinal research, which climbed to 16,3 µg/L between the 4th and 7th days of ICU admission (179). In the 2nd wave, besides the worsening of patients results, no significant differences were found between groups of patients. Finally, in the 3rd wave, significant differences were once again verified between median DDs results of both groups (*P*<0,001) and between the two groups median PLT counts (*P*=0,005). PLTs were significantly reduced in the group of deceased patients, indicating an increased risk of disease severity and mortality (210). In fact, once again, the survival curves only showed significant results regarding median PLT counts in the 3rd wave (**Figure 4.2.3.5D**).

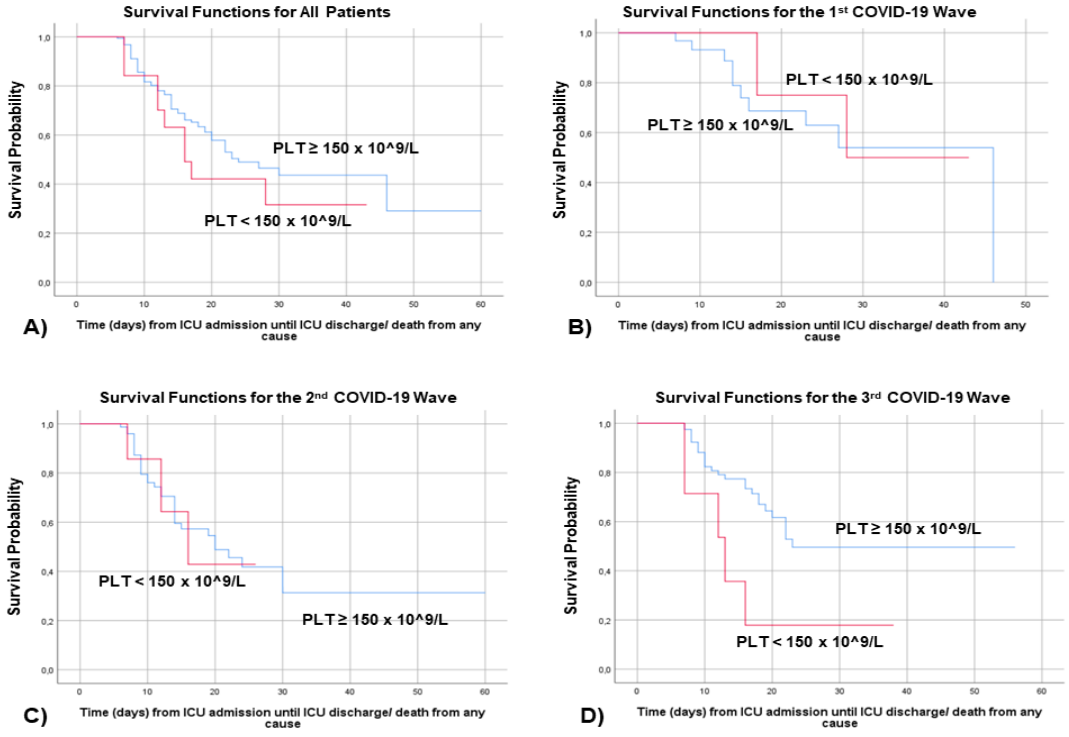


Figure 4.2.3.5. Survival curves from all patients with COVID-19 according to the PLT threshold of 150 x10^9/L in the 7th day of ICU interment (**A**) and separate survival curves for each COVID-19 wave (**B, C** and **D**).

Ultimately, the survival rate of patients with PLT counts inferior to $150x10^9$/L was significantly lower (*P*=0,017) than that of patients with PLT counts equal or superior to $150x10^9$/L, in the 3rd COVID-19 wave (**Table 4.2.3.8**).

4.2.3.8. *Comparisons between survival functions of all patients with and without decreased PLT counts, and separate analysis for all COVID-19 waves*

	All patients	**1st Covid-19 Wave**	**2nd Covid-19 Wave**	**3rd Covid-19 Wave**
	***P* Value**	***P* Value**	***P* Value**	***P* Value**
Log Rank (Mantel-Cox)	0,323	0,793	0,934	**0,017**
Breslow (Generalized Wilcoxon)	0,316	0,512	0,953	**0,013**
Tarone-Ware	0,312	0,623	0,943	**0,012**

Univariate logistic regression for all patients in each COVID-19 wave was applied to determine which biomarkers were related to the disease's outcome in the 7th day of ICU admission. Similarly to the 2nd day of ICU admission, statistically significant results were obtained for PLT counts in the 3rd COVID-19 wave. Thus, for every unit increase in PLT counts, the risk of death decreased by 0,7%. Once again, these results were consistent with the former survival analysis for the 3rd wave.

