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A meta-summary and bioinformatic analysis identified interleukin 6 as a master regulator of COVID-19 severity biomarkers

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ABSTRACT

With the rising demand for improved COVID-19 disease monitoring and prognostic markers, studies have aimed to identify biomarkers using a range of screening methods. However, the selection of biomarkers for validation from large datasets may result in potentially important biomarkers being overlooked when datasets are considered in isolation. Here, we have utilized a *meta*-summary approach to investigate COVID-19 biomarker datasets to identify conserved biomarkers of COVID-19 severity. This approach identified a panel of 17 proteins that showed a consistent direction of change across two or more datasets. Furthermore, bioinformatics analysis of these proteins highlighted a range of enriched biological processes that include inflammatory responses and compromised integrity of physiological systems including cardiovascular, neurological, and metabolic. A panel of upstream regulators of the COVID-19 severity biomarkers were identified, including chemical compounds currently under investigation for COVID-19 treatment. One of the upstream regulators, interleukin 6 (IL6), was identified as a "master regulator" of the severity biomarkers. COVID-19 disease severity is intensified due to the extreme viral immunological reaction that results in increased inflammatory biomarkers and cytokine storm. Since IL6 is the primary stimulator of cytokines, it could be used independently as a biomarker in determining COVID-19 disease progression, in addition to a potential therapeutic approach targeting IL6. The array of upstream regulators of the severity biomarkers identified here serve as attractive candidates for the development of new therapeutic approaches to treating COVID-19. In addition, the findings from this study highlight COVID-19 severity biomarkers which represent promising, robust biomarkers for future validation studies for their use in defining and monitoring disease severity and patient prognosis.

1. Introduction

A novel viral infection, COVID-19, was first identified in 2019 and has elicited a global pandemic and continues to be a serious public health challenge [1]. The identified viral pathogen, known as Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), has been shown to cause a broad spectrum of disease severity, with the majority confirmed as mild cases and a small percentage of the population experiencing severe effects such as pneumonia, acute respiratory distress syndrome (ARDS), sepsis, or multisystem organ failure (MOF) [2].

Patients with mild COVID-19 infection may experience flu-like symptoms including dry cough, fever, and body ache [3], while severe COVID-19 patients may present with dyspnea, rapid shallow breathing (respiratory rate $\geq$ 30/min), decreased blood oxygen saturation, and presence of $>$ 50 % lung infiltrates within 48 h [4]. Critically ill COVID-19 patients, however, may experience extreme deterioration in organ function, for example, respiratory failure, shock, and disseminated coagulopathy and may require mechanical ventilation and admission to the intensive care unit (ICU) [4]. COVID-19 disease severity is intensified due to the extreme viral immunological reaction that results in increased inflammatory biomarkers and cytokine storm, and in addition to the increased immunological reaction, reduced lymphocyte count further amplifies disease severity [5]. Patients that are compromised in either field are presented with an increased risk of severe infection.

It has become evident that comorbidities, such as chronic obstructive

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pulmonary disease (COPD), hypertension, cardiovascular disorders, and type 2 diabetes mellitus are associated with an increased risk of mortality from COVID-19 [6]. According to China's National Health Commission, about 75 % of patients that died from COVID-19 had one or more pre-existing comorbidity [7]. Other high-risk groups include elderly patients with chronic illness and immunocompromised patients; both are more likely to develop severe complications following COVID-19 infection [8]. A retrospective case study by Guan et al. reported the most typical comorbidity in COVID-19 patients being circulatory diseases and noted a direct correlation between the number of comorbidities and disease severity [9]. Patients with comorbidity tend to produce worse prognostic outcomes when compared to patients without pre-existing health conditions [9]; consequently, establishing a risk-assessment screening tool that addresses potential disease severity may help yield better prognostic values.

The currently established screening tool for COVID-19 is real-time RT-PCR, mainly for its convenient accessibility, rapid analysis, and accurate diagnostic results. The most common sample specimens are nasopharyngeal and oropharyngeal swabs, whereas intubated patients sometimes require tracheal aspiration, bronchial or bronchoalveolar samples [8]. Even when used as a reference standard, RT-PCR possesses limitations that may hinder its reliability for detecting SARS-CoV-2, for instance, the presence of false negatives in confirmed cases post-recovery and its inability in evaluating disease progression [10]; it, therefore, does not perform well in determining overall patient prognosis. Other studies aimed to identify different detection methods - for example, SAR-CoV-2 virus particle detection, nucleocapsid protein (NP) antigen detection assay, and antibody detection assays - to decrease frequent occurrences of false negatives and improve the overall COVID-19 detection performance [11]. However, the new approaches were not successful in outlining disease progression nor differentiating between disease severity.

Clinicians and public health workers have reported the essential need to understand SARS-CoV-2 disease progression to utilize health services and strategies in handling this pandemic [4]. With the rising demand for improved disease monitoring and prognostic markers, several studies aimed to identify potential COVID-19 biomarkers using a range of targeted assays and high-throughput proteomic screening methods. Selection of candidate biomarkers for validation studies from large datasets, however, may result in potentially important biomarkers being overlooked when datasets are considered in isolation. Indeed, in previous work, we interrogated published proteomic datasets to identify conserved molecular alterations in neuromuscular conditions and identified several proteins that were commonly dysregulated across multiple studies that had not previously been studied in association with those conditions [12–14]. In addition, this method also highlighted conserved molecular responses already proven to be directly relevant to disease pathogenesis [12–14], thus providing confidence that a multi-study comparison of proteomics data is a valid approach to finding disease-relevant biomarkers.

Here, we have applied the same concept to interrogate a range of COVID-19 biomarker datasets to identify potential biomarkers that track with COVID-19 severity and use bioinformatics tools to understand the pathways upon which they converge.

