Final Degree Project

Biochemistry and Molecular Biology

UNIVERSITAT ROVIRA i VIRGILI

INFLUENCE OF HYPERTENSION IN PATIENTS WITH UNFAVOURABLE COVID-19: A MULTI-OMICS STUDY

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This Final degree project has been based on the results obtained in my external Internship at the Infection and Immunity group (INIM) of the Institut d'Investigació Sanitària Pere Virgili (IISPV) under the supervision of Anna Rull Aixa and Alba Sánchez Morillo.

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ABBREVIATIONS

ACE2:	Angiotensin converting enzyme 2.
Ang II:	Angiotensin II.
AT1R:	Angiotensin II type 1 receptor.
AT2R	Angiotensin II type 2 receptor.
AUC:	Area under the curve.
BP:	Blood pressure.
ChoE:	Cholesterol esters.
COS:	Centre for Omic Sciences.
COPD:	Chronic obstructive pulmonary disease.
COVID-19:	Coronavirus disease.
CVD:	Cardiovascular disease.
DBP:	Diastolic blood pressure.
DG:	Diglyceride.
GSN:	Gelsolin.
HDL:	High density lipoprotein.
MAS:	Mitochondrial assembly receptor.
MERS:	Middle East respiratory syndrome.
MODS:	Multiorgan dysfunction syndrome.
NCD:	Non communicable disease.
NSP:	Non-structural protein.
PC:	Phosphatidylcholine.
PCR:	Polymerase chain reaction.
RAAS:	Renin-angiotensin-aldosterone system.
RNA:	Ribonucleic acid.
ROC:	Receiver operating characteristic.
ROS:	Reactive oxygen species.
RTC	Replication transcription complex.
SARS-CoV-2:	Severe acute respiratory syndrome coronavirus 2.
SBP:	Systolic blood pressure.
SM:	Sphingomyelin.
TG:	Triglyceride.
WHO:	World Health Organization.
ZA2G:	Zinc-alpha-2-glycoprotein.

1. ABSTRACT

Background: Coronavirus disease (COVID-19) is a disease that emerged in 2019 due to SARS-CoV-2 infection. Since the COVID-19 outbreak, preliminary research has shown that some comorbidities increase death and severe complications of the disease, with hypertension being the most common. Several previous studies have been able to hypothesize pathways by which these two diseases might be acting together. However, the reason why hypertension may worsen the outcome of COVID-19 patients is still unknown.

Objective: To find biomolecules and metabolic pathways that may explain the relationship between unfavourable COVID-19 and hypertension.

Methods: A total of 103 patients with COVID-19 were classified according to severity and divided into hypertensive (n=26) and non-hypertensive (n=77). Serum samples were collected at time of admission (acute phase) and four to eight weeks after (recovery phase), and multi-omics studies of proteins, lipids and metabolites were performed. Statistical analyses were performed using Metaboanalyst 5.0 and SPSS Statistics 25.0.

Results: Hypertension was present in 25.2% of COVID-19 patients as a comorbid disease. Hypertension was significantly related to COVID-19 severity (p=0.008), in fact, 84.6% of the hypertensive patients belonged to the unfavourable COVID-19 group (severe and critical). The COVID-19 unfavourable group with hypertension had significant differences in biomolecule profile compared to the COVID-19 unfavourable non-hypertensive group. A panel of biomolecules was obtained for the two phases of infection, obtaining a ROC curve with an AUC of 0.897 (95% CI: 0.776-1.000) in the acute phase, and a ROC curve with an AUC of 0.832 (95% CI: 0.720-0.945) in the recovery phase.

Conclusion: The results indicated that hypertension is a frequent comorbid disease in patients with unfavourable COVID-19, which significantly increases the severity of the disease. Gelsolin, myo-inositol and lipids being the most significant compounds in this study that could explain the clinical worsening of COVID-19 patients through different pathways such as immune system process, inflammation, oxidative stress and lipid metabolism.

Keywords: COVID-19 severity, hypertension, multi-omics, gelsolin, myo-inositol, lipids.

2. INTRODUCTION

In 2019 in Wuhan (China), has emerged a novel coronavirus disease (COVID-19), caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a highly transmissible and pathogenic virus which belong to the β coronavirus family [1]. It was declared a pandemic by the World Health Organization (WHO) on 11 March 2020. This virus spread rapidly and even faster with each variant. Since its emergence, the number of reported cases of COVID-19 has exceeded 760 million people worldwide, including more than 6 million confirmed deaths, data reported by WHO [2]. In Spain, that has been one of the countries in Europe most affected by the COVID-19 pandemic, a total of more than 13 million accumulated cases have been registered with 119,000 confirmed deaths [2].

2.1. History

Coronaviruses that infect humans were initially detected and identified during the 1960s [3]. There are coronaviruses that could cause common cold, but others from the same family cause acute respiratory distress syndrome, like the severe acute respiratory syndrome (SARS) coronavirus and the Middle East respiratory syndrome (MERS) coronavirus [4].

There have been two events in the past two decades where a crossover of coronaviruses between animal and human has occurred causing severe illness. The first one happened in the Guangdong province of China, in 2002, when a new β coronavirus was transmitted from bats to human and the Asian palm civet being the intermediary host. This virus was designated as SARS coronavirus (SARS-CoV), affected a total of 8,422 people mostly from China and Hong Kong and caused 916 deaths, with a mortality rate of 11% [5]. Then, in 2012, in Saudi Arabia emerged the MERS coronavirus (MERS-CoV), also with bat origin, but in this case the intermediary host was dromedary camels. This virus affected 2,494 people and caused 858 deaths, with a 34% of fatality rate [5].

The most recent case of coronavirus was in late 2019, the designated SARS-CoV-2 emerged in Wuhan (China) and caused an outbreak of unusual viral pneumonia. Concretely, on December 8 (2019) the first case of COVID-19 was reported in Wuhan. Cases of pneumonia continued to be reported throughout December, it was not until January 9 (2020) that China announced the identification of a novel coronavirus as the causative agent of the pneumonia outbreak. Being highly transmissible, this coronavirus spread fast around the world and on March 11, the WHO defined COVID-19 as a pandemic [6]. So far, this coronavirus has been more incident with a transmission rate of 3-4 while MERS-CoV and SARS-CoV transmission rate was <1 and 3, respectively [3], but less deadly than the previous ones. The fatality rate varies from country to

country but does not exceed 4.9%, and is 2.2% worldwide, information provided by Johns Hopkins Centre for Systems Science and Engineering (CSSE) [7]. This time were bats the origin source of SARS-CoV-2 and pangolins the intermediates hosts [3].

2.2. SARS-CoV-2 transmission

The human-to-human transmission can occur in different ways (Figure 1). One of them is the transmission via aerosols. It is known that the main way of transmission is through respiratory droplets released by an infected person, therefore sneezing and coughing causes SARS-CoV-2 to become airborne, infecting healthy individuals. This can occur through direct contact with the infected person or in spaces where infected aerosols are present. [3]. Also, transmission can also occur when people contact with contaminated inanimate objects, known as fomite transmission [3].

Other way is the Nosocomial-Related infections. Hospitals are an important source of secondary transmission of SARS-CoV-2, because they contain a large number of infected people. In a study by Santarpia et al. [8], surface of common items in the hospital room tested positive for SARS-CoV-2.

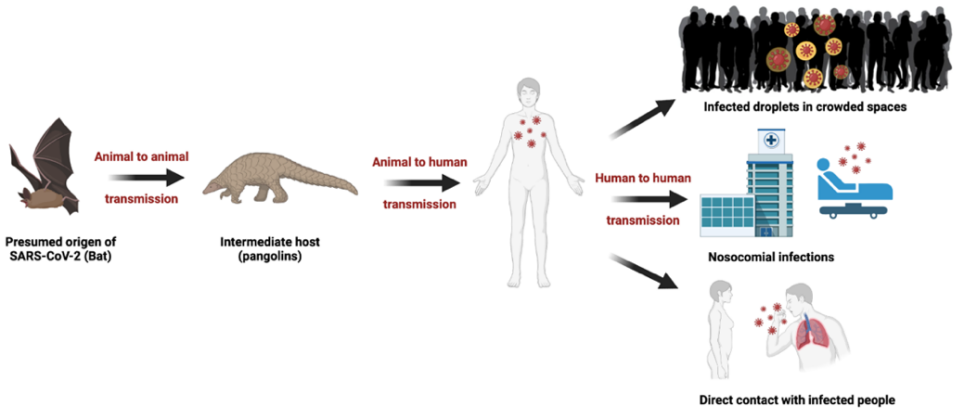


Figure 1. *Zoonotic transmission of SARS-CoV-2. Adapted from [3] with BioRender.com*

2.3. Coronavirus structure

Coronaviruses belongs to the family of Coronaviridae, subfamily *Coronavirinae.* This subfamily has four genera: *Alphacoronavirus, Betacoronavirus* (where SARS-CoV-2 belongs), *Gammacoronavirus* and *Deltacoronavirus* [9]. The genome of the coronaviruses is characterized by being a positive-sense single-stranded RNA (+ssRNA) with a length of 27–32 kb, which is larger than any other RNA viruses. Specifically, the genome size of SARS-CoV-2 is approximately 29.9 kb [9] and shares 79% genome sequence identity with SARS-CoV and 50% with MERS-CoV [10].
SARS-CoV-2 contains four structural proteins: spike protein (S), envelope protein (E), membrane protein (M) and nucleocapsid protein (N) (Figure 2). The nucleocapsid protein is forming the capsid outside the genome and this is further packed by an

envelope, which is associated with the other three structural proteins: membrane, spike and envelope protein. This virus also contains sixteen non-structural proteins (nsp1-16) [9],[11].

The spike glycoprotein (spike protein) mediates the entry of the coronavirus into host cells. This protein is composed of two functional subunits, including the S1 and S2 subunits. Binding to the receptor on host cell and fuse the membranes of viruses and host cells was the function of S1 subunit and S2 subunit, respectively [9].

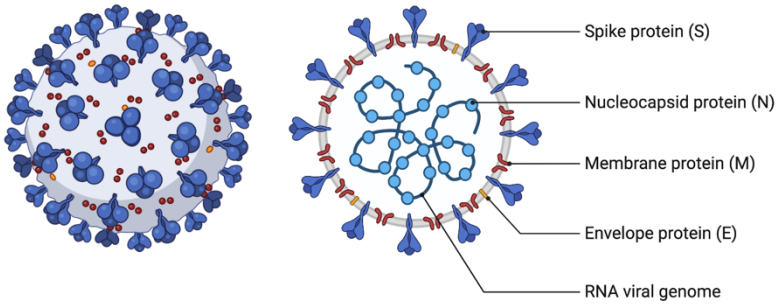


Figure 2. *SARS-CoV-2 structure. Created with BioRender.com*

2.4. SARS-CoV-2 infection

2.4.1. *Viral entry*

The membrane receptor of SARS-CoV-2 is the angiotensin converting enzyme 2 (ACE2). ACE2 is highly distributed throughout the different organs of the body, but its expression is particularly elevated in the epithelia of the lung and small intestine [12]. In addition, this protein is also expressed in the heart, kidneys, liver, brain and testicles [13]. Potential targets of SARS-CoV-2 infection are organs and tissues with higher expression of ACE2, thus, the distribution and abundance of this protein could be related to the clinical symptoms of COVID-19 [13].

A large number of spike protein (S-glycoprotein) are found on the surface of the virion membrane. These can bind to the ACE2 receptor on the surface of human cells, resulting in virus entry by endocytosis or direct membrane fusion [6]. Proteolysis of protein S of SARS-CoV-2 is necessary to activate the endocytic pathway of the virus. Host proteases participate in this proteolysis and activate SARS-CoV-2 entry, mediated by protease TMPRSS2 [6]. After fusion with the cell membrane, the viral genome RNA (+ sense) is released into the host cell cytoplasm. This RNA is translated by the host translation machinery, giving rise to two polyproteins (pp1a and pp1ab) that code for non-structural proteins, thus forming the replication-transcription complex (RTC). The RTC then replicates and synthesizes a set of subgenomic RNAs, which code for structural (S, E and M) and accessory proteins (that interfere with the host innate immune response) [12], [14]. After this, the generated RNA (+ sense), nucleocapsid proteins and envelope glycoproteins assemble through the endoplasmic reticulum (ER) and Golgi apparatus, resulting in the formation of a new virion. Finally, the virion-

containing vesicles fuse with the plasma membrane to release the virus by exocytosis (Figure 3) [12], [14].

