# CHARACTERIZING THE TYPE OF INTERLEUKIN-6 SIGNALING IN SEVERE COVID-19

by

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Dalhousie University is located in Mi'kma'ki, the
ancestral and unceded territory of the Mi'kmaq.
We are all Treaty people.

## DEDICATION

I dedicate this thesis to my lovely wife Sharmin. Thank you for tolerating me and being with me all the way.

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## ABSTRACT

Since the beginning of coronavirus disease-19 (COVID-19) pandemic many efforts were made to explore the role of interleukin-6 (IL-6) in COVID-19. I used severe non-COVID-19 to better understand the IL-6 signaling in severe COVID-19. I assess plasma concentration of twenty-five biomarkers in severe COVID-19 patients and showed interaction of pro-inflammatory biomarkers with IL-6. I further investigate the interaction between IL-6 signaling components [IL-6, soluble IL-6 receptor (sIL-6R) and soluble glycoprotein 130 (sgp130)] and explain the type of IL-6 signaling in severe COVID-19 and non-COVID-19 patients. A prediction model was also applied to classify the disease group based on performance of IL-6 biomarkers individually and together. My data provide evidence of differential IL-6 signaling in two the different severe disease models. Notably, from this study, it is evident that severe COVID-19 is characterized by dysregulated IL-6 trans-signaling while severe non-COVID-19 followed a pattern of IL-6 classical-signaling.

## LIST OF ABBREVIATIONS USED

| | |
|---|---|
| ACE2 | Angiotensin-converting enzyme 2 |
| Ang II | Angiotensin II |
| Ang 1-7 | Angiotensin 1-7 |
| Ang-2 | Angiopoietin-2 |
| ARDS | Acute respiratory distress syndrome |
| APC | Antigen-presenting cells |
| ADAM17 | A disintegrin and metalloprotease 17 |
| AUC | Area under the curve |
| BtCoV | Bat coronavirus |
| BuCoV | Bulbul coronavirus. |
| BAL | Bronchoalveolar lavage |
| BALF | Bronchoalveolar lavage fluid |
| BSF-2 | B-cell-stimulating factor 2 |
| B/T ratio | Binary/ternary complex ratio |
| CT | Computerized tomography |
| CoV | Coronavirus |
| CXCL | C-X-C motif chemokine ligand |
| CCL | Chemokine (C-C motif) ligand |
| COVID-19 | Coronavirus disease 2019 |
| CALI | COVID-19-associated lung injury |
| CAMs | Cell adhesion molecules |
| CRS | Cytokine release syndrome |

| | |
|---|---|
| cDNA | complementary deoxyribonucleic acid |
| CD4 | Clusters of differentiation 4 |
| DC | Dendritic cell |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme-linked Immunosorbent Assay |
| G-CSF | Granulocyte colony-stimulating factor |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| gp130 | Glycoprotein 130 |
| GISAID | Global Initiative on Sharing Avian Influenza Data |
| HSF | Hepatocyte-stimulating factor |
| HGF | Hybridoma growth factor |
| HCoV | Human coronavirus |
| IL | Interleukin |
| IL-1RA | Interleukin-1 receptor antagonist |
| ICAM | Intercellular adhesion molecule |
| IL-6R | IL-6 receptor |
| IRB | Institutional Research Board |
| ICU | Intensive care unit |
| INF | Interferon |
| kDa | Kilo dalton |
| LTC | long-term care |
| MCP | Monocyte chemoattractant protein |
| mRNA | Messenger ribonucleic acid |

| | |
|---|---|
| mol/L | Moles per litre |
| mIL-6R | Membrane bound IL-6 receptor |
| MPO | Myeloperoxidase |
| MERS-CoV | Middle East respiratory syndrome coronavirus |
| PCR | Polymerase chain reaction |
| rpm | Revolutions per minute |
| RNA | Ribonucleic acid |
| SARS | Severe acute respiratory syndrome |
| SARS-CoV | Severe acute respiratory syndrome coronavirus |
| SARS-CoV-2 | Severe acute respiratory syndrome coronavirus 2 |
| SOFA | Sequential Organ Failure Assessment |
| SD | Standard deviation |
| sgp130 | Soluble glycoprotein 130 |
| sIL-6R | Soluble IL-6 receptor |
| Th | T helper |
| Treg | T regulatory |
| TNF-α | Tumor necrosis factor-alpha |
| TGF-β | Transforming growth factor-β |
| TMPRSS2 | Transmembrane protease, serine 2 |
| VCAM | Vascular cell adhesion protein |
| VEGF | Vascular endothelial growth factors |
| WHO | World Health Organization |

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# CHAPTER 1: INTRODUCTION

## 1.1 Coronaviruses

Coronaviruses (CoVs) are a group of enveloped viruses with spherical or pleomorphic shape and a diameter of 80-120 nm. CoVs contain a non-segmented, single-stranded and positive-sense RNA genome. The CoVs genome is one of the largest among RNA viruses, ranging from 27 to 32 kilobases [1,2]. The viral envelope consists of a lipid bilayer that is supported by the membrane (M) and envelope (E) protein. Club-like projections constituted by the spike (S) protein are also anchored in the viral surface. Inside the envelope, the RNA genome is enclosed by nucleocapsid (N) protein in a beads-on-a-string fashion, scaffolding around 29,900 nucleotides of RNA [3,4].

According to the International Committee on Taxonomy of Viruses, coronaviruses belong to order *Nidovirales*, family *Coronaviridae*, subfamily *Coronavirinae* [1]. The subfamily is divided into four genera: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus* based on their serological and genomic structures. The genus *Betacoronavirus* is further divided into four distinct lineages: A, B, C, and D (Figure 1.1) [5]. Coronaviruses cause respiratory and intestinal infections in vertebrates such as mammals and birds. The alphacoronavirus and betacoronavirus infect only mammals while gammacoronavirus and deltacoronavirus mainly infect birds, with some exceptions such as mammalian infection [6].

### 1.1.1 History of Pathogenic Human Coronaviruses

Until now seven coronaviruses have been identified that infect humans and cause respiratory illness: HCoV-229E, HCoV-NL63, HCoV-OC43, HCoV-HKU1, SARS-CoV, MERS-CoV and SARS-CoV-2 [7,8]. Among the seven human coronaviruses, (HCoVs), HCoV-229E, and HCoV-NL63 belong to Alphacoronavirus, while HCoV-OC43 and HCoV-HKU1 belong to lineage A, SARS-CoV and SARS-CoV-2 to lineage B, and MERS-CoV to lineage C of Betacoronavirus (Figure 1.1) [1,2,7]. Human coronaviruses were not believed to be highly pathogenic to humans until the emergence of SARS-CoV in 2002. Before the emergence of SARS-CoV, HCoV-229E and HCoV-OC43 were the only known HCoVs to cause mild infection in the upper respiratory tract [9,10]. Two more pathogenic HCoVs, HCoV-NL63 and HCoV-HKU1, were identified in 2004 and 2005, respectively [11,12]. These four HCoVs (HCoV-229E, HCoV-OC43, HCoV-NL63, and HCoV-HKU1) are distributed globally and contribute to 15-30% of human common cold cases [13]. Although disease caused by these mild HCoVs are self-limiting and confined to the upper respiratory track, sometimes they can cause severe lower respiratory infections in immunocompromised patients and aged individuals [14,15]. SARS-CoV, MERS-CoV, and SARS-CoV-2 are highly pathogenic HCoVs, capable of severe lower respiratory infections which can result in fatal respiratory failure [16]. Elderly people or those who have ongoing medical conditions who become infected with these highly pathogenic HCoVs (SARS-CoV, MERS-CoV and SARS-CoV-2) are more susceptible to develop severe disease manifestations as well as fatal outcome [17,18]. SARS-CoV, MERS-CoV, HCoV-NL63, and HCoV-229E originated in bats whereas HCoV-OC43 and HCoV-HKU1

originated in rodents [9,10]. There are still many hypotheses regarding the origin of SARS-CoV-2; however, the most accepted theory is that it is a bat coronavirus [19].

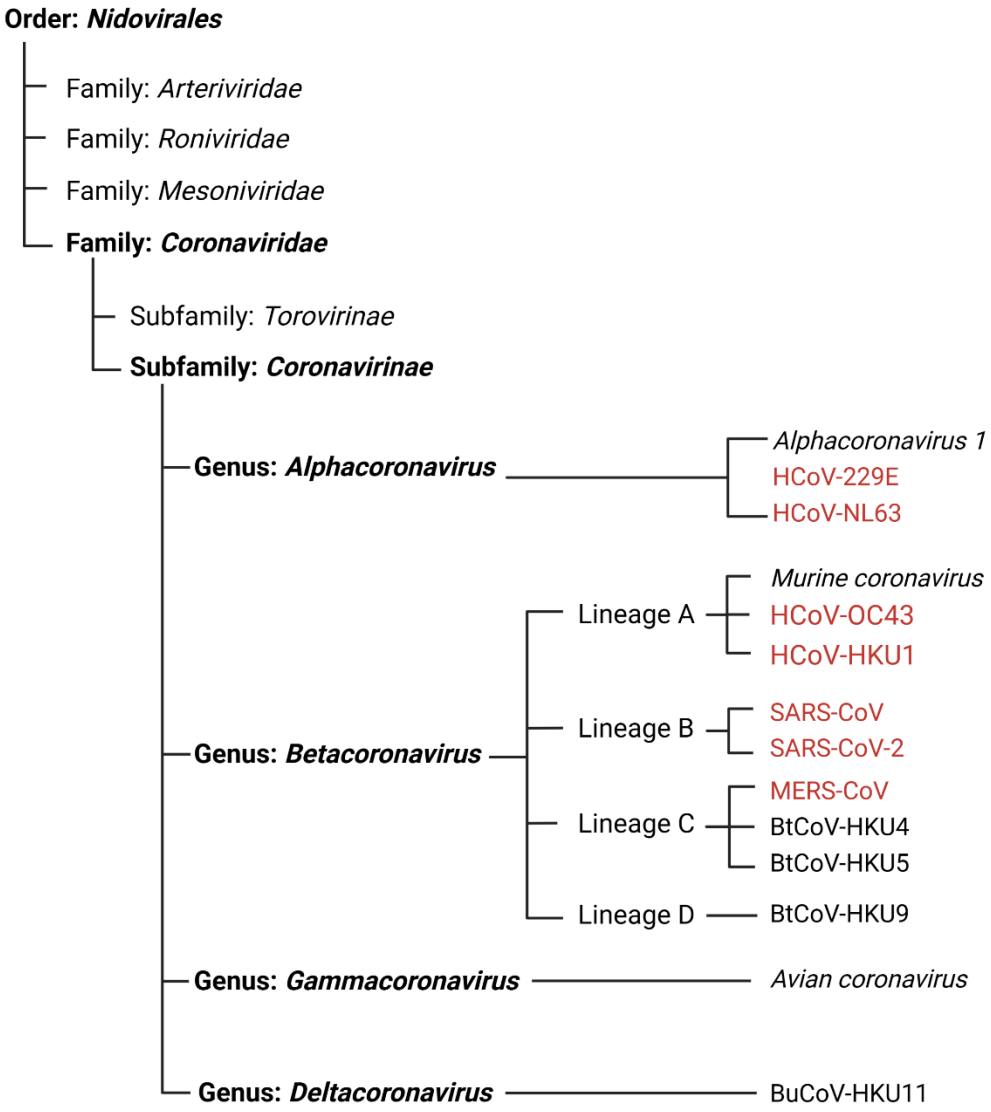

Order: Nidovirales
Family: Arteriviridae
Family: Roniviridae
Family: Mesoniviridae
Family: Coronaviridae
Subfamily: Torovirinae
Subfamily: Coronavirinae
Genus: Alphacoronavirus
Alphacoronavirus 1
HCoV-229E
HCoV-NL63
Genus: Betacoronavirus
Lineage A
Murine coronavirus
HCoV-OC43
HCoV-HKU1
Lineage B
SARS-CoV
SARS-CoV-2
Lineage C
MERS-CoV
BtCoV-HKU4
BtCoV-HKU5
Lineage D
BtCoV-HKU9
Genus: Gammacoronavirus
Avian coronavirus
Genus: Deltacoronavirus
BuCoV-HKU11

**Figure 1.1 Taxonomic classification of coronaviruses.** The seven known human coronaviruses are shown in red. Abbreviations: HCoV, human coronavirus; SARS-CoV, severe acute respiratory syndrome coronavirus; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; MERS-CoV, Middle East respiratory syndrome coronavirus; BtCoV, bat coronavirus; BuCoV, bulbul coronavirus. Figure adapted and modified from *Fung et al.* [1]

## 1.2 SARS: The First Pandemic of the 21st Century

Throughout history, humans have been struck by epidemics of infectious diseases. These epidemics caused terrible loss to life, the economy, society, and some have even changed the course of history. The successive outbreaks of bubonic plague, the Black Death, killed 50% or more of European populations during the Middle Ages. Epidemics that are not restricted to specific geographical regions, rather spread worldwide, are classified as pandemics. The influenza pandemic in 1918 is the notable viral pandemic of last century. At the end of World War I, because of the displacement of millions of people, inadequate healthcare systems, and the lack of antibiotics to treat secondary bacterial infections, the 1918 influenza pandemic was responsible for the deaths of tens millions of people in the last century [20]. We witnessed SARS (severe acute respiratory syndrome), the first pandemic of 21st century, in the fall of 2002 [21]. The first SARS outbreak was reported in November 2002 from Fosham City, Guangdong Province, China. Subsequently, from Guangdong Province, SARS spread to other provinces in China as well as throughout the world. During the period from November 2002 until the end of the pandemic in July 2003, SARS was reported in 29 countries with 8,437 cases resulting in 813 deaths [22,23]. SARS-CoV, a zoonotic betacoronavirus, was the causative agent of the SARS pandemic. SARS-CoV originated in horseshoe bats, which later adapted to palm civets as an intermediate host before infecting humans as a dead-end host [24]. The incubation period of SARS is 4-6 days before clinical symptoms develop. Disease manifestation in children is less severe than disease in adults, while adults with comorbidities are more likely to develop fatal outcome [25,26].

### 1.2.1 Immunopathogenesis of SARS

SARS patients develop influenza-like symptoms such as headache, malaise, high fever, rigors, and sore throat [27]. Some of these patients also have watery diarrhoea. Two-thirds of SARS-CoV-infected patients experience atypical pneumonia with shortness of breath [25,28]. Severe cases lead to fatal respiratory failure and acute respiratory distress syndrome, which are the common causes of death in SARS patients [25]. Patients with respiratory distress have abnormal chest radiographs or computerized tomography (CT). Most notably, chest imaging findings include patchy infiltrates, opacities, and areas of consolidation [21]. Angiotensin-converting enzyme 2 (ACE2) is the host cell-surface receptor for SARS-CoV [29]. ACE2 plays a critical role in the prevention of lung injury and blood pressure homeostasis by converting angiotensin (Ang) II to Ang 1-7. SARS-CoV downregulates the expression of ACE2 and thereby suppresses the protective effect of ACE2 [30,31]. Damage to lungs can be directly by viral invasion or indirectly through expression of immune mediators [32,33].

In most cases, immune dysregulation is the underlying cause of SARS-CoV pathogenesis. Virus infection might trigger intense pro-inflammatory responses; however, excessive levels of pro-inflammatory cytokines and chemokines are detected in severe cases while the virus is cleared by the immune system [27,33]. Disease severity is associated with cytokine release syndrome, immune dysfunction, and depletion of the lymphocyte population [34–36]. Lymphopaenia is the most common feature of severe SARS cases [37]. Infection of macrophages and lymphocytes is a crucial step in establishing SARS-CoV-induced pathogenesis [37]. Innate immune response acts as the first line of defence of our immune systems and later activates the adaptive immune

response. SARS-CoV interferes with the innate immune system by down-regulating type I interferons (INFs) [38], while infected macrophages and dendritic cells (DCs) are found to express high amounts of pro-inflammatory cytokines including interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-1 (IL-1), and tumor necrosis factor-alpha (TNF-α) [36,39]. Chemokines such as C-X-C motif chemokine ligand 10 (CXCL10) and CCL2 are upregulated in the plasma of SARS patients, which might be involved in the influx of monocytes and macrophages in severe patients [40,41]. These upregulated cytokines and chemokines are also responsible for endothelial dysfunction and vascular damage resulting from an aberrant inflammatory cascade [39]. Autopsies of SARS patients show hallmarks of apoptosis in infected lung, spleen, and thyroid tissues [42]. Direct infection of SARS-CoV and/or pro-inflammatory cytokines such as TNF-α can be responsible for inducing apoptosis [27]. Despite pro-inflammatory cytokines, anti-inflammatory mediators such as transforming growth factor-β (TGF-β) and prostaglandin $E_2$, were found at higher levels in the serum of SARS patients [43].

### 1.3 SARS-CoV-2 and Coronavirus Disease-2019

Several healthcare facilities in Wuhan, in Hubei province in China, reported multiple pneumonia cases of unknown cause in late December 2019 [44]. Most of these cases were epidemiologically linked to the Huanan Seafood Wholesale Market in Wuhan city, which is famous for not only seafood but also the trading of live wild animals [45–47]. On 31 December 2019, the Wuhan Municipal Health Commission made a public statement regarding a pneumonia outbreak of unidentified cause and informed the World Health Organization (WHO) [48]. Chinese scientists collected bronchoalveolar lavage

fluid samples from severe pneumonia patients and, through virus isolation and metagenomic RNA sequencing, they identified that the causative agent of this disease was a novel betacoronavirus that had not been previously identified [44,49,50]. The complete genome sequence of this novel coronavirus, determined by multiple research institutes, was first published on the Global Initiative on Sharing Avian Influenza Data (GISAID) database on 12 January 2020 [51]. Meanwhile, reports were coming in about new patients with no connection to the Huanan Seafood Wholesale Market, including infections in family clusters and nosocomial infections in healthcare facilities [52,53]. All this evidence indicated a human-to-human transmission route of this novel coronavirus [54]. With these events occurring during the celebration of the lunar new year and people travelling to see family and friends, this novel coronavirus rapidly spread to other cities in Hubei province as well as to other parts of China. All 34 provinces of China were affected by this novel coronavirus in only one month [55]. On 30 January 2020, the novel coronavirus outbreak was declared a "public health emergency of international concern" by the WHO [56]. On 11 February 2020, the International Committee on Taxonomy of Viruses named the novel coronavirus SARS-CoV-2, and the WHO named the disease caused by SARS-CoV-2 as COVID-19 [57]. COVID-19 reached its epidemic peak in China in February while an increasing number of countries reported the international spread of COVID-19 with larger clusters of infections in late February [58]. Due to the high transmissibility of SARS-CoV-2, COVID-19 had spread rapidly worldwide with international travel. On 11 March 2020, the WHO declared the COVID-19 outbreak a pandemic [59]. According to the COVID-19 dashboard of the Center for System Science and Engineering at Johns Hopkins University, as of 28 December 2022, a total of 228 countries and territories from all six continents had

reported more than 658 million COVID-19 cases resulting in 6,684,090 deaths [60]. The origin of SARS-CoV-2 and its natural host is still a topic of great scientific debate. As mentioned previously, bats are the natural hosts of alphacoronavirus and betacoronavirus. Until now, the closest relative to SARS-CoV-2 is a bat coronavirus named RaTG13, detected in *Rhinolophus affinis* from Yunnan province, China. Full-length genome sequence data shows that RaTG13 is 96.2% identical to SARS-CoV-2 [50,55]. According to some research groups, pangolins, another potential natural reservoir of zoonotic viruses, could be linked to the origin of SARS-CoV-2. Several SARS-CoV-2-linked viruses have been identified in tissues of Malayan pangolins exhibiting 92.4% sequence similarity [61,62].

### 1.3.1 Immunopathogenesis of COVID-19

Clinical manifestations of COVID-19 include fever, sore throat, cough, and shortness of breath. Most SARS-CoV-2-infected individuals will not develop severe COVID-19 [63], with around 80% of them developing only mild illnesses and ~20% requiring hospitalization [64]. COVID-19 disease severity has been attributed to direct damage by viral evasion and unregulated inflammatory responses [65–67]. Pro-inflammatory mediators in COVID-19 are not that greatly elevated when compared to other inflammatory disorders; therefore, it is believed that severity in COVID-19 is due to dysregulated pro-inflammatory immune response rather than elevated response [68,69]. The clinical progression from SARS-CoV-2 infection to severe COVID-19 includes a series of events, such as i) upper and lower respiratory tract infection, ii) COVID-19-

associated lung injury (CALI), iii) systemic inflammatory response syndrome, and iv) systemic failure [70].