The following analysis had the goal of determining possible differences between waves, regarding the median levels of each biomarker in every group of patients. In the group of discharged patients, only median DDs results showed significant differences between COVID-19 waves (*P*=0,037). For the group of deceased patients, differences were found regarding median FIB levels (*P*=0,009) (**Table 4.2.3.9**). Just like for the analysis of the 2nd day of ICU admission, the obtained differences were most likely a result of the increased median DD results in the 1st wave, mainly in comparison to the ones from the 3rd wave.

Table 4.2.3.9. *Coagulation-related biomarkers' distributions with significant differences across COVID-19 waves, for discharged patients in the 7th day of ICU admission*

	DDs levels comparison between:	***P* Value**
Discharged patients	**All COVID-19 Waves (**n=123**)**	**0,037†**
	2nd and 1st COVID-19 Waves	1,000†
	3rd and 1st COVID-19 Waves	0,061†
	3rd and 2nd COVID-19 Waves	0,151†
	FIB comparison between:	***P* Value**
	All COVID-19 Waves (n=58**)**	**0,009†**
	2nd and 1st COVID-19 Waves	**0,017†**
	2nd and 3rd COVID-19 Waves	0,068†
	3rd and 1st COVID-19 Waves	1,000†

The changes in FIB values between waves were a result of the significant lower levels in the 2nd COVID-19 wave, in comparison with the 1st one (*P*=0,017) (**Figure 4.2.3.6**). Thus, one can conclude that once again discharged patients demonstrated worse results in the 1st COVID-19 wave.

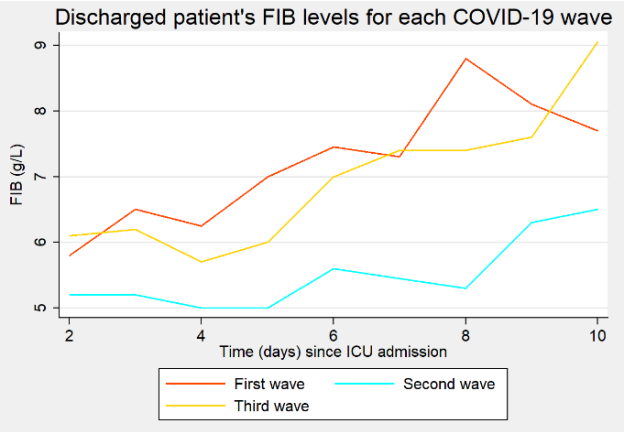


Figure 4.2.3.6. FIB (g/L) median time course by COVID-19 wave, for discharged patients, between the 2nd and 10th days of ICU admission.

To conclude the analysis of coagulation biomarkers, comparisons between the 2nd and 7th days of ICU admission (**Table 4.2.3.10**) were obtained to evaluate the patients' evolution.

Table 4.2.3.10. *Comparisons of coagulation-related biomarkers of all patients, the ones discharged from the ICU and those who died between the 2nd and 7th days of ICU admission for each COVID-19 wave*

		INR		PLTs x10⁹/L		DDs (µg/L)		FIB (g/L)	
		n	P Value	n	P Value	n	P Value	n	P Value
1st COVID-19 Wave	**Discharged patients**	25	0,798	26	**0,003**	25	0,276	11	**0,005**
	Deceased patients	12	0,754	12	1,000	12	0,530	7	0,735
	All patients	37	0,964	38	**0,010**	37	0,635	18	**0,013**
2nd COVID-19 Wave	**Discharged patients**	49	**0,004**	52	**0,003**	45	0,196	20	0,305
	Deceased patients	34	**0,025**	34	0,108	30	0,926	9	0,066
	All patients	83	**<0,001**	86	**0,001**	75	0,337	29	0,079
3rd COVID-19 Wave	**Discharged patients**	56	**0,001**	57	**0,002**	41	0,928	16	**0,008**
	Deceased patients	32	**<0,001**	32	0,256	28	**0,019**	10	**0,013**
	All patients	88	**<0,001**	89	**0,001**	69	0,120	26	**<0,001**

P-values represent the comparison of the median of a given biomarker on the 2nd day with that of the 7th day of ICU admission. All *P* values obtained through related-samples Wilcoxon matched-pair signed rank test.