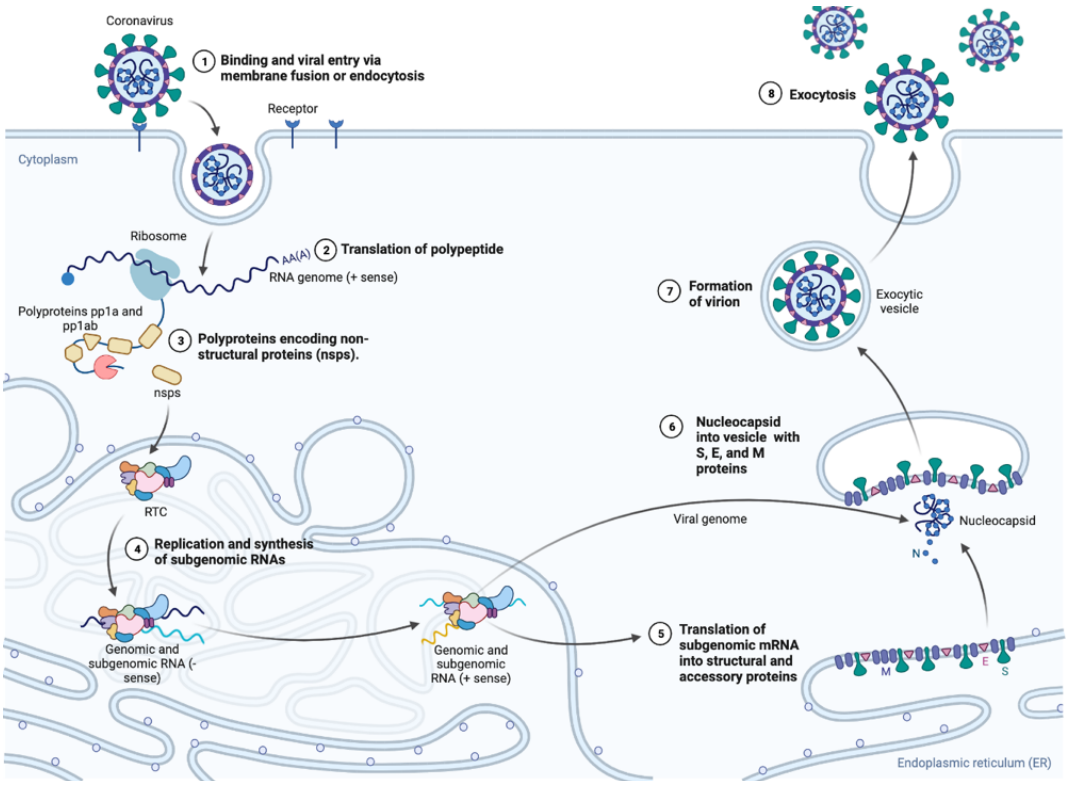


Figure 3. *SARS-CoV-2 entrance to host human cell (simplified), steps from one to eight. Adapted from [12] with BioRender.com*

In the virus entry, it is important to note that lipids play a very important role. SARS-CoV-2 protein S can bind cholesterol on HDL particles, and HDL uptake by SR-B1 (scavenger receptor, class B type 1) facilitates viral entry into cells that coexpress the ACE2 receptor [15]. Furthermore, successful fusion depends on the composition of the viral envelope and the host membrane, as lipid properties affect membrane fluidity and curvature. For example, cholesterol increases membrane fluidity and promotes the negative curvature that is critical for viral fusion, while lysophospholipids (LPL) promote positive curvature and inhibit fusion [15]. In addition, ACE2 locates with lipid rafts (microdomains enriched in cholesterol and glycosphingolipids) of host cell membranes, serve as platforms and facilitate viral entry [15], [16].

Once the virus infects the host, an antiviral and proinflammatory response occurs. Proinflammatory cytokines and chemokines recruit inflammatory cells to foci of infection, resulting in neutrophils and cytotoxic T lymphocytes, along with these cytokines, inducing lung tissue damage and stimulating fibrosis [12].

2.4.2. *Symptomatology*

There is a long and different symptomatology related to COVID-19, depending on the stage and severity of the disease, from no symptoms to critical illness. Most people experience fever (83–99%), cough (59–82%), fatigue (44–70%), anorexia (40–84%), shortness of breath (31–40%) and myalgia (11–35%) (Figure 4) [17]. In addition, other nonspecific symptoms have also been reported, such as sore throat, nasal congestion, headache, diarrhea, nausea and vomiting. Also, cases of loss of smell (anosmia) or taste (ageusia) appear before the onset of respiratory symptoms [17].

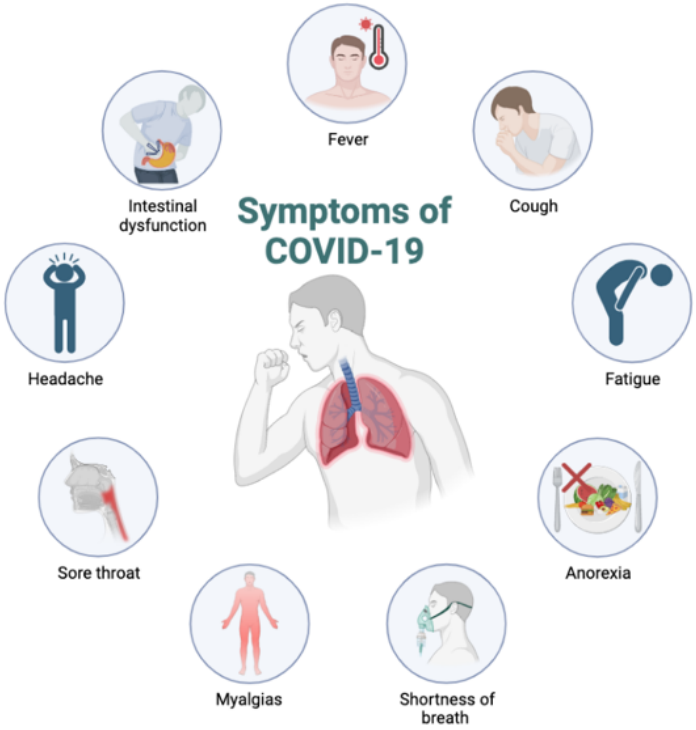


Figure 4*: Clinical symptoms of COVID-19. Created with BioRender.com*

WHO has made a classification of the disease severity into four classes (excluding asymptomatic patients) based on the symptoms presented [17]:

- Mild disease, people without evidence of viral pneumonia or hypoxia.
- Moderate disease, with clinical signs of pneumonia (fever, cough, dyspnea, fast breathing) but no signs of severe pneumonia.
- Severe disease, patients with severe pneumonia.
- Critical disease, those who present acute respiratory distress syndrome (ARDS), sepsis, septic shock or acute thrombosis.

2.4.3. *Risk factors*

All ages are susceptible to COVID-19 disease, but the median age of SARS-CoV-2 infection is around 50 years. In general, older males (>60 years) are more at risk of developing severe respiratory disease that requires hospitalization or may even lead to death [6]. The outcomes worse by the presence of comorbidities, such as diabetes, cancer, obesity, kidney diseases, pulmonary disease or arterial hypertension [6]. But hypertension is the most common pre-existing condition in COVID-19 patients [18].

2.5. Arterial hypertension

Systemic arterial hypertension (hereafter referred as hypertension) is a disease that is considered a chronic noncommunicable disease (NCD) and is associated with increased risk of cardiovascular disease (CVD) [19]. Hypertension is characterized by persistently elevated blood pressure (BP) in the systemic arteries. This BP is expressed as the ratio of systolic BP to diastolic BP. Systolic BP refers to the pressure exerted by the blood when the heart contracts, and diastolic BP refers to the pressure of the blood when the heart relaxes [19], [20]. It is caused by various factors, such as genetic, epigenetic, environmental and social factors. Its cut-off point is defined by a systolic blood pressure (SBP) ≥ 140, and a diastolic blood pressure (DBP) ≥ 90 mm Hg^3 [19], [20].

Hypertension is a leading cause of death and disability from cardiovascular events and stroke. It affects more than one billion adults worldwide, meaning more than 30% of the adult population is affected and is the leading cause of premature death worldwide. About half of those with hypertension do not know they have the condition, and many others do not receive adequate treatment, exposing them to avoidable medical complications and even death [19], [20].

Blood pressure is determined by different factors of the cardiovascular system, such as blood volume, cardiac output (amount of blood pumped by the heart per minute) and arterial tone balance (affected by intravascular volume and neurohormonal systems). The maintenance of physiological blood pressure levels requires a series of complex interactions between various elements, including natriuretic peptides, the sympathetic nervous system (SNS), the immune system and the renin-angiotensin-aldosterone system (RAAS). Malfunction or alteration of such factors leads (or may lead) to increased mean BP [19]. In the RAAS, clinical evidence has been found indicating a possible relationship between COVID-19 pathogenesis and hypertension [18].

2.6. Relationship of COVID-19 and hypertension

Hypertension is the most prevalent risk factor and comorbidity in COVID-19 patients. Prevalence of hypertension ranged between 25% and 45%. Several studies report that

hypertension significantly increases the risk of hospitalization and death in patients infected with SARS-CoV-2 [18], [20], [21]. In addition, an observational study has shown that most patients with COVID-19 have pre-existing hypertension prior to infection [22].

The association between COVID-19 and hypertension should not necessarily imply a causal relationship, since there is a high prevalence in terms of elevated blood pressure, with an incidence of 25% of the adult population and a peak of >60% in the elderly population [23]. Furthermore, hypertension is more frequent and is usually accompanied by other comorbidities, which increases the risk of worsening due to COVID-19 [23]. Thus, the individual role of hypertension in the worsening of SARS-CoV-2 infected patients should be studied.

Through the use of omic sciences, such as lipidomics, metabolomics, proteomics and genomics, it has been possible to study the mechanisms by which these two diseases could be related [18], [22]. These sciences allow you to study a large number of compounds involved in the functioning of the organism. As a result, some studies include possible hypotheses by which these two diseases are related to give a worse outcome.

COVID-19 and hypertension share some mechanisms that could explain this relationship of worsening health in infected persons with this comorbidity. The mechanisms that have been hypothesised to be the most significant are: role of ACE2 and the renin-angiotensin-aldosterone system (RAAS), role of inflammation and immune activation and role of metabolism and disorders of the gastrointestinal tract.

2.6.1 Role of ACE2 and RAAS

Angiotensin-converting enzyme 2 (ACE2) is the cellular receptor for SARS-CoV-2, but has other functions in the organism, such as being a regulator of RAAS, which plays an important role in blood pressure regulation [24].

The RAAS is regulated by two hormones, the angiotensin II (Ang II) and angiotensin I (Ang I). In particular, Ang II, a hypertensive hormone, is a vasoactive peptide that has inflammatory and vasoconstrictive properties and plays a fundamental role in the development of hypertension and its sequelae [18].

As shown in the Figure 5, the ACE/Ang II/Ang II type 1 receptor (AT1R) axis has a positive role in the regulation of RAAS. ACE converts Ang I to Ang II, which stimulates aldosterone release and causes an increment of blood pressure, and also activates AT1R inducing vasoconstriction. On the contrary, the ACE2/Ang (1-7)/ Ang II type 2 receptor (AT2R) axis negatively regulates this system. ACE2 counteracts the action of ACE. ACE2 metabolizes Ang I to Ang (1-9) and Ang II to Ang (1-7). Both Ang (1-9) and Ang (1-7) bind to and activate the AT2R, causing vasodilation and lowering blood

pressure. Furthermore, Ang (1-7) binds to the mitochondrial assembly receptor (MAS) receptor producing protective effects in various target organs, for example by reducing cardiac hypertrophy or reducing pulmonary tissue damage and inflammation [18] (Figure 5).

ACE/Ang II/AT1R and ACE2/Ang (1-7)/AT2R are co-expressed in tissues such as heart, lung and kidney. It is very important for maintaining physiological functions that these two axes are in balance, as an activation of ACE/Ang II/AT1R or deactivation of ACE2/Ang (1-7)/AT2R can lead to organ damage, also affecting an excessively high or low blood pressure [18], [23]. In SARS-CoV-2 infection, when the spike protein of the virus binds to its ACE2 receptor, it causes downregulation of this receptor, thus reducing its potential protective effects and also preventing it from being able to convert Ang II, causing an increment of the levels of this hormone, overactivity of the conventional ACE/Ang II/AT1R axis and decreased Ang (1–7) concentrations. This causes an increase blood pressure, and less organ protection (Figure 5). All this, in a context of pre-existing hypertension prior to infection, could result in an evident state of health deterioration [22], [25].

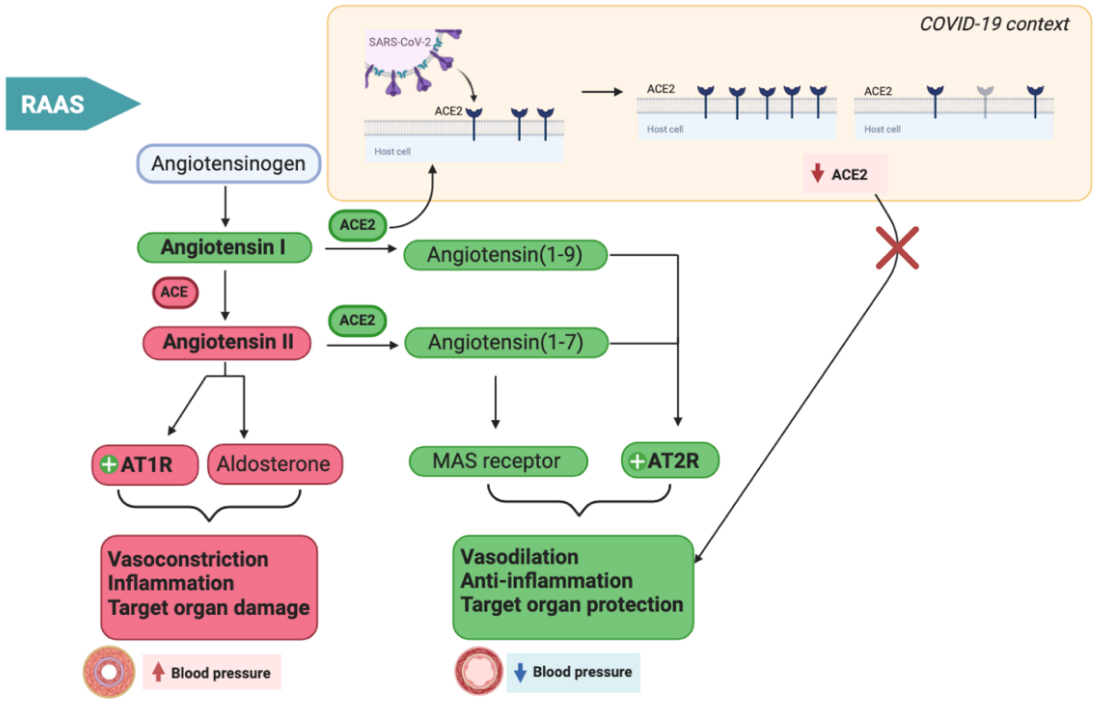


Figure 5. *Role of RAAS in the regulation of blood pressure and possible relationship with COVID-19 RAAS pathway. The red balloons indicate the components of the axis that are responsible for raising blood pressure, while the green balloons are the components of the axis that lower blood pressure. RAAS: renin-angiotensin-aldosterone system; ACE: angiotensin converting enzyme; ACE2: angiotensin converting enzyme 2; AT1R: angiotensin II type 1 receptor; AT2R: angiotensin II type 2 receptor. Adapted from [18] with BioRender.com*

2.6.2 Role of inflammation and immune activation

It is known that SARS-CoV-2 infection activates the innate and adaptive immune responses, that results in the release of proinflammatory factors, and causes hyperinflammation or "cytokine storms" (very high serum levels of cytokines) (Figure 6) [18]. In critically ill COVID-19 patients, an uncontrolled innate response and an altered adaptive immune response result in shock, tissue damage, or multiorgan failure [18].