SARS-CoV-2 establishes infection in the upper respiratory tract using ACE2 as the main cell surface receptor to invade host cells. Upon infection, SARS-CoV-2 has been reported to downregulate the expression of ACE2- and IFN-I-mediated host antiviral activity [36,71]. As soon as SARS-CoV-2 reaches the lungs, high numbers of lymphocytes initiate a pro-inflammatory cascade to eliminate virus [70,72,73]. In some cases, lung epithelial and alveolar macrophages trigger exacerbated local inflammation, resulting in upregulation of pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, IL-10, and TNF and chemokines such as CCL2, CXCL1, CXCL3, and CXCL10 [74–77]. These cytokines and chemokines trigger autocrine and paracrine release of pro-inflammatory mediators, and subsequently the recruitment of monocytes, neutrophils, and leukocytes in the lungs of COVID-19 patients [74,78]. Bronchoalveolar lavage fluid (BALF) samples from COVID-19 patients have shown a strong association between the enrichment of macrophages-neutrophils and elevated levels of pro-inflammatory mediators such as IL-6, IL-8, TNF, IL-1β, CCL2, CCL4, CXCL10, IL17, MCP (monocyte chemoattractant protein)-1, G-CSF (granulocyte colony-stimulating factor), GM-CSF (granulocyte-macrophage colony-stimulating factor), and IL-1RA (IL-1 receptor antagonist) [71,73,74,79,80]. While uncontrolled local inflammation initiates CALI, pro-inflammatory cytokines such as IL-6 and TNF are also shown to upregulate the endothelial cell adhesion molecules (CAMs), ICAM (intercellular adhesion molecule), VCAM (vascular cell adhesion protein), Ang-2 (angiopoietin-2), and VEGF (vascular endothelial growth factors) [81]. These proteins

trigger the impairment of lung glycocalyx thus disturbing the endothelial integrity and increasing permeability [82]. Consequently, virus is then allowed to enter systemic circulation and attack other organs that express the SARS-CoV-2 entry receptor ACE2 [70,83,84]. Acute lung injury-derived pro-inflammatory cytokines stimulate bone marrow to release immature granulocytes into circulation [85]. Release of IL-6, TNF-α, and IL-1β can impair the function of other organ systems [86,87]. When released into systemic circulation, these cytokines can hyperactivate endothelial and epithelial cells, monocytes/macrophages, and lymphocytes, which can result in cytokine release syndrome (CRS) [83,88]. Increased vascular leakage, dysfunction, and systemic inflammation might lead to hypotension and multi-organ failure, including acute respiratory distress syndrome (ARDS) [89,90].

### 1.4 Common Features of SARS and COVID-19

Clinical manifestation of both SARS and COVID-19 are similar with common influenza-like symptoms [63]. However, the mortality rate of SARS is higher than that of COVID-19 even though COVID-19 mortality greatly varies in different countries and severity increases with age. Chest radiology by X-ray/CT for presence of pneumonia caused by SARS-CoV and SARS-CoV-2 is similar. One notable difference is that in severe COVID-19 cases, both lungs are infected simultaneously while in SARS, unilateral involvement of lungs is more common [36,91]. An ample pro-inflammatory response in viral infection plays a crucial role in viral clearance and subsequent recovery, while a dysregulated pro-inflammatory response can cause catastrophic outcomes [35]. In the evolutionarily pathway, HCoVs have acquired the ability to encode several proteins that

help them to evade host response and trigger immune dysregulation [34,92,93]. Positive correlation between elevated pro-inflammatory cytokines and chemokines and severe SARS and COVID-19 have been confirmed, which indicates infection with SARS-CoV and SARS-CoV-2 leads to immune dysregulation, and subsequently, hypercytokinemia and ARDS [34,68,78,94]. Apart from the elevated pro-inflammatory cytokines and chemokines mentioned above, type-2 anti-inflammatory cytokines are also found to be elevated in COVID-19 similar to what was seen in SARS. However, elevation of anti-inflammatory cytokines in severe SARS and COVID-19 showed no obvious benefits, probably because type-2 cytokines also upregulate TMPRSS2, which may impair with the protective effect of type-2 cytokines [95].

### 1.5 Interleukin-6 draws most attention in both pandemics

As mentioned previously, aberrant immune response may underlie the severity of SARS and COVID-19. In both pandemics, among all pro-inflammatory cytokines, IL-6 expression and regulation are of particular interest to researchers and clinicians due to its involvement in multiple biological functions such as endothelial dysfunction, local and systemic inflammation, TNF-mediated apoptosis, B cell activation, etc. [96–99]. The prominent role of IL-6 in inflammatory diseases (e.g., rheumatoid arthritis, systemic sclerosis, systemic lupus erythematosus, ankylosing spondylitis, psoriasis, and Crohn's disease) was known even before the outbreak of SARS-CoV-2 [100–102]. Upon SARS-CoV and SARS-CoV-2 infection, the rapid rise of IL-6 has been observed to be associated with disease progression [97,103]. IL-6 is one of the key cytokines released by activated macrophages in response to infection. Several studies have shown an association between

excess IL-6 and severe SARS and COVID-19 [104–106]. In last two to three decades, with the development of our understanding of infection and inflammation, several inhibitors (tocilizumab, sarilumab and satralizumab) have also been developed to inhibit IL-6 signaling [107]. Wide acceptance of IL-6 as a crucial cytokine in CRS further encouraged clinicians to promote the use of IL-6 signaling inhibitors in severe COVID-19 during this pandemic. In complete IL-6 signaling, IL-6 first binds and forms a dimer with an IL-6 receptor (IL-6R). This binary complex then associates with a second receptor, glycoprotein 130 (gp130), and initiates IL-6 signaling [96]. Both the IL-6 receptor and glycoprotein 130 receptor have two distinct membrane-bound and soluble forms (Figure 1.2). As a pluripotent cytokine, IL-6 can perform both pro- and anti-inflammatory roles depending on the type of receptor with which it is binding. Fine-tuning of IL-6, the soluble IL-6 receptor (sIL-6R), and the soluble 130 receptor (sgp130) determines the type of IL-6 signaling [64].

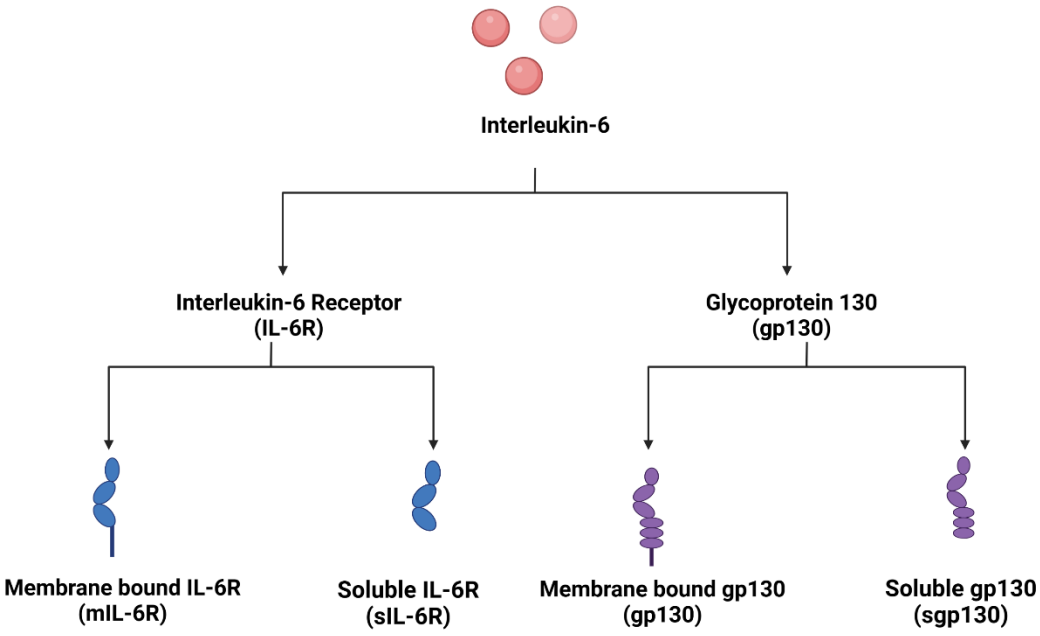



**Figure 1.2 Cognate and soluble mediators of IL-6 signaling.** Complete IL-6 signaling includes the binding of IL-6 with an IL-6 receptor and later association with a gp130 receptor. Both the IL-6 receptor and gp130 receptor have membrane-bound and soluble versions. The function of both soluble receptors (sIL-6R and sgp130) is to maintain a balance with IL-6 to direct the type of IL-6 signaling.

### 1.5.1 Interleukin-6

IL-6 is a 21-26 kDa soluble mediator belonging to the type 1 cytokine family with pleiotropic biological functions [108]. At first, IL-6 was known as B-cell-stimulating factor 2 (BSF-2) based on its ability to stimulate B-cells in antibody production [109]. In the 1980s, the various functions of IL-6 were studied by different groups and distinct names were proposed based on their functions. Apart from being called BSF-2, the same molecule is also known as hepatocyte-stimulating factor (HSF) for the effect of acute phase protein synthesis on hepatocytes; hybridoma growth factor (HGF) for enhancing the growth of myeloma cells; or interferon-β2 (IFN- β2) because of its antiviral activity [110,111]. In 1986, when cDNA of BSF-2 was first cloned, it was found that different research groups were studying the same identical molecules with different names, resulting in the designation of a single name, IL-6 [112,113]. Because of its pleiotropic nature, IL-6 is engaged in a vast number of fundamental processes of cell growth and cell activation, bone metabolism, immune response, and inflammation [111,114]. IL-6, in combination with TGF-β, stimulates the differentiation of T helper 17 (Th17) cells from naïve $CD4^+$ T cells as well as inhibits the differentiation of T regulatory (Treg) cells [115,116]. IL-6 also induces the activation of cytotoxic T cells from $CD8^+$ T cells [117]; stimulates higher expression of VEGF and cell adhesion molecules, resulting enhanced angiogenesis; and induces elevated vascular permeability and vascular leakage [118,119]. IL-6 is an important biomarker in understanding disease development as it is secreted in response to infection-, inflammation-, or stress-triggering factors. In healthy individuals, plasma levels of IL-6 concentration range between 2-10 pg/mL. In addition to immune cells, IL-6 can be

expressed by endothelial cells, epithelial cells, fibroblasts, and many other cells, depending on the stimulation and receptor binding [120,121].

### 1.5.2 Interleukin-6 Receptor

To perform biological activity, IL-6 binds with its transmembrane glycoprotein, named membrane-bound IL-6 receptor (mIL-6R), also designated as IL-6 receptor alpha or gp180 or CD126. mIL-6R expresses on just a few cell types, including leukocytes, hepatocytes, and some epithelial cells, which thereby restricts IL-6 signaling to within these cell types only [114,122]. A soluble form of IL-6 receptor (sIL-6R) has been found in circulation generated mainly by proteolytic cleavage of mIL-6R and to a minor extent by alternative splicing [123]. ADAM17 is the main protease protein involved in proteolytic cleavage of mIL-6R. This cleavage process is referred as ectodomain shedding [124]. sIL-6R is present in human plasma in a range of 25-145 ng/mL. sIL-6R binds with IL-6 to a higher affinity than that of mIL-6R and forms a binary complex (IL-6+sIL-6R) and is hence capable of stimulating cells to IL-6 signaling which do not express mIL-6R [83,101].

### 1.5.3 Glycoprotein 130

IL-6 and IL-6R complex (mIL-6R or sIL-6R) binds with gp130 or IL-6 receptor beta or IL-6 signal transducer or CD130 for transmitting the signal to the cytoplasmic domain [122,125]. gp130 receptor is ubiquitously expressed by all cells of the human body, including endothelial cells and fibroblasts [126]. In 1993, Narazaki et al. discovered a soluble form of gp130 (sgp130) in human serum that acts as a natural inhibitor of the IL-6+sIL-6R binary complex and forms a neutralized ternary complex (IL-6+sIL-6R+sgp130)

after binding with the binary complex [127,128]. Monocytes express high amounts of sgp130 by differential processing of gp130 mRNA but this expression is completely lost when monocytes are differentiated into macrophages. sgp130 exists at concentrations between 100-400 ng/mL in healthy human serum [129]. IL-6 has greater binding affinity to sIL-6R than mIL-6R, while sgp130 has specific binding affinity to the IL-6+sIL-6R binary complex only, and no binding affinity with either IL-6 or with sIL-6R alone [130].

### 1.5.4 Types of IL-6 Signaling

#### 1.5.4.1 IL-6 Trans-Signaling

During infection when antigen-presenting cells (APC) trigger the pro-inflammatory cascade, IL-6 trans-signaling appears first, later followed by classical signaling. Hence IL-6 trans-signaling is mainly a pro-inflammatory pathway that activates pro-inflammatory cytokines [114,131]. IL-6 trans-signaling is initiated by the binding of IL-6 with sIL-6R, forming a binary complex (IL-6+sIL-6R). Notably, sgp130 is attracted with the formation of the binary complex and neutralizes the binary complex by forming a ternary complex (IL-6+sIL-6R+sgp130) [120,132]. However, inhibition by sgp130 is not enough to trap all binary complexes, with the result that the free IL-6+sIL-6R binary complex can bind to any cells as gp130 is ubiquitously expressed by all cells (Figure 1.3A). Therefore, any nearby cells, such as endothelial cells without mIL-6R, are capable of inducing trans-signaling-mediated pro-inflammatory responses [132].

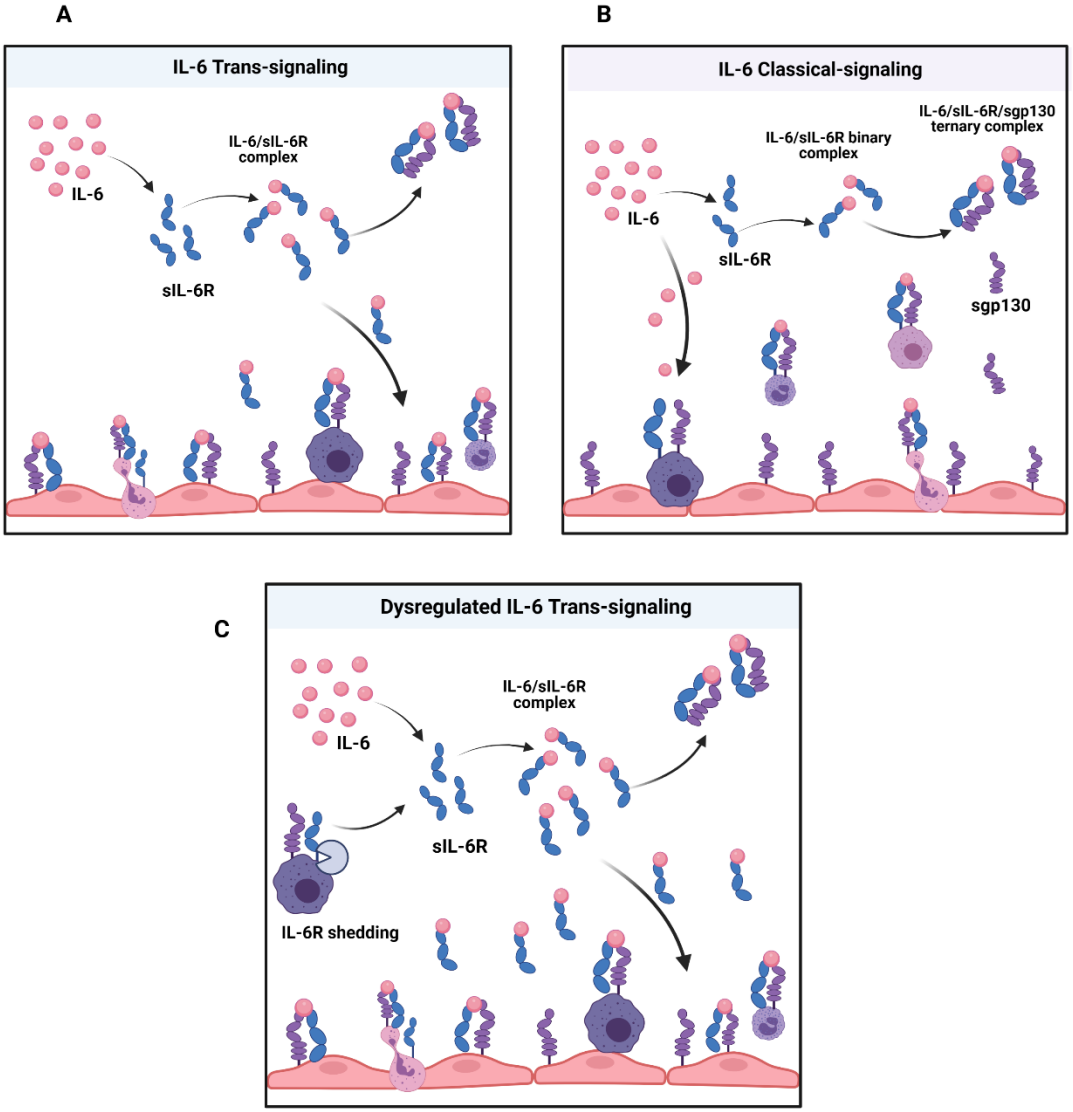
#### 1.5.4.2 IL-6 Classical signaling

Classical signaling takes place when most of the IL-6+sIL-6R binary complex is neutralized by sgp130 and frees IL-6 capable of binding to mIL-6R (Figure 1.3B) [83,133,134]. In the classical signaling pathway, IL-6 binds with its mIL-6R and subsequently with gp130 and triggers a downstream cascade, thereby maintaining this as a unique pathway to only those cells which express mIL-6R. IL-6 cis-signaling is anti-inflammatory by nature and characterized by activation of TGF-β, IL-10, IL-4, and IL-2 cytokines mainly, as well as by controlled expression of IL-6 [114,135,136]. IL-4 initiates polarization towards the M2 macrophage and TGF-β induces differentiation of naïve CD4+ T cells into regulatory T cells (Treg). The combination of TGF-β and IL-6 enhances production of IL-10 but neither TGF-β nor IL-6 alone can enhance IL-10 production [110].

In the event of infection or inflammation, IL-6 concentration can massively increase a million-fold and reach up to μg/mL, whereas sIL-6R can increase only two- to five-fold from its initial concentration [83,137]. Rapid increase in sIL-6R level is thought to be a result of membrane shedding of mIL-6R by ADAM17 [138]. Viral infections such as influenza and SARS-CoV-2 are shown to upregulate sIL-6R concentration by activating ADAM-17 [139,140]. It is hypothesized that in the presence of shedding events, sIL-6R concentration is upregulated and IL-6 fails to exceed the molar concentration of sIL-6R. Consequently, most of the IL-6 is attracted to form a binary complex with sIL-6R and sgp130 fails to trap these rapid, abundant binary complexes; consequently, polarization from IL-6 trans-signaling to cis-signaling is hampered, which is known as dysregulated IL-6 trans-signaling (Figure 1.3 C). Dysregulated trans-signaling causes overactivation of

endothelial cells, resulting in increased vascular permeability, endothelial dysfunction, fibrosis, and disseminated intravascular coagulation [83].

In both classical and trans-signaling IL-6 and its receptor complex (membrane bound or soluble form) associate with gp130 on cell surface to initiate the signal transduction for IL-6 production via JAK-STAT pathway [141]. Association with gp130 first activate Janus kinase (JAK) which is a tyrosine kinase family member. Activation of this kinase further leads to tyrosine phosphorylation and activation of signal transducer and activator of transcription 3 (STAT3) [142]. STAT3 further forms a dimer and transmit the transcription signal from the cell surface to nucleus [144]. The functional cis-regulatory elements in human IL-6 promoter includes binding sites for IRF-1, AP-1, C/EBP, Sp1 and NF-κB [110,146]. Cis-regulatory elements are activated by the stimulation with IL-1, TNF and TLR-mediated signal which leads to activation of IL-6 promoter. Viral proteins are also reported to enhance DNA binding activity of transcription factor NF-κB resulting higher IL-6 mRNA expression [148].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IL-6
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sgp130
C
Dysregulated IL-6 Trans-signaling
IL-6/sIL-6R complex
IL-6
sIL-6R
IL-6R shedding

**Figure 1.3 Graphical presentation of IL-6 signaling. A)** During IL-6 trans-signaling, most of the binary complexes (IL-6+sIL-6R) are neutralized by sgp130 (purple). The free binary complex binds with the nearby gp130 receptor expressed by any cells, such as endothelial cells. IL-6 trans-signaling triggers a pro-inflammatory cascade. **B)** In case of classical signaling, elevated level of sgp130 neutralized the available binary complexes. Free IL-6 binds with the membrane-bound IL-6 receptor, which is exclusively expressed only by limited cells (e.g., leukocytes, hepatocytes, and some epithelial cells). IL-6 classical signaling is anti-inflammatory. **C)** In the case of IL-6 receptor shedding, elevated levels of binary complexes are expected to form, which may result in dysregulated IL-6 trans-signaling. This event will result in uncontrolled pro-inflammatory responses.

