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Reviews

Discovery Proteomics for COVID-19: Where We Are Now

Angela McArdle, Kirstin E. Washington, Blandine Chazarin Orgel, Aleksandra Binek, Danica-Mae Manalo, Alejandro Rivas, Matthew Ayres, Rakhi Pandey, Connor Phebus, Koen Raedschelders, Justyna Fert-Bober,[§] and Jennifer E. Van Eyk*[,§]

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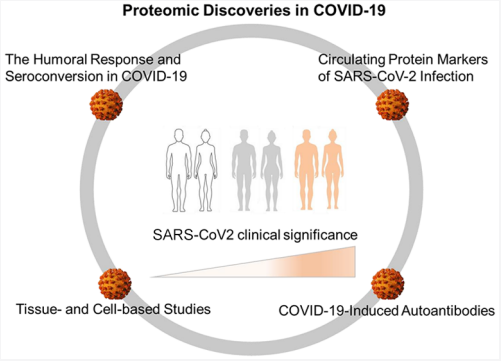
ABSTRACT: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a highly transmissible coronavirus responsible for the pandemic coronavirus disease 2019 (COVID-19), which has had a devastating impact on society. Here, we summarize proteomic research that has helped elucidate hallmark proteins associated with the disease with respect to both short- and long-term diagnosis and prognosis. Additionally, we review the highly variable humoral response associated with COVID-19 and the increased risk of autoimmunity.



KEYWORDS: *proteomics, COVID-19, SARS-CoV-2, biomarkers, plasma, biofluids, mass spectrometry*

INTRODUCTION

In early 2020, after a December 2019 outbreak in China, the World Health Organization (WHO) identified SARS-CoV-2 as a new type of *Betacoronavirus*. The virus initially known as 2019-nCoV was renamed as SARS-CoV-2 by the Coronavirus Study Group of the International Committee for the Taxonomy of Viruses.[1] In March 2020, the WHO named the resulting disease coronavirus disease-19 (COVID-19)[2] and later declared the COVID-19 outbreak to have reached pandemic status. SARS-CoV-2 was shown to spread mainly via respiratory droplets produced from coughing, sneezing, singing, and talking.[3] Quilty et al. proposed that up to 40% of COVID-19 cases exported from China went undetected at airports worldwide due to the absence of severe COVID-19 symptoms, resulting in potentially multiple chains of undetected human-to-human transmission.[4,5] Due to the symptom overlap between mild COVID-19-positive and influenza, Silverman et al. suggested that up to 80% of Americans who sought care for flu-like illnesses in March 2020 may have been infected with SARS-CoV-2.[6] The prevalence of SARS-CoV-2 in many other countries also may have been underestimated in the early months of the pandemic due to limited or inaccurate testing and undetected asymptomatic cases in early months.

Severe COVID-19 leads to acute respiratory failure and, in some cases, to death. It can be associated with an acute phase response often discussed as a "cytokine storm", prothrombotic immunopathology, and profound lymphopenia, which can culminate in multiple organ dysfunction and death.[7,8] The viral infection can result in lung, heart, and brain damage, which may increase the risk of long-term health problems. Additionally, patients infected by the more localized SARS virus epidemics were prone to developing infection-related long-term symptoms like chronic fatigue syndrome, which is characterized by extreme fatigue that worsens with physical or mental activity. On the basis of this observation and due to the similarity between SARS and SARS-CoV-2, SARS-like long-term effects can be expected for patients suffering from COVID-19.[9] Importantly, it has been proposed that SARS-CoV-2 may trigger autoimmunity phenomena through cross-reactivity with host cells and organs. Some patients have been reported to develop autoimmune diseases, such as Guillain-Barré syndrome or systemic lupus erythematosus, after SARS-CoV-2 infection.[10]

Now, in the second year of the pandemic, the scientific community continues to learn about COVID-19 and is trying to explain the variation of symptoms among age, gender, and comorbidities, particularly with new variants of SARS-CoV-2 emerging.[11] Circulating biomarkers have always played an

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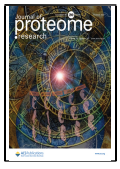