Regarding INR results, for comparisons between day 2 and 7 of ICU admission, no significant differences were found for any of the groups in the 1st COVID-19 wave, besides the worsening of the results (**Figure 4.2.3.7A, B**). In the 2nd and 3rd waves, INR results were significantly different for all patients (*P*<0,001) between the two analyzed days (**Figure 4.2.3.7A**). In the 2nd COVID-19 wave, both the discharged (*P*=0,004) and deceased (*P*=0,025) patients groups showed significant differences between the two days, since the INR results increased (**Figure 4.2.3.7C**). In the analyzed time frame, discharged patients results reached a peak approximately in the 5th day of ICU admission (slightly over the normality range) and started to decrease. On the other hand, deceased patients' results reached a peak on the 6th day of ICU admission and stabilized, until starting to decrease two days later. Although, their results were abnormally elevated during the entire analyzed period, in comparison with discharged patients, features that have been previously reported (179). In the 3rd wave, once again both the discharged (*P*=0,001) and deceased (*P*<0,001) patients groups showed significant differences between the two days, given that the INR results increased, even getting slightly outside normality ranges for

deceased patients (**Figure 4.2.3.8D**). Comparably to what happened in the 1st wave, in the 3rd one no significant differences were observed between discharged and deceased patients.

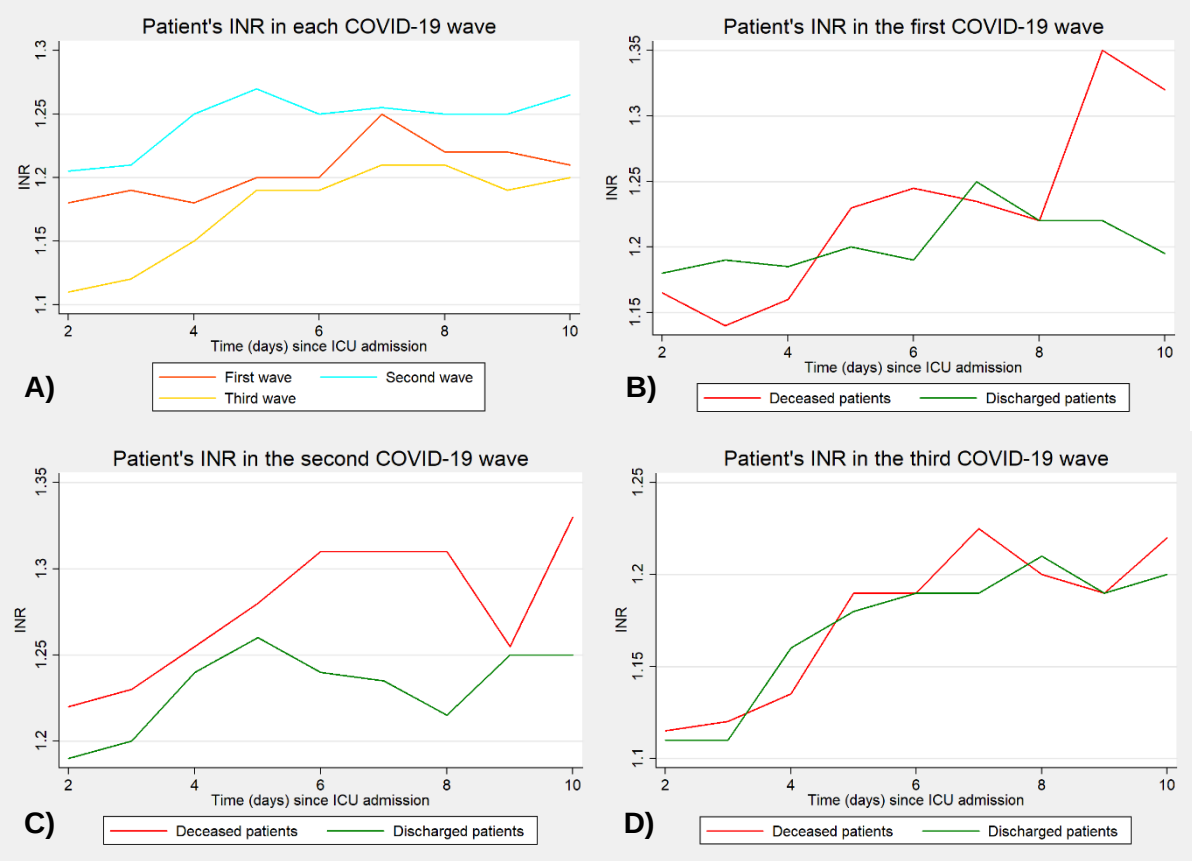


Figure 4.2.3.7. Median time course for patients' INR in each COVID-19 wave (**A**) and for the groups of discharged and deceased patients in the 1st (**B**), 2nd (**C**) and 3rd (**D**) COVID-19 waves, between the 2nd and 10th days of ICU admission.