2. Methods

2.1. Identification of COVID-19 biomarker studies

A literature search was performed on PubMed library database to compare eligible peer-reviewed studies to identify a set of differentially expressed proteins (DEPs) as potential COVID-19 biomarkers. A combination of search terms was used: "COVID" AND "severity" AND "biomarker", "COVID" AND "severity" AND "proteom*", and "COVID" AND "severity" AND "protein" AND "biomarkers". The search conducted included studies published up to August 2021; studies were selected if they identified DEPs in severe COVID patients using a proteomic based approach, including unbiased quantitative proteomics and targeted, ELISA-based immunoassays. Initially, ten studies were included, but one was excluded due to data comparing only severe and critical disease, leaving nine studies in total. These nine studies measured the degree of protein expression in severe COVID patients and compared them to healthy controls or non-severe patients.

2.2. Comparison of COVID-19 biomarker studies

Each of the nine studies utilized a different protein identification method and applied analysis, and thus, we established a *meta*-summary approach to aggregate the data and determine frequency of DEPs across the studies included. Firstly, we noted DEPs in severely infected COVID-19 patients and included them in our analysis; proteins that did not show a significant change between severely infected patients and healthy controls or non-severe patients were disregarded. Additionally, data comparing different levels of severity (i.e., critical vs severe cases) were also disregarded as results did not establish an association with a baseline reference (i.e., healthy controls or non-severe cases). Furthermore, selected data were restricted to proteins showing consistent differential expression in the same direction across time within the same study, resulting in a unified protein expression. Secondly, the collected data were added to a Microsoft Excel Spreadsheet; then, using the UniProt Database, proteins identified were converted to official gene symbols to minimize error and facilitate an accurate method of comparison [15]. Data from across all studies were then combined for comparison using Microsoft Excel PivotTable, allowing for the identification of repeated DEPs across studies, after which, a cut-off value was applied to the PivotTable to focus on proteins detected with the greatest frequency across multiple studies. Lastly, the direction of change for the final list of proteins in each study was identified and noted, which was expressed as the degree of protein expression in the most severe COVID-19 disease state studied compared to the less severe COVID-19 state or healthy condition.

2.3. Bioinformatics analysis

The Database for Annotation, Visualization and Integrated Discovery (DAVID 6.8) [16,17] platform was used to identify gene ontology terms that were enriched among proteins with a consistent direction of change across the COVID-19 biomarker studies. The analysis was conducted separately for the upregulated and downregulated proteins and the species was selected as "Homo sapiens". Biological process and molecular function gene ontology terms were reported if they had at least three proteins assigned and with a p-value < 0.05.

The Ingenuity Pathway Analysis (IPA) application (Ingenuity Systems, Silicon Valley, CA; Krämer et al., 2014) was used to gain further insights into the various biological systems that may have been impacted by changes in protein expression in severe vs less severe COVID-19 infection. Proteins that were consistently increased and decreased across two or more COVID-19 severity studies were inputted into IPA with an arbitrary fold change of 2 and −2, respectively. The Diseases and Functions Analysis identified the biological functions and/or diseases that were most significant from the data set. All proteins from the dataset that were associated with biological functions and/or diseases in the Ingenuity Knowledge Base were considered for the analysis. A right-tailed Fisher's Exact Test was used to calculate a p-value determining the probability that each biological function and/or disease assigned to that data set is due to chance alone and the final list of biological functions and/or disease terms was ranked accordingly to the resulting p-value.

The same dataset of proteins that were consistently increased and decreased across two or more COVID-19 severity studies was used for network generation in IPA. Each identifier was mapped to its corresponding object in Ingenuity's Knowledge Base and these proteins were overlaid onto a global molecular network developed from information

contained in the Ingenuity Knowledge Base. Networks were then algorithmically generated based on their connectivity. The Functional Analysis of a network identified the biological functions and/or diseases that were most significant to the proteins in the network. A right-tailed Fisher's Exact Test was used to calculate a p-value determining the probability that each biological function and/or disease assigned to that network is due to chance alone. The resulting networks are a graphical representation of the molecular relationships between proteins, where proteins are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). All edges are supported by at least one reference from the literature, from a textbook, or from canonical information stored in the Ingenuity Knowledge Base.

IPA Upstream Regulator Analysis was used to identify the upstream regulators that may be responsible for expression changes observed in the list of proteins that were consistently increased and decreased across two or more COVID-19 severity studies. The software predicts which upstream regulators are activated or inhibited to explain the up-regulated and down-regulated proteins observed in the dataset using a z-score algorithm. The algorithm is designed to reduce the chance that random data will generate significant predictions [18] and is based on expected causal effects between upstream regulators and targets derived from the curated literature in the Ingenuity® Knowledge Base.

3. Results

3.1. Overview of COVID-19 biomarker studies

Nine studies of COVID-19 were selected for comparison, with a total of 6,802 differentially expressed proteins identified (Table 1) [19–27]. A number of methods were used to extract and identify proteins in different studies, including label-free quantitative mass spectrometry [19,26,27], TMT quantitative mass spectrometry [24,25], and immunoassays [20–22,25]. Samples were collected from a total of 531 patients and 157 healthy controls' blood plasma/serum that were later subdivided into groups based on their disease severity. Studies comprised of different comparison groups in detecting differentially expressed proteins; for example, Mild vs Healthy Controls [19,25], Severe vs Healthy Controls [19,25], Moderate vs Severe vs Critical [20], ICU vs Non-ICU [21], WHO 3 (Hospitalized) vs WHO 4&5 (Oxygen ± NIV) vs WHO 6&7 (Ventilated) [22], Non-survivors vs Survivors [23,27], Severe vs Non-severe [24,26], and Fatal vs Healthy Controls [25].