Table 1. Previous Review Papers on the Topics of Proteomics and COVID-19

title	DOI	reference
Utility of proteomics in emerging and re-emerging infectious diseases caused by RNA viruses	10.1021/acs.jproteome.0c00380	74
Proteomics and informatics for understanding phases and identifying biomarkers in COVID-19 disease	10.1021/acs.jproteome.0c00326	75
Perspective on proteomics for virus detection in clinical samples	10.1021/acs.jproteome.0c00674	76
Mass spectrometry techniques in emerging pathogens studies: COVID-19 perspectives	10.1021/jasms.0c00238	77
The acute respiratory distress syndrome biomarker pipeline: crippling gaps between discovery and clinical utility	10.1016/j.trsl.2020.06.010	78
The role of biomarkers in diagnosis of COVID-19—A systematic review	10.1016/j.lfs.2020.117788	79
Predictors of COVID-19 severity: A literature review	10.1002/rmv.2146	80
Hematologic, biochemical, and immune biomarker abnormalities associated with severe illness and mortality in coronavirus disease 2019 (COVID-19): A meta-analysis	10.1515/cclm-2020-0369	81
Biochemical biomarkers alterations in Coronavirus Disease 2019 (COVID-19)	10.1515/dx-2020-0057	82
Biomarkers of COVID-19 and technologies to combat SARS-CoV-2	10.1016/j.abst.2020.08.001	83
A comprehensive overview of proteomics approach for COVID 19: new perspectives in target therapy strategies	10.1007/s42485-020-00052-9	12
Proteomics in the COVID-19 battlefield: First semester check-up	10.1002/pmic.202000198	84
A real-time dashboard of clinical trials for COVID-19	10.1016/S2589-7500(20)30086-8	85
Review of trials currently testing treatment and prevention of COVID-19	10.1016/j.cmi.2020.05.019	86

important role in clinical decision-making for infectious diseases, including evaluating severity of disease, effectiveness of treatment, and the appropriate allocation of resources. In the present review, we focus on the application of proteomic studies, in both human tissue and biofluids, to better define COVID-19 diagnostic criteria and management as well as tailoring potential therapies to this disease.

Proteomic Discoveries in COVID-19

The clinical spectrum of COVID-19 is complex. It is reported that approximately 80% of those infected have a self-limiting case; however, clinical severity ranges from asymptomatic to severe respiratory failure and in cases, fatality as a result of multiorgan dysfunction.[12,13] Interindividual clinical variability has also been described across age, gender, ethnicity, and in those with predisposing comorbidities including obesity, diabetes, and cancer. Described by Williamson et al., COVID-19-related death has been associated with being male (hazard ratio (HR) 1.59 (95% confidence interval 1.53−1.65)); greater age and deprivation (both with a strong gradient); diabetes; severe asthma; and various other medical conditions. Compared with people of white ethnicity, Black and South Asian people were at higher risk, even after adjustment for other factors (HR 1.48 (1.29−1.69) and 1.45 (1.32−1.58), respectively).[14] Regardless of the reported correlation between severity of diseases and black and minority ethnicity, the heterogeneity of the disorder highlights the need for individualized medical care with respect to detecting and monitoring the progress of the infection as well as the response to treatment. Further still, since COVID-19 is a newly emerging disease, there is little known about the long-term health effects; therefore longitudinal analysis is necessary to establish definitions around this.[15] Proteomic approaches are suited for studying COVID-19 since they can provide quantitation and differential expression of proteins and their post-translational modifications (PTMs), and thus can represent drivers of pathological cellular mechanisms and individual disease status. Here we provide a review of proteomic discoveries in the human studies of COVID-19. See Table 1 for previous review papers on the topics of proteomics and COVID-19, and Table 2 for diagnostic and prognostic protein biomarkers of COVID-19.

Tissue- and Cell-Based Studies

Proteomic mass spectrometry (MS)-based analysis of lung tissue, the primary site of infection, has been fundamental for our understanding of disease pathogenesis. An early study by Wu et al. on lung parenchyma from 9 patients who died of COVID-19 during the first wave of the pandemic in Wuhan (China) revealed differential expression of 637 proteins in cases versus controls.[16] Strikingly, levels of proteins such as cathepsin B and L and several S100 proteins were markedly increased in quantity in the cases compared to controls. These cytoplasmic proteins are mainly secreted by neutrophils and macrophages and play a major role in structural remodeling and leukocyte recruitment. The authors corroborated their findings with transcriptional data, with resultant RNA transcripts showing concurrent expression changes to proteins in cases and controls. Interestingly, pathway analysis of the genetic data revealed the major pathways driving pathogenesis were those involved in the generation of neutrophil extracellular traps, which is a unique mechanism of cell death that allows neutrophils to kill extracellular pathogens while minimizing host cell injury.