In all COVID-19 waves, patients' results significantly increased between the 2nd and 7th days of ICU admission, besides only peaking after the 8th day (**Figure 4.2.3.8A**). According to Ruchond's *et al.* (210) research, PLTs showed an increasing trend in the group of survivors, but a downward trend or low results for the non-survivors. The same was verified in this study regarding the discharged patients' results in the 1st and 3rd waves, but in the 2nd one, after approximately the 5th day of ICU admission, PLTs counts stated to decrease while remaining between normality ranges (**Figure 4.2.3.8B-D**). Thus, in all COVID-19 waves, discharged patients' results were significantly different between the 2nd and the 7th days of ICU admission (*P*=0,003 in the 1st and 2nd waves and 0,002 in the 3rd). For deceased patients, a downward trend was only observed for the first five days of ICU admission in the 1st wave, but besides lower than for discharged patients, PLTs counts also kept on increasing in this group of patients (**Figure 4.2.3.8B-D**). Despite their increasing trend in the 2nd and 3rd COVID-19 waves, no significant differences were detected between the results of 2nd and 7th days of ICU admission (*P*=0,108 and 0,256 respectively).

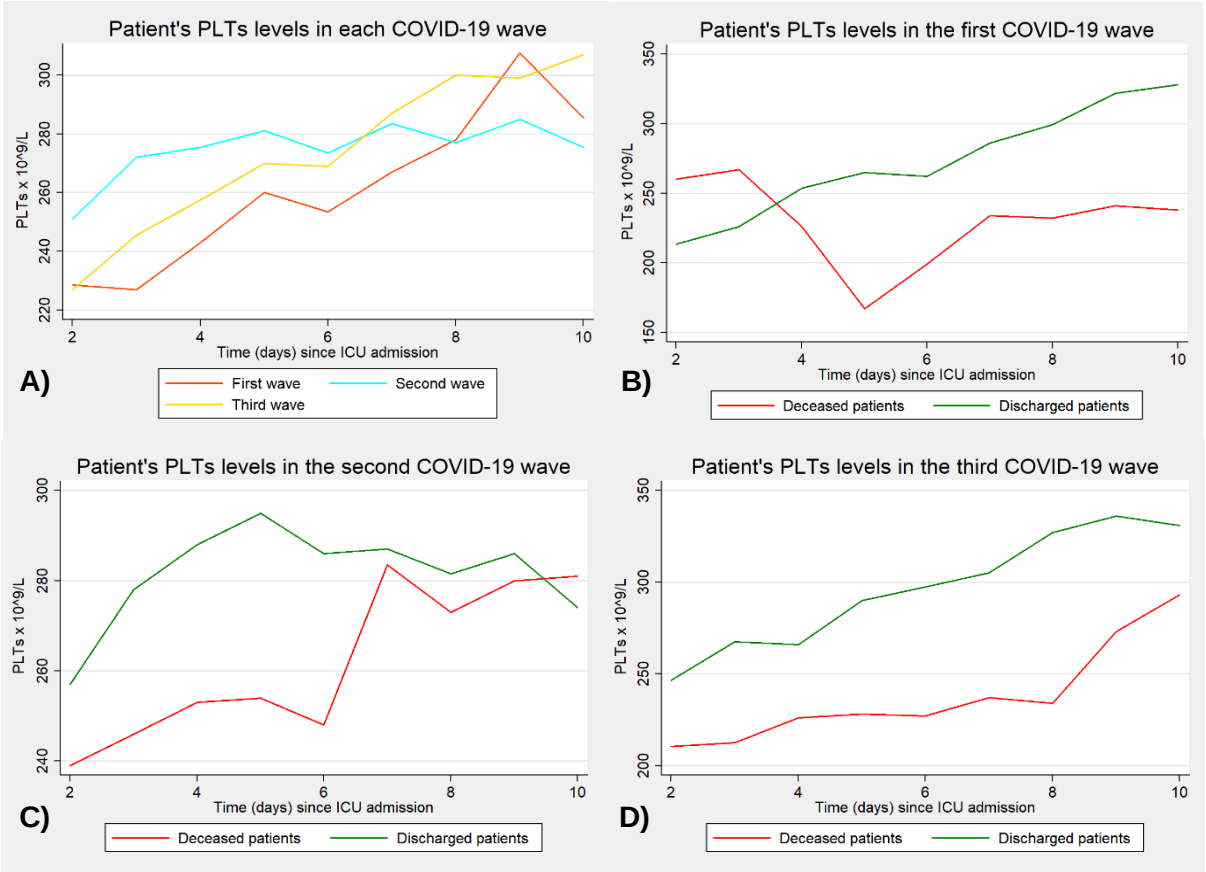


Figure 4.2.3.8. Median time course for patients' PLTs levels in each COVID-19 wave (**A**) and for the groups of discharged and deceased patients in the 1st (**B**), 2nd (**C**) and 3rd (**D**) COVID-19 waves, between the 2nd and 10th days of ICU admission.