In addition to this, hypertension has also been shown to activate both the innate and adaptive immune systems, leading to the release of cytokines, contributing to increased cytokine storm, further increasing hyperinflammation [18], [23], [25]. Cytokines released by immune cells promote an acute cardiac inflammatory response and induce infiltration and activation of immune cells in the myocardium. This event promotes vascular dysfunction and organ damage [22] and produces oxidative stress [23].
A study performed by Trump et al. [25] showed that an elevated immune activity due to hypertension is a contributing factor to the increased risk of more critical COVID-19 outcome. They observed a distinct inflammatory predisposition in immune cells in hypertensive patients that were associated with a critical course of SARS-CoV-2 infection. For example, macrophages and neutrophils from these patients showed increased expression of the proinflammatory cytokines [25].

In brief, the activated innate immune response and chronic inflammation in hypertensive patients weaken their initial immunity to combat COVID-19. This overactivated immune response damages organs and may result in multiorgan failure [18].

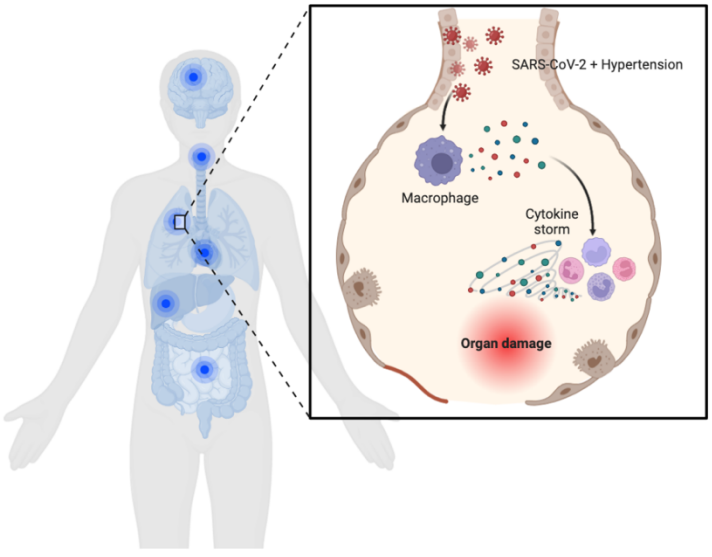


Figure 6. *Formation of cytokine storm and organ damage due to SARS-CoV-2 infection and hypertension. Stepwise pathway of how the virus and hypertension overstimulate immune cells, such as macrophages, which release an excess of cytokines (cytokine storm) leading to organ damage. Created with BioRender.com*

2.6.3 Role of metabolism and gastrointestinal tract disorders

Both SARS-CoV-2 and hypertension play an important role in gastrointestinal tract as ACE2 is also expressed there, implying it to be another site of SARS-CoV-2 infection [26]. For this reason, gastrointestinal upset is a symptom of patients with COVID-19, as mentioned above. ACE2 is an important regulator involved in the maintenance of intestinal microbial homeostasis [26]. In addition, hypertension exerts adverse effects on the gut microbiota, which may be worsened by COVID-19. An imbalance in the intestinal microbiota has been detected in hypertensive patients, as a decrease in short-chain fatty acids, such as butyrate and acetate that possess antiinflammatory properties, and also increasing intestinal permeability was observed (Figure 7) [18], [27]. There is also evidence that SARS-CoV-2 could destroy the gut-blood barrier, affecting the host response to inflammation and leading to multiorgan dysfunction and septic shock [18] (Figure 7).

For these reasons, the increased severity of COVID-19 in hypertension, among other factors, may be due to cumulative depletion or displacement of favorable bacteria in the gut [18].

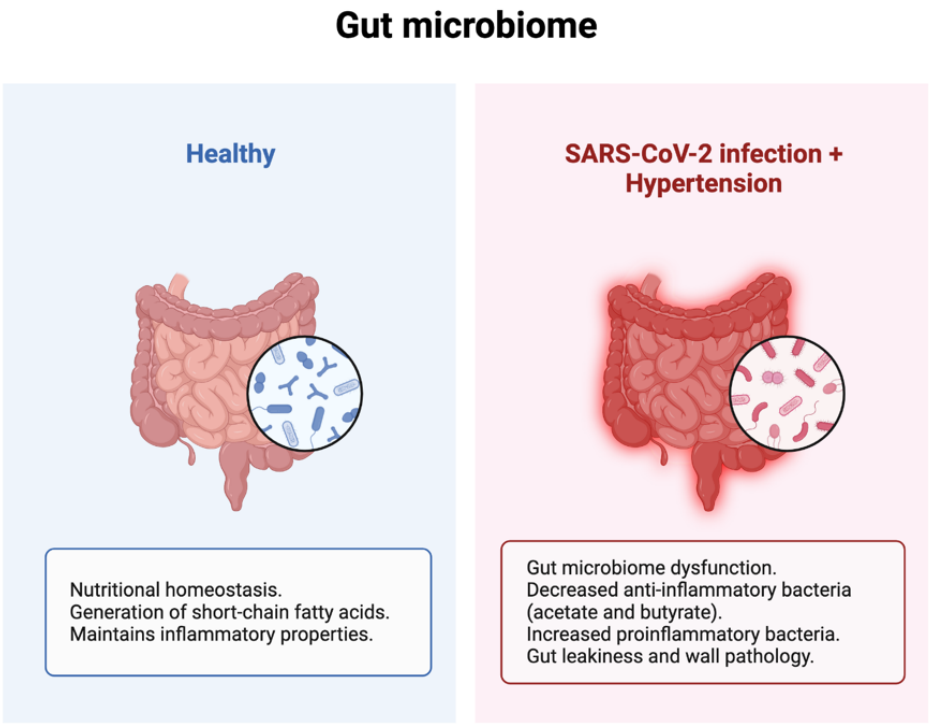


Figure 7. *Comparison of healthy gut (left) and gut from a hypertensive person infected by SARS-CoV-2 (right). Created with BioRender.com*

3. HYPOTHESIS AND OBJECTIVES

Hypertensive patients are more vulnerable to develop severe COVID-19 complications through different joint mechanisms. The altered biomolecules and metabolic pathways by which a person with hypertension and infected by SARS-CoV-2 may have a poor prognosis of the disease are not fully known.

The main hypothesis of this study is that COVID-19 patients present more complications due to hypertension. In addition, it is also hypothesized that patients with hypertension and unfavourable COVID-19 have different lipidomic, metabolomic and proteomic profiles than unfavourable COVID-19 patients without hypertension, which may explain the relationship between COVID-19 severity and hypertension.

The main objective is to find biomolecules or a profile of biomolecules that can be used to explain the relationship between the two diseases and how hypertension could be aggravating a SARS-CoV-2 infection, both in the acute and recovery phase, by lipidomic, metabolomic and proteomic study.

Our secondary objectives would be:

- Identify the metabolic pathways or biological process involved in the hypertension and COVID-19.

- To find biomarker panels that differentiate between unfavourable COVID-19 individuals with and without hypertension to predict a worse outcome and to identify possible therapeutic targets for hypertensive patients with COVID-19.

4. MATERIALS AND METHODS

The methodology applied in the following study is summarised in Figure 9.

4.1. Study design and classification criteria

The study cohort included 103 patients with SARS-CoV-2 infection confirmed by Polymerase Chain Reaction (PCR) within the first 21 days of the infection from two different hospitals: Hospital Universitari de Tarragona Joan XXIII and Hospital Universitari Vall d'Hebron de Barcelona. All patients were recruited between March 2020 and February 2021 (from the first to third waves). According to the inclusion criteria described in "Diagnosis and Treatment Protocol for COVID-19 Patients (version 8 trial)" [28] COVID-19 patients were classified into three groups of severity: mild (n=40), severe (n=34) and critical (n=29) (Figure 8). Classification criteria were shown in Table S1. Blood samples were extracted to patients in two time points: the first was on admission to the hospital (baseline or acute phase) and the second was four to eight weeks after admission (recovery phase). None of the patients included in the study had received the SARS-CoV-2 vaccine at the time of blood sampling. All the information was collected and stored in a database, which contains data related to hospitalization, such as symptoms presented at the time of admission, radiological findings, degree of pneumonia, oxygen therapy required, medical treatment received, comorbidities, biochemical data as well as demographic data and previous diseases of interest. Among these 103 COVID-19 patients, 26 of them had hypertension and 77 were normotensives (Figure 8).

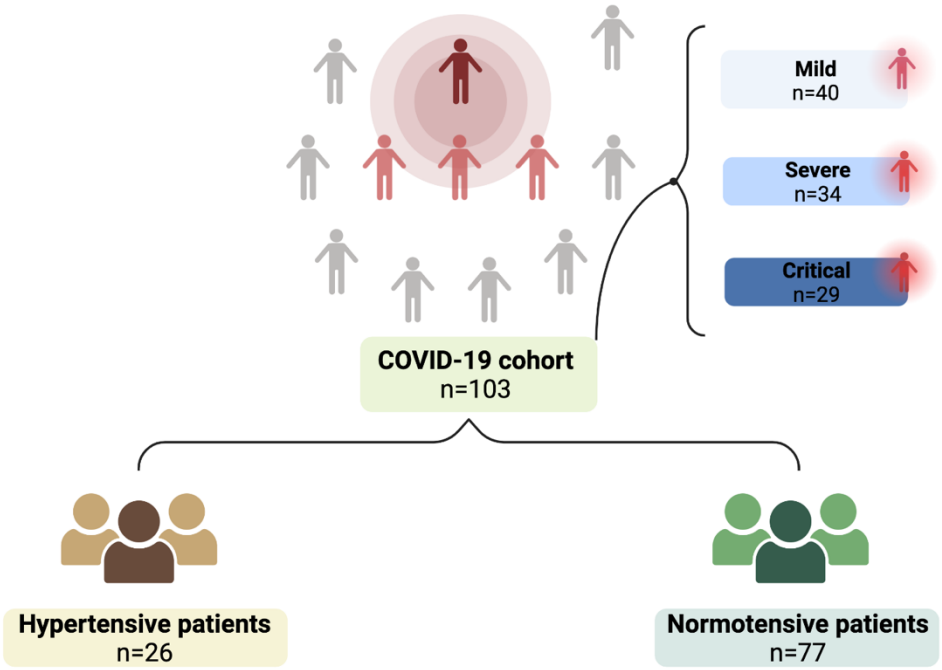


Figure 8. *Study cohort and its subgroups. This COVID-19 cohort was composed by 103 patients and was divided into two groups, hypertensive (n=26) and non-hypertensive (n=77). The COVID-19 patients were classified according to severity: mild (n=40), severe (n=34) and critical (n=29). The n corresponds to the number of patients per group. Created with BioRender.com*

4.2. Samples recruitment

The sampling protocol performed included standard biochemical parameters, blood cell count, and clinical evaluation at inclusion and four to eight weeks after inclusion. Once the samples were collected, abundant protein depletion, metabolite extraction and lipid extraction protocols were performed. Serum samples were stored at a temperature of -80ºC at BioBank of Institut d'Investigació Sanitària Pere Virgili (IISPV) facilities until the moment of use for multi-omics analysis.

4.3. Ethics

Protocols were performed in agreement with the recommendations of the Ethical and Scientific Committees from each participating institution and were approved by the Committee for Ethical Clinical Research following the rules of Good Clinical Practice from the IISPV (079/2020, CEIm IISPV) and from the Vall d´Hebron Hospital (PR(AG)192/2020). The CEIm IISPV is an independent committee, formed by health and non-health professionals, which supervises the correct application of the ethical principles that govern clinical trials and research projects that are carried out in our area, specifically in its methodology, ethics and laws. All subjects or their relatives gave written informed consent in accordance with the Declaration of Helsinki.

4.4. Serum analysis by multi-omics technology

The samples collected were sent to the Centre for Omic Sciences (COS) of Eurecat, as it is a scientific infrastructure equipped with advanced tools and technologies in the fields of multi-omics. Proteomics, lipidomics and metabolomics analyses were performed on serum samples from COVID-19 patients at the COS.

For proteomics, first, prior to analysis, depletion of the most abundant plasma proteins (Albumin, IgG, antitrypsin, IgA, transferrin, haptoglobin and fibrinogen) was performed to increase the number of identified/quantified proteins by Human-7 Multiple Affinity Removal Spin (MARS) cartridge. Protein digestion with Trypsin/Lys-C Protease Mix and the use of peptide 11-plex TMT labelling was performed to compare protein expression. Finally, tandem mass spectrometry (MS/MS) analysis and protein identification and quantification were performed by Proteome Discoverer software using multidimensional protein identification technology (MudPIT).

For lipidomics, a liquid-liquid extraction with chloroform:methanol (2:1) based on Folch procedure was performed for the extraction of hydrophobic lipids. Then, were analysed by Ultra-High Performance Liquid Chromatography coupled with Quadrupole Time-of-Flight (UHPLC-qTOF). The identification of lipid species was performed by matching their accurate mass and tandem mass spectrum, to Metlin-PCDL (Personal Compound Database and Library) from Agilent, which contains more than 40,000 metabolites and lipids. After, these were semi quantified using one internal standard for each lipid family.

Finally, for metabolomics a protein precipitation extraction was performed by adding eight volumes of methanol:water (8:2) to plasma samples. The derivatized compounds were analysed by Gas Chromatography coupled with Quadrupole Time-of-Flight (GC-qTOF). The chromatographic separation was based on Fiehn Method and ionization was done by electronic impact (EI). In addition of targeted compounds from central carbon metabolism, a screening for the identification of more metabolites was performed by matching their EI mass spectrum and retention time to metabolomic Fiehn library (from Agilent) which contains more than 1,400 metabolites. After, these were semi-quantified in terms of internal standard response ratio [29].