These observations were further validated through histological analysis of parenchyma, which showed evidence of neutrophilic infiltration into diseased tissue. A report by Leng et al. found 641 differentially expressed proteins in lung tissue from 2 newly deceased COVID-19 patients in comparison to controls.[17] In agreement with the reports from the Wu et al. study, levels of S100 proteins and extracellular matrix proteins (matrix metalloproteinases 2, 8, and 9) were increased in diseased tissue. Another key finding was that proteins upstream [toll like receptor 4, Tumor necrosis factor receptor−associated factor, B cell activating factor, CD4] and downstream [interleukin-6 (IL6), -8 (IL8), tumor necrosis factor-α (TNF-α), interferon-β (INF-β), intercellular adhesion molecule 1 (ICAM1)] to nuclear factor κB were elevated in infected tissue. Additionally, levels of pro-angiogenic proteins such as ephrin receptor and vascular endothelial growth factor receptor were diminished while proteins involved in coagulation cascade were upregulated. The results from this study underlined the pathological mechanism in diseased tissue, including overproduction of proinflammatory cytokines, increased the risk of clot formation, platelet activation, fibrosis, and multiorgan failure that may eventually lead to death among SARS-CoV-2-positive patients.[17]

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Table 2. Diagnostic and Prognostic Protein Biomarkers of COVID-19

biomarker	diagnostic/prognostic	matrix	cohort	platform	reference
RPQGLPNNTASWFTALTQHGK (peptide originating from SARS-CoV-2 nucleoprotein)	Diagnostic	Gargle	Proof of concept study	Targeted LC-MS/MS (PRM) Pharmafludics-Orbitrap Fusion Tribrid. 3 h Gradient	[43]
SARS CoV-2 RNA extracts	Diagnostic	Respiratory swab	n = 36 COVID pos vs n = 42 respiratory disease control	Lateral flow-based assay (40 min turnaround)	[87]
IgG, IgA, and IgM	Diagnostic	Whole Blood (DBS)	n = 31 DBS serum matched (COVID) 17 DBS only healthy	ELISA	10.3201/eid2612.203309[88]
Nucleocapsid protein of SARS-CoV-2	Diagnostic	Urine	239 COVID pos (Wuhan) and 20 from Chongqing	Immunochromatographic assay 10 min turn around	10.1101/2020.03.07.20032524[89]
Adjunctive test viral RNA and IgM and IgG	Diagnostic only: antibodies concentrations exhibited no differences among three (moderate, severe, critical) subgroups	Nasopharyngeal swabs and serum samples	133 COVID-19 patients, there were 44 moderate cases, 52 severe cases, and 37 critical cases with no differences in gender and age among three subgroups	ELISA + RT-PCR	10.1016/j.intimp.2020.106746[90]
(CSF white blood cell count, neopterin, β2-microglobulin (β2M), and immunoglobulin G-index), blood-brain-barrier (BBB) integrity (albumin ratio), and axonal injury (CSF neurofilament light chain protein [NfL]	detected...	CSF	n = 6 moderate to severe	light beam analyzer and immunoassay	10.1212/WNL.0000000000010977[91]
List of proteins associated with altered coagulation and complement and IL-6 pathways	Diagnostic	serum	n = 49 of which 33 were COVID-19 positive	timsTOF Evosep	10.1021/acs.jproteome.0c00365[37]
27 proteins complement factors, the coagulation system, inflammation modulators, and pro-inflammatory factors upstream and downstream of interleukin 6	Prognostic	serum and plasma	n = 199	LC-MS/MS SWATH	10.1016/j.cels.2020.05.012[92]
Urine tests revealed that low β2-microglobulin and liver-type fatty acid-binding protein levels were associated with mild disease, whereas high levels were associated with severe disease	Prognostic	Urine	n = 12 severe, n = 13 moderate, n = 33 mild	Immunoassay	10.1097/CCE.0000000000000170[93]
IL-6 strong predictor of fatal outcome	Prognostic	Blood test	n = 611 included. Analysis on n = 433	Flow cytometry	10.1016/j.jaci.2020.07.009[36]
IL-6 correlated with CT results, also patients with severe disease showed significantly elevated level of D-dimer, ESR, LDH, CRP, and ferritin than those with nonsevere disease	Prognostic	Plasma	n = 69 severe	ELISA	10.15252/emmm.202012421[39]
IL-2, IL-6, IL-8, IL-10 increased in severe cases; Lymphocyte count decreased with increased severity, IL-2R/lymphocytes were superior compared with other markers for the identification of COVID-19 with critical illness	Prognostic	Serum	n = 168 mild, n = 169 severe, n = 52 critical	ELISA	10.1111/cei.13450[35]
Chemical parameters of urine such as urine occult blood (BLOOD), proteinuria (PRO), bilirubin, urobilinogen, potential of hydrogen (pH), specific gravity (SG), ketone (KET), urine glucose (GLU-U), nitrite and leukocytes (LEU)	Prognostic	Urine	45 healthy, 67 mild, 42 severe, 10 critical	Chemical parameters of urine such as urine occult blood (BLOOD), proteinuria (PRO), bilirubin, urobilinogen, potential of hydrogen (pH), specific gravity (SG), ketone (KET), urine glucose (GLU-U), nitrite, and leukocytes (LEU)	10.1515/cclm-2020-0220[94]
56 proteins distinguish between mild and severe: pathways = complement activation, regulation of immune response, cellular oxidant detoxification, cellular response to hypoxia, and oxidative stress induced apoptosis	Prognostic	Urine	32 healthy, 6 COVID-19 pos: 3 severe, 3 mild	LC-MS/MS (Lumos DDA)	10.1101/2020.05.02.20088666[95]
93 proteins and 204 metabolites differentially expressed between severe and nonsevere	Prognostic	Serum	n = 28 healthy, n = 25 disease control, n = 37 nonsevere, n = 28 severe	DDA QeHFx and for metabo-UPHLC and waters instrument	10.1016/j.cell.2020.05.032[33]