Maximum median DD levels were higher for all patients in the 1st wave, throughout the analyzed period of time, and increasing trends were verified in all the waves (**Figure 4.2.3.9A**). DDs were only significantly different between the 2nd and 7th days of ICU admission for the group of deceased patients, in the 3rd COVID-19 wave (*P*=0,019) (**Figure 4.2.3.9D**). Until the 7th day of ICU admission, deceased patients' DDs results kept on increasing. Discharged patients' results also increased but in a very subtle way in the 1st and 3rd waves. In both groups, maximum values were obtained around the 6th and 7th days of ICU admission (**Figure 4.2.3.9B-D**). In other research, DDs levels reached maximum values between the 1st and 3rd days of hospitalization and after that decreased. This happened for both discharged and deceased patients, although the deceased ones had higher results at any studied time point (179). These findings are very distinct from the ones in the present study, except for the fact that discharged patients also had, almost always, inferior results than the deceased ones, mainly in the 1st and 3rd waves. In another report, maximum DD results were achieved around the 7th day of hospitalization, for both survivors and non-survivors, and higher levels and velocity of DD level increase were obtained for non-survivors. The authors also reached the conclusion that the dynamics of this biomarker may help monitor the disease progression (228).

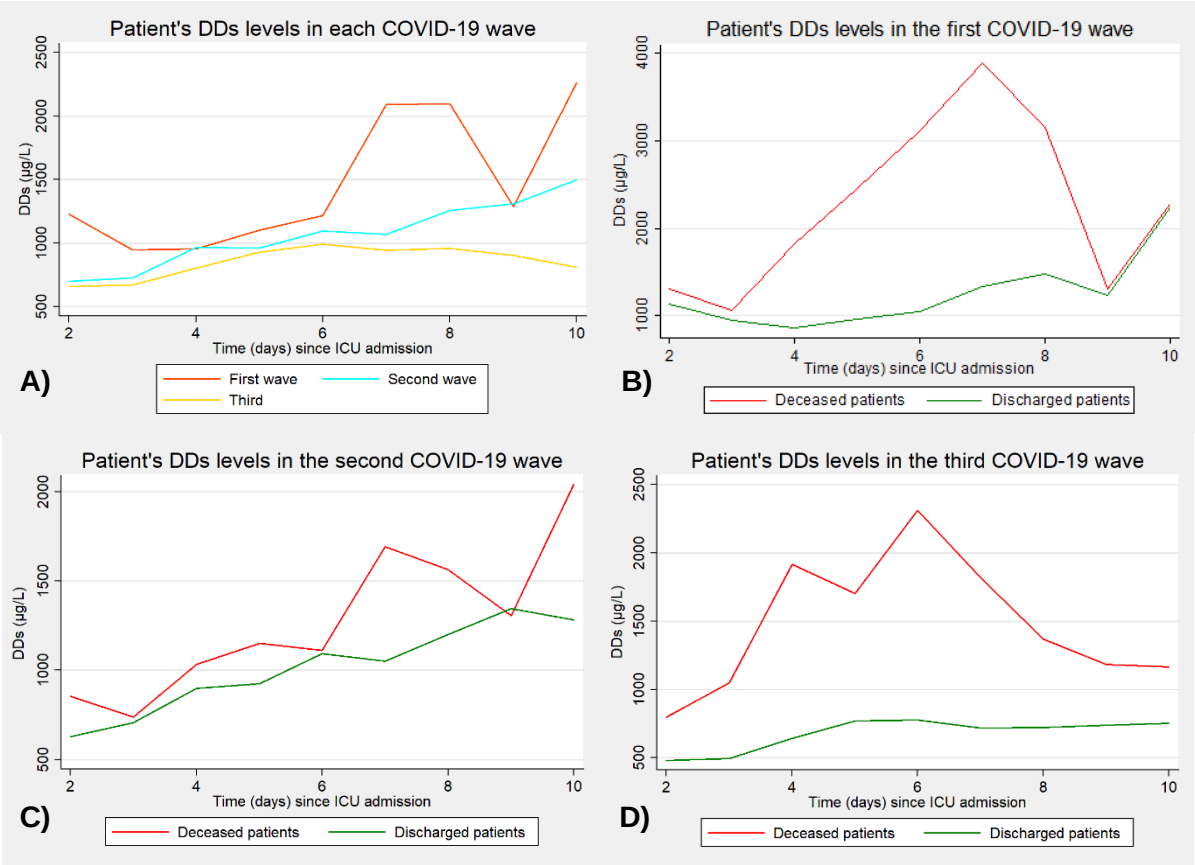


Figure 4.2.3.9. Median time course for patients' DDs levels in each COVID-19 wave (**A**) and for the groups of discharged and deceased patients in the 1st (**B**), 2nd (**C**) and 3rd (**D**) COVID-19 waves, between the 2nd and 10th days of ICU admission.