## 1.6 Knowledge Gap regarding IL-6 and Severe COVID-19

Since the beginning of the COVID-19 pandemic, many studies reported high IL-6 levels in severe COVID-19 patients when compared to the IL-6 levels of healthy individuals, which inspired clinicians to consider inhibition of IL-6 signaling as a therapeutic intervention [143,145,147,149]. The approach used by available interventions, such as tocilizumab and sarilimab, is called global inhibition, where they inhibit both pro- and anti-inflammatory responses mediated by IL-6 [141,150]. Unfortunately, widespread acceptance of IL-6 as the keystone pro-inflammatory cytokine, along with no clinical evidence regarding the fine-tuning of the IL-6, sIL-6R and sgp130 axis in maintaining pro- and anti-inflammatory IL-6 cascade, and the absence of any therapeutic alternatives, the off-label use of tocilizumab for severe COVID-19 treatment has been promoted. However, tocilizumab failed to meet its primary endpoint in clinical trials [151,152].

Severe forms of COVID-19 are characterized by an imbalance between pro- and anti-inflammatory responses [153]. Studies suggest that IL-6 levels in severe COVID-19 patients might be higher than IL-6 levels in healthy control subjects, yet lower than levels of IL-6 in other severe non-COVID-19 patients with cytokine dysregulated syndrome [97,154,155]. Several studies have shown how the dysregulation of IL-6 signaling components (IL-6, sIL-6R and sgp130) are associated with cytokine release syndrome (CRS), cardiovascular disease, and inflammatory diseases [126,156,157]. To investigate the limitation of current IL-6 inhibition-based therapeutics, researchers have pointed out that, without knowing the type of IL-6 signaling in severe COVID-19, blind intervention based on cytokine elevation may worsen an already compromised immune system [158,159]. Our understanding of the IL-6 pathway in severe COVID-19 is still incomplete

because most studies are based on circulating IL-6 concentration only, without acknowledging its signaling components (sIL-6R and sgp130).

## 1.7 Rationale and Objectives

As current immunomodulatory treatment management including IL-6 inhibition in severe COVID-19 is based on previous experiences of severe non-COVID-19 patients [159,160], I investigated the types of IL-6 signaling in severe COVID-19 patients using severe non-COVID-19 patients as controls. I examined the differences in IL-6 signaling between critically ill COVID-19 and non-COVID-19 patients with sepsis symptoms (hereinafter severe COVID-19 and severe non-COVID-19, respectively). I studied the IL-6 signaling components IL-6, sIL-6R and sgp130 to characterize the types of IL-6 signaling in both disease groups. I also calculated the binary/ternary complex ratio (B/T ratio) which demonstrates the interaction of the three IL-6 signaling components as well as the IL-6 signaling type. Therefore, my objectives for this project were as follows: 1) characterizing the type of IL-6 signaling between the two critically ill patients groups, and 2) confirming that the IL-6 signaling components IL-6, sIL-6R and sgp130 are essential to determining the type of IL-6 signaling rather IL-6 alone.

# CHAPTER 2 MATERIALS AND METHODS

## 2.1 Ethics statement

The critically ill patient cohort obtained an institutional Research Board (IRB) approval granted by SJH/TUH Joint Research Ethics Committee and The Health Research Consent Declaration Committee (HRCDC) under the register REC: 2020-05 List 74 17 and project ID 0428. The COVID-19 negative samples are from a cohort that obtained an IRB approval at Dalhousie University and is covered under the protocol "Sentinel surveillance for severe outcomes of laboratory-confirmed influenza in adults for the annual influenza season and for confirmed and suspected cases of COVID-19/SARS-CoV-2 acute respiratory disease" REB#1,020,727.

## 2.2 Patient criteria and recruitment

All the samples used for COVID-19 biomarker analysis and characterizing the type of IL-6 signaling in this study were obtained from a prospective cohort of critically ill with sepsis symptoms patients admitted to a 40-bed mixed medical and surgical intensive care unit (ICU) at St James's Hospital in Dublin, Ireland, from September 2020 to March 2021. Per the definition of Sepsis-3, sepsis is defined as an acute change in the total SOFA score ≥2 points that is a result of the infection. The baseline SOFA score is assumed to be 0 in patients not known to have pre-existing organ dysfunction. Patients over 18 years of age admitted to the ICU who met the criteria for the definition of sepsis mentioned above were recruited into the study. Appendix 1 shows the actual number days patients had spent in the ICU when they were enrolled in the study. Patients were consented after receiving a

consultation and explanation of the study with an information leaflet. Patients who were mechanically ventilated at time of admission or enrollment were enrolled via consultation with their documented next-of-kin, who provided proxy consent in line with local ethics and consent declaration guidelines. Patients were excluded if they refused to consent or withdrew consent for the study. COVID-19 sepsis was confirmed with SARS-CoV-2 infection detected by polymerase chain reaction test performed in the hospital. A total of 50 PCR-confirmed severe COVID-19 and 30 severe non-COVID-19 patients were recruited during the enrollment period. Clinical and demographic characteristics of the critically ill patients are shown in appendix 2. Plasma samples from 11 COVID-19 negative individuals were also used to establish the baseline value for biomarkers screened from severe COVID-19 patients. These samples were collected from a non-profit long-term care (LTC) home in Halifax, Nova Scotia, during the first wave of COVID-19 between 26 and 28 April 2020. Clinical characteristics of enrolled SARS-CoV-2 negative individuals are shown in appendix 3. COVID-19 negative individuals were further screened for the presence of SARS-CoV-2 spike IgG and IgM, which are indicators of any previous SARS-CoV-2 infection before sampling.

### 2.3 Sample collection and storage

Whole blood samples were collected in EDTA tubes per local protocols at 3 time points from critically ill patients. Time point 1 (T1), on admission to ICU as soon as the criteria for the definition of sepsis was met; time point 2 (T2), 3-5 days following T1; time point 3 (T3), at day 14 from T1, if the patient survived and had not been discharged from the ICU due to ongoing sepsis symptoms. A total of 121 samples (T1:50; T2:46; T3:25)

were collected from severe COVID-19 patients and 70 samples (T1:30; T2:26; T3:14) were collected from severe non-COVID-19 patients. COVID-19 negative individuals were sampled once. Whole blood samples were processed no later than 2 hours after sample collection. Whole blood was centrifuged at 3400 rpm for 12 minutes to isolate plasma. Isolated plasma was aliquoted in 4-6 cryovials and stored in a -80°C freezer until further use.

## 2.4 Biomarker profiling

### 2.4.1 Multiplex cytokine assay

A panel of 25 biomarkers was analysed using the Ella-SimplePlex$^{TM}$ immunoassay (San Jose, California, USA). I chose these biomarkers based on previous evidence of their biological involvement in SARS and COVID-19 immunopathogenesis [161,162]. I further divided them into nine groups based on their biological function similarities during infection. Biomarkers were grouped as follows: Endothelial dysfunction: ICAM-1, E-selectin, VCAM-1, ANG-2, VEGF-C; neutrophil degranulation: myeloperoxidase (MPO), Lipocalin-2/NGAL; chemotaxis: CCL-2, CXCL-10; T helper 1 (Th1) response: IFN-γ, IL-15, IL-12, IL-2; pro-inflammatory (Th17) cytokine: IL-6, IL-17A, IL-1b; anti-inflammatory (Th2) cytokines: IL-4, IL-10, IL-1ra; T cell apoptosis: TNF-α, PD-L1/B7-H1; T cell survival:IL-7, Granzyme B; granulocyte mobilization: GM-CSF, G-CSF. Plasma samples were diluted using the sample diluent provided with the kit. Assay was performed according to the manufacturer's protocol.

### 2.4.2 Enzyme-linked Immunosorbent Assay (ELISA)

IL-6 signaling receptor biomarkers sIL-6R and sgp130 were measured using commercially available human sIL-6R ELISA (Cat: BMS214TEN, Invitrogen™, USA) and human sgp130 (IL6ST) ELISA (Cat: EHIL6STX10, Invitrogen™, USA) kits, respectively. Plasma samples were diluted 250X and 1000X for sIL-6R and sgp130 ELISA, respectively. Assays were performed according to the manufacturer's protocol.

## 2.5 Derivation of molar concentration of IL-6 signaling components and the binary and ternary complexes

Considering that IL-6, sIL-6R, and sgp130 interact in circulation on a molar basis, I calculated the molar (M) concentration of these biomarkers for all time points from each patient. To obtain the individual molar concentration, i.e., moles per litre (mol/L) of IL6, sIL6R, and sgp130, I divided the concentrations of individual biomarkers derived from immunoassay by their respective molecular weights in kilo Dalton (kD), i.e., IL6 by 23.7, sIL6R by 50, and sgp130 by 100. Later, I expressed their concentrations in nanomole/L (nmol/L). Next I estimated the nanomolar concentration of binary (IL-6:sIL-6R) complexes with equation 1 and ternary (IL-6:sIL:6R:sgp130) complexes with equation 2, below. This formula was first proposed by Müller-Newen *et al.* in 1998 [163] and later adapted by other research groups [156,164].

$$[\boldsymbol{IL6{:}\,sIL6R}] = \boldsymbol{0.5[sIL6R]_i + 0.5[IL6]_i + 0.5\,K_{D1} - 0.5([sIL6R]_i^2 + [IL6]_i^2 + 2[IL6]_i K_{D1} + K_{D1}^2)^{0.5}} \quad (\boldsymbol{equation\ 1})$$

$$[\boldsymbol{IL6{:}\,sIL6R{:}\,sgp130}] = \boldsymbol{0.5[sgp130]_i + 0.5[IL6{:}\,sIL6R]_i + 0.5K_{D2} - 0.5([sgp130]_i^2 + [IL6{:}\,sIL6R]_i^2 + 2[IL6{:}\,sIL6R]_i K_{D2} + K_{D2}^2)^{0.5}} \quad (\boldsymbol{equation\ 2})$$

where IL6, sIL6, and sgp130 are the nanomolar concentrations of the biomarkers of each patient. IL6:sIL6 represents the binary complex of these two biomarkers while IL6:sIL6:sgp130 represents the ternary complex among all three biomarkers. $\boldsymbol{K_{D1}}$ and $\boldsymbol{K_{D2}}$ represent the dissociation constants for the binary and ternary complex; that is, 0.5 and 0.05 nmol/L respectively. I derived the binary/ternary complex ratio (B/T ratio) by dividing equation 1 by equation 2. Here the value of the B/T ratio indicates free binary complexes, capable of systemic inflammation. Therefore, a high B/T ratio represents higher availability of free binary complexes.

### 2.6 Statistical Analyses

GraphPad Prism 9.4.0 (San Diego, USA) was used to assess the biomarkers at different time points. A p-value <0.05 was considered statistically significant and error bars indicate standard deviation (SD). I also used Support Vector Machines (SVM) to assess the disease model prediction capacity of the IL-6 signaling components (IL-6+sIL-6R+sgp130) and its individual components. The linear kernel was chosen. The classifier was implemented in Python (version 3.9.7) through the sklearn library (version 1.1.2). Each SVM model was trained and tested with concentrations of biomarkers in a proportion of

0.9/0.1 (train and test, respectively) in a stratified 10-fold cross-validation process. The metrics accuracy, precision, recall, specificity, receiver operator characteristic (ROC), and area under the curve (AUC) were obtained in each validation fold of each classification model and further used to assess the predictions. All of the performance metrics are found in the sklearn.metrics package.

# CHAPTER 3 RESULTS

## 3.1 Plasma biomarkers for endothelial dysfunction and pro-inflammatory responses are significantly higher in critically ill COVID-19 patients

To examine the level of pro- and anti-inflammatory biomarkers and their interaction in COVID-19 patients, first I examined the levels of 25 immune mediators in critically ill COVID-19 patients at three different time points. These biomarkers were selected because of their involvement in the inflammatory cascade during the infection as mentioned by previous studies [161,162]. ICAM -1, E-selectin, VCAM-1, ANG-2, VEGF-C were recognized biomarkers for endothelial activation and dysfunction [165,166]. IL-17A, IL-6, and IL-1β are Th17 cytokines, known for their pro-inflammatory function during infection [167]. Th2 cytokines (IL-4, IL-10, IL-1ra) were chosen because of their anti-inflammatory function [168,169]. Chemotaxis biomarkers such as CXCL-10 and CCL-2 are strong recruiters of macrophages, monocytes, neutrophils, and T cells to the site of inflammation [170,171].

Th1 cytokines promote cell-mediated immunity. I therefore checked circulating levels of IFN-γ, IL-2, IL-12, and IL-15 as representative biomarkers of Th1 response in severe COVID-19 [169,172]. GM-CSF contributes to macrophage survival and diminishes alveolar apoptosis while G-CSF promotes migration of neutrophils from bone marrow to peripheral blood [173,174]. Lipocalin-2 functions as a growth factor, stimulating immune cells proliferation. Its expression was found to be higher during sepsis onset [175]. Upregulation of MPO is positively associated with cytokine storms [176]. PD-L1 supresses the immune system by binding with PD1, thereby transmitting an inhibitory signal [177].

TNF-α mediates inflammatory responses by regulating growth and differentiation of several immune cells through the apoptosis process [178]. IL-7 stimulates cellular differentiation of hematopoietic stem cells into lymphoid progenitor cells [179].

During the COVID-19 pandemic many of these biomarkers were mentioned in different studies in order to assess their involvement in COVID-19 pathogenesis [180–182]. Our lab has been screening these panels of biomarkers in COVID-19 patients since the beginning of the pandemic [162]. Since the main focus of my thesis is to characterize the types of IL-6 signaling in COVID-19 patients, I previously screened the concentrations of these panels of twenty-five biomarkers. Profiling of individual biomarkers will not only exhibit their changes at different timepoints but also reveal their interactions with IL-6 in critically ill COVID-19 patients. This interaction will further help to explain the effects of the types of IL-6 signaling activated in severe COVID-19 immunopathogenesis.

My data shows that most of the biomarkers for endothelial dysfunction were highly upregulated in severe COVID-19 patients (Figure 3.1A). Plasma levels of Ang-2 ($p < 0.001$), E-selectin ($p = 0.0097$), and ICAM-1 ($p = 0.0024$) were also elevated significantly from T1 to T3, as well as higher than the baseline values observed in COVID-19 negative individuals. No significant changes were noticed in VCAM-1 levels from T1 to T3 though plasma VACM-1 was significantly higher than that at baseline at all three time points (Figure 3.1A). Plasma VEGF-C was lower than that at baseline; however, no statistically significant reduction was observed from T1 to T3 (Figure 3.1A).

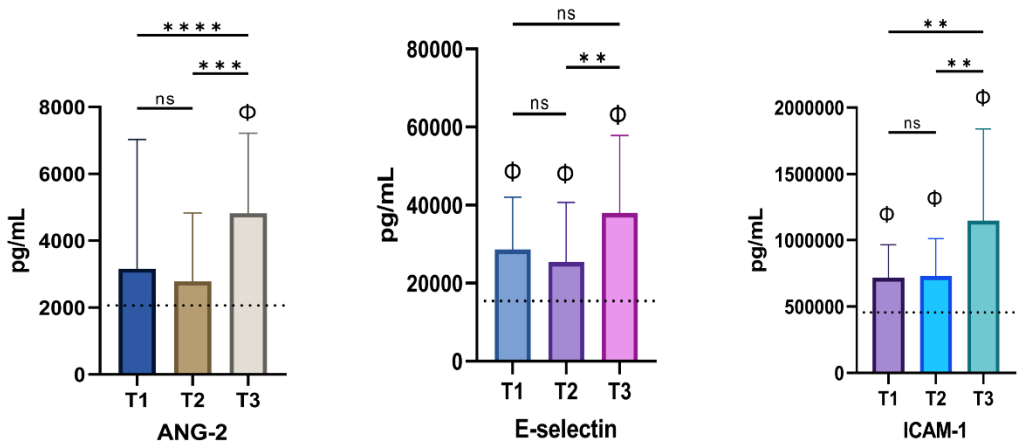
IL-6 and IL-17A were significantly higher than baseline levels while IL-6 increased significantly ($p < 0.0001$) from T1 to T3 (Figure 3.1B), but no significant changes were observed in IL-17A and IL-1β levels at different time points. As mentioned in other studies,

my data also showed acute phase upregulation of anti-inflammatory cytokines (IL-10 and IL-4) compared to baseline levels [183,184]. In my samples, IL-4 and IL-10 were significantly higher than baseline since T1; however, their levels started to decrease in subsequent time points (Figure 3.1C). IL-1ra, an inhibitor of IL-1β, was shown to increase from T1 to T3 ($p = 0.0004$) (Figure 3.1C).

Rapid increase of circulating CXCL-10 concentration followed by significant reductions ($p < 0.0001$) was observed in severe COVID-19 patients (Figure 3.1D). No statistically significant changes were observed in CCL-2 levels from T1 to T3; however, mean CCL-2 concentrations increased from T1 to T3, which is significantly higher than the baseline value (Figure 3.1D). SARS-CoV-2 is reported to downregulate the antiviral activity of interferons [185]. No significant changes in IFN-γ were detected in severe COVID-19 patients (Figure 3.1E). IL-12 regulates Th1 differentiation and maintenance. A significant reduction ($p < 0.0001$) in IL-12 was observed in severe COVID-19 patients after enrollment. No significant changes were observed in IL-2 and IL-15 levels during the study period (Figure 3.1E). Among other biomarkers, severe COVID-19 patients showed significant reductions ($p < 0.0001$) in GM-CSF (Figure 3.1F) and elevations ($p = 0.0005$) in TNF-α (Figure 3.1G). MPO remained higher than the baseline levels (Figure 3.1H) while IL-7 was downregulated over time (Figure 3.1I).