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Table 2. continued

biomarker	diagnostic/prognostic	matrix	cohort	platform	reference
Peptide Sequences for Nucleoprotein (DQVILLNK + ADETQALPQR) and Spike Glycoprotein (NIDGYFK + FQTLALHR)	Diagnostic	Culture Lung Epithelial Cell Mucus Secretions	Proof of concept study	LC-MS/MS DDA and PRM (Q-Exactive HF-X)	10.1021/acs.analchem.0c02288[96]
Machine-learning-based selection of 11 proteins with combinations for clinical outcome prediction; COVID-19 positive vs Healthy prediction [orosomucoid-1/alpha-1-acid glycoprotein-1(ORM1/AGP1), ORM2, fetuin-B (FETUB), and cholesteryl estertransfer protein (CETP)]; Severity prediction with or without fatal outcome (CETP, S100A9, C-reactive protein); Mild vs Severe Symptom Outcome Prediction [zinc-a2-glycoprotein 1 (AZGP1), ORM2, and complement factor I (CFI)]; Convalescence prediction [serine proteinase inhibitor A3/a1-antichymotrypsin (SER-PINA3/ACT), lymphocyte cytosolic protein 1/L-plastin (LCP1/LPL), and peptidase inhibitor 16 (PI16)]; Host plasma protein alterations (Elevated ORM1, ORM2, S100A9, CRP, AZGP1, CFI, SERPINA3/ACT, LCP1/LPL; Reduced FETUB, CETP, and PI16)	Prognostic	Plasma	Initial cohort for Machine Learning: n = 30 (Fatal outcome = 5; Severe symptoms = 7; Mild symptoms = 10; Healthy patients without COVID-19 = 8); Expanded cohort for biomarker validation: n = 160 (fatal = 40, severe = 40, mild = 40, healthy = 40)	Tandem Mass Tag DDA LC-MS/MS (Q-Exactive HF-X)	[32]
COVID20: 20 urinary peptides for prognosis of critical and fatal outcomes of COVID-19 disease	Prognostic	Urine	Pilot: COVID-19 patients (n = 15), Matched controls (n = 45)	P/ACE-MDQ CE micrOTOF and Dionex Ultimate 3000 RSLS nanoflow system or Beckman CE, with Orbitrap Q Exactive plus	[44]
Early disease stage inflammation-associated proteins and sustained apoptosis and hyperinflammation-associated proteins differentiate severe (ICU) from moderate (non-ICU) patients [ICU have elevated OSM, S100A12, IL-6, HGF, and CCL7] and distinguish hospitalized (ICU and non-ICU) from healthy controls [hospitalized have elevated CASP8, IFNγ, IL-18R, and CCL8]	Prognostic	Plasma	n = 15 severe, n = 25 moderate, n = 119 healthy controls (n = 18 clinical blood analyses, n = 18 plasma proteins, n = 70 antibody reactivities)	Olink and Legendplex	[42]