3.2. Multi-study identification of conserved molecular response to COVID-19

Proteins identified across the nine studies showed either increased or decreased expression through different levels of disease severity. Therefore, studies were arranged according to the comparison groups presented to determine the direction of gene expression in those patients and thus, the conserved molecular response to COVID-19 (Table S1). Proteins that showed differential expression in five or more studies were initially selected for comparison. This revealed 80 proteins that were differentially expressed in COVID-19 vs healthy or severe vs less severe COVID-19, 45 of which were consistently changed in the same direction across five or more studies (Table S2) and 35 of which showed opposing directions of change in different studies (Table S3). Of the 45 proteins showing a consistent direction of expression change, twenty-four were increased in expression while 21 proteins were decreased in expression. C-reactive protein, for example, was consistently increased in eight comparison groups: Mild vs Healthy [19,25], Severe vs Healthy [19,25], ICU vs non-ICU [21], WHO 4–7 vs WHO 3 [22], Severe vs non-Severe [24], and Fatal vs Healthy [25] (Table S2). On the contrary, fibrinogen was differentially expressed in eight studies but with opposing directions of expression, being decreased in three—Mild vs Healthy [19], Severe vs Healthy [19], ICU vs non-ICU [21]—and increased in five comparison groups—WHO 4–7 vs WHO 3 [22], Mild vs Healthy [25], Severe vs Healthy [25], Fatal vs Healthy [25], and Severe vs non-severe [26] (Table S3). Comparisons presented in Tables S2 and S3 therefore show proteins that were differentially expressed in severe and mild COVID-19 patients compared to healthy controls or in severe vs less severe COVID-19; as a result, these proteins may serve as general biomarkers of COVID-19 infection regardless of disease severity.

Gene ontology analysis using DAVID software identified a range of biological and molecular process terms that were enriched among the proteins with a consistent direction of change across the COVID-19 biomarker studies from Table S2 (Fig. 1). Perhaps unsurprisingly, the top biological process term was "inflammatory response" with six proteins increased in expression in severe COVID-19 vs less severe or healthy mapping to it (i.e., C-reactive protein (CRP), S100 calcium-binding protein A8 and A9 (S100A8; S100A9), orosomucoid 1(ORM1), pro-platelet basic protein (PPBP), and alpha 1-antichymotrypsin

Table 1
Overview of COVID-19 biomarker studies. The publications included within this study that aimed to identify biomarkers of COVID-19. DEPs - differentially expressed proteins; ICU - intensive care unit; WHO – World Health Organisation.

Comparison	Method	Sample type	DEPs Included	Reference
Mild vs Control	Label-free quantification using mass spectrometry	Plasma	4,915	Chen et al. 2020 (mild) [19]
Severe vs Control	Label-free quantitative proteomics	Plasma	5,514	Chen et al. 2020 (severe) [19]
Moderate vs severe vs critical	Immunoassay	Serum	7	Ghazanfari et al. 2021 [20]
Severe vs Non-severe (ICU v non-ICU)	ELISA/ spectrophotometry / immunoturbidimetry / chemiluminescence	Serum	11	Kaya et al. 2021 [21]
WHO 3 (hospitalised) vs WHO 4&5 (Oxygen ± NIV) vs WHO 6&7 (Ventilated)	ELISA / automated analysers	Serum	8	Keddie et al. 2020 [22]
Non-survivors vs Survivors (at different times)	–	Serum	2	Li et al. 2021 [23]
Severe vs Non-severe	TMTpro quantification using mass spectrometry	Serum	41	Shen et al. 2020 [24]
Mild vs Healthy	TMT quantification using mass spectrometry	Plasma	174	Shu et al. 2020 (mild) [25]
Severe vs Healthy	TMT quantification using mass spectrometry	Plasma	192	Shu et al. 2020 (severe) [25]
Fatal vs Healthy	TMT quantification using mass spectrometry	Plasma	195	Shu et al. 2020 (fatal) [25]
Severe vs Non-severe	Label-free quantification using mass spectrometry	Plasma	38	Suvarna et al. 2021 [26]
Non-survivors vs Survivors	Label-free quantification using mass spectrometry	Serum/ plasma	11	Völlmy et al. 2021 [27]