After screening the circulatory levels of all 25 biomarkers, I further checked the correlation between these biomarkers at each time point (Figures 3.1 J, K and L represent T1, T2, and T3 respectively). Correlation coefficient r value indicates the strength of relationship between variables. r value ranges from -1 to +1 where positive value indicates positively correlated and negative value indicates negative correlation while r=0 means no

correlation. Correlation matrix revealed that IL-6 is strongly correlated with the following pro-inflammatory biomarkers: ICAM-1, E-selectin, Ang-2, CCL-2, IL-1β, TNF-α, PD-L1/B7-H1, and IL-1ra. This data indicates that IL-6 is an important biomarker in severe COVID-19 immunopathogenesis that has significant interaction with biomarkers for vascular dysfunction and pro-inflammatory cytokines in severe SARS-CoV-2 infections [39,86,170,186]. However, none of these studies confirmed the types of IL-6 signaling involved in severe cases.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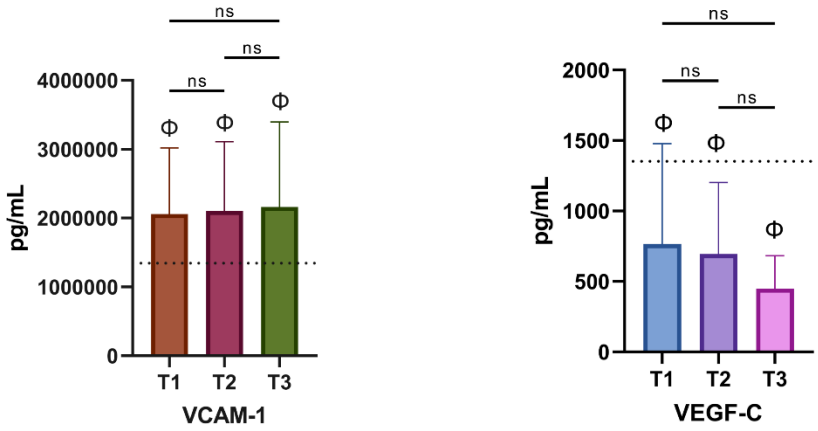
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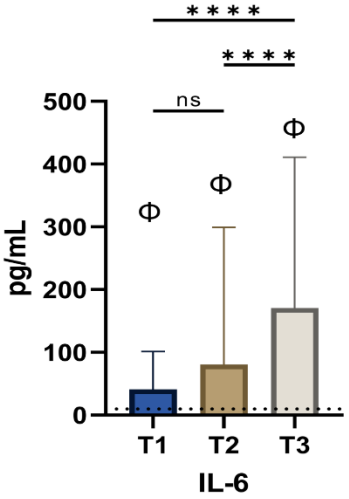
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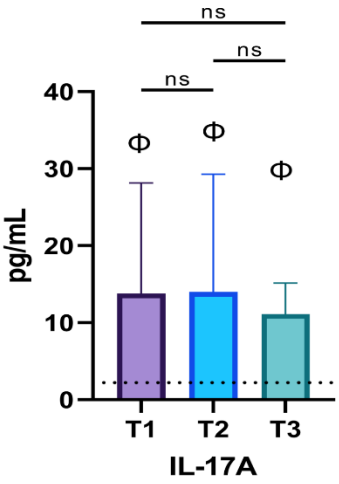
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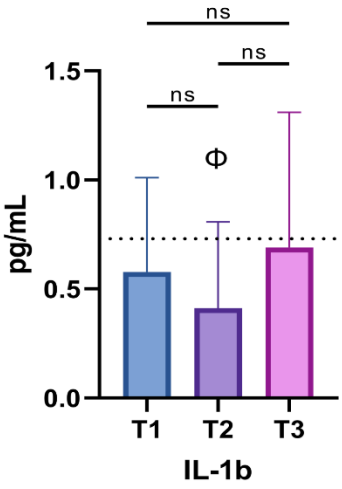
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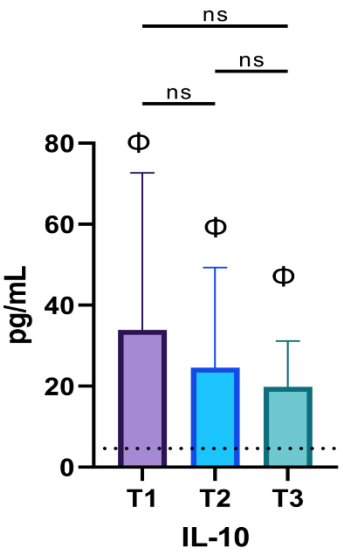
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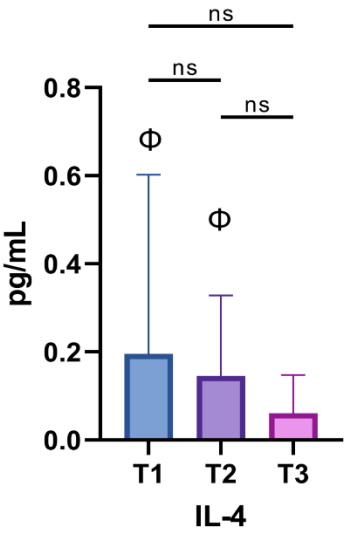
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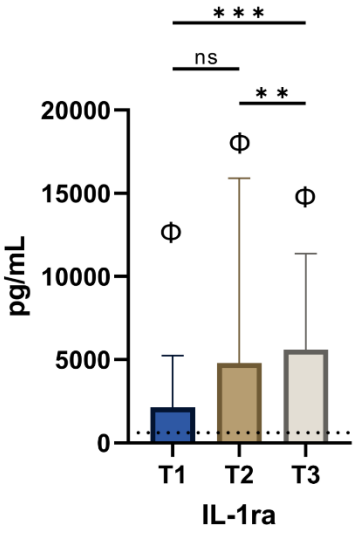
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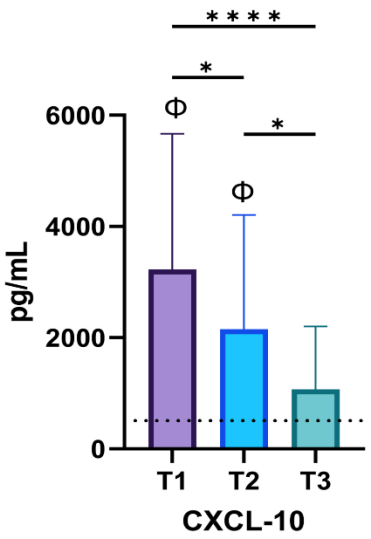
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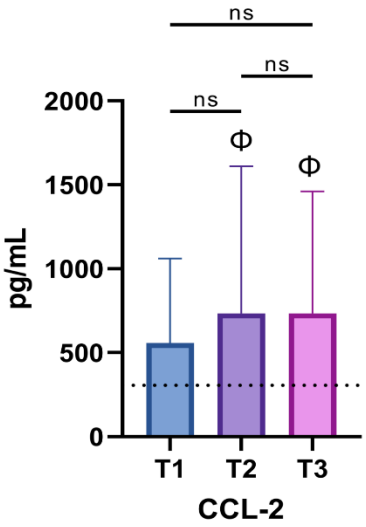
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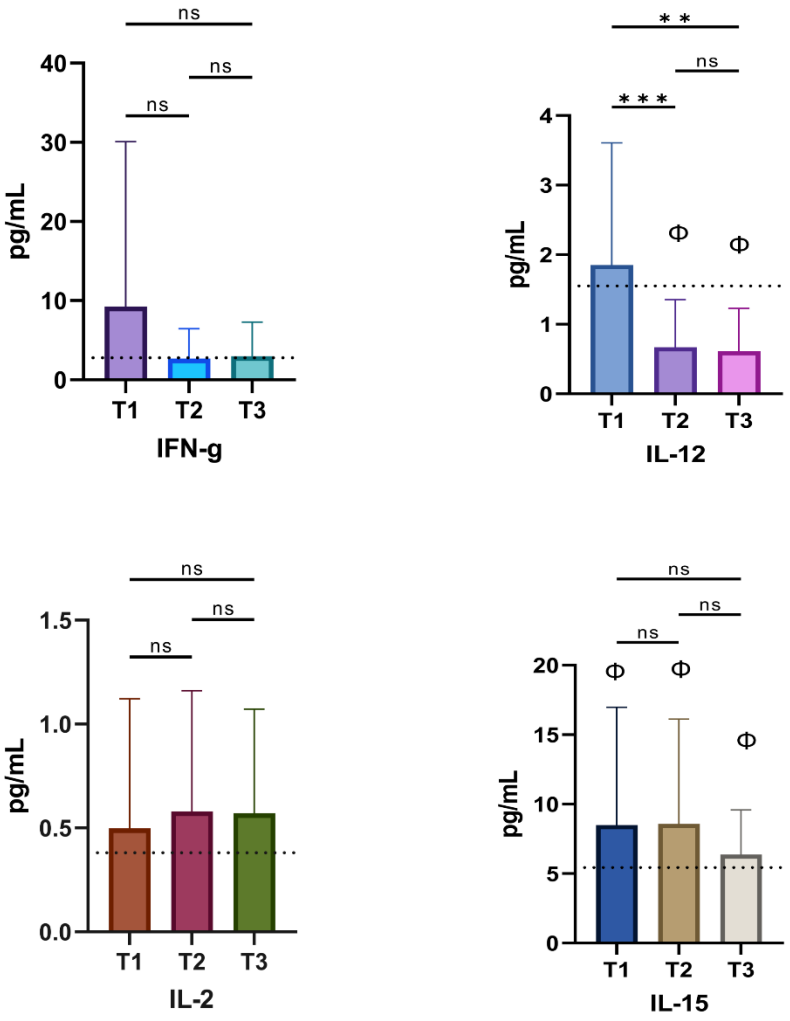
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IL-15

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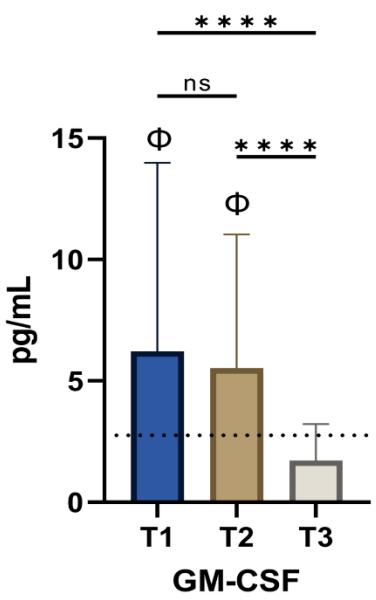
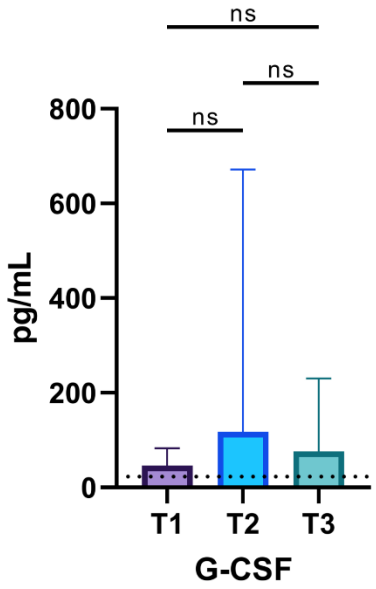

****
ns
****
Φ
Φ
15
10
5
0
pg/mL
T1
T2
T3
GM-CSF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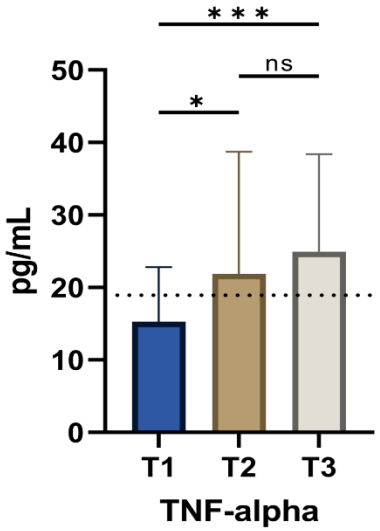
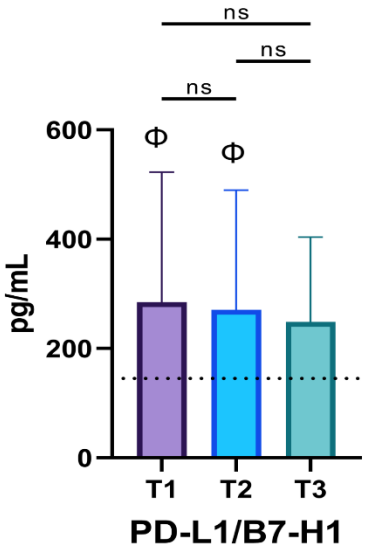
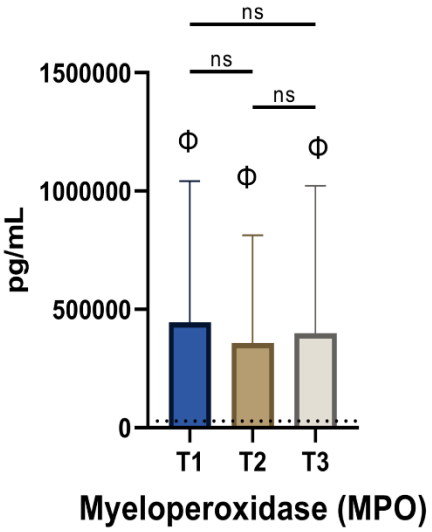
ns
ns
800
600
400
200
0
pg/mL
T1
T2
T3
G-CSF


* * *
ns
*
50
40
30
20
10
0
pg/mL
T1
T2
T3
TNF-alpha

**G**


ns
ns
ns
600
400
200
0
pg/mL
Φ
Φ
T1
T2
T3
PD-L1/B7-H1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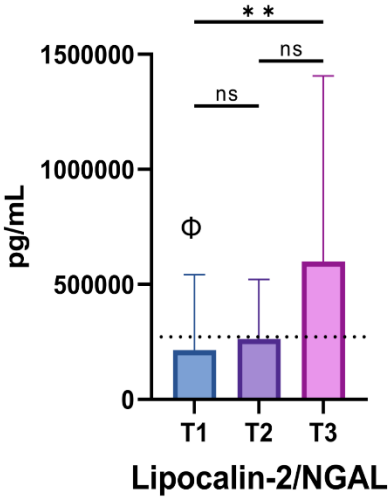
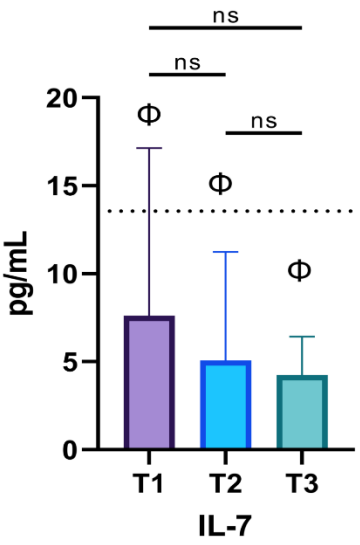
ns
ns
1500000
1000000
500000
0
pg/mL
Φ
Φ
Φ
T1
T2
T3
Myeloperoxidase (MPO)

**H**


* *
ns
ns
1500000
1000000
500000
0
pg/mL
Φ
T1
T2
T3
Lipocalin-2/NGAL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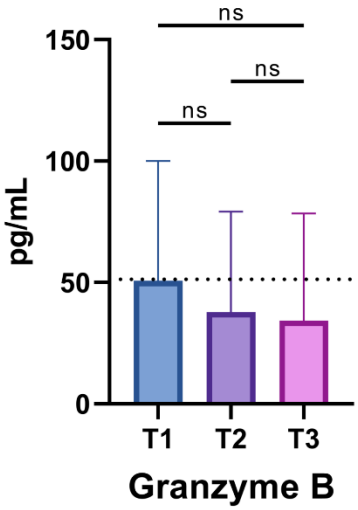
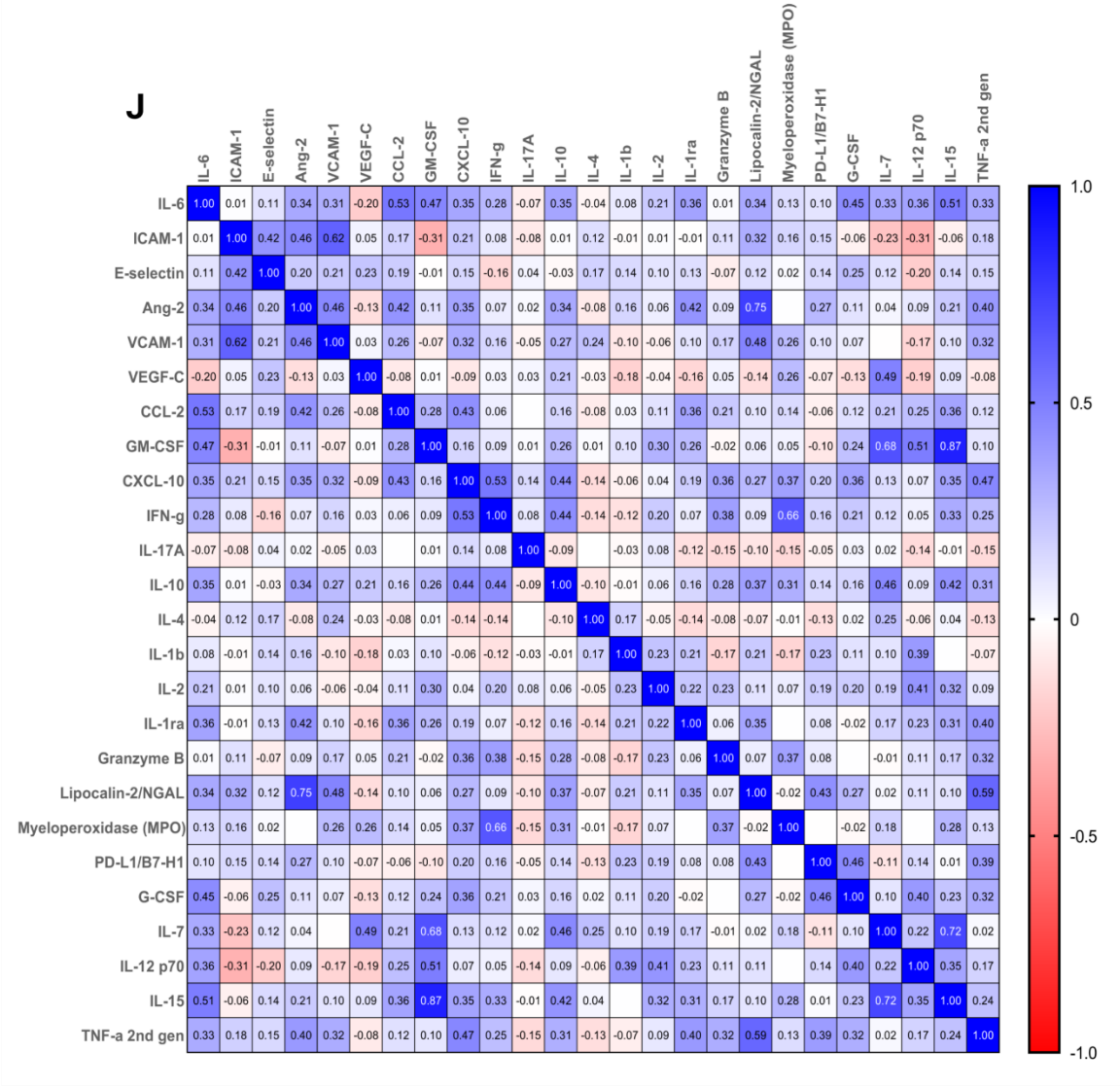
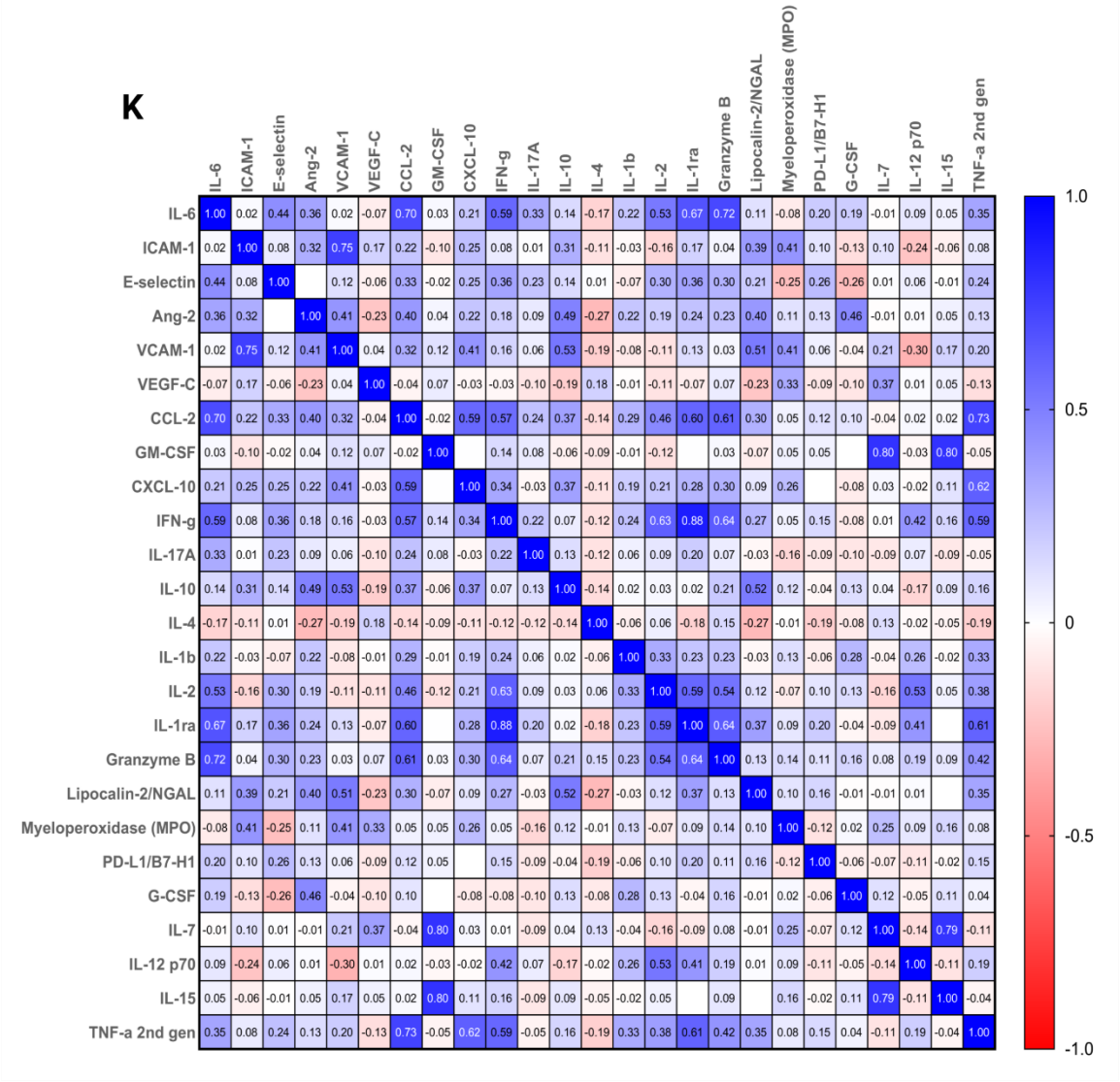
ns
ns
Φ
Φ
Φ
20
15
10
5
0
pg/mL
T1
T2
T3
IL-7