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Proteomic analysis of infected cells have provided key insight into pathogenic molecular pathways driving the infection. There have been several papers published which are described in reviews.[18,19] One highlight is the work by Bojkova et al. in which the response of SARS-CoV2 infection of human colon epithelial cell line, Caco-2, labeled with stable isotope amino acids (SILAC), showed activation of the host cell translational machinery resulting in 2715 newly synthesized/translated proteins with a subset of 459 proteins with abundance trajectories similar to the viral proteins.[20] These were enriched for metabolomic pathways involved in nucleotide metabolism. Importantly, it was found that perturbing the cellular model with inhibitors (at nontoxic concentrations) of protein translation, RNA splicing, glycolysis, and nucleotide synthesis prevents viral replication. Reanalysis of this data set[20] using alternative bioinformatic tools supported their findings but also found infection-responsive proteins were linked to both the inflammatory response and chromosome segregation during mitosis.[21] Many studies have been published, but we have focused on a few interactome studies that uncovered novel interactions between viral and host proteins as indicators of potentially unusual or new functional insights. Of interest was the identification of shared host binding partners of two hCoV nonstructural proteins (Nsp2 and Nsp4) in cells infected with SARS-CoV-1, SARS-CoV-2, or hCoV-OC43, an endemic strain associated with the common cold.[22] The common host proteins bound to Nsp4 included N-linked glycosylation machinery, unfolded protein response associated proteins, and antiviral innate immune signaling factors while both Nsp2 and Nsp4 interactors with mitochondria-associated ER membrane proteins. In another study, hemeagglutinin-tagged viral proteins for SARS-CoV-2 and SARS-CoV were expressed in a lung-derived human cell line, and the host alteration in transcriptome, proteome, and the ubiquitinome and phosphoproteome also supported SARS-CoV-2 proteins interaction with protein complexes involved in endoplasmic reticulum−Golgi trafficking and transport.[23] Interestingly, the data collectively pointed toward (among other pathways) the TGF-β pathway, which was specifically dysregulated by SARS-CoV-2 ORF8 while SARS-CoV-2 ORF3 specifically dysregulated autophagy.[23] Additional PTM-focused proteomic data indicated a phospho-signature enriched on SLC35 (Sialic acid transport) and SUMO family proteins, while ubiquitination data indicated SARS-CoV-2 specifically increased modification of autophagy-related factors and EGFR pathways. However, perhaps most interesting was that the PTM mapping showed that the majority of viral proteins were also post-translationally modified. For example, 21 of the 27 detected SARS coronavirus proteins, were ubiquitinated with Nsp 2 and Nsp 3 being most heavily modified.[23] This leads to the question of whether monitoring PTM status of the bait proteins during interactome studies is required to tease out any PTM-specific regulatory mechanism that may be invoked. Another interesting study identified the substrates of the SARS-CoV-2 proteases, a papain like (PL) protease and the main protease (Mpro also named 3CLpro), a chymotrypsin-like cysteine protease by recombinantly expressed proteases in lung epithelial and endothelial cell lysates using LC-based N-terminal identification.[24] For example, SARS-CoV-2 Mpro cleaved optineurin (OPTN), a protein involved in ubiquitination, autophagy, protein trafficking, and maintenance of the apparatus, but also importantly, in the activation of NF-κB pathway, antiviral, and antibacterial signaling. Most impressive was the broad potential involvement of ubiquitination or numerous E3 ubiquitin ligases cleaved by the tested Mpros, suggesting the PTM status, including proteolysis, could be adding a layer of biological complexity over that of transcriptional regulation by the viral infection.