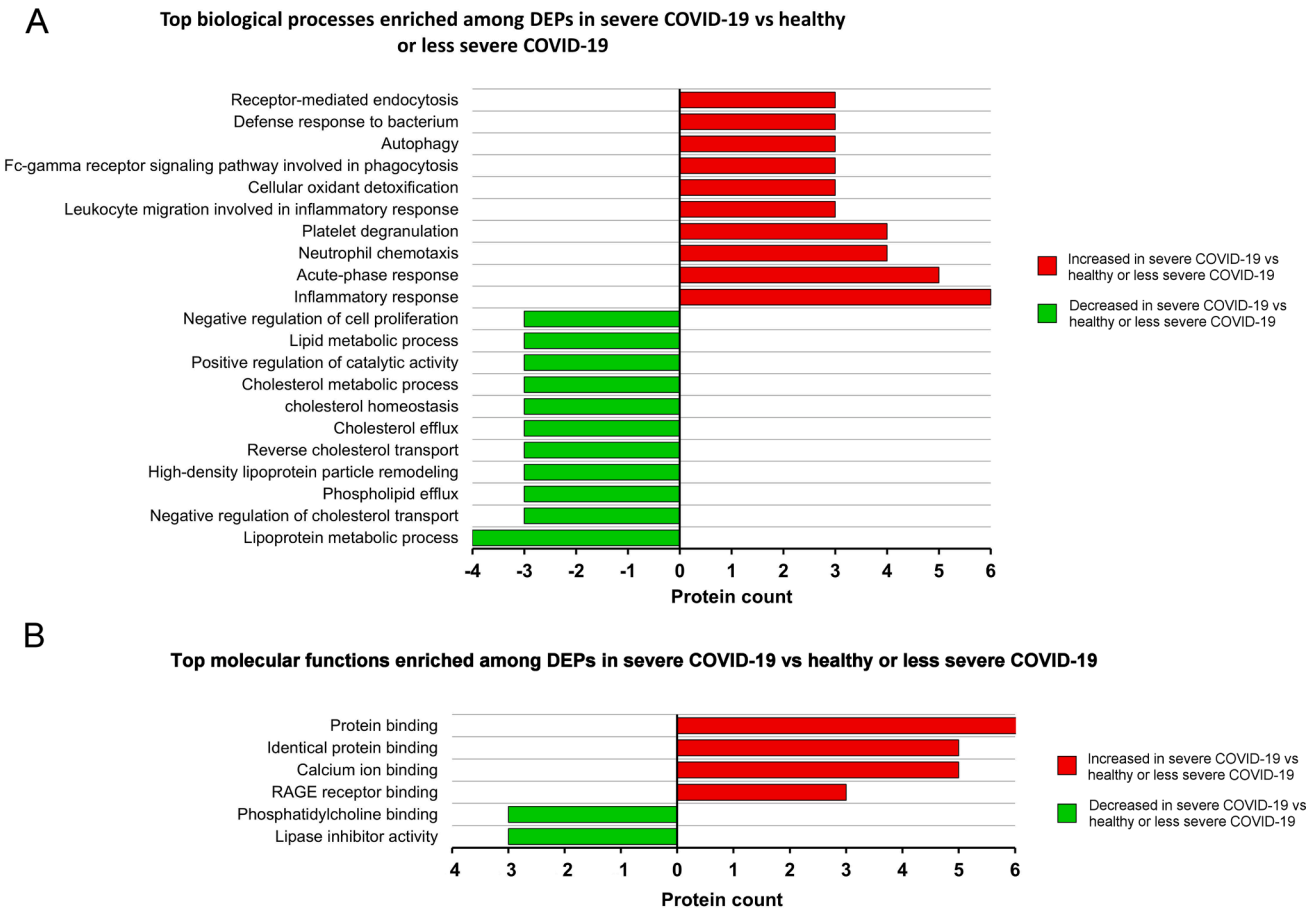


Fig. 1. Bioinformatics analysis of the 45 proteins changed in expression in the same direction in blood samples from patients with severe COVID-19 vs less severe or healthy individuals. Gene ontology analysis of the differentially expressed proteins from Table S2 using DAVID identified enriched terms associated with (A) biological process and (B) molecular function. Only terms with three or more proteins mapped to them are shown. DEPs: differentially expressed proteins.

(SERPINA3)) (Fig. 1A). Other enriched biological process terms among the increased proteins included "acute phase response" (n = 5 proteins), "neutrophil chemotaxis" (n = 4 proteins) and "platelet degranulation" (n = 4 proteins). Enriched molecular process terms among these proteins included "protein binding" (n = 6), calcium ion binding (n = 5) and RAGE receptor binding (n = 3) (Fig. 1B). Proteins decreased in expression in severe COVID-19 vs less severe or healthy mostly mapped to terms relating to metabolic processes and cholesterol / lipid transport and remodeling, with the top hit being "lipoprotein metabolic process" (n = 4 proteins) (Fig. 1A), "phosphatidylcholine binding" (n = 3 proteins) and "lipase inhibitor activity" (n = 3 proteins) (Fig. 1B).

3.3. *Multi-study identification of potential biomarkers of COVID-19 severity*

Whilst the potential panel of core biomarkers of COVID-19 infection identified above may also correlate with disease severity, the heterogeneity of the datasets made it unreliable to quantitatively compare the degree of differential expression across each of the studies to draw such conclusions. Therefore, to identify potential COVID-19 severity biomarkers more clearly and robustly the focus of our investigation was narrowed to study proteins that were identified in comparisons of only severe vs less-severe COVID-19 infection. Since this limited the pool of studies to just seven and significantly reduced the total number of differentially expressed proteins from 6802 to 18, we chose to identify proteins in more than one study rather than the more stringent cut-off used above. Following these filtering steps, 17 resulting proteins were identified as potential COVID-19 severity biomarkers (Table 2), of which 11 proteins were increased and six were decreased in expression in severe vs less severe COVID-19 patient blood samples. One protein, kallistatin, was expressed in opposite directions in two studies [24,26] and was therefore excluded from further analysis. Five of the 17 proteins were increased in three separate studies of severe vs less severe COVID-19 patient blood samples (i.e., fibrinogen (FGG) [21,22,26], alpha-1-antichymotrypsin (SERPINA3) [24,26,27], interleukin 10 (IL10) [20,22,23], c-reactive protein (CRP) [21,22,24], d-dimer [20,21,24]) and one was decreased in severe vs less severe COVID-19 patient blood samples (i.e. insulin-like growth factor-binding protein 3 (IGFBP3) [24,26,27]).

DAVID bioinformatics analysis of the 11 increased and 6 decreased proteins from Table 2 returned few gene ontology matches, none of which mapped to more than two proteins, likely because of the numbers of proteins in each dataset. We therefore undertook IPA bioinformatics analysis to gain insights into the biological systems that may have been impacted by changes in protein expression in severe vs less severe COVID-19 infection. Unlike DAVID, IPA permits the upload of fold changes alongside differentially expressed protein identifiers, meaning that the proteins increased and decreased in expression could be analysed as a whole dataset while retaining information about the direction of expression change. Serving to provide excellent proof of concept for this approach, the top disease or biofunction hit returned by IPA was "severe COVID-19" with a p-value of 1.02E-13 (Fig. 2A). The proteins associated with this term are albumin (ALB), apolipoprotein M (APOM), properdin (CFP), c-reactive protein (CRP), histidine-rich glycoprotein (HRG), interleukin-10 (IL10), interleukin-6 (IL6) and alpha-1-antichymotrypsin (SERPINA3). A range of other enriched diseases and

Table 2
The conserved molecular response to COVID-19. Individual proteins that were differentially expressed across two or more separate comparisons are shown, along with the number of studies they were identified in ("repeat hits").