4.5. Statistical analysis

First of all, the study database was created and organized. Statistical significance of clinical variables, such as sex, age, COVID-19 severity, comorbidities, COVID-19 symptoms or oxygen requirement, regarding hypertension (grouping variable), was calculated with the $\chi 2$ test for categorical data. Then, the severe and critical groups were grouped together to create an unfavourable COVID-19 group to focus on the severity of the disease, leading the two main groups of this study: COVID-19 unfavourable hypertensive and COVID-19 unfavourable non-hypertensive patients. Significant differences between hypertension groups were performed by the Mann-Whitney U test (non-parametric test for two independent samples). This analysis was done for both values obtained at entry (baseline) and after four to eight weeks. The recovery phase data were normalized by calculating the ratio between the data at four to eight weeks divided by the data at the time of admission (baseline). All the Statistical analyses described were performed using SPSS Statistics (version 25.0, SPSS Inc., Chicago, IL). The results were considered statistically significant at $P < 0.05$. Graphical representations were generated with Metaboanalyst 5.0 and STRING 11.5 software.
STRING is a database of known and predicted protein-protein interactions, included direct (physical) and indirect (functional) associations, and allows to construct a protein network. STRING software was used to analyse possible correlations between significant proteins obtained.

Metaboanalyst is a platform dedicated to multi-omic data analysis, visualization, and functional interpretation through an easy web interface. Volcano Plot, Heatmap, Random Forest and Joint pathways analysis and representations were done using Metaboanalyst 5.0 software. A Volcano Plot was performed to see the evolution of the different compounds over time (from admission to four to eight weeks later) and which of them varied (increasing or decreasing) significantly according to the group. Volcano plot is a statistical analysis that combines results from Fold Change (FC) analysis and T-tests into one single graph, allowing intuitively to select significant features based on either biological significance, statistical significance, or both. A Heatmap was done to analyse which metabolites were significantly downregulated or upregulated in our groups. Heatmap provides intuitive visualization of a data table to identify features that

are high or low. Each coloured cell on the map corresponds to a concentration value in the data table. Then, Random Forest analyses were performed to determine the proteins, lipids and metabolites with higher accuracy in classifying patients according to hypertensive group. Random forest is a regression technique that uses randomization, among other processes, to achieve a high degree of predictive accuracy. The joint pathway analysis is based on linking the protein, metabolite and lipid data with the different metabolic pathways and biological processes that are affected, resulting in a graph showing bubbles with different colours and sizes depending on the significance. The enrichment analysis module (Joint pathway) conducts analysis for human and mammalian species using multiple libraries that encompass approximately 6,300 groups of metabolite sets to help identify significant associations and enrichments between metabolite sets and the biological context under study.

Finally, Binary logistic regression models and Receiver operating characteristic (ROC) curves were generated by SPSS Statistics 25.0 to evaluate the potential accuracy of the selected biomarkers to discriminate patients as hypertensive or non-hypertensive with unfavourable COVID-19. The selected biomarkers were chosen from the top selected proteins, metabolites and lipids in Random Forest analyses. The key objective is to discover biomarkers that have the ability to classify various conditions or diseases. These biomarkers, whether a single metabolite or a group of metabolites, should exhibit high sensitivity (true-positive rate) and specificity (true-negative rate). To accomplish this, predictive models are constructed using one or multiple biomarkers, and their performance and reliability are assessed to effectively classify new patients as either diseased or healthy.

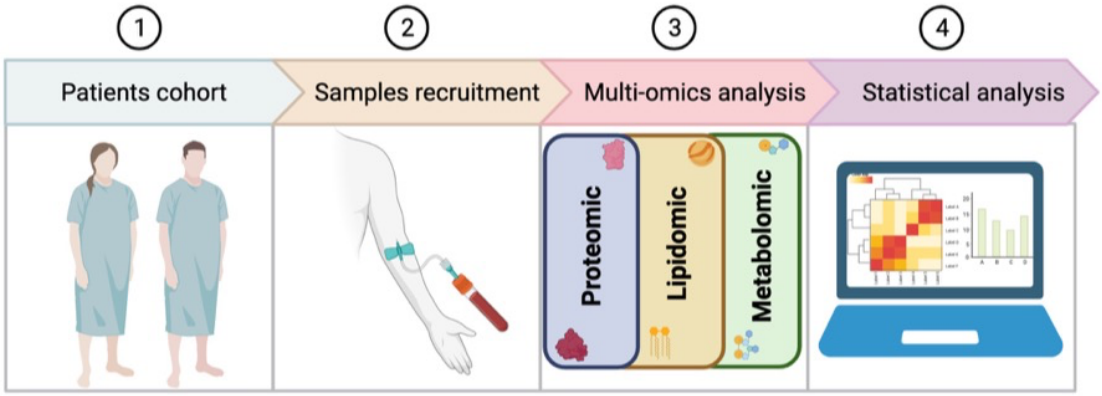


Figure 9. *Methodology of the study. Created with BioRender.com*

5. RESULTS

5.1. Characteristics of the patient cohort

The cohort comprised 103 nonvaccinated patients with a COVID-19-positive diagnosis was classified into hypertensive (n=26) and non-hypertensive (n=77). As expected, in this cohort, the median age is higher in patients with hypertension (66.5) compared to patients without hypertension (49). In addition, male sex predominated in the hypertensive group (61.5%) and females were more abundant in the non-hypertensive group (63.6%). Factors such as age, sex, exercise, oxygen requirement, pneumonia and severity of COVID-19 were significant in patients with hypertension. Comorbidities such as cardiovascular events and obesity were significantly related to hypertensive patients (Table 1).

These patients had other comorbidities such as obesity, chronic obstructive pulmonary disease (COPD), obesity, cancer, Human Immunodeficiency Virus (HIV) and other cardiovascular events, but only obesity and cardiovascular events were significantly high in hypertension patients. In terms of symptoms reported in acute infection, pneumonia was highly prevalent in hypertensive patients (84.6%) compared to those without hypertension (57.1%), according to that the severity COVID-19 was significantly related to hypertension group. Most hypertensive patients had severe (n=14) and critical (n=8) COVID-19, meaning 22 patients with an unfavourable prognosis (84.6%), in contrast to almost half (46.8%) of non-hypertensive patients with mild cases of COVID-19. Moreover, the oxygen requirement had a significant relationship with being hypertensive (80.8%), compared to only 48.1% of normotensive patients (p=0.004). Finally, the patients who practice exercise were mostly normotensive (p=0.019) (Table 1).

Six patients died from COVID-19, three of whom belonged to the hypertensive group and three to the non-hypertensive, according with the non-significant result between groups, although these six patients belonged to the critical group of COVID-19 (Table 1).

Table 1. *Demographic and clinical features of study cohort.*

Variables	COVID-19 study cohort		
	Hypertensive (n=26)	**Non-hypertensive** (n=77)	**P value**
Sex	Women: 10 (38.5) Men: 16 (61.5)	Women: 49 (63.6) Men: 28 (36.4)	0.025
Age	66.5 (55.2-74.7)	49 (38.0-59.0)	<0.0001
Comorbidities – no. (%)			
Obesity	9 (34.6)	10 (13.0)	0.014
Cardiovascular disease	7 (26.9)	3 (3.9)	0.001
COPD	0 (0)	2 (2.6)	n.s
Cancer	1 (3.8)	3 (3.9)	n.s
HIV	0 (0)	0 (0)	n.s
COVID-19 Symptoms			
Fever	21 (80.8)	54 (70.1)	n.s
Cough	16 (61.5)	55 (71.4)	n.s
Pneumonia	22 (84.6)	44 (57.1)	0.017
Respiratory insufficiency	14 (53.8)	29 (37.7)	n.s
Arthromyalgias	3 (11.5)	15 (19.5)	n.s
Dyspnea	9 (34.6)	32 (41.6)	n.s
Thoracic pain	4 (15.4)	6 (7.8)	n.s
Cephalea	3 (11.5)	21 (27.3)	n.s
Ageusia	0 (0)	5 (6.5)	n.s
Dysgeusia	0 (0)	2 (2.6)	n.s
Diarrhea	8 (30.8)	19 (24.7)	n.s
Nausea/vomiting	3 (11.5)	5 (6.5)	n.s
Odynophagia	4 (15.4)	10 (13.0)	n.s
Asthenia	6 (23.1)	14 (18.2)	n.s
Anosmia	0 (0)	8 (10.4)	n.s
Severity of COVID-19	Mild: 4 (15.4) Severe: 14 (53.8) Critical: 8 (30.8)	Mild: 36 (46.8) Severe: 20 (26.0) Critical: 21 (27.3)	0.008
Oxygen therapy			
Oxygen required	21 (80.8)	37 (48.1)	0.004
Lifestyle			
Exercise	7 (26.9)	44 (57.1)	0.019
Alcohol	3 (11.5)	8 (10.4)	n.s
Smoker	2 (7.7)	6 (7.8)	n.s
Mortality			
Exitus	3 (11.5)	3 (3.9)	n.s

Data are presented as n (%) or median (25th-75th interquartile range). P values were computed using non-parametric Mann-Whitney test for continuous data and $\chi 2$ test for categorical data. P value < 0.05 was considered significant. COPD, chronic obstructive pulmonary disease; HIV, Human Immunodeficiency Virus; n.s non-significant.

Having confirmed that hypertension is significantly related to the severity of COVID-19 and therefore they have a direct relationship, from here on our study will focus on unfavourable COVID-19 patients (n=63), that correspond to the severe and critical ones, in order to study the role of hypertension in these patients. As shown in Table 1, there are only four patients who are COVID-19 mild, therefore they had to be discarded in this study, since it would not be possible to make a good comparative study. Then, 22 were hypertensive and 41 were non-hypertensive in this new cohort (Figure 10).

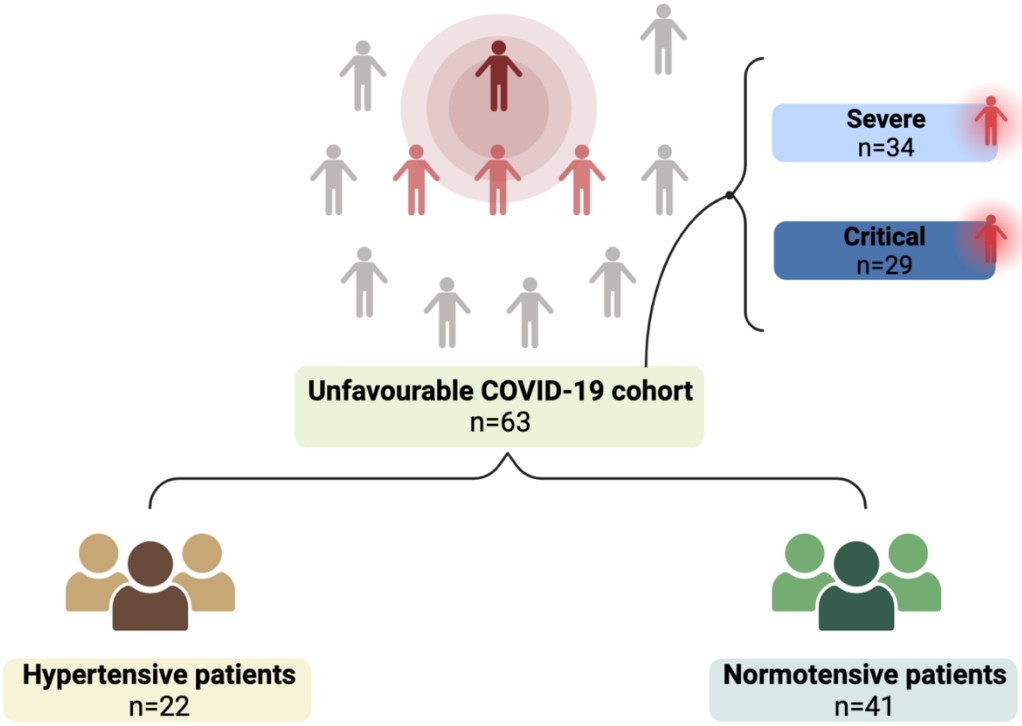


Figure 10. *New study cohort and its subgroups. The 63 unfavourable COVID-19 patients correspond to severe (n=34) and critical (n=29) groups. This COVID-19 cohort was divided into two groups, hypertensive (n=22) and non-hypertensive (n=77). The n corresponds to the number of patients per group. Created with BioRender.com*

Therefore, from here on, we made a comparison between these two groups (COVID-19 unfavourable hypertensives and COVID-19 unfavourable non-hypertensives) and we studied their metabolic, lipidomic and proteomic differences at two different points, in the acute phase and in the recovery phase of the infection, to analyse what factors could be related to this worsening of COVID-19 patients with hypertension.

5.2. Biochemical differences in hypertensive patients between the acute and recovery phase of unfavourable COVID-19

Once the samples were collected at the beginning (acute phase) and four to eight weeks later (recovery phase) of the SARS-CoV-2 infection, we analysed the differences between these two stages of infection in hypertension and normotensive patients with unfavourable COVID-19 to identify what molecular relationship hypertension might have with COVID-19 and why hypertensive patients have a worse prognosis.

5.2.1. Biomolecules

A Mann-Whitney U test was performed on lipids, proteins and metabolites levels using SPSS Statistics 25.0 to investigate which compounds, in each phase of the infection, could be altered due to hypertension and unfavourable COVID-19. This analysis determined 18 proteins, 3 metabolites and 30 lipids in the acute phase, and 8 proteins, 5 metabolites and 15 lipids in the recovery phase, with a significant increase or decrease in relation to hypertension and COVID-19 severity. All analyses performed hereafter were done using these significant biomolecules.