Regarding FIB levels, upward trends, especially after the 4th day of ICU admission were observed in all COVID-19 waves (**Figure 4.2.3.10A**), but significant differences between the 2nd and 7th days of ICU admission were only obtained for the 1st and 2nd waves (*P*=0,013 and <0,001 respectively). Discharged patients from the first wave (*P*=0,005), as well as both discharged and deceased patients from the third wave (*P*=0,008 and 0,013 respectively), also showed significant differences between the two analyzed days. Although, increasing trends were observed in most of the cases (**Figure 4.2.3.10B-D**), with values above normality ranges since the 2nd day of ICU admission. According to Eljilany *et al.* (229) initial markers of coagulopathy in COVID-19 are DD levels, while late markers include INR, increased PLTs and decreased FIB levels. In the acute phase, FIB levels are expected to increase as part of the inflammatory response (as it was observed in the present study) followed by a decrease when DIC occurs (230). This last stage wasn't observed in the time period analyzed in this study.

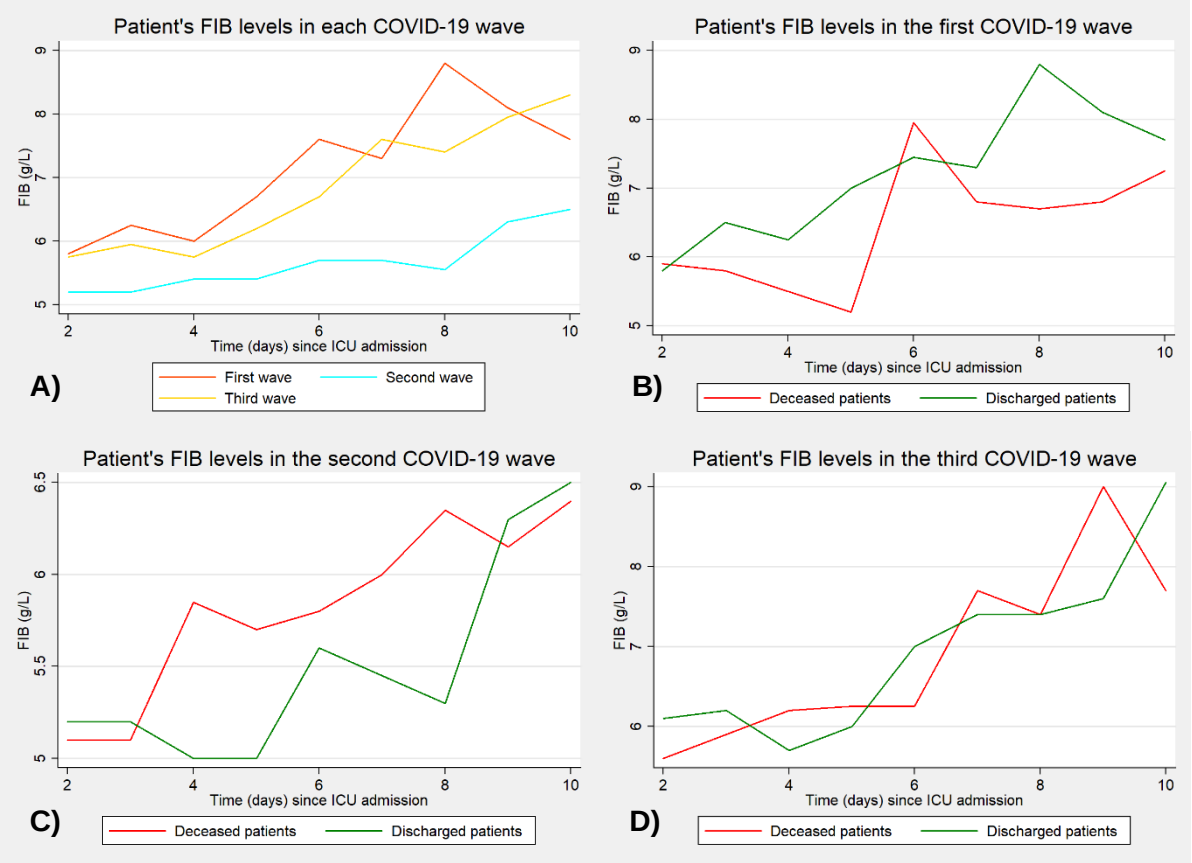


Figure 4.2.3.10. Median time course for patients' FIB levels in each COVID-19 wave (**A**) and for the groups of discharged and deceased patients in the 1st (**B**), 2nd (**C**) and 3rd (**D**) COVID-19 waves, between the 2nd and 10th days of ICU admission.