Gene name	Protein Name	Number of repeat hits	Direction of change	Comparison	Reference
FGG	Fibrinogen	3	Increased	ICU vs non-ICU	[21]
				Severe vs non-Severe	[26]
				WHO 4–7 vs WHO 3	[22]
SERPINA3	Alpha-1-antichymotrypsin	3	Increased	Severe vs non-Severe	[24,26]
				non-Survivors vs Survivors	[27]
IL10	IL-10	3	Increased	Critical vs Severe vs Moderate	[20]
				WHO 4–7 vs WHO 3	[22]
				non-Survivors vs Survivors	[23]
CRP	C-reactive protein	3	Increased	ICU vs non-ICU	[21]
				WHO 4–7 vs WHO 3	[22]
				Severe vs non-Severe	[24]
D-Dimer	D-dimer	3	Increased	Critical vs Severe vs Moderate	[20]
				ICU vs non-ICU	[21]
				Severe vs non-Severe	[24]
IGFBP3	Insulin-like growth factor-binding protein 3	3	Decreased	Severe vs non-Severe	[24,26]
				non-Survivors vs Survivors	[27]
IL6	Interleukin-6	2	Increased	WHO 4–7 vs WHO 3	[22]
				non-Survivors vs Survivors	[23]
PCT	Procalcitonin	2	Increased	Critical vs Severe vs Moderate	[20]
				ICU vs non-ICU	[21]
LCP1	Plastin-2	2	Increased	Severe vs non-Severe	[24,26]
APOM	Apolipoprotein M	2	Decreased	Severe vs non-Severe	[24,26]
SERPING1	Plasma protease C1 inhibitor	2	Increased	Severe vs non-Severe	[24,26]
CFP	Properdin	2	Decreased	Severe vs non-Severe	[24,26]
ITIH2	Inter-alpha-trypsin inhibitor heavy chain H2	2	Decreased	Severe vs non-Severe	[24]
				non-Survivors vs Survivors	[27]
ALB	Albumin	2	Decreased	ICU vs non-ICU	[21]
				Severe vs non-Severe	[24]
LDH	Lactate dehydrogenase	2	Increased	ICU vs non-ICU	[21]
				WHO 4–7 vs WHO 3	[22]
Ferritin	Ferritin	2	Increased	ICU vs non-ICU	[21]
				WHO 4–7 vs WHO 3	[22]
HRG	Histidine-rich glycoprotein	2	Decreased	Severe vs non-Severe	[24]
				non-Survivors vs Survivors	[27]
SERPINA4	Kallistatin	2	Decreased	Severe vs non-Severe	[24]
			Increased	Severe vs non-Severe	[26]

biofunctions were also identified including organismal injury and abnormalities (e.g., occlusion of artery and thrombus), immune system response (e.g., degranulation of cells and adhesion of immune cells), and neurological disease; the latter having the largest (n = 10) number of proteins from Table 2 assigned to it. Subsequent network analysis using IPA identified three networks with which the potential COVID-19 severity biomarkers are associated (Fig. 2B-D). The largest network (Fig. 2B) included eight of the potential COVID-19 severity biomarkers and was associated with "cardiovascular disease, infectious disease, and protein synthesis" biofunctions. The two smaller networks each included four of the potential biomarkers and were associated with "cellular development, cellular growth and proliferation, and embryonic development" (Fig. 2C) and "endocrine system disorders, inflammatory response, and metabolic disease" (Fig. 2D).

3.4. Identification of upstream regulators of potential COVID-19 severity biomarkers

Upstream regulator analysis using IPA revealed a number of molecules of different types, including chemical drugs, cytokines and transcription regulators with potential to target the differentially expressed proteins identified in Table 2 (a full list is provided in Table S4). One of these, IL6, had the highest positive activation z-score of 2.79 and was identified as an upstream regulator that can explain observed expression changes of eight proteins differentially expressed in severe vs less-severe COVID-19 (i.e., ALB, CFP, CRP, FGG, IGFBP3, IL10, IL6 and SERPINA3) (Fig. 3A). The z-score determined IL6 to be in an activated state based on the regulation direction associated with its relationship to the eight differentially expressed proteins. Causal network analysis in IPA subsequently identified IL6 as a "master regulator" of the seven differentially expressed proteins (excluding itself) and was the only differentially expressed protein from Table 2 to have been assigned master regulator status (Table S5). Potential drug targets of the IL6 activation network include IL6 receptor inhibitor, clazakizumab, siltuximab, tocilizumab, ziltivekimab, and anti-IL6 monoclonal antibody (Fig. 3A). The second most activated upstream regulator after IL6 was the nuclear factor kappa-light-chain-enhancer of activated B cells complex (NF-kB complex). With a positive activation score of 2.38, the NF-kB complex is predicted to explain observed expression changes of six proteins differentially expressed in severe vs less-severe COVID-19 (i.e., SERPINA3, IL6, CRP, IL10, FTH1 and FGG) (Fig. 3B). Potential drug targets of the NF-kB complex activation network include triflusal, NF-kappaB decoy, dexanabinol, NK-kappaB inhibitor, thalidomide and combinations of prednisone, dexamethasone, bortezomib or rituximab with thalidomide (Fig. 3B).

4. Discussion

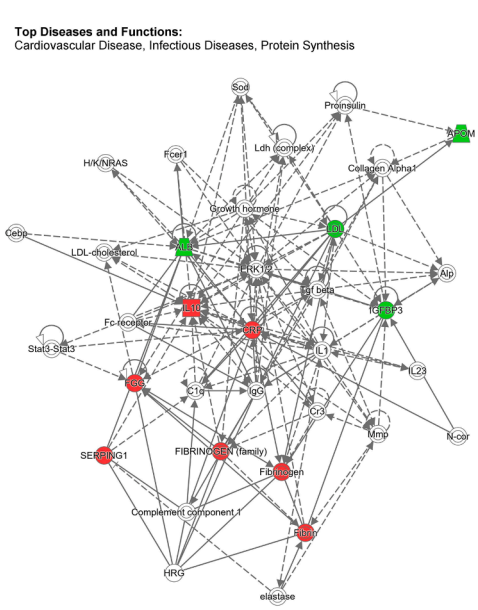
In this study, we identified proteins that were commonly dysregulated across multiple studies of blood samples from patients with severe COVID-19 compared to those with less severe symptoms. These biomarkers were shown to be involved in a range of biological processes that include inflammatory responses, acute phase response, neutrophil chemotaxis, and platelet degranulation. Knowledge of these processes and molecular functions identified further established potential applications in defining disease severity, survival rates, and overall patient prognosis and introduced the possibility of disease modification by narrowing drug selection to a specified targeted immune response.