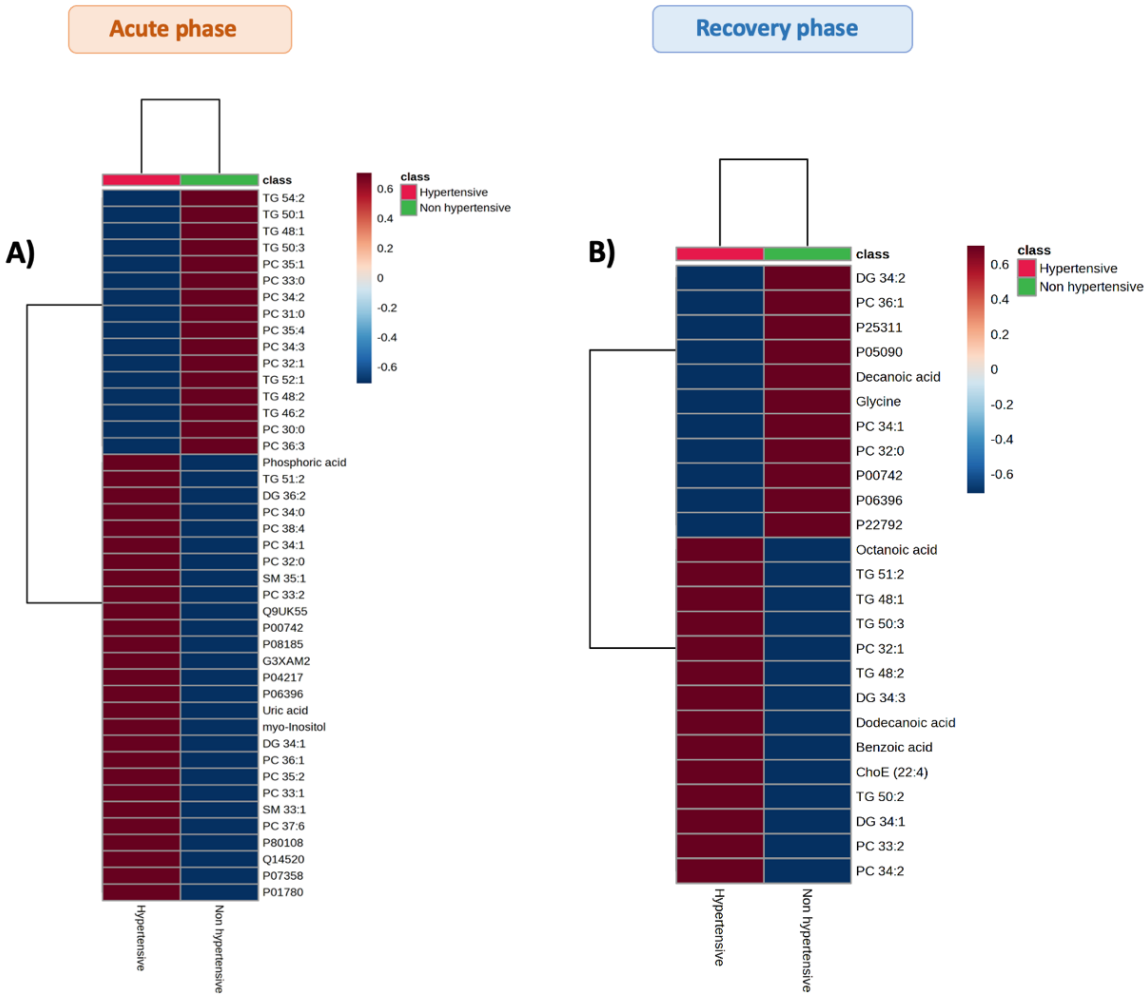


Figure 11. *Distribution of significant biomolecules in accordance with hypertension in unfavourable COVID-19 patients. Significant biomolecules* ***A)*** *in the acute phase of SARS-CoV-2 infection and* ***B)*** *in the recovery phase of SARS-CoV-2 infection. Heatmaps were made using Metaboanalyst 5.0 and show at the left the hypertensive group and non-hypertensive group in the right. Mean values for each compound (rows) in each group (columns) are color-coded based on relative abundance, low (blue) and high (red). Significant biomolecules (p values < 0.05) were determined by the nonparametric Mann-Whitney test. TG: Triglyceride; DG: Diglyceride; SM: Sphingomyelin; ChoE: cholesterol esters; PC: Phosphatidylcholine.*

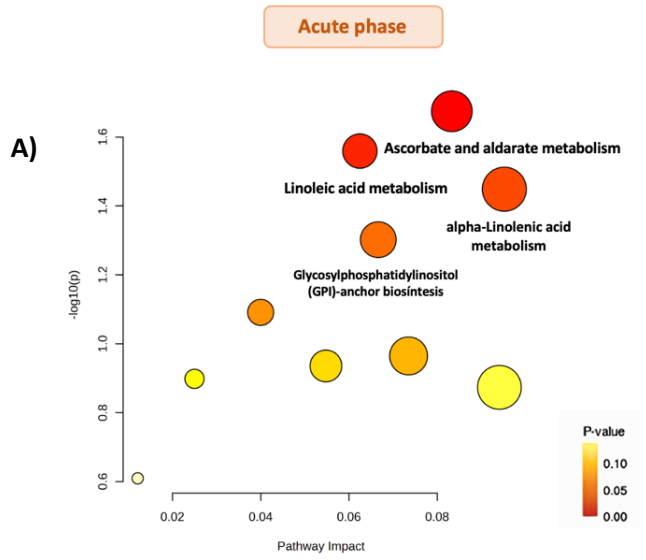
As shown in Heatmaps represented using Metaboanalyst 5.0 (Figure 11), a greater number of molecules was significant in the acute phase (Figure 11A) in comparison to the recovery phase (Figure 11B).

In the acute phase, of the 43 compounds, sixteen compounds were significantly decreased and 27 were increased in hypertensive patients (Figure 11A). In the non-hypertensive group, all the biomolecules that increased significantly compared to the hypertensive group were lipids, concretely most of triglycerides, whereas in the hypertensive group increased lipids, metabolites and proteins. Therefore, all the compounds that decreased in hypertensive patients were lipids, contrary to normotensives.

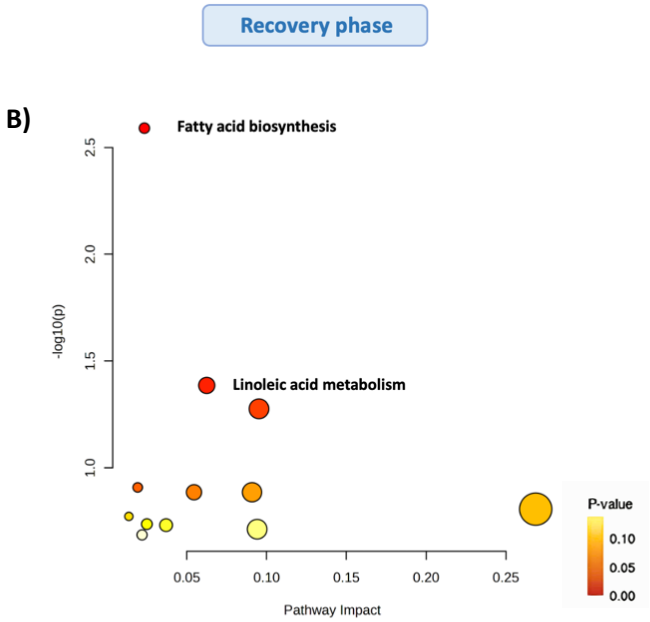
In the recovery phase, a total of 25 compounds were found to be differentially expressed in COVID-19 unfavourable hypertensives and non-hypertensives. Eleven of these compounds were found to be decreased and 14 increased in hypertensive patients. In contrast to the acute phase, in the recovery phase most lipids and all triglycerides increased in the hypertensive group. In this phase all proteins are decreased in the hypertensive group, contrary to normotensives that increased (Figure 11B).

5.2.2. Metabolic pathways

In relation to the enriched pathways in which biomolecules related to hypertension and unfavourable COVID-19 were involved, a Joint-Pathways analysis was performed by Metaboanalyst 5.0 with the same significant components as previous analysis. To investigate how hypertension could worsen the prognosis of COVID-19, it is important to know which metabolic pathways were affected by both diseases.



Pathway name	Match	Impact	P-value	-log(P)
Ascorbate and aldarate metabolism	1/13	0.083	0.021	1.675
Linoleic acid metabolism	1/17	0.063	0.028	1.559
alpha-Linolenic acid metabolism	1/22	0.095	0.036	1.449
Glycosylphosphatidylinositol (GPI)-anchor biosynthesis	1/31	0.067	0.049	1.302



Pathway name	Match	Impact	P-value	-log(P)
Fatty acid biosynthesis	3/129	0.023	0.003	2.590
Linoleic acid metabolism	1/17	0.063	0.041	1.386

Figure 12. *Metabolic pathways related to hypertension and unfavourable COVID-19. Joint pathway analysis using Metaboanalyst 5.0, representing metabolic pathways sorted by pathway impact and -log10 (p) **A)** in the acute phase and **B)** in the recovery phase of COVID-19. Of the metabolic pathways represented, only those with a P value <0.05 were considered (bubbles displaying name of pathway). The size of bubbles shows pathway impact value, and the colour denotes the level of significance by means of p-values. Inserted tables showing the name of the significant metabolic pathways with their respective data.*

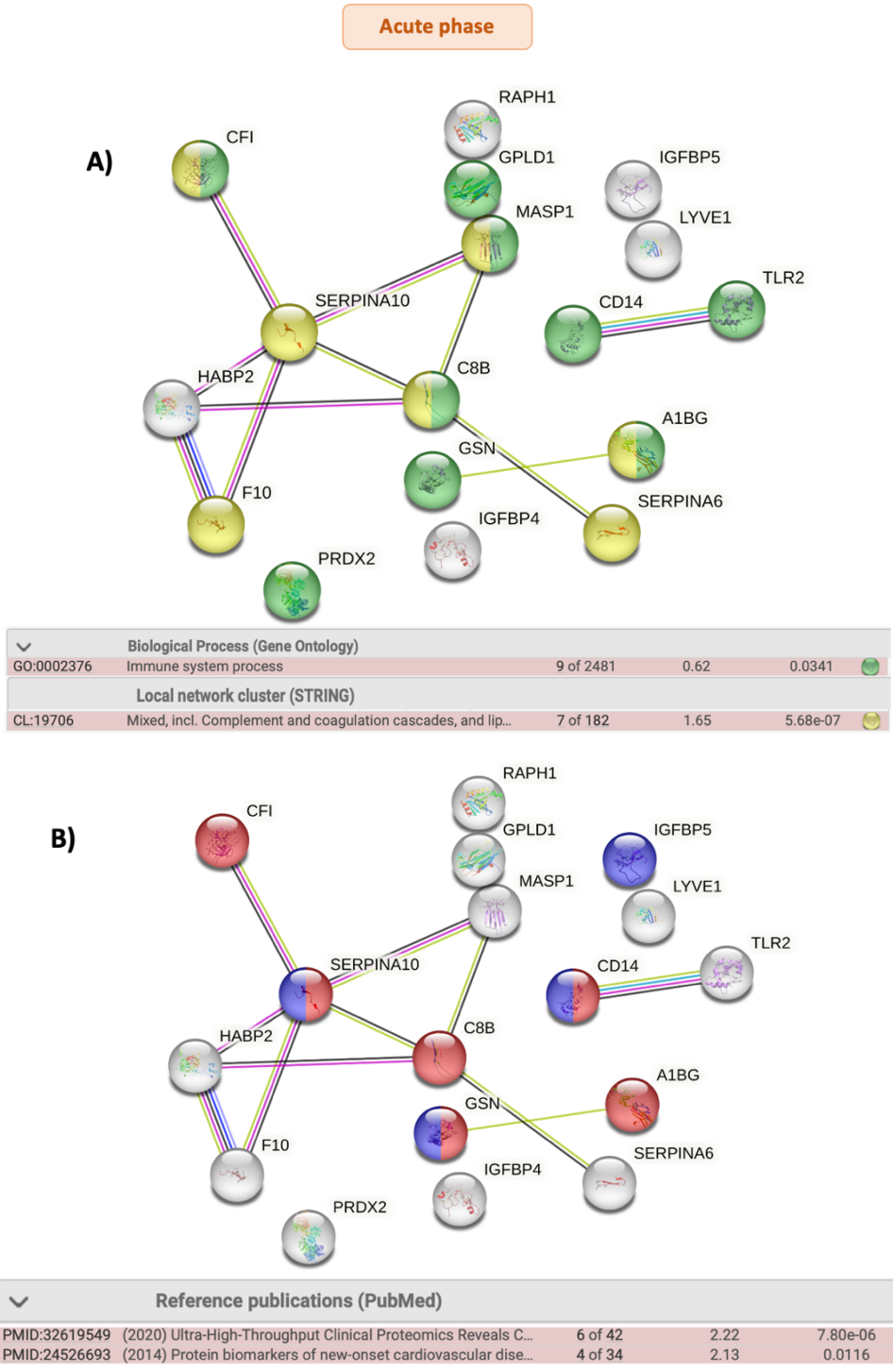
As shown in Figure 12, in the acute phase of the infection there were more metabolic pathways affected compared to the recovery phase, as biomolecules in the previous analysis. This finding may indicate that the organism is more altered in hypertensive people when the SARS-CoV-2 infection is still acute.

Specifically, in the acute phase, four metabolic pathways, Ascorbate and aldarate metabolism, Linoleic acid metabolism, alpha-Linolenic acid metabolism and Glycosylphosphatidylinositol (GPI)-anchor biosynthesis, were significantly altered, ordered from most to least significant. Myo-inositol was involved in Ascorbate and aldarate metabolism, phosphatidylcholine in Linoleic acid metabolism and in alpha-Linolenic acid metabolism and finally, the protein Glycosylphosphatidylinositol Specific Phospholipase D1 (GDP1) in Glycosylphosphatidylinositol (GPI)-anchor biosynthesis (Figure 12A).

On the other hand, in the recovery phase, we found only two metabolic pathways that were significantly altered due to hypertension and COVID-19 severity. These pathways were fatty acid biosynthesis and linoleic acid metabolism. Of note, these two metabolic

pathways were lipid pathways, mainly responsible for fatty acid synthesis. In this case, Dodecanoic acid, Decanoic acid and Octanoic acid were involved in Fatty acid biosynthesis, and the Phosphatidylcholine in the metabolism of Linoleic acid (Figure 12B).