Circulating Protein Markers of SARS-CoV-2 Infection

In addition to studies in tissues and cells, proteomic analyses of circulating biofluids have elucidated much about the host proteome response to COVID-19. Since biofluids can be collected noninvasively, there has been a steady growth in the number of proteomic reports in liquid biopsies. These studies have not only helped unravel the pathological mechanisms driving infection, but importantly, have also resulted in the identification of potential diagnostic and prognostic biomarkers. Such biomarkers are crucial for containing the pandemic and it has been widely acknowledged that existing diagnostic assays are lacking sensitivity and specificity.[25] The SARS-CoV-2 RNA tests has been associated with a concerning level of false negatives, in part due to lack of established reference ranges in a diverse population, but also because of sample collection and transportation methods.[26] In contrast to the RNA assay, the serology test has been associated with an alarming number of false positives, largely owing to cross reactivity with antibodies from a previous infection with other strains of the SARS virus.[25,27] Additionally, it takes time to mount an antibody response and dependence on immunoassays may result in delayed or missed diagnosis of vulnerable individuals. Finally, the presence of antibodies against SARS-CoV-2 protein(s) does not infer immunity or designate whether the host is still a source of transmission.[28] Thus, although some reports suggest the serology test can be used as a standalone diagnostic, it is recommended that it be used as a companion diagnostic. Indeed, it has been demonstrated that using the antibody assay in adjunction with the nucleic acid test is more sensitive compared to testing for RNA alone.[29] Accurate detection is vital for identifying those who may need medical attention, but is also important from an economic standpoint, to guide who should self-isolate from those who can return to work. Even with an accurate method for detecting infection with SARS-CoV-2, there is a further complexity because no assay is yet FDA-approved and available to define the progression of the disease or the response to treatment. Consequently, the healthcare system faces overwhelming pressure and difficult decision-making when trying to segregate which individuals will develop a mild case from those who will require intensive care.[30] Moreover, early referral to intensive care treatment units has been shown to improve outcome for patients with severe COVID-19.[31] An effective means of monitoring disease is further emphasized with the release of COVID-19 vaccine, reopening activities indoors, as well as emergence of new viral strains. Clearly there is a need for new biomarkers and several proteomic studies focused on this aspect of COVID-19 are described in what follows.

A study by Shu et al. describes analysis of 10 mild as well as 7 severe and 5 fatal COVID-19 positive individuals whose blood samples were collected during hospitalization at 4 and 2 time points, respectively, were compared to 8 healthy controls. LC-MS/MS quantitative analysis revealed 860 proteins corresponding to 8472 peptides. A total of 195 proteins were found to be significantly differentially expressed between cases compared to controls. Pathway analysis revealed extensive