Proteins associated with coagulation were among the most significantly expressed markers in severe COVID-19 patients. As described

A

Categories	Diseases or Functions Annotation	p-value	Activation z-score	Molecules	# Molecules
Infectious Diseases	Severe COVID-19	1.02E-13		ALB, APOM, CFP, CRP, HRG, IL10, IL6, SERPINA3	8
Infectious Diseases,Inflammatory Disease	Systemic inflammatory response syndrome	2.81E-09		ALB, CRP, IL10, IL6	4
Cardiovascular Disease,Organismal Injury and Abnormalities	Occlusion of artery	8.04E-09	0	ALB, APOM, CRP, FGG, HRG, IL10, IL6, SERPINA3	8
Cellular Compromise,Inflammatory Response	Degranulation of cells	1.05E-08		ALB, CFP, FGG, FTH1, HRG, IL10, SERPINA3, SERPING1	8
Cell-To-Cell Signaling and Interaction,Hematological System Development and Function,Immune Cell Trafficking	Adhesion of immune cells	1.07E-08	-0.364	CFP, CRP, FGG, IL10, IL6, LCP1, SERPING1	7
Cell Death and Survival	Cell death of lymphoma cell lines	1.20E-08	-1.929	ALB, FTH1, IGFBP3, IL10, IL6, SERPINA3	6
Cellular Compromise,Inflammatory Response	Degranulation of blood platelets	1.32E-08		ALB, FGG, HRG, SERPINA3, SERPING1	5
Cell-To-Cell Signaling and Interaction	Binding of myeloid cells	1.33E-08	-0.579	CFP, CRP, FGG, IL10, IL6, LCP1	6
Neurological Disease	Progressive neurological disorder	1.39E-08		ALB, CRP, FTH1, HRG, IGFBP3, IL10, IL6, LDHA, SERPINA3, SERPING1	10
Cardiovascular Disease,Hematological Disease,Organismal Injury and Abnormalities	Thrombus	1.48E-08		ALB, CRP, FGG, HRG, IL10, IL6	6

B



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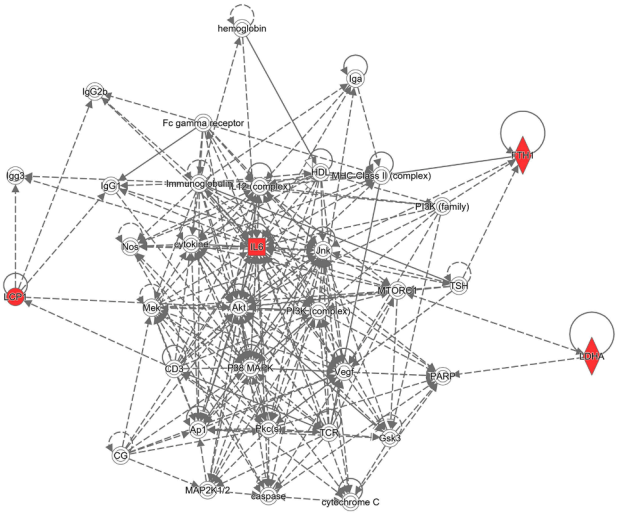

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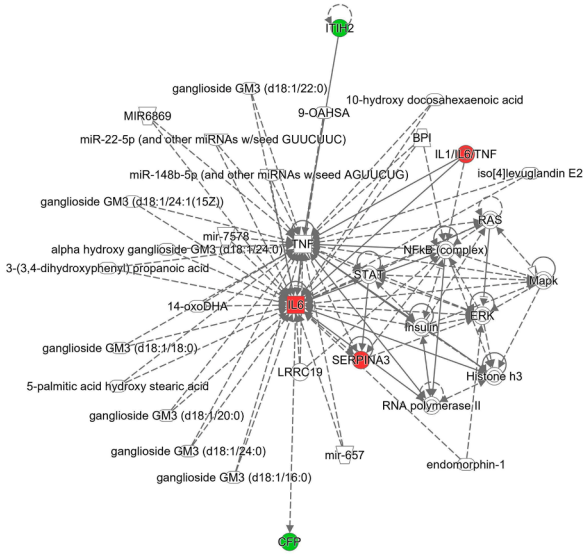

Fig. 2. IPA bioinformatics analysis of potential COVID-19 severity blood biomarkers. (A) the top 10 diseases or functions that were enriched among the 17 proteins that are potential COVID-19 severity biomarkers (from Table 2). The p-value indicates the probability that each biological function and/or disease assigned to that annotation is due to chance alone, and the activation z-score gives an indication of whether proteins assigned to annotations are likely to be in an activated (positive score) or inhibited state (negative score). Proteins (i.e., "molecules") are represented by their official gene symbols. (B, C and D) the three interaction networks identified by IPA based on their connectivity. Proteins increased in severe vs less severe COVID-19 are represented as red nodes and proteins decreased in severe vs less severe COVID-19 are shown as green nodes. The biological relationship between two nodes is represented as an edge (line), with direct interactions shown as a solid line and indirect as a dashed line. Lines without arrows represent chemical-protein interactions, correlation, or protein–protein interactions; lines with a solid grey arrow represent activation, causation, expression, localization, membership, modification, molecular cleavage, phosphorylation, regulation of binding or transcription; lines with a white arrowhead represent translocation. Node shapes denote the type of molecule (a full list is available here: https://qiagen.secure.force.com/KnowledgeBase/KnowledgeIPAPage?id=kA41i000000L5rTCAS). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

above, disseminated coagulopathy is one of the extreme deteriorations that may be experienced in severe COVID-19 cases [4], with the two commonly involved factors being fibrinogen and D-dimer [28]. Here, fibrinogen gamma chain (FGG) and D-dimer were found to be increased in expression in more than one study of patients with severe COVID-19 compared to less severe, indicating an elevated thrombotic tendency with severe COVID-19 symptoms. A retrospective study by Liao et al. reported that 50 % of 466 COVID-19 patients had increased D-dimer levels, with a gradual decrease in FGG levels in non-survivors as the disease progresses, which implies the continuous consumption of coagulation factors [28]. More recently, a bioinformatics analysis study highlighted a negative correlation with IL-6, D-dimer, prothrombin time (PT), thrombin time (TT), and CRP in severe cases; this further correlates with the consistent findings of this study [29] Consequently, hemostatic biomarkers are likely to be useful for monitoring on a case-to-case basis for any conditional deteriorations. Knowledge of the biological pathways with which COVID-19 severity biomarkers are associated also yields important clues regarding potential therapeutic approaches.