Also, we wanted to investigate what connection could have the proteins involved in hypertensive and unfavourable COVID-19 in order to elucidate which other pathways might be affected due to the two diseases. The significant proteins by the Mann-Whitney test were analysed using STRING. The results were shown in the Figure 13.



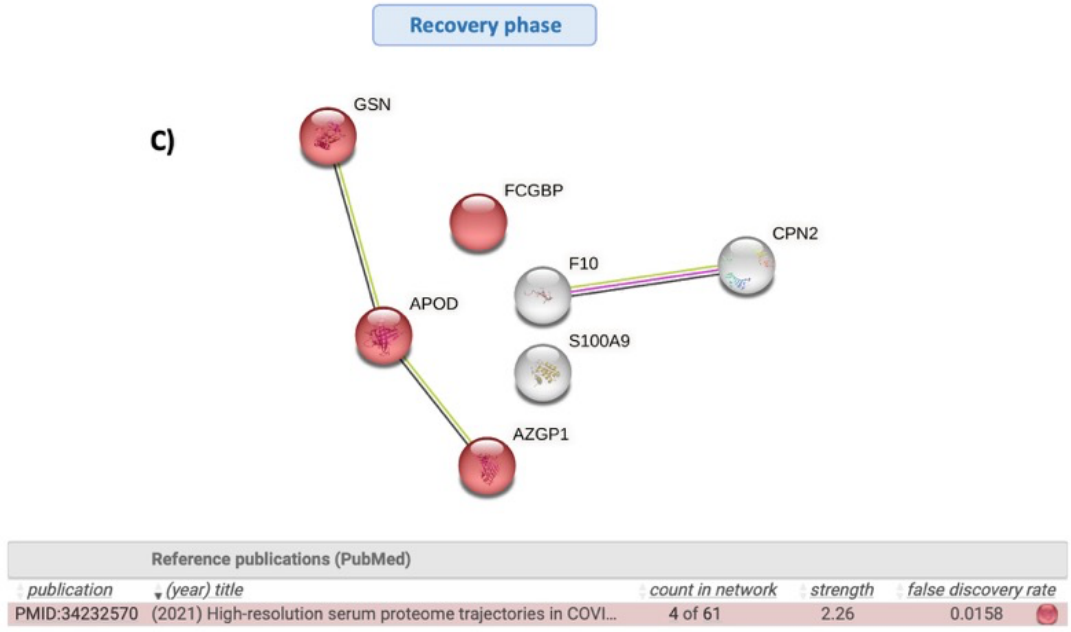


Figure 13. *Protein networks in patients with hypertension and unfavourable COVID-19 by STRING. Proteins named by gene. The colors of the balls indicate which proteins participate in the same process.* ***A)*** *and* ***B)*** *Protein interconnection in the acute phase and* ***C)*** *in the recovery phase.* ***A)*** *In green: proteins involved in immune system processing; in yellow: proteins that belong to the Complement and coagulation cascades and lipoprotein particles;* ***B)*** *in red: proteins that have been mentioned in the publication entitled "Ultra-High-Throughput Clinical Proteomics Reveals Classifiers of COVID-19 Infection"; in violet: proteins that have been mentioned in "Protein biomarkers of new-onset cardiovascular disease: prospective study from the systems approach to biomarker research in cardiovascular disease initiative";* ***C)*** *in red: proteins mentioned in the article entitled "High-resolution serum proteome trajectories in COVID-19 reveal patient-specific seroconversion". Significant proteins (p values < 0.05) were determined by the nonparametric Mann-Whitney test.*

There was more protein data in the acute phase than in the recovery phase. In the acute phase, we found seventeen significant proteins and some of them were interconnected, whereas in the recovery phase only seven were significant. Twelve of them were involved in immune system processes or in the complement and coagulation cascades and lipoprotein particles in acute phase. This is interesting since these are processes that occur both in hypertension and COVID-19, even more so if infection is severe. Complement Factor I (CFI), Mannan-binding lectin serine protease 1 (MASP1), Complement component C8 beta chain (C8B) and Alpha-1B-glycoprotein (A1BG) pertained to both processes (Figure 13A). In addition, some of these proteins have already been mentioned in previous two publications, one focused on COVID-19 and the other on cardiovascular events, including hypertension. Three of these proteins [Protein Z-dependent protease inhibitor (SERPINA10), Monocyte differentiation antigen CD14 (CD14) and Gelsolin (GSN)] were found in both articles (Figure 13B). This could

be important for our study as these proteins are related in the two diseases we are studying.

In the recovery phase, of the seven proteins shown in the Figure 13C, four [GSN, Apolipoprotein D (APOD), Zinc-alpha-2-glycoprotein (AZGP1) and IgGFc-binding protein (FCGBP)] were mentioned in a publication based on the seroconversion phase of COVID-19. Interestingly, the seroconversion phase is the moment in which the patient begins to acquire antibodies, that is, weeks after infection, in accordance with the recovery phase that we are studying (Figure 13C).

5.2.3. Biomarkers based on hypertension and unfavourable COVID-19

Random Forest were performed with the data of significant biomolecules to visualize the 15 compounds in the acute phase and the recovery phase, could best distinguish between unfavourable COVID-19 patients with hypertension from those without hypertension. This is important to study which are the key biomolecules or biological pathways involved in these two diseases.

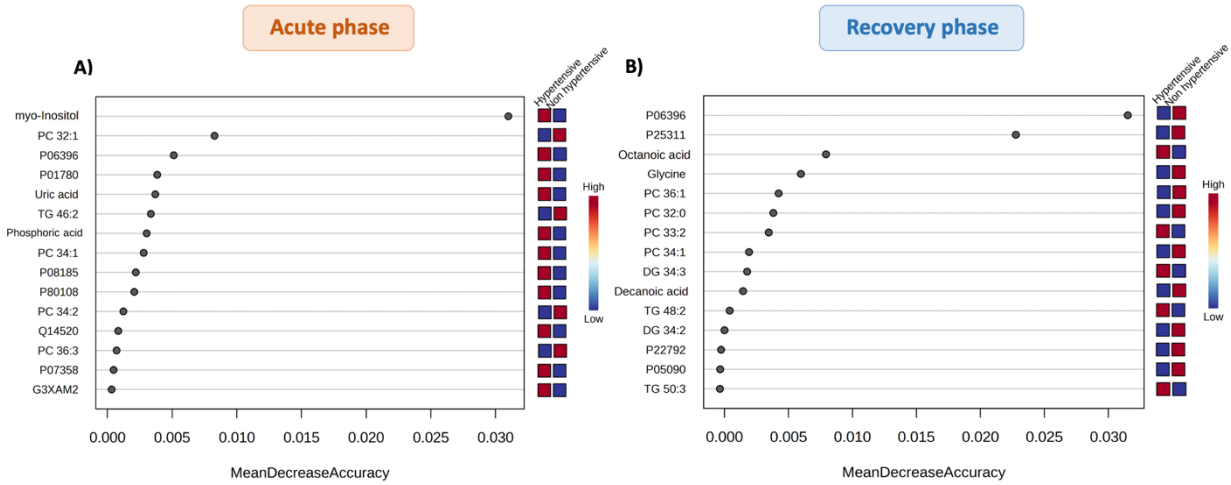


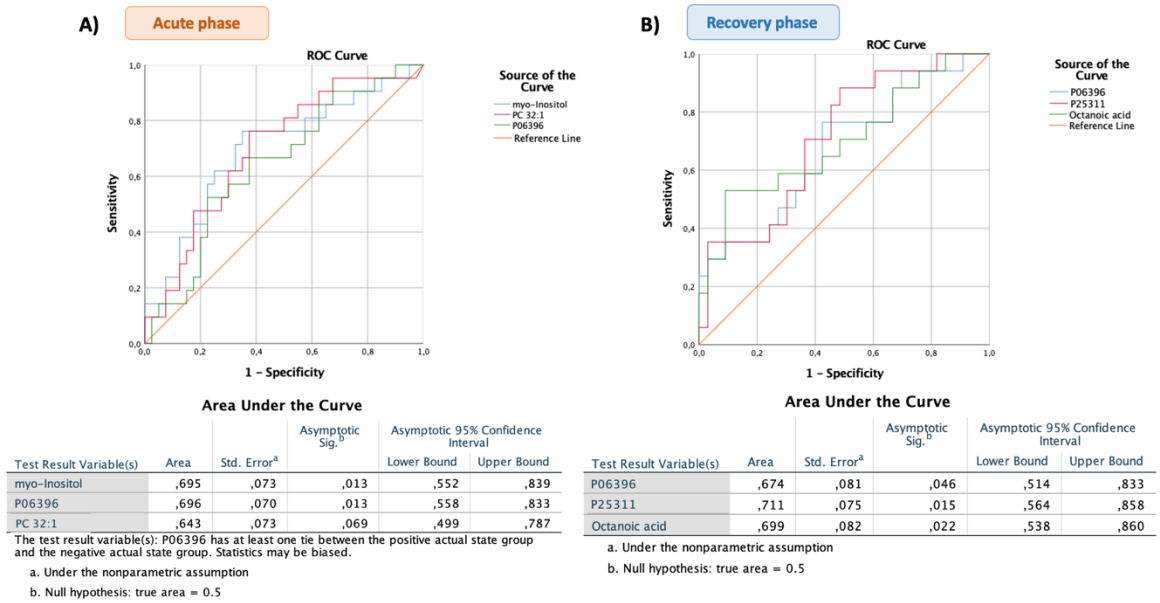
Figure 14. *Main biomolecules that best distinguish between the two groups according to hypertension and unfavourable COVID-19. Random forest of significant proteins, metabolites and lipids by Metaboanalyst 5.0 shows the 15 compounds ranked by classification accuracy to distinguish between hypertensive and non-hypertensive patients with unfavourable COVID-19* ***A)*** *belong to the acute phase and* ***B)*** *to the recovery phase. The colours represent the accuracy power on the group (red for high and blue for low). Y-axis indicates the name of the compounds and X-axis the mean decrease accuracy. PC: Phosphatidylcholine; TG: Triglyceride; DG: Diglyceride.*

In the acute phase, myo-inositol differentiated the two groups better than the others, followed by Phosphatidylcholine 32:0 (PC 32:0) and protein Gelsolin (P06396). Myo-inositol and Gelsolin were increased in COVID-19-unfavourable hypertensive patients

and decreased in non-hypertensives. In contrast, PC 32:1 was decreased in hypertensives whereas increased in normotensives (Figure 14A).

In the recovery phase, the compounds that are able to distinguish better between the two groups were the Gelsolin protein (P06396), Zinc-alpha-2-glycoprotein (ZA2G, P25311) and Octanoic acid. The Gelsolin protein was the best discriminatory compound, and this protein was decreased in COVID-19 unfavourable hypertensive patients, as the protein Zinc-alpha-2-glycoprotein (ZA2G, P25311). Contrary, Octanoic acid was increased in the hypertensive group and, therefore, decreased in the non-hypertensive group (Figure 14B).

Finally, we wanted to identify key biomarkers that could best distinguish between unfavourable COVID-19 patients with hypertension from without hypertension and could be predict when a patient with hypertension and COVID-19 progresses unfavourably. For this purpose, the top three compounds of each Random Forest representation (Figure 14A and Figure 14B) were selected to perform ROC curves: myo-inositol, Phosphatidylcholine 32:0 (PC 32:1) and Gelsolin protein (P06396) for the acute phase, and Gelsolin protein (P06396), Zinc-alpha-2-glycoprotein (P25311) and Octanoic acid for the recovery phase.



Area Under the Curve

Test Result Variable(s)	Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
myo-Inositol	,695	,073	,013	,552	,839
P06396	,696	,070	,013	,558	,833
PC 32:1	,643	,073	,069	,499	,787

The test result variable(s): P06396 has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased.

a. Under the nonparametric assumption

b. Null hypothesis: true area = 0.5

Area Under the Curve

Test Result Variable(s)	Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
P06396	,674	,081	,046	,514	,833
P25311	,711	,075	,015	,564	,858
Octanoic acid	,699	,082	,022	,538	,860

a. Under the nonparametric assumption

b. Null hypothesis: true area = 0.5

Figure 15. *Receiver operating characteristic (ROC) curves analysis to distinguish hypertensive patients with COVID-19 unfavourable from non-hypertensives.* ***A)*** *ROC curves of myo-inositol, PC 32:1 and P06396 for acute phase.* ***B)*** *ROC curves of P06396, P25311 and Octanoic acid for recovery phase. PC: Phosphatidylcholine.*

In the acute phase, the three compounds give similar values of area under the curve (AUC): 0.695 (95% confidence interval (CI): 0.552-0.839) in Myo-inositol, 0.696 (95% CI: 0.558-0.833) in Gelsolin, and 0.643 (95% CI: 0.499-0.787) in PC 32:1 (Figure 15A).

In the recovery phase, the three compounds also give similar AUCs: 0.674 (95% CI: 0.514-0.833) in Gelsolin (P06396), 0.711 (95% CI: 0.564-0.858) in Zinc-alpha-2-glycoprotein (P25311) and 0.699 (95% CI: 0.538-0.860) in Octanoic acid (Figure 15B).

We wanted better AUC values for the ROC curves. Therefore, ROC curves were made by binary logistic regression analysis with the three compounds of each phase, in order to see if the combination increases the discriminatory power.