I


ns
ns
ns
150
100
50
0
pg/mL
T1
T2
T3
Granzyme B


J
IL-6 ICAM-1 E-selectin Ang-2 VCAM-1 VEGF-C CCL-2 GM-CSF CXCL-10 IFN-g IL-17A IL-10 IL-4 IL-1b IL-2 IL-1ra Granzyme B Lipocalin-2/NGAL Myeloperoxidase (MPO) PD-L1/B7-H1 G-CSF IL-7 IL-12 p70 IL-15 TNF-a 2nd gen
IL-6 1.00 0.01 0.11 0.34 0.31 -0.20 0.53 0.47 0.35 0.28 -0.07 0.35 -0.04 0.08 0.21 0.36 0.01 0.34 0.13 0.10 0.45 0.33 0.36 0.51 0.33
ICAM-1 0.01 1.00 0.42 0.46 0.62 0.05 0.17 -0.31 0.21 0.08 -0.08 0.01 0.12 -0.01 0.01 -0.01 0.11 0.32 0.16 0.15 -0.06 -0.23 -0.31 -0.06 0.18
E-selectin 0.11 0.42 1.00 0.20 0.21 0.23 0.19 -0.01 0.15 -0.16 0.04 -0.03 0.17 0.14 0.10 0.13 -0.07 0.12 0.02 0.14 0.25 0.12 -0.20 0.14 0.15
Ang-2 0.34 0.46 0.20 1.00 0.46 -0.13 0.42 0.11 0.35 0.07 0.02 0.34 -0.08 0.16 0.06 0.42 0.09 0.75 0.27 0.11 0.04 0.09 0.21 0.40
VCAM-1 0.31 0.62 0.21 0.46 1.00 0.03 0.26 -0.07 0.32 0.16 -0.05 0.27 0.24 -0.10 -0.06 0.10 0.17 0.48 0.26 0.10 0.07 -0.17 0.10 0.32
VEGF-C -0.20 0.05 0.23 -0.13 0.03 1.00 -0.08 0.01 -0.09 0.03 0.03 0.21 -0.03 -0.18 -0.04 -0.16 0.05 -0.14 0.26 -0.07 -0.13 0.49 -0.19 0.09 -0.08
CCL-2 0.53 0.17 0.19 0.42 0.26 -0.08 1.00 0.28 0.43 0.06 0.16 -0.08 0.03 0.11 0.36 0.21 0.10 0.14 -0.06 0.12 0.21 0.25 0.36 0.12
GM-CSF 0.47 -0.31 -0.01 0.11 -0.07 0.01 0.28 1.00 0.16 0.09 0.01 0.26 0.01 0.10 0.30 0.26 -0.02 0.06 0.05 -0.10 0.24 0.68 0.51 0.87 0.10
CXCL-10 0.35 0.21 0.15 0.35 0.32 -0.09 0.43 0.16 1.00 0.53 0.14 0.44 -0.14 -0.06 0.04 0.19 0.36 0.27 0.37 0.20 0.36 0.13 0.07 0.35 0.47
IFN-g 0.28 0.08 -0.16 0.07 0.16 0.03 0.06 0.09 0.53 1.00 0.08 0.44 -0.14 -0.12 0.20 0.07 0.38 0.09 0.66 0.16 0.21 0.12 0.05 0.33 0.25
IL-17A -0.07 -0.08 0.04 0.02 -0.05 0.03 0.01 0.14 0.08 1.00 -0.09 -0.03 0.08 -0.12 -0.15 -0.10 -0.15 -0.05 0.03 0.02 -0.14 -0.01 -0.15
IL-10 0.35 0.01 -0.03 0.34 0.27 0.21 0.16 0.26 0.44 0.44 -0.09 1.00 -0.10 -0.01 0.06 0.16 0.28 0.37 0.31 0.14 0.16 0.46 0.09 0.42 0.31
IL-4 -0.04 0.12 0.17 -0.08 0.24 -0.03 -0.08 0.01 -0.14 -0.14 -0.10 1.00 0.17 -0.05 -0.14 -0.08 -0.07 -0.01 -0.13 0.02 0.25 -0.06 0.04 -0.13
IL-1b 0.08 -0.01 0.14 0.16 -0.10 -0.18 0.03 0.10 -0.06 -0.12 -0.03 -0.01 0.17 1.00 0.23 0.21 -0.17 0.21 -0.17 0.23 0.11 0.10 0.39 -0.07
IL-2 0.21 0.01 0.10 0.06 -0.06 -0.04 0.11 0.30 0.04 0.20 0.08 0.06 -0.05 0.23 1.00 0.22 0.23 0.11 0.07 0.19 0.20 0.19 0.41 0.32 0.09
IL-1ra 0.36 -0.01 0.13 0.42 0.10 -0.16 0.36 0.26 0.19 0.07 -0.12 0.16 -0.14 0.21 0.22 1.00 0.06 0.35 0.08 -0.02 0.17 0.23 0.31 0.40
Granzyme B 0.01 0.11 -0.07 0.09 0.17 0.05 0.21 -0.02 0.36 0.38 -0.15 0.28 -0.08 -0.17 0.23 0.06 1.00 0.07 0.37 0.08 -0.01 0.11 0.17 0.32
Lipocalin-2/NGAL 0.34 0.32 0.12 0.75 0.48 -0.14 0.10 0.06 0.27 0.09 -0.10 0.37 -0.07 0.21 0.11 0.35 0.07 1.00 -0.02 0.43 0.27 0.02 0.11 0.10 0.59
Myeloperoxidase (MPO) 0.13 0.16 0.02 0.26 0.26 0.14 0.05 0.37 0.66 -0.15 0.31 -0.01 -0.17 0.07 0.37 -0.02 1.00 -0.02 0.18 0.28 0.13
PD-L1/B7-H1 0.10 0.15 0.14 0.27 0.10 -0.07 -0.06 -0.10 0.20 0.16 -0.05 0.14 -0.13 0.23 0.19 0.08 0.08 0.43 1.00 0.46 -0.11 0.14 0.01 0.39
G-CSF 0.45 -0.06 0.25 0.11 0.07 -0.13 0.12 0.24 0.36 0.21 0.03 0.16 0.02 0.11 0.20 -0.02 0.27 -0.02 0.46 1.00 0.10 0.40 0.23 0.32
IL-7 0.33 -0.23 0.12 0.04 0.49 0.21 0.68 0.13 0.12 0.02 0.46 0.25 0.10 0.19 0.17 -0.01 0.02 0.18 -0.11 0.10 1.00 0.22 0.72 0.02
IL-12 p70 0.36 -0.31 -0.20 0.09 -0.17 -0.19 0.25 0.51 0.07 0.05 -0.14 0.09 -0.06 0.39 0.41 0.23 0.11 0.11 0.14 0.40 0.22 1.00 0.35 0.17
IL-15 0.51 -0.06 0.14 0.21 0.10 0.09 0.36 0.87 0.35 0.33 -0.01 0.42 0.04 0.32 0.31 0.17 0.10 0.28 0.01 0.23 0.72 0.35 1.00 0.24
TNF-a 2nd gen 0.33 0.18 0.15 0.40 0.32 -0.08 0.12 0.10 0.47 0.25 -0.15 0.31 -0.13 -0.07 0.09 0.40 0.32 0.59 0.13 0.39 0.32 0.02 0.17 0.24 1.00
1.0
0.5
0
-0.5
-1.0

**K**


IL-6 ICAM-1 E-selectin Ang-2 VCAM-1 VEGF-C CCL-2 GM-CSF CXCL-10 IFN-g IL-17A IL-10 IL-4 IL-1b IL-2 IL-1ra Granzyme B Lipocalin-2/NGAL Myeloperoxidase (MPO) PD-L1/B7-H1 G-CSF IL-7 IL-12 p70 IL-15 TNF-a 2nd gen
1.0
0.5
0
-0.5
-1.0

**L**

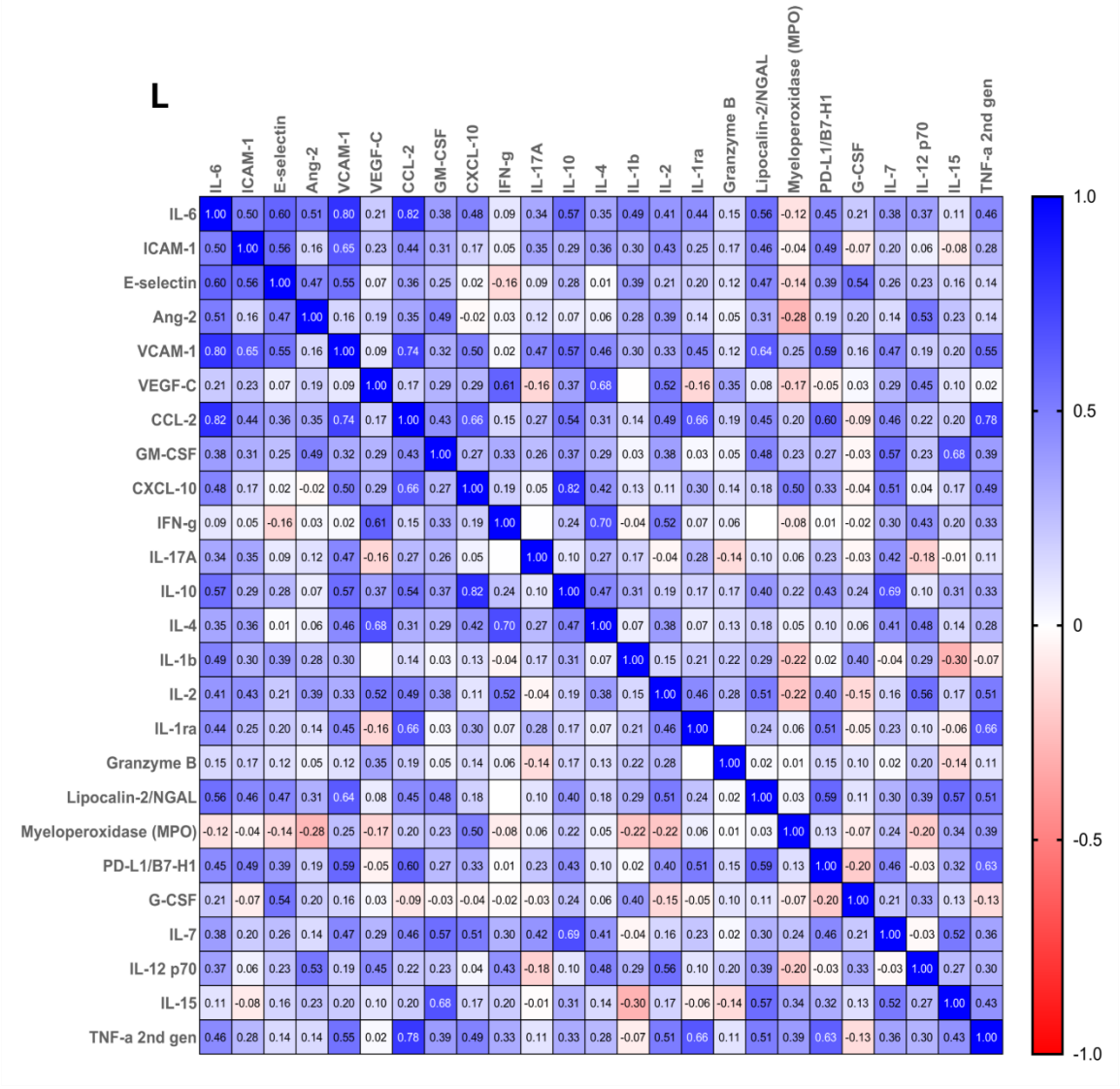

IL-6 ICAM-1 E-selectin Ang-2 VCAM-1 VEGF-C CCL-2 GM-CSF CXCL-10 IFN-g IL-17A IL-10 IL-4 IL-1b IL-2 IL-1ra Granzyme B Lipocalin-2/NGAL Myeloperoxidase (MPO) PD-L1/B7-H1 G-CSF IL-7 IL-12 p70 IL-15 TNF-a 2nd gen
IL-6 1.00 0.50 0.60 0.51 0.80 0.21 0.82 0.38 0.48 0.09 0.34 0.57 0.35 0.49 0.41 0.44 0.15 0.56 -0.12 0.45 0.21 0.38 0.37 0.11 0.46
ICAM-1 0.50 1.00 0.56 0.16 0.65 0.23 0.44 0.31 0.17 0.05 0.35 0.29 0.36 0.30 0.43 0.25 0.17 0.46 -0.04 0.49 -0.07 0.20 0.06 -0.08 0.28
E-selectin 0.60 0.56 1.00 0.47 0.55 0.07 0.36 0.25 0.02 -0.16 0.09 0.28 0.01 0.39 0.21 0.20 0.12 0.47 -0.14 0.39 0.54 0.26 0.23 0.16 0.14
Ang-2 0.51 0.16 0.47 1.00 0.16 0.19 0.35 0.49 -0.02 0.03 0.12 0.07 0.06 0.28 0.39 0.14 0.05 0.31 -0.28 0.19 0.20 0.14 0.53 0.23 0.14
VCAM-1 0.80 0.65 0.55 0.16 1.00 0.09 0.74 0.32 0.50 0.02 0.47 0.57 0.46 0.30 0.33 0.45 0.12 0.64 0.25 0.59 0.16 0.47 0.19 0.20 0.55
VEGF-C 0.21 0.23 0.07 0.19 0.09 1.00 0.17 0.29 0.29 0.61 -0.16 0.37 0.68 0.52 -0.16 0.35 0.08 -0.17 -0.05 0.03 0.29 0.45 0.10 0.02
CCL-2 0.82 0.44 0.36 0.35 0.74 0.17 1.00 0.43 0.66 0.15 0.27 0.54 0.31 0.14 0.49 0.66 0.19 0.45 0.20 0.60 -0.09 0.46 0.22 0.20 0.78
GM-CSF 0.38 0.31 0.25 0.49 0.32 0.29 0.43 1.00 0.27 0.33 0.26 0.37 0.29 0.03 0.38 0.03 0.05 0.48 0.23 0.27 -0.03 0.57 0.23 0.68 0.39
CXCL-10 0.48 0.17 0.02 -0.02 0.50 0.29 0.66 0.27 1.00 0.19 0.05 0.82 0.42 0.13 0.11 0.30 0.14 0.18 0.50 0.33 -0.04 0.51 0.04 0.17 0.49
IFN-g 0.09 0.05 -0.16 0.03 0.02 0.61 0.15 0.33 0.19 1.00 0.24 0.70 -0.04 0.52 0.07 0.06 -0.08 0.01 -0.02 0.30 0.43 0.20 0.33
IL-17A 0.34 0.35 0.09 0.12 0.47 -0.16 0.27 0.26 0.05 1.00 0.10 0.27 0.17 -0.04 0.28 -0.14 0.10 0.06 0.23 -0.03 0.42 -0.18 -0.01 0.11
IL-10 0.57 0.29 0.28 0.07 0.57 0.37 0.54 0.37 0.82 0.24 0.10 1.00 0.47 0.31 0.19 0.17 0.17 0.40 0.22 0.43 0.24 0.69 0.10 0.31 0.33
IL-4 0.35 0.36 0.01 0.06 0.46 0.68 0.31 0.29 0.42 0.70 0.27 0.47 1.00 0.07 0.38 0.07 0.13 0.18 0.05 0.10 0.06 0.41 0.48 0.14 0.28
IL-1b 0.49 0.30 0.39 0.28 0.30 0.14 0.03 0.13 -0.04 0.17 0.31 0.07 1.00 0.15 0.21 0.22 0.29 -0.22 0.02 0.40 -0.04 0.29 -0.30 -0.07
IL-2 0.41 0.43 0.21 0.39 0.33 0.52 0.49 0.38 0.11 0.52 -0.04 0.19 0.38 0.15 1.00 0.46 0.28 0.51 -0.22 0.40 -0.15 0.16 0.56 0.17 0.51
IL-1ra 0.44 0.25 0.20 0.14 0.45 -0.16 0.66 0.03 0.30 0.07 0.28 0.17 0.07 0.21 0.46 1.00 0.24 0.06 0.51 -0.05 0.23 0.10 -0.06 0.66
Granzyme B 0.15 0.17 0.12 0.05 0.12 0.35 0.19 0.05 0.14 0.06 -0.14 0.17 0.13 0.22 0.28 1.00 0.02 0.01 0.15 0.10 0.02 0.20 -0.14 0.11
Lipocalin-2/NGAL 0.56 0.46 0.47 0.31 0.64 0.08 0.45 0.48 0.18 0.10 0.40 0.18 0.29 0.51 0.24 0.02 1.00 0.03 0.59 0.11 0.30 0.39 0.57 0.51
Myeloperoxidase (MPO) -0.12 -0.04 -0.14 -0.28 0.25 -0.17 0.20 0.23 0.50 -0.08 0.06 0.22 0.05 -0.22 -0.22 0.06 0.01 0.03 1.00 0.13 -0.07 0.24 -0.20 0.34 0.39
PD-L1/B7-H1 0.45 0.49 0.39 0.19 0.59 -0.05 0.60 0.27 0.33 0.01 0.23 0.43 0.10 0.02 0.40 0.51 0.15 0.59 0.13 1.00 -0.20 0.46 -0.03 0.32 0.63
G-CSF 0.21 -0.07 0.54 0.20 0.16 0.03 -0.09 -0.03 -0.04 -0.02 -0.03 0.24 0.06 0.40 -0.15 -0.05 0.10 0.11 -0.07 -0.20 1.00 0.21 0.33 0.13 -0.13
IL-7 0.38 0.20 0.26 0.14 0.47 0.29 0.46 0.57 0.51 0.30 0.42 0.69 0.41 -0.04 0.16 0.23 0.02 0.30 0.24 0.46 0.21 1.00 -0.03 0.52 0.36
IL-12 p70 0.37 0.06 0.23 0.53 0.19 0.45 0.22 0.23 0.04 0.43 -0.18 0.10 0.48 0.29 0.56 0.10 0.20 0.39 -0.20 -0.03 0.33 -0.03 1.00 0.27 0.30
IL-15 0.11 -0.08 0.16 0.23 0.20 0.10 0.20 0.68 0.17 0.20 -0.01 0.31 0.14 -0.30 0.17 -0.06 -0.14 0.57 0.34 0.32 0.13 0.52 0.27 1.00 0.43
TNF-a 2nd gen 0.46 0.28 0.14 0.14 0.55 0.02 0.78 0.39 0.49 0.33 0.11 0.33 0.28 -0.07 0.51 0.66 0.11 0.51 0.39 0.63 -0.13 0.36 0.30 0.43 1.00
1.0
0.5
0
-0.5
-1.0

**Figure 3.1 Levels of plasma biomarkers indicating elevated inflammatory biomarkers in severe COVID-19 patients.** Plasma samples were collected at three time points. Each column bar represents a time point (T1, n=50; T2, n=46; T3, n=25). Biomarkers were quantified with Ella simple plex immunoassay. 50 µl of diluted plasma samples were used for the assay. All dilutions were performed using the sample diluent provided with the kit. Dotted line (…..) represents the baseline value measured from SARS-CoV-2-negative individuals. Error bars indicate standard deviation. Statistical significance was assessed with a Kruskal-Wallis test with Dunn's correction for multiple comparisons. P value classification (ns-non-significant, $p > 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$). ϕ indicates significant differences with baseline value.