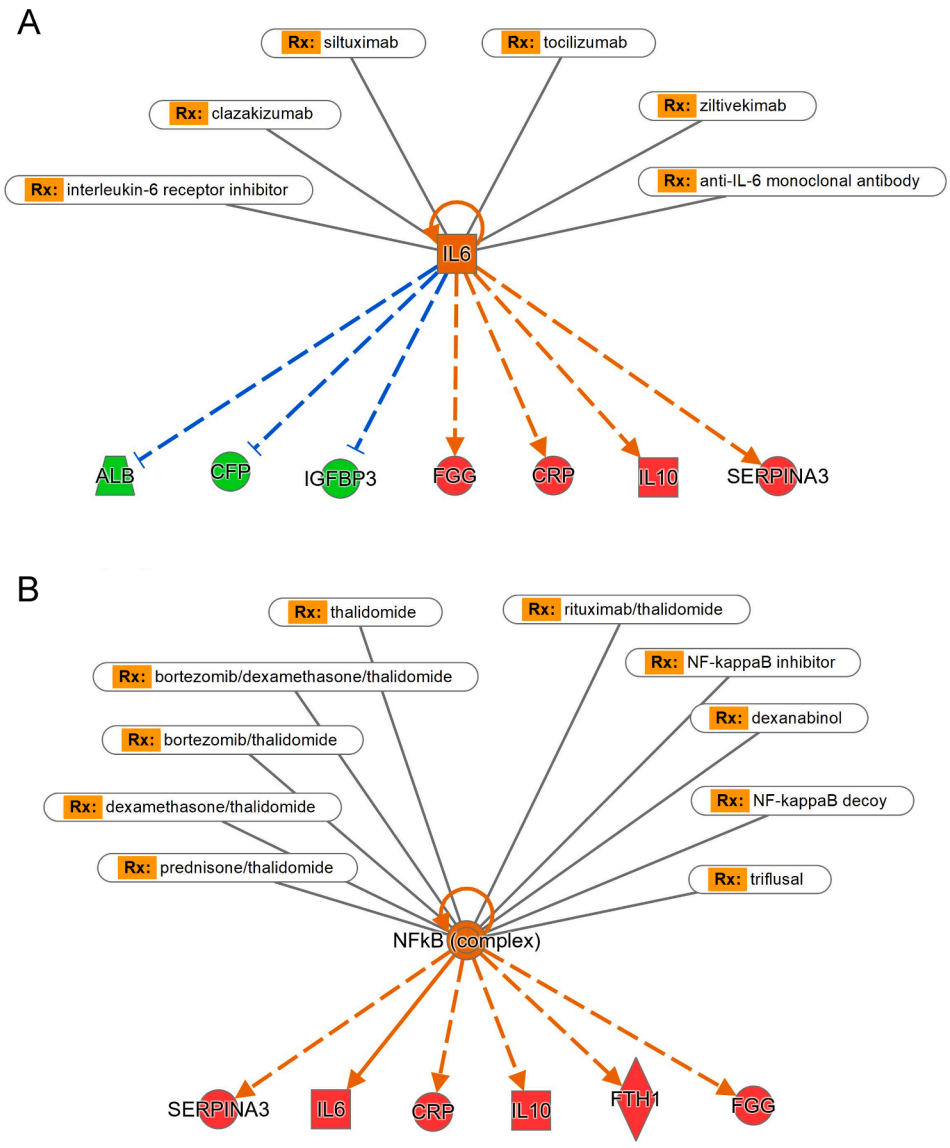

Fig. 3. Top upstream regulators of potential COVID-19 severity biomarkers. IL6 (A) and NFkB complex (B) were identified as two upstream regulators that can explain observed expression changes of proteins differentially expressed in severe vs less-severe COVID-19. The blue lines represent predicted indirect inhibition or ubiquitination, and the red lines represent predicted indirect activation, causation, expression, localization, membership, modification, molecular cleavage, phosphorylation, regulation of binding or transcription. Drugs with potential to target each regulator are displayed in an ellipse with Rx followed by drug name. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Heparin, for example, is a commonly used anticoagulant that was identified here as an upstream regulator of three COVID-19 severity biomarkers (Table S4), and it is already well known that COVID-19 patients can respond well to anticoagulation therapy. The current and most widely utilized treatment is low molecular weight heparin (LMWH), which in light of recent data, has been advised to be administered prophylactically to all COVID patients. A study of 449 patients with severe COVID-19 who received prophylactic LMWH exhibited significantly better prognosis ($P = 0.029$) under a sepsis-induced coagulopathy (SIC) score of ≥ 4 [30,31].

Acute-phase response is another biological process that was significantly associated with the biomarkers of severe COVID-19, one of which is c-reactive protein (CRP) which was increased in severe vs less severe cases. In a retrospective study, the increased expression of CRP had a significant association ($P < 0.001$) with severe clinical presentation of COVID-19 and size of lung lesions [32], and it has been shown to directly correlate with increased disease severity, associated with increased inflammation [32,33]. CRP is rapidly elevated in acute viral infections to eliminate pathogens via the complement system and enhanced phagocytosis of macrophages [33]; it also had a notable increase in other viral infections, including SARS, MERS, influenza A, and H1N1 [34]. IL6 regulates the secretion of CRP via transcription factors in hepatocytes, making it a significant byproduct in cytokine storm—which further increases the risk of multi-organ damage [34].

IL6 is a cytokine that triggers the body's defence system via stimulating acute phase reactants, hematopoiesis, and humoral immune system in response to infection or tissue damage; hence, IL6 was reported as a key mediator for shock, respiratory failure, and multi-organ failure in severe COVID-19 patients [35,36]. In our analysis, IL6 was assigned master regulator of seven other proteins, highlighting it as having a critical role in regulating the expression of other biological markers during COVID-19 infection. A systematic review and *meta*-analysis detected a threefold increase in serum IL6 in complicated/severe COVID cases, further implying that severity of cases results from hyperactivation of host immune response such in cytokine storm and cytokine-induced sepsis (CIS) [36]. Along with COVID infections, IL6 plays an essential role in other viral infections; in an experiment with influenza A-infected mice, IL6 was shown to contribute to the immunity of mice by promoting T cell response and tissue remodelling and regulation of inflammatory resolution and phagocytic activities [37]. Since IL6 is the primary stimulator of cytokines, it may have potential for use independently as a biomarker in determining COVID-19 disease progression, in addition to a potential therapeutic approach targeting IL6.