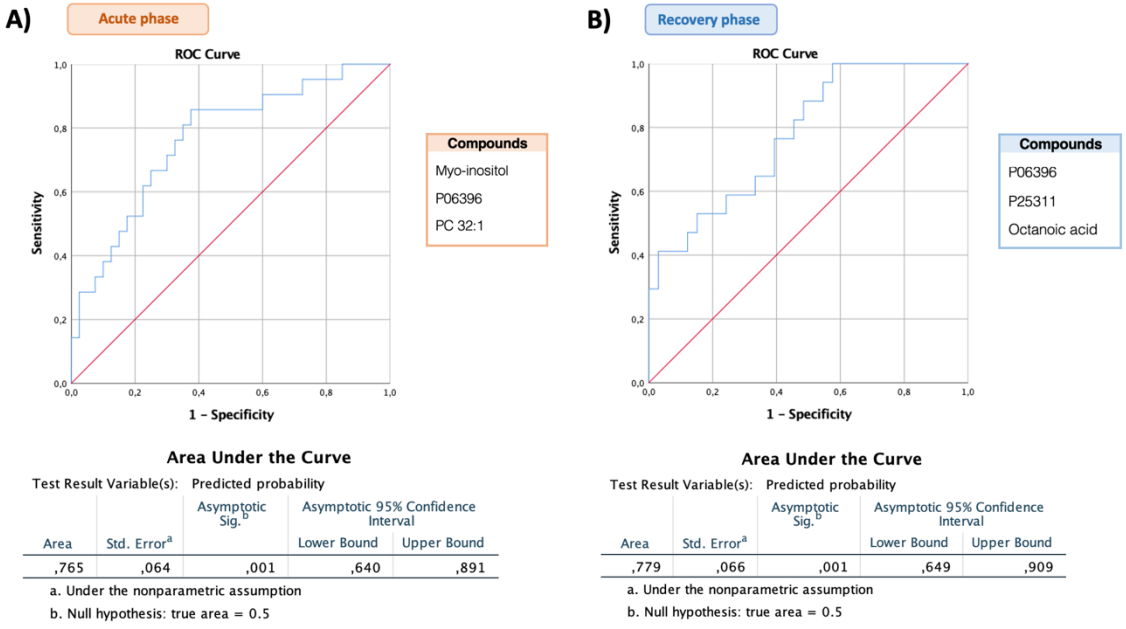


Figure 16. *Receiver operating characteristic (ROC) curves analysis by binary logistic regression. ROC curves of three different compounds combined to distinguish hypertensive patients with COVID-19 unfavourable from non-hypertensives.* ***A)*** *ROC curve for acute phase.* ***B)*** *ROC curve for recovery phase. PC: Phosphatidylcholine.*

The combination of myo-inositol, Gelsolin (P06396) and PC 32:1 results in a ROC curve with an AUC of 0.765 (95% CI: 0.640-0.891). This AUC value is higher than the AUC of the individual biomolecules, indicating that the combination discriminated significantly better between the two groups in the acute phase than the individual compounds (Figure 16A).

In the recovery phase, the combination of Gelsolin (P06396), Zinc-alpha-2-glycoprotein (P25311) and Octanoic acid improved the ROC curve with an AUC of 0.779 (95% CI: 0.649-0.909). This AUC value is also higher than the AUC of the individual biomolecules (Figure 16B).

We wanted to improve even more the AUC values, so many more combinations of the compounds that appeared in the Random Forest have been tested, apart from the first three. The set of the first six compounds of the Random Forest (Figure 14A and Figure 14B) were selected and ROC curves and binary logistic regression analysis were performed to assess their accuracy (Figure 17).

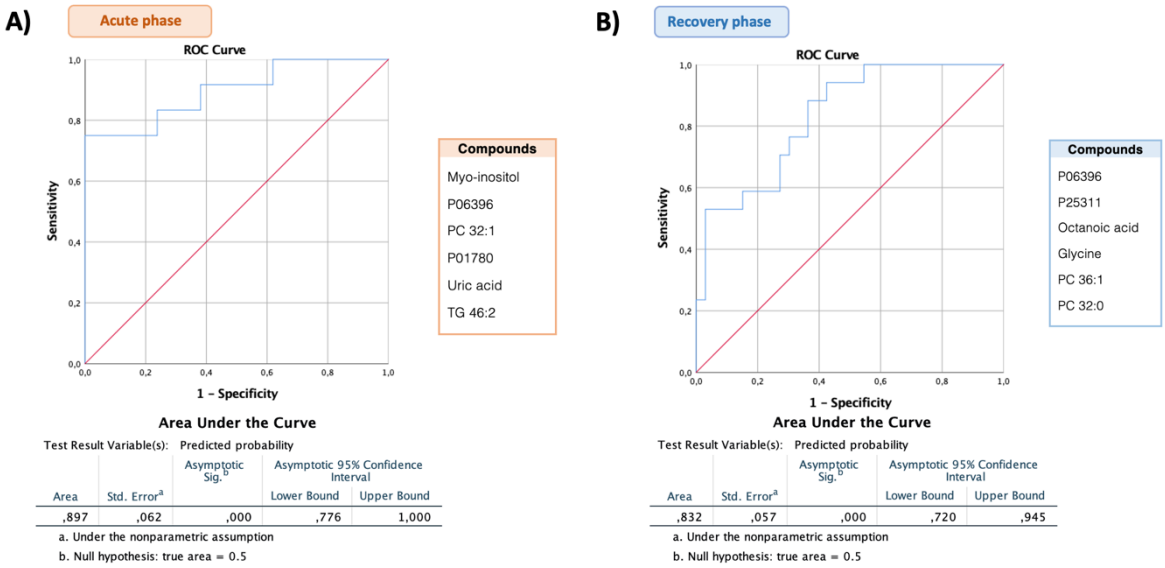


Figure 17. *Receiver operating characteristic (ROC) curves and binary logistic regression analysis. ROC curves of six different compounds combined to distinguish hypertensive patients with unfavourable COVID-19 from non-hypertensives.* ***A)*** *ROC curve for acute phase,* ***B)*** *ROC curve for recovery phase. PC: Phosphatidylcholine; TG: Triglyceride.*

The combination of myo-inositol, P06396, PC 32:1, Immunoglobulin heavy variable 3-7 (P01780), Uric acid and TG 46:2 showed a ROC curve with a good AUC value of 0.897 (95% CI: 0.776-1.000), indicating that this panel has a great potential predictive of hypertensive patients with unfavourable prognosis of COVID-19 (Figure 17A). These biomarkers could help to identify hypertensive patients who may develop an unfavourable course in COVID-19 in early stage of infection.

On the other hand, the combination of P06396, P25311, Octanoic acid, Glycine, PC 36:1 and PC 32:0 shows a ROC curve with a good AUC value of 0.832 (95% CI: 0.720-0.945), indicating that this panel apart from helping us to see differences in healing because it is from the recovery phase, can also anticipate other complications derived from the recovery phase.

As we can observe, the Gelsolin protein (P06396), was found to be important at both infection times, so it may have a greater significance in this study. Therefore, the representation of its concentrations in the two groups and in the two points of the study were shown in Figure 18. As can be observed, in the acute phase (Figure 18A) gelsolin was expressed more in unfavourable COVID-19 hypertensive patients, and on the contrary, in the recovery phase (Figure 18B), this protein is found to be more expressed

in the non-hypertensive patients. These results correlate with those obtained by Random forest (Figure 14).

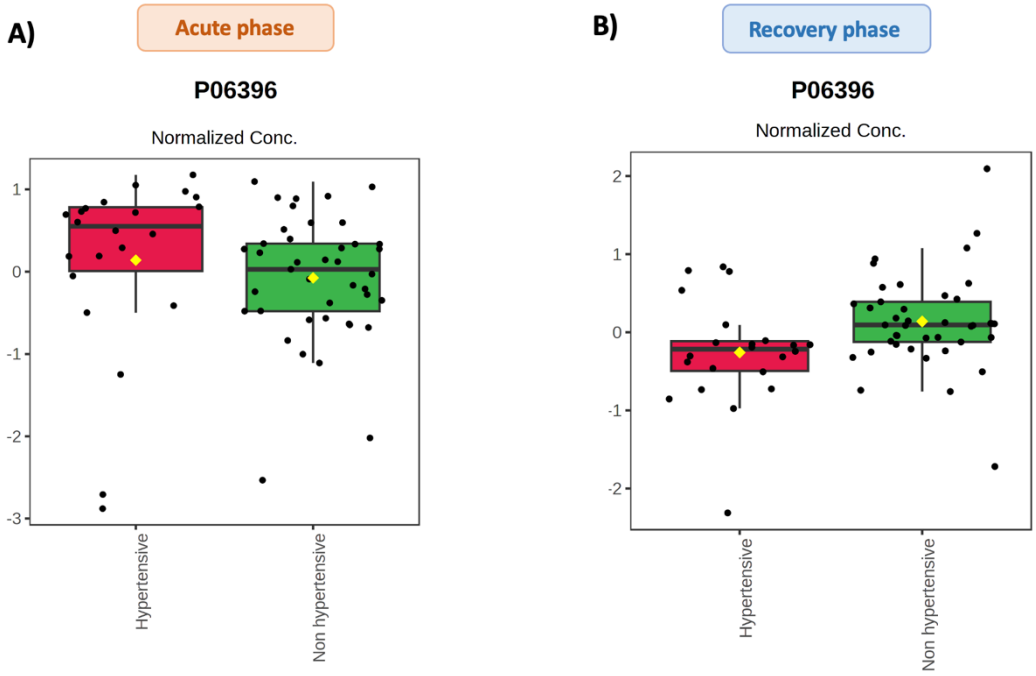


Figure 18. *Box plot of Gelsolin protein (P06396) in unfavourable COVID-19 hypertensive and non-hypertensive patients. Normalized concentrations corresponding to* ***A)*** *the acute phase of COVID-19 and* ***B)*** *to the recovery phase. The red rectangles represented the concentration of hypertensive patients, whereas the green rectangles belonged to non-hypertensive patients. Boxes demonstrating interquartile range, horizontal line indicating the median, and vertical line showing the 95% confidence interval. Conc.: concentration.*

5.3. *Biochemical evolution in hypertensive patients with unfavourable COVID-19*

Once the differences between the two study groups were seen in the two phases of the infection (acute phase and recovery phase), evolution studies were performed to analyse the possible changes of the biomolecules, not only in the separate phases, but from the acute phase to the recovery phase. A Volcano Plot analysis was performed for metabolomic, proteomic and lipidomic data by Metaboanalyst 5.0, to see which biomolecules changed significantly over time, from the acute phase (time of admission) to the recovery phase (four to eight weeks later). These analyses were based on serum concentrations of 239 proteins, 78 metabolites and 112 lipids in the two phases of infection.

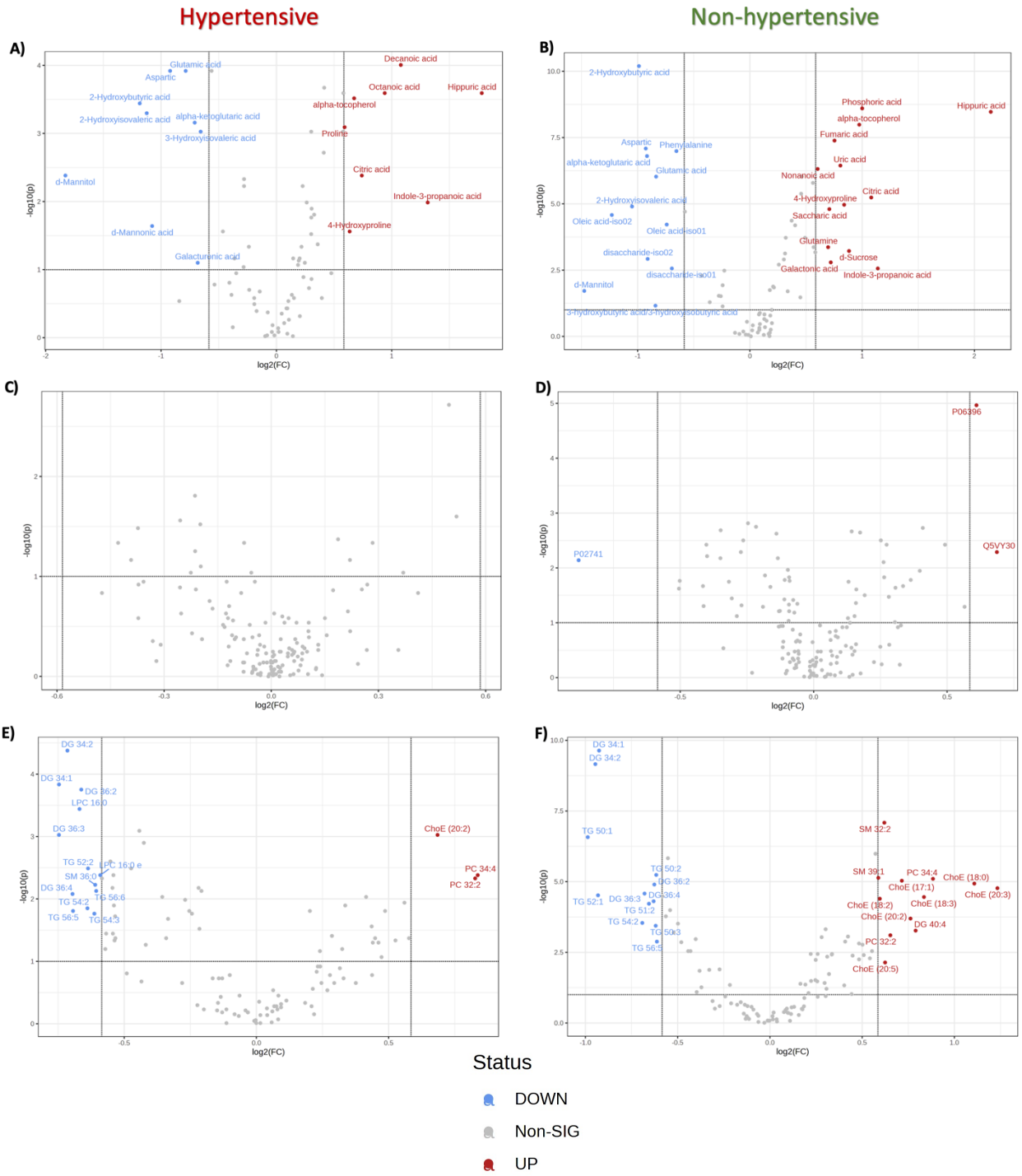


Figure 19. *The evolution of proteomic, metabolomic and lipidomic profiles from acute phase to recovery phase of COVID-19 considering hypertension. Volcano Plot analysis was performed by Metaboanalyst 5.0 of metabolomic (**A and B**), proteomic (**C and D**) and lipidomic (**E and F**) data from unfavourable patients of COVID-19 at the acute phase and recovery phase with hypertension (n=22) (**A, C, E**) and without hypertension (n=41) (**B, D, F**). X-axis corresponds to log2 (Fold Change) and y-axis to −log10 (p-value) revealing biomolecules with a p value <0.05 and Fold Change >1.5. The red colour corresponds to those molecules that have significantly increased over time, the blue colour to molecules that have significantly decreased and the grey dots to molecules that have not varied significantly. TG: Triglyceride; DG: Diglyceride; SM: Sphingomyelin; ChoE: Cholesterol Esters; PC: Phosphatidylcholine.*

In the Figure 19, we observed six volcano plots distributed by omics (proteomics, metabolomics and lipidomics) and by hypertension groups. Concretely, the Figure 19A and 19B shown the comparative evolution of metabolomics of unfavourable COVID-19 patients with hypertension and without hypertension, respectively. As observed, there was more movement of metabolites in the non-hypertensive patients. In fact, in hypertensive patients (Figure 19A) eight metabolites increased significantly and nine decreased, while in non-hypertensive patients (Figure 19B) thirteen metabolites increased and twelve decreased over time. Apart from these differences, many of the metabolites that changed in the hypertensive group coincided with the metabolites that also change in the same way in the non-hypertensive group. Besides, Decanoic acid, Octanoic acid and Proline were increased in the hypertensive group but not in the normotensive group, and 3-hydroxyisovaleric acid, d-Mannoic acid and Galacturonic acid decreased in the hypertensive group, while they do not appear in the non-hypertensive group. These metabolites seem to be related to hypertension and unfavourable prognostic of COVID-19.