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enrichment in processes involved in inflammation, immune cell migration and degranulation, complement system, coagulation cascades, and energy metabolism. Notably, platelet degranulation and the complement and coagulation cascades were the most enriched, with proteins involved in these processes more dramatically altered in fatal and severe cases versus mild cases. Interestingly, two SARS-CoV-2-encoded proteins, nsP2 and nsP7, were identified in the plasma from 5 out of 7 severe and 2 out of 5 fatal cases, but these proteins were undetectable in mild cases or healthy controls, suggesting that these two viral proteins may contribute to pathogenesis. The data was further mined, and a machine learning strategy was devised to generate a 4-protein panel [orosomucoid-1/alpha-1-acid glycoprotein-1 (ORM1/AGP1), ORM2, fetuin-B (FETUB), and cholesteryl ester transfer protein (CETP)], which could segregate fatal, severe, mild cases and healthy controls with areas under the curve of 0.95, 0.91, 0.97 and 0.98, respectively. Results were validated following LC-MS/MS analysis in independent cohort of 9 fatal, 6 severe, 6 mild, and 5 controls. In addition to identifying proteins for classification of disease, it was also possible to identify proteins predictive of clinical outcome. Proteins CETP, S100A9, and C-reactive protein (CRP) could segregate severe from fatal outcome (AUC 0.92). Additionally, proteins zinc-a2-glycoprotein 1 (AZGP1), ORM2, and complement factor I (CFI) could distinguish between mild and severe outcome (AUC 1.0). The authors further confirmed the LC-MS/MS data by ELISA analysis of the identified markers in an additional cohort that included 40 fatal, 40 severe, 40 mild, and 40 healthy cases.[32] Like Shu et al., another study by Shen et al. also described pathway enrichment for proteins involved in immune cell activation platelet degranulation, activation of complement system pathway, and dysregulated metabolism pathways.[33] A combined proteomic and metabolomic approach was used to support the identification of prognostic markers in sera from 46 individuals with COVID-19 and 53 controls. A machine-learning model was generated using proteomic and metabolomic measurements from a training cohort of 18 nonsevere and 13 severe patients. The model correctly classified severe patients with an accuracy of 93.5. The predictive accuracy of the molecular signature was attributed to 22 proteins and 7 metabolites, and the panel was further validated using 10 independent patients, 7 of which were correctly classified. To further validate the predictive classifier, targeted mass spectrometric assays for the 22 proteins and 7 metabolites were developed and applied to a second test cohort containing 19 SARS-CoV-2 positive patients. This analysis led to the correct assignment of 16 out of 19 patients. During each round of analysis, the authors described the likelihood that misclassifications were due to the fact that individuals were receiving treatments for other comorbidities prior to or during time of admission.[33] In another LC-MS/MS based study, Messner et al. describe the development of an ultrahigh throughput proteomic workflow and its application to plasma samples with examples focused on COVID-19. An exploratory discovery analysis was performed in serum of 199 random, assumed-to-be healthy individuals versus 31 SARS-CoV-2-positive individuals.[34] Those with COVID-19 differed in disease severity (as defined by the WHO ordinal outcome scale of clinical improvement). LC-MS/MS analysis of test samples resulted in quantification of 229 unique proteins, of which 37 were found to be significantly differentially expressed. Cluster analysis demonstrated that based on these protein expression levels, the various WHO grades of COVID-19 could be deciphered. A validation study was performed in 17 SARS-CoV-2-positive patients versus 15 healthy controls, and the differential expression of 27 out of 37 proteins was confirmed. An interesting observation from this study was that 2 patients clinically classified as severe clustered in with mild-COVID-19 cases based on their proteomic profile. A retrospective analysis of the clinical data revealed that one of these individuals was misdiagnosed with influenza type B while the other started chemotherapy 10 days prior to infection. This finding suggests that the proteomic data may be more objective than currently available clinical classifiers and should be considered as a supplemental tool for clinicians. This study also identified that many of the proteins found to be upregulated in COVID-19 cases are implicated in Interlukin-6-driven inflammatory response. This is not unsurprising since IL-6 is reported as a potent predictor of COVID-19 severity.[34−36] In keeping with this, D'Alessandro et al. performed an interesting study in 49 individuals, 33 of which were SARS-CoV-2 positive. The goal was to investigate the influence of circulating levels of interlukin-6 (an indicator of disease severity) on abundance levels of proteins in serum. LC-MS/MS analysis revealed that several proteins were correlated with levels of Interlukin-6, notably C-reactive protein. Pathway analyses performed on protein clusters in sera of SARS-CoV-2-postitive patients classified by Interleukin-6 levels, indicated significant enrichments of clusters of proteins related to the coagulation and complement cascades, immunoglobulins, antimicrobial enzymes, apolipoproteins, and other transporters. The authors emphasized the link between the hypercoagulative status of those with SARS-CoV-2 positive patients could be linked to disease severity as measured by levels of IL-6 and suggested the use of pro-fibrinolytic agents as potential therapeutic strategy.[37]