Considering the total longitudinal data corresponding to the UCI stay, this time associations between coagulation-related biomarkers and death were tested by means of univariate GEEs models (**Table 4.2.1.12**). In the 2nd wave, for patients with increased INR the risk of death increased 1,0%. In the 3rd wave, for every unit increase in INR, the risk increased 1,4% and for patients with decreased PLTs, the risk of death increased 3,9%.

Table 4.2.3.11. *Statistically significant results from univariate GEEs models for coagulation-related biomarkers in each COVID-19 wave*

		OR	95% CI	P Value
2nd COVID-19 Wave	**Increased INR**	1,010	1,002-1018	0,010
3rd COVID-19 Wave	**Max. INR**	1,014	1,006-1,023	0,001
	Decreased PLTs	1,039	1,013-1,067	0,004

Chapter 5 – Conclusion and future perspectives

In terms of clinical and demographic features, deceased patients were considerably older (*P*<0,001), had more comorbidities (*P*=0,008), required more IMV (*P*<0,001), and spent less time in the hospital than discharged patients (*P*<0,001). More patients were admitted during the 3rd COVID-19 wave and less in the 1st one. There were no significant differences in mortality across the waves (*P*=0,729). Age (*P*=0,021) and BMI (*P*=0,013), on the other hand, varied between waves. Patients from the 1st wave were significantly older and relied more on IMV (*P*=0,023) and ECMO (*P*<0,001). Patients who needed IMV, had higher mortality (*P*<0,001), even in the ICU (*P*<0,001), and were admitted to the ICU (*P*<0,001) and hospitalized (*P*<0,001) for longer periods of time. Younger patients didn't require as much IMV (*P*=0,028) but relied on techniques like HFO.

Regarding the laboratory results of the 2nd day of ICU admission, no significant differences were observed between discharged and deceased patients from the 1st COVID-19 wave. Most of the detected differences were between the groups of patients belonging to the 2nd and 3rd waves, being that deceased patients had almost always worse results. In the 7th day of ICU admission, significant differences between groups regarding laboratory results were found in all COVID-19 waves. Through the survival analysis made for all biomarkers in the two mentioned days, it was possible to conclude that in the 3rd wave, mortality rates were higher for patients with hs-cTn I levels above normality ranges in the 2nd day of ICU admission (*P*=0,006), with LYM levels under normality ranges in the 7th day of ICU admission (*P*=0,048), and for patients with PLT levels were below normality ranges, either in the 2nd (*P*<0,001) or 7th days (*P*<0,001) of ICU admission. In the 2nd wave, higher mortality rates were only observed for patients with increased hs-cTn I levels (*P*=0,013) and LYM levels under normality ranges (*P*=0,033) in the 2nd day of ICU admission. Since in the 1st wave biomarkers' results weren't different between discharged and deceased patients, survival analysis didn't lead to any significant results.

Comparisons between the 2nd and 7th days of ICU admission led to the conclusion that, in the 7th day, hs-cTn I and LDH results were significantly lower for all patients, in all COVID-19 waves (except LDH in the 1st wave). Regarding inflammation biomarkers, in all waves, RBCs, HCT and HGB were significantly lower in the 7th day of ICU admission. Biomarkers with increasing trends included WBCs, NEUs, EOs and, controversially, LYMs. INR, DDs, FIB and PLTs were coagulation biomarkers which also displayed increasing trends between the 2nd and 7th days of ICU admission. Although, more preeminent differences were detected in the 2nd and 3rd COVID-19 waves.

Regarding comparisons between waves, when significant differences between the biomarkers were found in the 2nd day of ICU admission, most of time they seemed to be a result of the significantly worse results observed in the 1st wave. In the 7th day of ICU admission, those differences seemed to be caused by worse results both in the 1st and in the 3rd waves. Thus, in the 2nd wave results were better in comparison to the remaining two. Despite all the mentioned observations, the behavior of each wave was very different between each set of biomarkers, and when comparisons between groups of deceased and discharged patients were obtained for each wave.