Figure 19C and 19D of proteomic data showed a lower number of compounds compared to previous figures. In fact, in the COVID-19 unfavourable hypertensive group there was no change in protein expression (Figure 10C). In contrast, two proteins increased [Retinol-binding protein (Q5VY30) and Gelsolin (P06396)] and one protein decreased [C-reactive protein (P02741)] in the non-hypertensive group (Figure 19D).

Finally, the Figure 19E and 19F corresponded to lipidomic data. In unfavourable COVID-19 hypertensive patients (Figure 19E), three lipids were significantly increased and thirteen were decreased, and in non-hypertensives (Figure 19F), twelve lipids were significantly increased while twelve decreased. As in the metabolomics, more lipid movement is again observed in the non-hypertensive patients, which they only have the effect of unfavourable COVID-19. Furthermore, the three lipids that increased in the hypertensive group also increased in the non-hypertensive group. On the other hand, in the set of lipids that decreased in hypertensive group, six lipids [lysophosphatidylcholine 16:0 (LPC 16:0), triglyceride 52:2 (TG 52:2), LPC 16:0e, sphingomyelin 36:0 (SM 36:0), TG 56:6 and TG 54:3] were different compared to the non-hypertensive group (Figure 10E).

6. DISCUSSION

Since the emergence of SARS-CoV-2, it has been a worldwide health problem, causing a large number of deaths and sequelae. This is why the study of COVID-19 disease has been booming during the last 4 years. Thanks to these studies, it has been possible to discover that the existence of different comorbidities can produce a greater risk of suffering a critical state of COVID-19 [18], [22], [23]. Among these comorbidities is hypertension, which could be an important risk factor for COVID-19 patients [18], [22], [25], [30]. Hypertension is very prevalent, with millions of people affected worldwide, so the study of a possible relationship between these two diseases is very important to understand and try to improve the quality of life of people who suffer from these diseases. Although it was not yet clear whether hypertension could have a direct impact on the severity of COVID-19, in this study we have seen that hypertension does affect the severity of SARS-CoV-2 infection (Table 1), so this is an important finding that is consistent with previous reports [18], [22], [25], [30]. Here, the percentage of hypertensive patients among COVID-19 patients was 25 %, similar to previous studies (25%-40%) [18], [31]–[34], most of them close to 30%. In addition, a study by Jianhua Hu et al. [35] collected the percentages of severe COVID-19 patients who had hypertension from different studies, range from 23.7% to 78.7%. In our case, the percentage was 53.8%, a result within the described range in the other studies. In addition, it also shows that men are the sex most affected by hypertension, as in our study (61.5%) [35], [36] (Table 1).

Multiple complex mechanisms could be involved in the hypertension-mediated worsening of COVID-19 disease, which are still under investigation. In our study, we have focused on some of the compounds and pathways that could cause this alteration and linking hypertension to COVID-19. It is important to note that not all of the risk of COVID-19 severity can be attributed to hypertension, since there are other comorbidities and factors involved in the severity of this disease, but this condition is among the most important.

In this study, we described that there might be differences between the lipidomic, proteomic, and metabolomic panel of unfavourable COVID-19 patients with and without hypertension, which might indicate a relationship between a worse response to SARS-CoV-2 infection and hypertensive patients. That is, patients with hypertension were more likely to have a worse prognosis for COVID-19. These compounds and pathways have been studied both in acute phase and recovery phase and from one point to another (evolution), to study the changes discussed below.

In the acute phase (Figure 17A), the six most important biomolecules were myo-inositol, gelsolin (P06396), phosphatidylcholine 32:1, Immunoglobulin heavy variable 3-7 (P01780), uric acid and triglyceride 46:2. In the recovery phase (Figure 17B) the six

compounds with the highest discriminatory power were Gelsolin (P06396), Zinc-alpha-2-glycoprotein (P25311), Octanoic acid, Glycine, Phosphatidylcholine 36:1 and Phosphatidylcholine 32:0. Thanks to these two panels and other analyses, we were able to see which biomolecules and pathways might be involved in unfavourable progression of COVID-19 patients with hypertension.

The most notable protein in our study is gelsolin (P06396). Gelsolin is a principal actin-modulating protein. It is present in most human tissues, capable of binding, severing, and capping cytoskeletal actin, and is involved in cell motility, cell shape and metabolism [37]. This protein discriminated well the two groups both in the acute phase and in the recovery phase (Figure 14 and Figure 17), and was significantly present, its expression being high in the acute phase in unfavourable hypertensive COVID-19 and was higher in the recovery phase in unfavourable non-hypertensive COVID-19 patients. Moreover, as observed in the Figure 19, this protein is important in the evolution of unfavourable COVID-19 patients without hypertension which, as can be seen, increased its expression over time in these patients, unlike in hypertensive patients where there were no changes over time. But concretely, in the hypertensive group, gelsolin was higher in the acute phase and decreased in the recovery phase, contrary to normotensive group (Figure 18). Thus, it is a protein found in many physiological processes and it has been described in previous literature that is related to both pathological and beneficial processes [38]. It is a marker of innate immunity, and is involved in disease pathogenesis and viral infections, for example, inhibiting virus infection [38]. In relation to COVID-19, other authors described a decrease of gelsolin in severe COVID-19 patients compared to controls, which is related to a worse outcome, since its reduction decreases its protective role of organs, so it could cause a multiorgan dysfunction disease (MODS) increasing mortality [39], [40]. Moreover, it has been described that gelsolin decreases its levels at the time of admission and returns to normal levels after 30 days [40], which is related to an improved outcome and coincided with our results in evolution of normotensive unfavourable COVID-19 patients (Figure 18 and Figure 19). In contrast, hypertensive and unfavourable COVID-19 patients maintains gelsolin levels over time in the two phases, maintaining low levels in the recovery phase (compared to the levels of normotensive patients), which could be related to a worse outcome compared to the normotensive group [40]. The fact that gelsolin levels are higher in the acute phase in the hypertensive group compared to normotensives may be explained because muscles are the main source of gelsolin, among them the smooth and cardiac muscle, which in hypertension may be damaged and the release of this protein may occur [41]. In addition, as mentioned before, gelsolin also participates in pathological processes related to cardiac diseases, such as hypertension, where high levels of this protein produce cardiac hypertrophy, and heart disease [41], [42]. Therefore, this protein could indicate and produce worse outcomes in hypertensive people with COVID-19 compared to non-hypertensive people with COVID-19, since gelsolin could be acting in the course of hypertension and heart

disease, instead of practicing its beneficial effects on COVID-19 infection, and not recovering its levels after weeks.

Another compound that has been important in our study is the myo-inositol, which had a high discriminating power to differentiate the two groups, and was present at high levels in hypertensive patients, both facts in the acute phase. Myo-inositol, also known as inositol, is a carbocyclic sugar found in large quantities in the brain and other mammalian tissues. It plays a crucial role in transmitting cellular signals when exposed to various hormones, neurotransmitters, and growth factors, and is involved in the regulation of osmotic balance. The synthesis of myo-inositol occurs from glucose 6-phosphate (G6P) [43]. Reactive oxygen species (ROS) are able to stimulate myo-inositol production through different pathways [44]. A study previously described that myo-inositol levels were high in patients with hypertension because oxidative stress occurs due to an enhanced production of ROS [44]. Furthermore, it has also been seen that an up-regulated myo-inositol may contribute to vascular resistance in hypertensive patients [44], [45], in addition to the fact that Angiotensin II induces the formation of inositol [46]. This could also explain the increased levels of this compound in patients with hypertension. COVID-19 severe patients also undergo a state of oxidative stress [47], which may be aggravated by the hypertensive condition, worsening the clinical situation of the patients. In addition, the alteration of myo-inositol observed in our study affected the ascorbate aldarate metabolism (Figure 12A), which is described as a beneficial pathway for the organism, therefore this alteration impairs this beneficial action of eliminating oxygen free radicals [48]. All this could explain the worse prognosis of COVID-19 patients with hypertension, as they are in an even worse state of health with increased inflammation and oxidative stress. These two pathways (inflammation and oxidative stress) may be especially important in the acute phase of SARS-CoV-2 infection, and consequently the myo-inositol, indicating this exacerbated state of inflammation and oxidative stress in COVID-19 patients, aggravated by the presence of hypertension.

Other components that have been significantly altered in this study due to hypertension and unfavourable COVID-19 were lipids, such as triglycerides, diglycerides sphingomyelins, cholesterol esters or phosphatidylcholines. Patients with hypertension often suffer from dyslipidemia, presenting a higher level of lipids [49], [50]. Dyslipidemia and hypertension share common pathophysiological mechanisms, included causing damage to endothelial cells, promoting inflammation, inducing oxidative stress, and contributing to the development of atherosclerosis in the arteries [50]. This could contribute to viral infection since lipids are involved in the entry of SARS-CoV-2 and consequently, aggravate the clinical condition [15]. It should be noted that some of patients belonging to both groups in this study presented an alteration in lipid levels. In the normotensive group due to the unfavourable COVID-19 status, but in hypertensive group this alteration lasts over time, so the biosynthesis of fatty acids and that of linoleic

acid (which also participates in the biosynthesis of fatty acids), were altered in hypertensive patients in the recovery phase (Figure 12B). Therefore, hypertensive patients may present a greater alteration of lipids that worsens the infection. In addition, it has been described in previous studies that higher triglyceride (TG) levels are found in patients with a higher risk of death due to COVID-19, so that the concentrations of TG could be an indication of the severity of the disease [51]. In accordance, our results shown that TGs were increased mostly in the non-hypertensive group with unfavourable COVID-19 in the acute phase (Figure 11A). But after 4-8 weeks, TGs only increased in the hypertensive group (Figure 11B), which could indicate a worse prognosis in these patients and support our hypothesis.

To conclude, it should be noted that our study had some limitations. First, it is important to highlight that this study has a relatively low number of patients due to the difficulty of finding unvaccinated COVID-19 positive patients from the first waves. Concretely, in the cohort there were few COVID-19 mild hypertensive patients. Also, a control group of patients without COVID-19 was not represented to compare the results. Longer follow-up of patients would be needed to see the development of sequelae or consequences of hypertension in COVID-19 patients. Furthermore, although the proposal of associated biomarkers is interesting, their application in the clinic is neither immediate nor feasible. Finally, there is intrinsic variability between individuals, so there are many factors to take into account that can influence the study. Therefore, further studies correcting for possible limitations are needed to corroborate the results.

7. CONCLUSION

In conclusion, it has been proven that there is a significant relationship between the severity of COVID-19 and hypertension, so that hypertensive individuals are more likely to develop worst COVID-19 outcomes. The metabolic, lipidomic and proteomic differences between unfavourable COVID-19 patients with hypertension and without hypertension, could explain that hypertension, through different joint mechanisms with COVID-19, does play an important role in the course and worsening of the disease produced by SARS-CoV-2. Further research in this field could lead to possible treatments that address these mechanisms to improve the prognosis and quality of life of patients with hypertension and COVID-19.

The most prominent compounds that have been most related to unfavourable COVID-19 status and hypertension were gelsolin protein, myo-inositol and lipids, involved in different pathways, such as immune system process, inflammation, oxidative stress and lipid metabolism. This could indicate possible pathways to understand the mechanisms by which the two diseases act together and worsening clinical condition of the patients.

In this study, we have been able to determine for the first time the biomolecules and pathways involved in unfavourable COVID-19 patients with hypertension using multi-omics analysis in the acute and recovery phase, but further studies are needed to test whether these compounds could serve to improve or create new treatments. In addition, studies of the long-term impact of hypertension on COVID-19, such as possible sequelae and other consequences, are needed.

SUPPLEMENTARY MATERIALS

Supplementary Table S1. *Clinical classification criteria of COVID-19 patients* [28].

WHO classification	Group description	Group
WHO 1	No pneumonia.	Mild
WHO 2	Pneumonia without hospitalization.	Mild
WHO 3	Pneumonia with hospitalization and without O2 requirement.	Mild
WHO 4	Pneumonia with hospitalization and with O2 requirement, low-flow.	Severe
WHO 5	Pneumonia with hospitalization and with O2 requirement, non-invasive ventilation.	Severe
WHO 6	Severe pneumonia, ICU, O2 requirement, mechanical ventilation.	Critical
WHO 7	Severe pneumonia, ICU, O2 requirement, mechanical ventilation, vasopressors or dialysis.	Critical
WHO 8	Exitus.	Critical

WHO: World Health Organization; ICU: intensive care unit.

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