Since analysis above, evidence has emerged of several elevated

proinflammatory cytokines involved in cytokine storm in COVID-19 patients, including IL-6 and IL-12. The elevated IL-6 in severe cases further supports our findings. However, IL-12 was more prominent in mild rather than severe patients, while in severe patients, IL-12 had similar levels to the healthy controls [38]. Kimura et al. (2021) also confirmed the regulatory role played by IL-6 and other cytokines, such as IL-1β and TNF, in mediating differentially expressed proteins found in severe cases [39]. Furthermore, other immune-related protein biomarkers such as PZP, SELENOP, PON1, and CBP2 have recently been identified as potential prognostic COVID-19 biomarkers that correlate with disease recovery [40]. Taken together, there is convincing evidence that the immune system, particularly via proinflammatory cytokines, play a role in the body's response to COVID-19 infection. The precise mechanism(s) by which individual molecules are controlled over time certainly warrants further attention though, considering the IL-12 finding above, and that IL-6 levels are attenuated in other inflammatory conditions such as sepsis [41], which appears to be in contrast to whereas the cytokine release syndrome is associated with increased IL-6 expression, leading to an acute respiratory distress syndrome and mechanical ventilation in severe COVID-19 patients [42].

The array of upstream regulators of the severity biomarkers identified here are likely to provide a valuable resource for the design of new therapeutic approaches to treating COVID-19. In addition, potential drug targets of the IL6 activation network were highlighted, including several drugs that have already received attention in recent studies that have been intensively addressing the possibilities of IL6-targeted therapy for COVID-19. One systemic review assessed the use of one of these drugs, tocilizumab (TCZ), which is a monoclonal antibody that blocks IL6-IL6R receptor (IL6R) complex with the transmembrane protein, thus limiting the proinflammatory effects of IL6 [43]. The data provided a reasonable basis on the use of tocilizumab in severe and critical COVID-19 patients resulting in overall favourable outcomes; however, published evidence is still required to verify beneficial outcomes in suppressing proinflammatory cytokines. A cohort study by Luo et al. outlined the effectiveness of TCZ in treating cytokine storm and decreasing acute phase reactant levels [44]. In a randomized control trial that assessed interleukin-6 antagonists, critically ill COVID patients treated with tocilizumab and sarilumab exhibited effective outcomes compared with the traditional standard of care [45]. In another study by Salama et al., (2021), hospitalized COVID-19 patients with pneumonia who were not receiving mechanical ventilation were randomly given standard care as well as tocilizumab or placebo. The outcome monitored was either mechanical ventilation or death over a period of 28 days. The outcome detected showed a significant decrease in progression of mechanical ventilation and death in the tocilizumab group [46]. A first of its kind clinical trial established an IL-6-based mortality prediction model for COVID-19 patients using five factors: 1) peripheral blood oxygen saturation, 2) neutrophil/lymphocyte ratio, 3) lactate dehydrogenase, 4) IL-6, and 5) age [47]. Currently, the Food and Drug Administration (FDA) does not approve the use of TCZ in COVID-19 patients. Nonetheless, there are ongoing trials that aim to explore the further assessment of IL6 targeted therapeutical drugs, which include: tocilizumab, clazakizumab, siltuximab, and sarilumab [48]. While tumor necrosis factor (TNF), another key player in mediating cytokine storm, was not identified among the list of proteins commonly dysregulated in severe COVID-19, it was highlighted as an upstream regulator of nine of the proteins, and the major TNF signal transducer molecule, NF-kB was found to be the second most activated upstream regulator after IL6. The notion of blocking TNF by pharmacologically inhibiting NF-kB appears to be gaining attention as an attractive approach to therapy design for COVID-19 [49]. Several groups of compounds have been suggested to have potential for this application, including glucocorticoids, several of which, among other types of drugs, were identified here as potential targets of the NF-kB activation network. Clinical trials on TNF/NF-kB blockage are underway but are currently far less advanced than studies of IL6 targeting.

More recent studies of Alaiya et al. (2021), Mardani et al. (2022), and Tahery et al. (2021) showed an elevated levels of CRP alongside other biomarkers in severe vs asymptomatic COVID-19 comparison groups, suggesting the plausibility of CRP as a severity and mortality marker for SARS-CoV-2 infection [50–52].

In this study, biomarkers were identified based on them showing a consistent direction of expression change in the studies when compared with a baseline reference range; however, it was not possible to reliably compare the degree of protein expression across each study due to the heterogeneity within the datasets. Nevertheless, the strength of this approach is that proteins identified as being consistently altered in the same direction in different studies, despite the heterogeneity, are much more likely to represent promising, robust biomarkers for future validation studies. Ultimately, this helps set a consistent framework for detecting biomarkers when used to assess the severity of COVID-19 infection at the time that a patient presents with illness. Some of these biomarkers may also correlate with disease progression but further longitudinal studies are warranted to validate their use as prognostic biomarkers.

The findings from this study and many others bring forward the future implications of adopting robust methods in diagnosing COVID-19 cases and an early detection of severe cases. Current studies are already considering the possible application of lateral flow tests, drawn from patient's serum, in detecting severe COVID-19 cases [53]. Nevertheless, further evaluation of serological tests is required in the clinical setting to overcome controversies brought forward by many studies [53]. A plausible solution to accurately identify fast progressing COVID cases may require monitoring of biomarker validity to consider deteriorating comorbidities, vaccination status, and the possibility of the emergence of new variants.

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CRediT authorship contribution statement

Mohannad Ghanem: Conceptualization, Validation, Data curation, Resources, Writing – original draft, Writing – review & editing, Visualization. **Sharon J. Brown:** Software, Writing – review & editing, Visualization. **Aysha EAT Mohamed:** Writing – review & editing, Visualization. **Heidi R. Fuller:** Conceptualization, Methodology, Software, Validation, Data curation, Resources, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

We have shared the available data in the supplementary file

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2022.156011.

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