**(A)** biomarkers for endothelial dysfunction: ICAM-1, E-selectin, VCAM-1, ANG-2, and VEGF-C. **(B)** biomarkers for pro-inflammatory cytokines: IL-6, IL-17A, IL-1b. **(C)** anti-inflammatory cytokines: IL-4, IL-10, IL-1ra. **(D)** chemotaxis biomarkers: CCL-2, CXCL-10. **(E)** Th1 biomarkers: IFN-g, IL-15, IL-12, IL-2. **(F)** granulocytes mobilization biomarkers: GM-CSF, G-CSF. **(G)** T cell apoptosis biomarkers: TNF-alpha, PD-L1/B7-H1. **(H)** neutrophil degranulation biomarkers: MPO, Lipocalin-2/NGAL, (I) T cell survival biomarkers, IL-7, Granzyme-B,

**(J, K &L):** Heat map representing the Pearson correlation matrix R values between 25 biomarkers at 3 different time points. (J) Time point 1; (K) Time point 2; (L) Time point 3. Positive correlation at all three time points was observed between proinflammatory cytokine IL-6 with IL-1b, biomarkers for endothelial dysfunction (ICAM-1, E-selectin, Ang-2), biomarkers for T cell apoptosis (TNF-alpha, PD-L1/B7-H1), chemotaxis

biomarkers CCL2, and anti-inflammatory cytokine IL-1ra. Both chemotaxis biomarkers (CCL2 and CXCL-10) were also positively correlated.

## 3.2 IL-6 signaling components can successfully characterize the type of IL-6 signaling

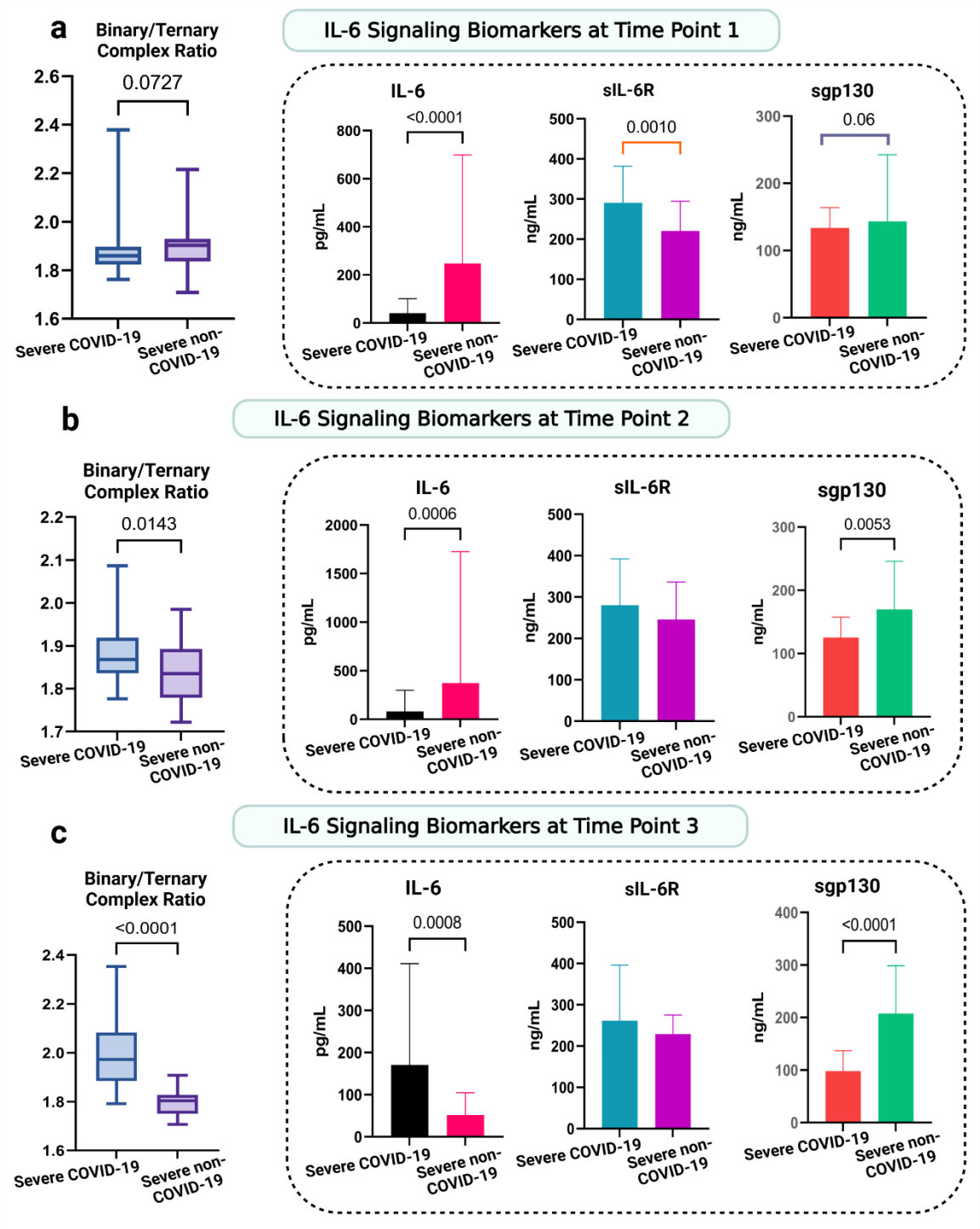
To characterize the type of IL-6 signaling between two critically ill patient groups, I investigated IL-6 signaling components (IL-6, sIL-6R, and sgp130) in severe COVID-19 and severe non-COVID-19 patients at three different time points. I also analyzed their B/T ratio to summarize the interaction of three IL-6 signaling biomarkers as well as the evolution of IL-6 signaling.

My data shows that, at T1 (Figure 3.2A), IL-6 was significantly higher in the severe non-COVID-19 group compared to the IL-6 in the severe COVID-19 group (247.827 pg/mL and 40.918 pg/mL for severe COVID-19 and severe non-COVID-19, respectively). However, sIL-6R, which is the essential molecule to form binary complexes and trigger IL-6 trans-signaling, was significantly higher in severe COVID-19 patients at T1. No statistical significance was found in either the binary complex neutralizing molecule sgp130 nor in the B/T ratio between the two groups (Figure 3.2A).

At T2, the IL-6 levels continued to increase (Figure: 3.2B) in both groups; however, IL-6 in the severe non-COVID-19 group was still significantly higher than that in the COVID-19 group (372.876 pg/mL in severe non-COVID-19 and 80.628 pg/mL in COVID-19). No statistical significance was observed in sIL-6R levels between the two groups but their mean concentration (280 ng/mL for severe COVID-19 and 245.49 for non-COVID-19) was higher than the healthy normal range (25-75 ng/mL). Interestingly, the severe COVID-19 group showed a significant decrease in sgp130, which is also reflected by a significant increase of B/T ratio in the severe COVID-19 group (Figure: 3.2B).

From T3 sampling (Figure 3.2C), severe COVID-19 patients were characterized by a continuous increase of IL-6 levels (170 pg/mL at T3) and decrease in the IL-6 trans-signaling inhibitor sgp130. On the other hand, non-COVID-19 patients showed continual upregulation of sgp130 and a sudden drop in IL-6 levels (52.03 pg/mL at T3). No statistical significance in sIL-6R levels was observed in both groups; however, the mean concentration (261.62 ng/mL and 228.81 ng/mL for COVID-19 and non-COVID-19 respectively) remained higher than mean concentration in the healthy range until T3. Significant differences were also observed in the B/T ratio between the two disease models. Table 1 summarizes the percentage of changes of all IL-6 signaling components at time point intervals.

To summarize the interaction of all three IL-6 signaling biomarkers from T1 to T3 and visualize the trend of IL-6 signaling, I further plotted the B/T ratio at three time points (Figure 3.2D). Longitudinal data of the B/T ratio showed that the severe COVID-19 group had a significant evolution from T1 to T3 while the ratio decreased significantly in the severe non-COVID group. An elevated B/T ratio indicates uncontrolled binary complexes triggering IL-6 trans-signaling, while a diminishing trend of B/T ratio in severe non-COVID-19 is the sign of down regulating IL-6 trans-signaling by sgp130.


a
Binary/Ternary Complex Ratio
0.0727
2.6
2.4
2.2
2.0
1.8
1.6
Severe COVID-19
Severe non-COVID-19
IL-6 Signaling Biomarkers at Time Point 1
IL-6
<0.0001
pg/mL
800
600
400
200
0
sIL-6R
0.0010
ng/mL
500
400
300
200
100
0
sgp130
0.06
ng/mL
300
200
100
0
b
Binary/Ternary Complex Ratio
0.0143
2.2
2.1
2.0
1.9
1.8
1.7
IL-6 Signaling Biomarkers at Time Point 2
IL-6
0.0006
pg/mL
2000
1500
1000
500
0
sIL-6R
ng/mL
500
400
300
200
100
0
sgp130
0.0053
ng/mL
300
200
100
0
c
Binary/Ternary Complex Ratio
<0.0001
2.4
2.2
2.0
1.8
1.6
IL-6 Signaling Biomarkers at Time Point 3
IL-6
0.0008
pg/mL
500
400
300
200
100
0
sIL-6R
ng/mL
500
400
300
200
100
0
sgp130
<0.0001
ng/mL
400
300
200
100
0

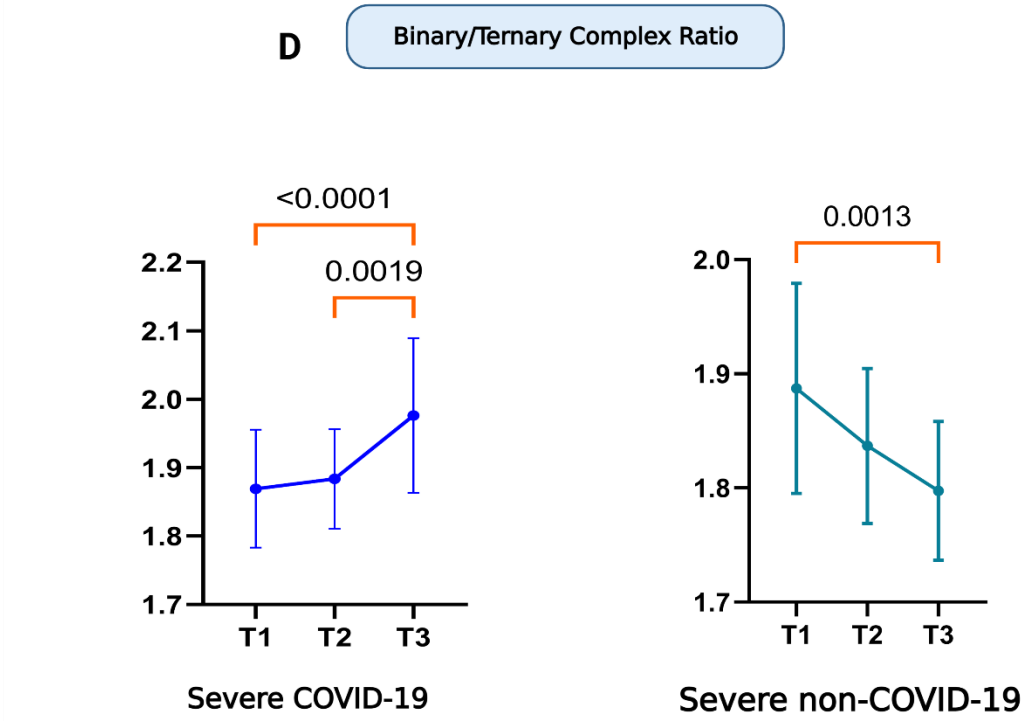

D
Binary/Ternary Complex Ratio
<0.0001
0.0019
2.2
2.1
2.0
1.9
1.8
1.7
T1
T2
T3
Severe COVID-19
0.0013
2.0
1.9
1.8
1.7
T1
T2
T3
Severe non-COVID-19

**Figure 3.2 Expression of IL-6 signaling components explain the differential IL-6 signaling in two critically ill disease groups.** sIL-6R and sgp130 were measured by solid-phase sandwich ELISA kit. Dilution for sIL-6R and sgp130 were 250- and 1000-fold, respectively. Statistical significance was assessed with a two-tailed unpaired Mann-Whitney test. **A.** At time point 1 (severe COVID-19, n= 50; severe non-COVID-19, n= 30), no statistical significance was found in the binary/ternary complex ratio between the two patient groups. Responsible biomarkers (IL-6, sIL-6R and sgp130) making up the binary/ternary complex ratio showed significant differences. IL-6 was significantly higher ($p < 0.0001$) in the non-COVID-19 group but both groups had no significant differences in sgp130 concentrations. The COVID-19 sepsis group had significantly higher ($P = 0.0010$) levels of sIL-6R compared to the non-COVID-19 group at T1, at the onset of sepsis symptoms. **B.** At time point 2 (COVID-19, n= 46; non-COVID-19, n= 26), the COVID-19 group had significantly higher ($P= 0.0143$) binary/ternary complex ratios compared to the non-COVID-19 group. IL-6 was still significantly higher ($P = 0.0006$) in the non-COVID-19 group. Significant differences ($P = 0.0053$) were observed in sgp130 levels between the two groups with reductions in COVID-19 and increases in non-COVID-19. **C.** At time point 3 (severe COVID-19, n= 25; severe non-COVID-19, n= 14), the significant difference of the binary/ternary ratio between the two groups is progressing ($P < 0.0001$). Significant decrease of IL-6 levels ($P= 0.0004$) and increase of sgp130 levels ($p < 0.0001$) were observed in the non-COVID-19 group. No significant difference in sIL-6R levels.

**D):** Trend of Binary/Ternary complex ratio explains the dysregulation as well as the type of IL-6 signaling. Statistical significance was assessed with a Kruskal-Wallis test with Dunn's correction for multiple comparisons.

| Time point difference | B/T Ratio | IL-6 | sIL-6R | sgp130 | Group |
|---|---|---|---|---|---|
| **T2-T1** | 0.76 | 49.25 | -3.78 | -6.52 | **Severe COVID-19** |
| **T3-T2** | 4.88 | 52.71 | -7.10 | -27.84 | |
| **T2-T1** | -2.75 | 33.54 | 10.15 | 15.64 | **Severe Non-COVID-19** |
| **T3-T2** | -2.35 | -616.68 | -7.29 | 18.09 | |

**Table 1: Percentages of changes of IL-6 signaling components between time point intervals.** (+) indicates elevation and (-) represents reduction.

## 3.3 IL-6 signaling biomarkers together are the best predictors to classify a critically ill disease model

Prediction modeling was attempted using the biomarkers that compose the IL-6 signaling. The objective was to determine whether IL-6 signaling biomarkers individually or together can classify patients into either severe COVID-19 or severe non-COVID-19 (Figure 3.3). For this prediction analysis, I used the last time point data for each patient because, after the onset of sepsis symptoms, the last time point data is the closest to an outcome (deceased or discharged or stayed in ICU) for each patient in this study. A support vector machine consisting of a linear kernel was used for predicting the type of disease model undergoing a stratified 10-fold cross-validation process. The best AUROC score was found to be $0.83 \pm 0.10$ with the input set for the three IL-6 signaling biomarkers together (IL-6, sIL-6R, sgp130) (Figure 3.3A). When I employed the individual biomarkers in the same classificatory rationale, the AUROC obtained was $0.56 \pm 0.21$, $0.39 \pm 0.22$, and $0.78 \pm 0.11$ for IL6, sIL6, and sgp130, respectively. This data confirms that severe COVID-19 and non-COVID-19 patients are best classified when we consider all three IL-6 signaling components together, rather than considering only IL-6 or the other two signaling components individually.