The estimated crude OR, regarding the risk of death, allowed to conclude that in the 2nd COVID-19 wave, for the 2nd day of ICU admission, patients with increased hs-cTn I (OR=2,412; *P*=0,028), decreased RBCs (OR=2,390; *P*=0,045), higher PLRs (OR=1,002; *P*=0,007) or NLRs (OR=1,057; *P*=0,004) had an increased risk of death. For patients from the 3rd COVID-19 wave, higher risks were obtained in cases of increased hs-cTn I (OR=2,789; *P*=0,020), higher LDH levels (OR=1,003; *P*=0,001), higher CRP levels (OR=1,004; *P*=0,029), lower PLT counts (OR=0,994; *P*=0,006) or decreased PLTs (OR=4,333; *P*=0,007). For the 7th day of ICU admission, patients in the 2nd wave had a greater risk of death when in the presence of higher CRP levels (OR=1,005; *P*=0,014), decreased RBCs (OR=5,333; *P*=0,035), decreased HCT (OR=8,854; *P*=0,042), lower levels of HGB (OR=0,734; *P*=0,012), higher levels of WBC (OR=1,105; *P*=0,017) or NEU (OR=1,131; *P*=0,007), increased NEU (OR=5,019; *P*=0,017), decreased EO (OR=3,214; *P*=0,011) or higher NLRs (OR=1,076; *P*=0,002). For patients in the 3rd wave, those with higher PCT (OR=1,326; *P*=0,031), CRP (OR=1,005; *P*=0,022) or WBC levels (OR=1,118; *P*=0,018), decreased LYMs (OR=4,435; *P*=0,002), higher NEU levels (OR=1,166; *P*=0,004), higher NLRs (OR=1,076; *P*=0,002) or lower levels of PLTs (OR=1,003; *P*=0,006), were also at higher risk of death.

Considering the longitudinal data, in the 1st wave, patients with increased hs-cTn I levels (OR=1,004; *P*=0,019), in risk of CS (OR=1,004; *P*=0,017), with increased myoglobin levels (OR=1,024; *P*=0,002) and lower EO counts (OR=0,997; *P*=0,043), were at a greater risk of dying. In the 2nd wave, the risk of death was also higher for patients with increased hs-cTn I levels (OR=1,017; *P*=0,002), in risk of CS (OR=1,021; *P*=0,006) and with increased myoglobin levels (OR=1,189; *P*=0,015), and for patients with decreased EOs (OR=1,009; *P*<0,001), higher WBC (OR=1,001; *P*=0,001) and NEU counts (OR=1,002; *P*<0,001), with increased WBCs (OR=1,015; *P*<0,001) and NEUs (OR=1,018; *P*<0,001), with lower LYM counts (OR=0,985; *P*<0,001), decreased LYMs (OR=1,014; *P*=0,002) and increased INR (OR=1,010; *P*=0,010). Finally, in the 3rd wave, patients with increased hs-cTn I (OR=1,021; *P*=0,003), increased CK (OR=1,023; *P*<0,001), decreased EOs (OR=1,014; *P*=0,001), lower EO counts (OR=0,968; *P*<0,001), higher WBC (OR=1,001; *P*=0,032) and NEU counts (OR=1,002; *P*=0,006), increased NEUs (OR=1,013; *P*=0,021), lower LYM counts (OR=0,984; *P*=0,004) and decreased LYMs (OR=1,027; *P*<0,001), higher INR (OR=1,014; *P*=0,001), and decreased PLTs (OR=1,039; *P*=0,004), were also in higher risk of death.

Aside from the positive results, more research is needed to develop reliable biomarkers for COVID-19's outcome. This study had some limitations, starting with the study sample, that was made up entirely of patients from a single ICU/hospital and quite small when considering that most of the research was focused on three COVID-19 wave groups. Thus, a larger sample size might increase the statistical power of the study. Regarding the clinal and demographical analysis, several information wasn't available, such as the severity of certain cardiac, liver, and renal disorders, the type of medicine patients were taking, and, most crucially, the severity of COVID-19 disease. In the future, it may be critical to consider disease severity in order to generate more trustworthy results and provide information on how to respond in each situation. Furthermore, the impact of therapies like IMV, ECMO, and HFO, as well as medication, on the patients' clinical course was not reported or included in the statistical

analysis. Future research should look into the impact of these factors as well as the early use of antivirals, immunomodulating medicines, vaccines, and other treatments on the disease's progression. In terms of laboratory data, not all biomarkers' results were available at any time point during the patients' ICU stay. There were even days with no measurements for certain patients, mostly after the 7th day of ICU admission. Another limitation was time, since most of it was directed into modeling data and creating databases as complete as possible, several biomarkers related to other organs weren't analyzed. Besides all the former mentioned future work, other possibilities involve including multivariate analysis and the application of more complex prediction models in order to generate stronger results and predict the patients' outcome.

Despite the above-mentioned limitations, this study provides useful information for prognostic evaluation that can be used to guide treatment and monitoring. Most importantly, it consists of valuable information, that took a lot of time to gather and organize into usable databases, and that can be employed as the foundation of a variety of future research.

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