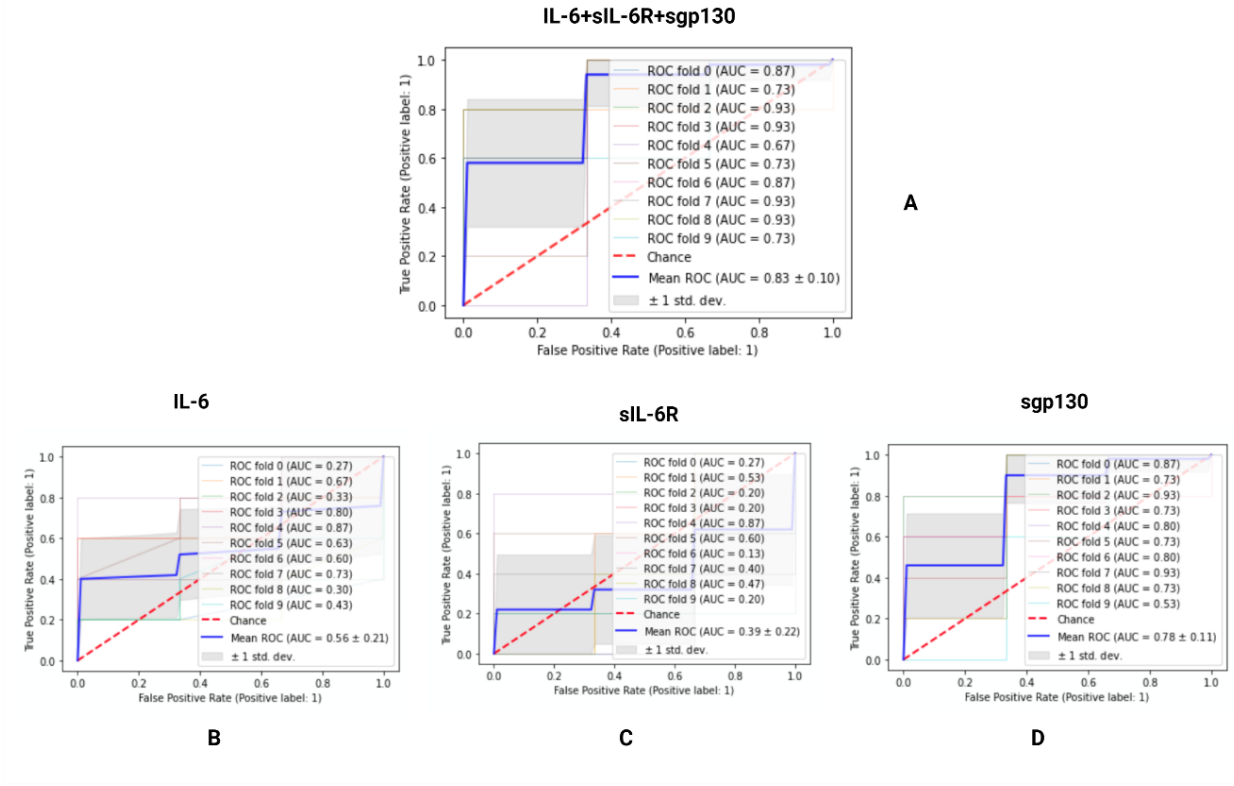
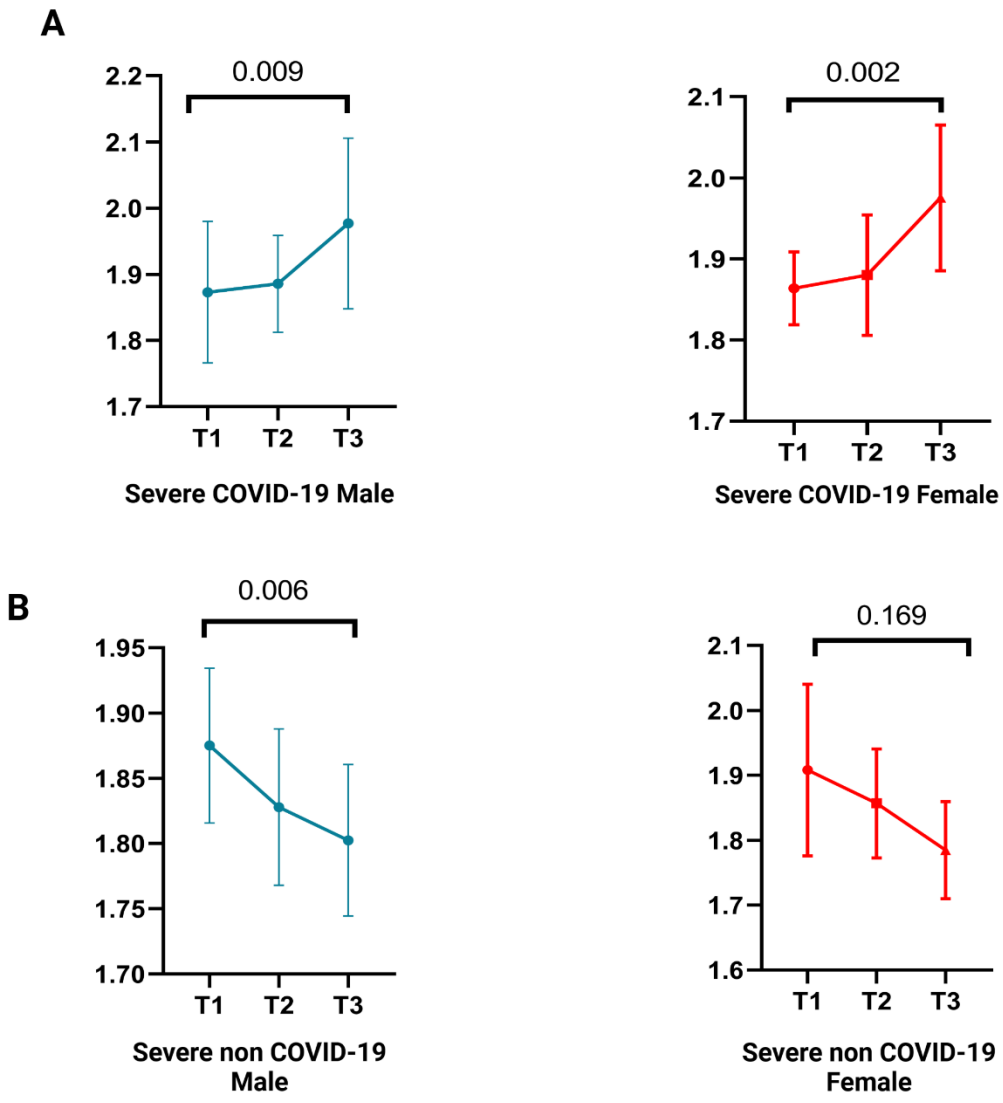
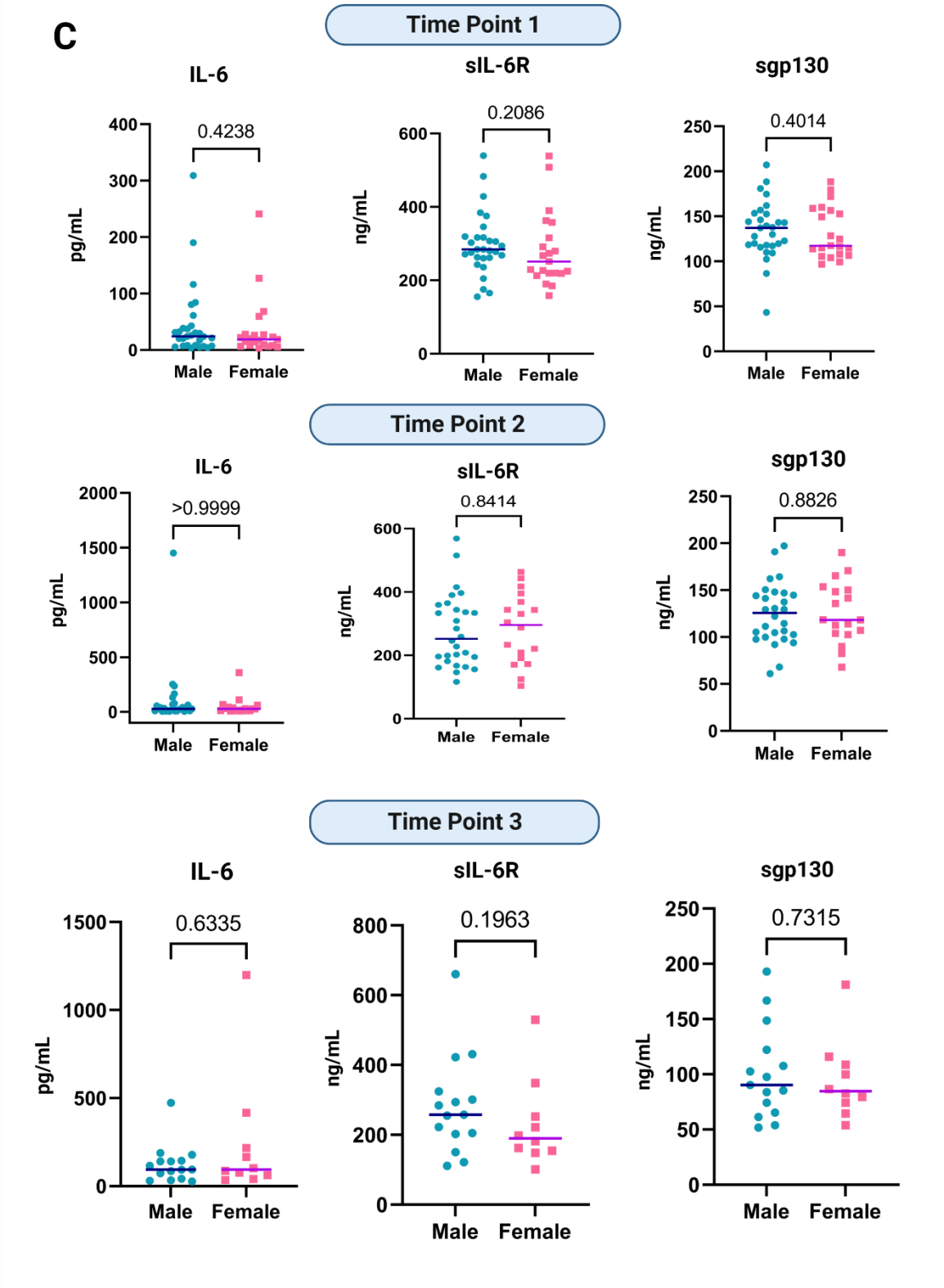

IL-6+sIL-6R+sgp130
True Positive Rate (Positive label: 1)
False Positive Rate (Positive label: 1)
ROC fold 0 (AUC = 0.87)
ROC fold 1 (AUC = 0.73)
ROC fold 2 (AUC = 0.93)
ROC fold 3 (AUC = 0.93)
ROC fold 4 (AUC = 0.67)
ROC fold 5 (AUC = 0.73)
ROC fold 6 (AUC = 0.87)
ROC fold 7 (AUC = 0.93)
ROC fold 8 (AUC = 0.93)
ROC fold 9 (AUC = 0.73)
Chance
Mean ROC (AUC = 0.83 ± 0.10)
± 1 std. dev.
A
IL-6
ROC fold 0 (AUC = 0.27)
ROC fold 1 (AUC = 0.67)
ROC fold 2 (AUC = 0.33)
ROC fold 3 (AUC = 0.80)
ROC fold 4 (AUC = 0.87)
ROC fold 5 (AUC = 0.63)
ROC fold 6 (AUC = 0.60)
ROC fold 7 (AUC = 0.73)
ROC fold 8 (AUC = 0.30)
ROC fold 9 (AUC = 0.43)
Chance
Mean ROC (AUC = 0.56 ± 0.21)
± 1 std. dev.
B
sIL-6R
ROC fold 0 (AUC = 0.27)
ROC fold 1 (AUC = 0.53)
ROC fold 2 (AUC = 0.20)
ROC fold 3 (AUC = 0.20)
ROC fold 4 (AUC = 0.87)
ROC fold 5 (AUC = 0.60)
ROC fold 6 (AUC = 0.13)
ROC fold 7 (AUC = 0.40)
ROC fold 8 (AUC = 0.47)
ROC fold 9 (AUC = 0.20)
Chance
Mean ROC (AUC = 0.39 ± 0.22)
± 1 std. dev.
C
sgp130
ROC fold 0 (AUC = 0.87)
ROC fold 1 (AUC = 0.73)
ROC fold 2 (AUC = 0.93)
ROC fold 3 (AUC = 0.73)
ROC fold 4 (AUC = 0.80)
ROC fold 5 (AUC = 0.73)
ROC fold 6 (AUC = 0.80)
ROC fold 7 (AUC = 0.93)
ROC fold 8 (AUC = 0.73)
ROC fold 9 (AUC = 0.53)
Chance
Mean ROC (AUC = 0.78 ± 0.11)
± 1 std. dev.
D

**Figure 3.3 Support Vector Machines employed in IL6 signaling biomarkers in classifying patients of non-COVID-19 sepsis and COVID-19 sepsis**. Linear SVMs were used. I evaluated the test performance through the Area Under the Curve (AUC) measurement of a stratified 10-fold cross-validation procedure (90% train, 10% test) to conclude that: **A)** the best classificatory performance (AUC = 0.83) was obtained through a combination of the individual concentration of the biomarkers IL6, sIL6R, and sgp130; **B)** the individual concentration of IL6 alone could not distinguish between viral and non-viral sepsis; **C)** the individual concentration of sIL6R alone could not distinguish between viral and non-viral sepsis; and **D)** sgp130 alone also decreased in satisfactory classification performance (AUC = 0.78).

### 3.4 Sex difference has no influence on the type of IL-6 signaling

I further investigated to determine whether sex difference has any influence on the type of IL-6 signaling within the same disease group. I checked the B/T ratios and IL-6 signaling biomarkers (IL-6, sIL-6R, sgp130) between males and females in the severe COVID-19 and severe non-COVID-19 groups separately. My data showed that the B/T ratio in both males and females in the severe COVID-19 group followed a similar trend of evolution from T1 to T3, which indicates IL-6 trans-signaling in both sexes (Figure 3.4A). On the other hand, in the severe non-COVID-19 group, both males and females showed a diminishing trend in the B/T ratio from T1 to T3, suggesting IL-6 classical signaling (Figure 3.4B). Sex difference showed no significant impact on the levels of IL-6 signaling biomarkers (IL-6, sIL-6R, sgp130) between our enrolled patients from both disease groups (Figures 3.4C and D represent severe COVID-19 and severe non-COVID-19 respectively).


A
0.009
2.2
2.1
2.0
1.9
1.8
1.7
T1
T2
T3
Severe COVID-19 Male
0.002
2.1
2.0
1.9
1.8
1.7
T1
T2
T3
Severe COVID-19 Female
B
0.006
1.95
1.90
1.85
1.80
1.75
1.70
T1
T2
T3
Severe non COVID-19 Male
0.169
2.1
2.0
1.9
1.8
1.7
1.6
T1
T2
T3
Severe non COVID-19 Female


C
Time Point 1
IL-6
0.4238
pg/mL
400
300
200
100
0
Male
Female
sIL-6R
0.2086
ng/mL
600
400
200
0
Male
Female
sgp130
0.4014
ng/mL
250
200
150
100
50
0
Male
Female
Time Point 2
IL-6
>0.9999
pg/mL
2000
1500
1000
500
0
Male
Female
sIL-6R
0.8414
ng/mL
600
400
200
0
Male
Female
sgp130
0.8826
ng/mL
250
200
150
100
50
0
Male
Female
Time Point 3
IL-6
0.6335
pg/mL
1500
1000
500
0
Male
Female
sIL-6R
0.1963
ng/mL
800
600
400
200
0
Male
Female
sgp130
0.7315
ng/mL
250
200
150
100
50
0
Male
Female

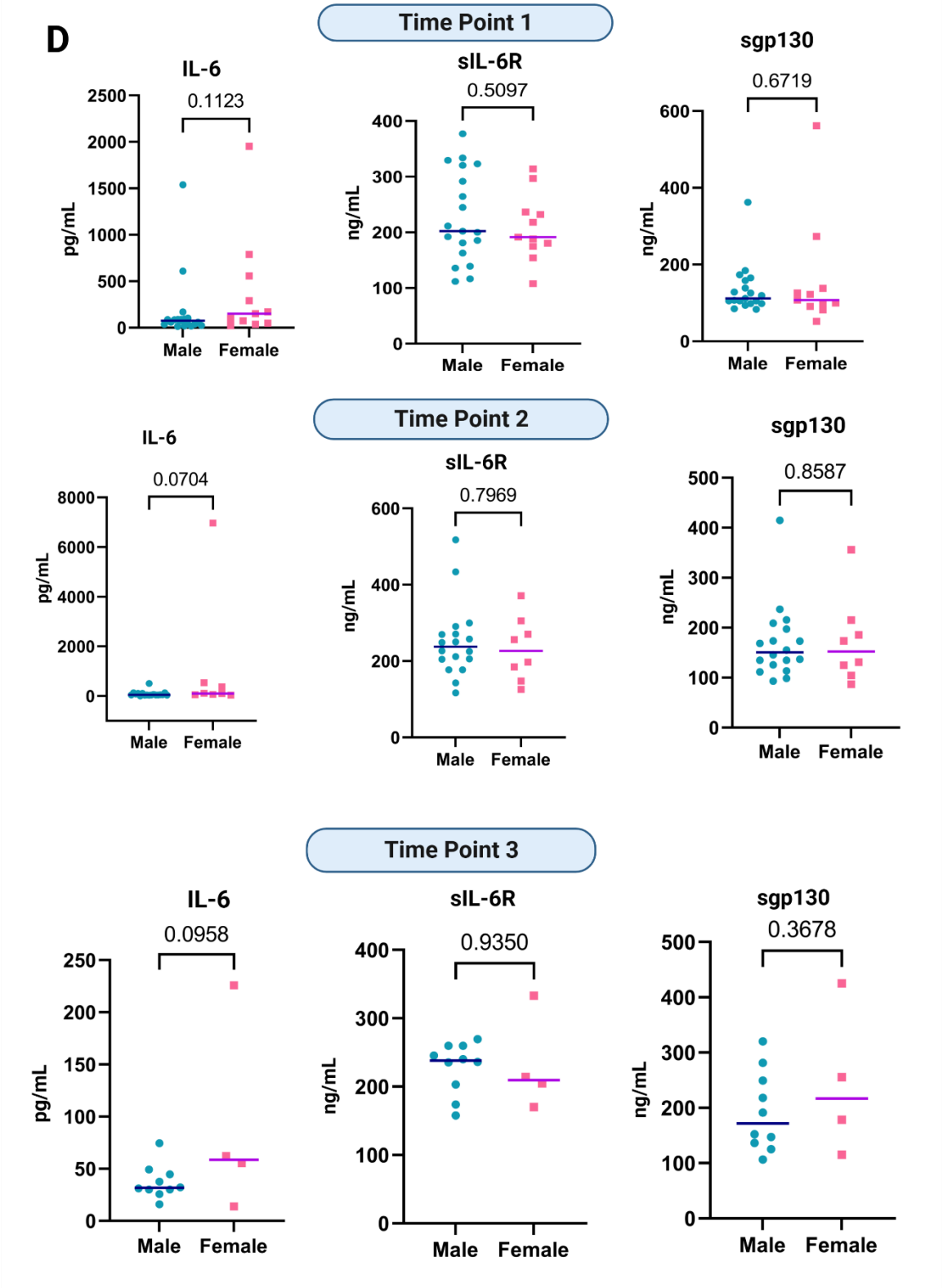

D
Time Point 1
IL-6
0.1123
pg/mL
2500
2000
1500
1000
500
0
Male
Female
sIL-6R
0.5097
ng/mL
400
300
200
100
0
Male
Female
sgp130
0.6719
ng/mL
600
400
200
0
Male
Female
Time Point 2
IL-6
0.0704
pg/mL
8000
6000
4000
2000
0
Male
Female
sIL-6R
0.7969
ng/mL
600
400
200
0
Male
Female
sgp130
0.8587
ng/mL
500
400
300
200
100
0
Male
Female
Time Point 3
IL-6
0.0958
pg/mL
250
200
150
100
50
0
Male
Female
sIL-6R
0.9350
ng/mL
400
300
200
100
0
Male
Female
sgp130
0.3678
ng/mL
500
400
300
200
100
0
Male
Female

**Figure 3.4 Sex difference showed no impact on type of IL-6 signaling.** A) Increasing B/T complex ratio was observed in both males and females in the severe COVID-19 group. High B/T complex ratio indicates IL-6 trans-signaling. Statistical significance was assessed with a Kruskal-Wallis test with Dunn's correction for multiple comparisons. B) Both males and females in the severe non-COVID-19 groups showed reductions in the B/T complex ratio, which is the pattern of IL-6 classical signaling. Statistical significance was assessed with a one-way ANOVA with Tukey's multiple comparisons test. (C) Levels of IL-6 signaling components between males and females from the severe COVID-19 group showed no significant difference at different time points. (D) IL-6 signaling components between males and females in the severe non-COVID-19 group also showed no significant difference at different time points. Statistical significance was assessed with a two-tailed unpaired Mann-Whitney test and two-tailed unpaired t-test.

# CHAPTER 4 DISCUSSION

In the past two years, multiple studies have investigated critically ill COVID-19 and non-COVID-19 patients in parallel to have a better understanding of inflammatory cytokine dysregulation in COVID-19 compared to other critically ill non-COVID-19 patients. These studies have reported IL-6 as a key pro-inflammatory cytokine, elevated in critically ill COVID-19 patients; however, IL-6 levels in COVID-19 patients is still lower than that in severe non-COVID-19 patients [103,155,159,187]. In this research, I have shown that characterizing the type of IL-6 signaling explains severe COVID-19 more accurately than assessing just individual concentrations of IL-6.

## 4.1 Systemic inflammation in severe COVID-19 is associated with IL-6-mediated inflammatory dysfunction

Sepsis is the severe form of systemic inflammation characterized by an imbalance between uncontrolled expression of pro-inflammatory biomarkers. In this study, COVID-19 patients were critically ill with sepsis symptoms, which is an ideal group to study systemic inflammation. Biomarker profiling revealed that pro-inflammatory mediators are significantly upregulated in COVID-19 patients. I found that plasma biomarkers for endothelial activation were significantly elevated in severe COVID-19. Ang-2 overexpression disrupts endothelial junctional integrity and sensitizes endothelial cells to upregulate the expression of ICAM-1, VACM-1, or E-selectins [188]. Plasma ICAM-1 and VCAM-1 upregulation are hallmarks of endothelial inflammation [189,190]. E-selectin is also a more specific marker for endothelial activation [189,191]. Several previous studies

mentioned that increased expression of these endothelial markers is influenced by IL-6 [192,193]. I also showed that the plasma level of Th17 group pro-inflammatory biomarkers are higher than the baseline value. A high level of IL-17A was reported to downregulate IL-10 while IL-1β influenced the production of IL-17A [136]. My data showed a downward trend of IL-10 level while IL-17A remained higher than the baseline level. Multiple studies showed that elevated levels of IL-6 influence the expression of inflammatory markers such as Ang-2, ICAM-1, VACM-1, and TNF-α in sepsis patients [170,190,194,195]. In my samples, I also found these elevated pro-inflammatory biomarkers are strongly correlated with IL-6.

Previous data on SARS-CoV showed that a rapid increase in CXCL-10 is associated with IL-6-mediated endothelial dysfunction as well as failure to respond to immunological treatments [170,196,197]. My data showed a similar trend in CXCL-10 expression in severe SARS-CoV-2 infection. IL-2 is a potent inhibitor of Th17-mediated inflammatory responses whereas IL-15 has a role in viral clearance [198,199]. No significant changes were observed in IL-2 and IL-15 levels while concentrations of anti-inflammatory cytokines IL-10 and IL-4 were also found to decrease overtime. Reduced GM-CSF was reported to be related to the failure to clear surfactant from the lungs [200] and TNF-α is a pleiotropic pro-inflammatory cytokine originally associated with T cell apoptosis [201]. I found that GM-CSF levels in the plasma of severe COVID-19 patients decreased significantly while TNF-α level was upregulated.

My findings on biomarker profiling are aligned with those of previous studies which showed the presence of elevated pro-inflammatory biomarkers in severe COVID-19. Moreover, from multiple timepoint data, I further confirmed that upregulation of these

pro-inflammatory biomarkers are correlated with circulatory levels of IL-6. This data indicates that systemic inflammation and dysregulated pro-inflammatory responses in severe COVID-19 is regulated by IL-6; however, this evidence does not provide confirmation about the type of IL-6 signaling in COVID-19.

### 4.2 Dysregulated IL-6 trans-signaling is a unique feature of severe COVID-19

During acute inflammation, IL-6 can increase up to a million-fold and it ranges from pg/ml to μg/ml while sIL-6R can increase only 2- to 5-fold. This is the first study to show that, at the onset of sepsis symptoms, severe COVID-19 patients have significantly elevated sIL-6R levels compared to levels in non-COVID-19 patients and that these levels are almost 4-fold higher than those in the healthy maximum range. Rapid increase of sIL-6R levels upon viral infection has been reported previously [202]. Viral infection triggers activation of A disintegrin and metalloprotease 17 (ADAM-17), which is the shedding protease of the membrane-bound IL-6 receptor [203,204]. Activated ADAM-17, following a rapid and reversible mechanism, travel to the cell surface to shed membrane-bound IL-6 receptors, resulting in elevated sIL-6R [205]. Recent data by Patra et al. describe how SARS-CoV-2 infection triggers ADAM-17-mediated production of sIL-6R in vitro [139]. sIL-6R is the prerequisite for forming the IL-6:sIL-6R binary complex for enduring IL-6 trans-signaling. I have also reported a rapid increase of sIL-6R levels in a COVID-19 group, which is aligned with previous findings. Moreover, from multiple time point data, I showed evidence of uninterrupted growth of the B/T ratio in severe COVID-19 patients while the severe non-COVID-19 group showed the opposite. A high B/T ratio indicates that binary complexes are not fully neutralized, enabling free binary complexes capable of

systemic inflammation [126]. As mentioned previously, during infection and inflammation, IL-6 trans-signaling appears first and is later followed by classical signaling. Any failure of our immune system to divert the trans-signaling to classical signaling will result in uncontrolled upregulation of IL-6 [114,137]. In this study, I showed that IL-6 was initially lower in the severe COVID-19 group, but with the downregulation of the binary complex neutralizing agent (i.e., sgp130) in the COVID-19 group, IL-6 increased until end of the study. These observations are undoubtedly evidence of dysregulated IL-6 trans-signaling. My data not only supports the previous studies showing that elevated IL-6 in severe COVID-19 is correlated with inflammatory markers, but also confirms the type of IL-6 signaling that is active in severe COVID-19 cases.

My findings are also aligned with those of previous studies where researchers showed rapid increase of IL-6 in severe non-COVID-19 [96,154]. With subsequent time point data, my results validate previous findings by confirming that IL-6 trans-signaling is neutralized in severe non-COVID-19 with the upregulation of sgp130. Furthermore, I have reported a significant down-regulation of IL-6 levels at T3, which is confirmation that IL-6 classical signaling is functioning. In my sampling group, there was no significant difference in the B/T ratio between the severe COVID-19 and non-COVID-19 groups at the beginning of sepsis symptoms, but the ratio decreased significantly in the non-COVID-19 group in the first four days and continued to decrease until the end of the study. This result clearly showed that IL-6 trans-signaling may be active at the onset of non-COVID-19 sepsis but disease prognosis is not related to the IL-6 trans-signaling-mediated pro-inflammatory cascade.

### 4.3 Circulating IL-6 level individually is not enough to explain IL-6 signaling

For many years, researchers have considered circulating IL-6 levels to explain the IL-6-mediated inflammatory signaling without acknowledging its dual function as a pluripotent cytokine [101,132]. After the emergence of the COVID-19 pandemic, IL-6-based inhibitors were proposed for therapeutic care of severe patients [150,151,206]. Use of IL-6 inhibitors in severe COVID-19 is based on previous therapeutic experience and available data on circulating IL-6 levels in other non-COVID-19 cytokine disorders. Notably, these data are based on IL-6 elevation without considering the type of IL-6 signaling [68,158]. However, these IL-6 inhibitors (e.g tocilizumab, sarilumab) have not shown expected outcomes in severe COVID-19 treatment [207,208]. Until now, evidence of the type of IL-6 signaling in severe COVID-19 has been lacking. In this study, I have confirmed that circulating IL-6 concentrations can only explain the correlation of IL-6 with other inflammatory biomarkers but can not explain the type of signaling that might be helpful for therapeutic approaches. IL-6, sIL-6R, and sgp130 are three biomarkers that determine the fate of IL-6 signaling, which is evident in the work presented here. I have shown that IL-6 signaling components (IL-6, sIL-6R, and sgp130) are not only essential to determine the type of IL-6 signaling but also key to determine the B/T ratio. The B/T ratio not only summarizes the interaction of these biomarkers but can also be useful in understanding the evolution of IL-6 signaling in a timely manner. Moreover, from the prediction model presented, I have also shown that COVID-19 versus non-COVID-19 sepsis can be distinguishable if we consider IL-6 signaling components together. All this evidence confirms that individual cytokine elevation data is not enough to explain the disease mechanism in COVID-19.

## 4.4 Sex difference and IL-6 signaling

Several studies have reported that males are at higher risk for COVID-19 severity and fatality [209–211]. Previous data also showed that, after infection, males produce higher levels of IL-6 compared to females [212,213]. Therefore, after I confirmed the type of IL-6 signaling involved in severe COVID-19, I also investigated whether the type of IL-6 signaling is similar or dissimilar between males and females. My data confirmed that both severe COVID-19 and non-COVID-19 males and females had similar types of IL-6 signaling in the same disease group. Though the number of severe male patients was higher in this cohort, no significant difference was observed in circulating levels of IL-6, sIL-6R, and sgp130 between males and females. Both male and female severe COVID-19 patients exhibited upward trends in the B/T ratio from T1 to T3 while non-COVID-19 patients showed the opposite. Therefore, I further confirm that regardless of sex differences, severe COVID-19 followed a dysregulated IL-6 trans-signaling.

## 4.5 Limitations of This Study

In this cohort, I used severe non-COVID-19 sepsis group as a comparator for severe COVID-19 sepsis. Therefore, it is a comparison of IL-6 signaling between two critically ill patient group with systemic inflammation. This study was exclusively limited to ICU patients where some patients died in ICU during the study period (10 COVID-19 and 7 non-COVID-19), some were discharged from ICU (13 COVID-19 and 12 non-COVID-19) and some patients stayed in ICU until end of this study (27 COVID-19 and 11 non-COVID-19). Those who were discharged from ICU they had improvement in their sepsis symptoms and moved to ward treatment until recovery. As mentioned in methodology section,

patients were enrolled in ICU as soon as they meet sepsis criteria and followed up for later timepoints if they were in ICU with sepsis symptoms. Therefore, none of the enrolled patients in this cohort were truly recovered. In this study I did not have ward, asymptomatic or non-COVID-19 healthy control to characterize their IL-6 signaling against severe COVID-19 patients. Moreover, as mentioned I did not have equal number of patients at each timepoint therefore during the analysis of sex influence on IL-6 signaling, I did not have a large sample size in each sex group at all time points, which can be considered a limitation of this analysis.

## 4.6 Concluding Remarks and Future Perspective

Severe COVID-19 is characterized by a respiratory distress syndrome accompanied by dysregulated immune responses. Therefore, several immunomodulatory therapies including anti-IL-6 therapies have been proposed as intervention therapies in severe COVID-19. However, considering the challenges with existing anti-IL-6 inhibitors, researchers agreed that success with IL-6 inhibition requires intervention during a finite window of opportunity depending on the type of IL-6 signaling at that time [158]. My research identifies the types of IL-6 signaling in severe COVID-19 and proposes a strategy to characterize the IL-6 signaling type using IL-6 signaling components as well as the trend of the B/T ratio. Moreover, my research shows promise for future therapeutic strategies. sgp130 is known as our immune system's natural inhibitor to IL-6-mediated pro-inflammatory responses. My research shows clinical evidence that upregulation of sgp130 successfully reduces the B/T ratio in severe non-COVID-19 patients whereas severe COVID-19 patients failed to control the B/T ratio because of downregulation of sgp130.

Existing IL-6 inhibitors known for their global inhibition of IL-6 signaling failed to meet the expected clinical outcome in severe COVID-19; consequently, my research indicates opportunities for inhibitors designed for selective inhibition. Global inhibitors block both IL-6 trans-signaling and classical signaling mediated pro- and anti-inflammatory responses, whereas selective inhibitors may offer blocking of IL-6 trans-signaling only while allowing IL-6 classical signaling to control the pro-inflammatory responses.

In last few years, scientists have been trying to characterize the type of IL-6 signaling in different inflammatory diseases such as inflammatory bowel disease and osteoporosis. With use of synthetic sgp130, they are trying to selectively inhibit IL-6 trans-signaling while leaving IL-6 classical signaling to activate the regulatory function [214–216]. As my research confirms the type of IL-6 signaling and protective role of sgp130, future studies should consider designing *in vitro* and *in vivo* experiments focusing on the neutralization of IL-6 trans-signaling. One of the major challenges with current immunomodulatory treatments against COVID-19 is that they have a broader affect on other immune mediators' function. Therefore, we should consider *in vitro* and *in vivo* models where we can trigger IL-6 signaling upon SARS-CoV-2 infection and test the efficacy of sgp130 in neutralizing the hyper-inflammatory effect of IL-6 trans-signaling. Until now, no approved selective inhibitors of IL-6 signaling have been available for therapeutic use. A synthetic sgp130-based inhibitor named olamkicept (sgp130Fc) is under clinical trial against inflammatory bowel disease [217]. My research not only contributes to the understanding of IL-6 signaling but also provides initial evidence that off-label use of this type of selective inhibitor may show promise in severe COVID-19, once approved

after successful clinical trials. Therefore, this study should be considered the groundwork for planning future IL-6-based therapeutic inhibitors in COVID-19 treatment.

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**Appendix 1** Critically ill COVID-19 and non-COVID-19 patients were enrolled in the study as soon as they meet the sepsis criteria. The table shows the actual number of days' difference between when the patient was admitted to ICU and when enrolled in the study.

| ID | Patient group | Date of ICU admission | Date of Study enrollment | Actual day in ICU when enrolled in study (T1) |
|---|---|---|---|---|
| 1 | COVID-19 Sepsis | 2020-09-15 | 2020-09-16 | 1 |
| 2 | COVID-19 Sepsis | 2020-09-23 | 2020-09-25 | 2 |
| 3 | COVID-19 Sepsis | 2020-10-05 | 2020-10-07 | 2 |
| 4 | COVID-19 Sepsis | 2020-10-05 | 2020-10-07 | 2 |
| 5 | COVID-19 Sepsis | 2020-10-28 | 2020-10-29 | 1 |
| 6 | COVID-19 Sepsis | 2020-11-30 | 2020-12-01 | 1 |
| 7 | COVID-19 Sepsis | 2020-11-30 | 2020-12-01 | 1 |
| 8 | COVID-19 Sepsis | 2020-11-30 | 2020-12-01 | 1 |
| 9 | COVID-19 Sepsis | 2020-12-13 | 2020-12-14 | 1 |
| 10 | COVID-19 Sepsis | 2021-01-01 | 2021-01-05 | 4 |
| 11 | COVID-19 Sepsis | 2020-12-19 | 2020-12-22 | 3 |
| 12 | COVID-19 Sepsis | 2020-12-24 | 2020-12-28 | 4 |
| 13 | COVID-19 Sepsis | 2020-12-29 | 2020-12-31 | 2 |
| 14 | COVID-19 Sepsis | 2021-01-03 | 2021-01-04 | 1 |
| 15 | COVID-19 Sepsis | 2020-12-30 | 2021-01-06 | 7 |
| 16 | COVID-19 Sepsis | 2021-01-05 | 2021-01-06 | 1 |
| 17 | COVID-19 Sepsis | 2021-01-04 | 2021-01-06 | 2 |
| 18 | COVID-19 Sepsis | 2021-01-07 | 2021-01-08 | 1 |
| 19 | COVID-19 Sepsis | 2021-01-07 | 2021-01-08 | 1 |
| 20 | COVID-19 Sepsis | 2021-01-10 | 2021-01-11 | 1 |
| 21 | COVID-19 Sepsis | 2021-01-08 | 2021-01-11 | 3 |
| 22 | COVID-19 Sepsis | 2021-01-10 | 2021-01-12 | 2 |

| ID | Patient group | Date of ICU admission | Date of Study enrollment | Actual day in ICU when enrolled in study (T1) |
|---|---|---|---|---|
| 23 | COVID-19 Sepsis | 2021-01-12 | 2021-01-14 | 2 |
| 24 | COVID-19 Sepsis | 2021-01-13 | 2021-01-14 | 1 |
| 25 | COVID-19 Sepsis | 2021-01-14 | 2021-01-14 | 0 |
| 26 | COVID-19 Sepsis | 2021-01-12 | 2021-01-14 | 2 |
| 27 | COVID-19 Sepsis | 2021-01-14 | 2021-01-15 | 1 |
| 28 | COVID-19 Sepsis | 2021-01-13 | 2021-01-15 | 2 |
| 29 | COVID-19 Sepsis | 2021-01-13 | 2021-01-15 | 2 |
| 30 | COVID-19 Sepsis | 2021-01-18 | 2021-01-19 | 1 |
| 31 | COVID-19 Sepsis | 2021-01-19 | 2021-01-20 | 1 |
| 32 | COVID-19 Sepsis | 2021-01-21 | 2021-01-22 | 1 |
| 33 | COVID-19 Sepsis | 2021-01-27 | 2021-01-28 | 1 |
| 34 | COVID-19 Sepsis | 2021-01-27 | 2021-01-28 | 1 |
| 35 | COVID-19 Sepsis | 2021-01-29 | 2021-01-29 | 0 |
| 36 | COVID-19 Sepsis | 2021-02-01 | 2021-02-02 | 1 |
| 37 | COVID-19 Sepsis | 2021-02-03 | 2021-02-04 | 1 |
| 38 | COVID-19 Sepsis | 2021-02-04 | 2021-02-05 | 1 |
| 39 | COVID-19 Sepsis | 2021-02-04 | 2021-02-05 | 1 |
| 40 | COVID-19 Sepsis | 2021-02-07 | 2021-02-08 | 1 |
| 41 | COVID-19 Sepsis | 2021-02-09 | 2021-02-10 | 1 |
| 42 | COVID-19 Sepsis | 2021-02-13 | 2021-02-15 | 2 |
| 43 | COVID-19 Sepsis | 2021-02-16 | 2021-02-17 | 1 |
| 44 | COVID-19 Sepsis | 2021-02-17 | 2021-02-17 | 0 |
| 45 | COVID-19 Sepsis | 2021-02-18 | 2021-02-19 | 1 |
| 46 | COVID-19 Sepsis | 2021-02-21 | 2021-02-22 | 1 |
| 47 | COVID-19 Sepsis | 2021-02-25 | 2021-02-25 | 0 |
| 48 | COVID-19 Sepsis | 2021-02-25 | 2021-02-26 | 1 |
| 49 | COVID-19 Sepsis | 2021-03-09 | 2021-03-11 | 2 |

| ID | Patient group | Date of ICU admission | Date of Study enrollment | Actual day in ICU when enrolled in study (T1) |
|---|---|---|---|---|
| 50 | COVID-19 Sepsis | 2021-03-13 | 2021-03-15 | 2 |
| 51 | non-COVID-19 Sepsis | 2020-09-18 | 2020-09-22 | 4 |
| 52 | non-COVID-19 Sepsis | 2020-10-24 | 2020-10-28 | 4 |
| 53 | non-COVID-19 Sepsis | 2020-09-28 | 2020-09-30 | 2 |
| 54 | non-COVID-19 Sepsis | 2020-10-23 | 2020-10-24 | 1 |
| 55 | non-COVID-19 Sepsis | 2020-10-08 | 2020-10-16 | 8 |
| 56 | non-COVID-19 Sepsis | 2020-10-15 | 2020-10-22 | 7 |
| 57 | non-COVID-19 Sepsis | 2020-10-20 | 2020-10-28 | 8 |
| 58 | non-COVID-19 Sepsis | 2020-10-25 | 2020-10-28 | 3 |
| 59 | non-COVID-19 Sepsis | 2020-10-25 | 2020-10-28 | 3 |
| 60 | non-COVID-19 Sepsis | 2020-10-26 | 2020-11-03 | 8 |
| 61 | non-COVID-19 Sepsis | 2020-10-31 | 2020-11-03 | 3 |
| 62 | non-COVID-19 Sepsis | 2020-11-08 | 2020-11-10 | 2 |
| 63 | non-COVID-19 Sepsis | 2020-11-09 | 2020-11-18 | 9 |
| 64 | non-COVID-19 Sepsis | 2020-11-23 | 2020-11-24 | 1 |
| 65 | non-COVID-19 Sepsis | 2020-12-05 | 2020-12-07 | 2 |
| 66 | non-COVID-19 Sepsis | 2020-12-07 | 2020-12-08 | 1 |
| 67 | non-COVID-19 Sepsis | 2020-12-10 | 2020-12-10 | 0 |
| 68 | non-COVID-19 Sepsis | 2020-12-09 | 2020-12-14 | 5 |
| 69 | non-COVID-19 Sepsis | 2020-12-10 | 2020-12-17 | 7 |
| 70 | non-COVID-19 Sepsis | 2020-12-20 | 2020-12-22 | 2 |
| 71 | non-COVID-19 Sepsis | 2020-12-18 | 2020-12-22 | 4 |
| 72 | non-COVID-19 Sepsis | 2021-01-05 | 2021-01-06 | 1 |
| 73 | non-COVID-19 Sepsis | 2021-01-16 | 2021-01-18 | 2 |
| 74 | non-COVID-19 Sepsis | 2021-01-19 | 2021-01-19 | 0 |
| 75 | non-COVID-19 Sepsis | 2021-01-22 | 2021-01-22 | 0 |
| 76 | non-COVID-19 Sepsis | 2021-01-26 | 2021-01-28 | 2 |

| ID | Patient group | Date of ICU admission | Date of Study enrollment | Actual day in ICU when enrolled in study (T1) |
|---|---|---|---|---|
| 77 | non-COVID-19 Sepsis | 2021-02-09 | 2021-02-15 | 6 |
| 78 | non-COVID-19 Sepsis | 2021-02-23 | 2021-02-25 | 2 |
| 79 | non-COVID-19 Sepsis | 2021-02-25 | 2021-02-26 | 1 |
| 80 | non-COVID-19 Sepsis | 2021-03-14 | 2021-03-15 | 1 |

**Appendix 2:** Clinical and demographic characteristics of critically ill patients. Enrolled patients from two severe disease groups had no significant differences in their ages and weights. However, severe non-COVID-19 patients had high APACHE and SOFA scores at the time of enrollment. Hypertension was the most common comorbidity in severe COVID-19 patients followed by asthma, while the severe non-COVID-19 patients had mostly chronic obstructive pulmonary disease (COPD) followed by hypertension. Piperacillin/tazobactam was the most administered antibiotic treatment in ICU in both patient groups. Hydrocortisone was the predominant immunosuppressant drug used during ICU treatment.

| Clinical Characteristics | Severe COVID-19 | Severe non-COVID-19 | P Value |
|---|---|---|---|
| Population (n) | 50 | 30 | - |
| Age (mean ± standard deviation) | 65.48±11.02 | 63.13±12.12 | 0.46 |
| Female sex (n) | 21 | 11 | - |
| Weight (kg) | 85.05±19.20 | 81.02±23.30 | 0.41 |
| APACHE | 20±8.47 | 23.6±6.84 | 0.07 |
| SOFA (During enrollment, T1) | 7.16±3.35 | 8.73±3.70 | 0.05 |
| **Comorbidities [n (%)]** | | | |
| Hypertension | 26 (52) | 8 (27) | - |
| Asthma | 11 (22) | 1 (3) | - |
| Obesity | 6 (12) | 0 | - |
| Cancer | 2 (4) | 4 (13) | - |
| Alcoholism | 5 (10) | 2 (7) | - |
| Chronic obstructive pulmonary disease (COPD) | 8 (16) | 9 (30) | - |
| Diabetes Mellitus | 8 (16) | 3 (10) | - |
| **Treatment during ICU care** | | | |
| **Antibiotics [n (%)]** | | | |
| Amikacin | 14 (28) | 7 (23) | - |
| Clarithromycin | 25 (50) | 21 (70) | - |
| Piperacillin/tazobactam | 39 (78) | 25 (83) | - |
| Meropenem | 31 (62) | 18 (60) | - |
| Vancomycin | 36 (72) | 21 (70) | - |
| Linezolid | 24 (48) | 7 (23) | - |
| **Immunosuppressant [n (%)]** | | | |
| Dexamethasone | 8 (16) | 2 (7) | - |
| Hydrocortisone | 47 (94) | 10 (33) | - |
| Methylprednisolone | 3 (6) | 3 (10) | - |

**Appendix 3:** Clinical and demographic characteristics of SARS-CoV-2 negative individuals. Female population is higher is SARS-CoV-2 negative individuals and hypertension is most common comorbidity among enrolled elderly individuals.

| **Clinical Characteristics** | **SARS-CoV-2 Negative Individuals** |
|---|---|
| Population (n) | 11 |
| Age (mean ± standard deviation) | 83.09±12.24 |
| Female sex (n) | 8 |
| **Comorbidities [n (%)]** | |
| Hypertension | 4 (36.36) |
| Diabetes Mellitus | 1 (9.09) |
| Asthma | 2 (18.18) |
| Chronic obstructive pulmonary disease (COPD) | 2 (18.18) |