Although MS can provide novel information about proteins, immunoassays (and other capture reagent-based methods) can offer higher sensitivity, and thus, can be better suited to measure low-abundant proteins like cytokines. Today, immunoassays are commonly employed in clinical decision-making through clinical chemistry laboratories or as point of care devices.[38] Capture-based immunoassays have been used to study the multiplex-cytokine profile in SARS-CoV-2 positive patients.[35,36,39] In a study of severe SARS-CoV-2 positive patients (n = 69) and nonsevere disease patients (n = 11), Liu et al. show that severe-disease patients' baseline interleukin-6 levels correlated positively with both maximal body temperature during hospitalization and more progressed bilateral and interstitial abnormalities in chest computed tomography (CT) results. Patients with severe disease also showed significantly elevated levels of D-dimer, erythrocyte sedimentation rate, Lactate dehydrogenase, C-reactive protein and ferritin relative to those with nonsevere disease.[39] A report by Fraser et al. quantified 1161 plasma proteins using the O-link technology in 10 SARS-CoV-2 positive, 10 noninfected (SARS-CoV-2 negative) patients, and 10 healthy controls.[40] On the basis of differentially expressed proteins, those that tested positive could be segregated from those that tested negative for COVID-19 as well as healthy controls. Importantly, 6 proteins (CMRF-35-like molecule-1, CD83, FAM3B, Insulin Like Growth Factor 1 Receptor, Opticin, and Interleukin-12 receptor subunit beta-1) were found to accurately predict SARS-CoV-2-positive patient mortality. The authors described their study as limited based on the modest number of

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individuals included, but suggested it set a premise for future hypothesis-driven studies on larger cohorts. Previous work on the same cohort using a smaller immunoassay panel (57 proteins) revealed 17 proteins were significantly differentially expressed between SARS-CoV-2 positive and negative patients with the strongest predictors being 6 proteins (Tumor necrosis factor, Granzyme B, Heat shock protein 70, Interleukin-18, Interferon-gamma-inducible protein 10, and Elastase).[41]

In a longitudinal study of 40 hospitalized SARS-CoV-2 positive patients, Haljasmägi et al.[42] compared 101 proteins, antibodies, and known inflammation markers in plasma samples obtained from intensive care unit (ICU)-admitted patients (ages 42−92, mean age 66), and who were classified as severely ill (n = 15), and SARS-CoV-2 positive patients who did not require ICU admission (ages 21−92, mean age 65) but who were classified as moderately ill (n = 25). Patients categorized as moderately ill had hospital stays ranging from 1 to 44 days (median = 11 days), while severely ill patients stayed in the ICU for 1 to 54 days (median = 10 days), with most of the severe patients requiring mechanical ventilation within 10 days post-symptom-onset. Additionally, the study included a small group (n = 6) of SARS-CoV-2 positive patients with mild symptoms who were not admitted to the hospital. The authors also studied healthy controls, ages 23−87, who did not have a cough, fever, or recent infection for a month prior to being in the study (n = 119). The controls were divided into three analysis groups, (1) clinical blood analysis (n = 18), (2) plasma proteins (n = 18), and (3) antibody reactivities (n = 70). Haljasmägi et al. claimed that a cluster analysis based on 19 protein markers separated severe (ICU) patients from moderate (non-ICU) patients and distinguished hospitalized and nonhospitalized SARS-CoV-2 positive patients from healthy controls. The proteins used in the cluster analysis were those elevated in early stages of disease with levels that peaked within 24−72 h after hospitalization (IL-6, -8, -10, and C-X-C Motif Chemokine Ligand-10 and -11, C−C chemokine ligand-2, -7, -8, PD-L1, IL-18R1, and INF-γ) and markers with sustained elevated levels associated with inflammation and apoptosis (Caspase 8, Transforming growth factor beta-1, Interlukin-7, Colony stimulating factor-1, Vascular endothelial growth factor A, Hepatocyte growth factor, Tumor necrosis factor superfamily member 14, Oncostatin, and S100A12). The authors related elevated early stage markers to strong initial inflammatory response and monocyte-macrophage activation, while they related sustained markers to T-cell apoptosis and tissue destruction due to hyperinflammation syndrome. All hospitalized patients had elevated levels of Caspase 8, INF-γ, IL-18R1, and C−C chemokine ligand 8 relative to healthy controls. When comparing ICU-patients with non-ICU patients, severe (ICU) cases showed higher levels of oncostatin M (OSM) and S100A12 as well as IL-6, hepatocyte growth factor and C−C chemokine ligand 7, which were higher in the most severe ICU patients (4 ARDS-diagnosed, 1 died) relative to the rest of the ICU patients.[42]

Finally, although plasma and serum represent the ultimate target for biomarker discovery, COVID-19-based research studies have not been restricted to these matrices. Indeed, a targeted LC-MS/MS assay has been developed to detect the SARS-CoV-2 nucleocapsid protein from gargle solution.[43] Additionally, an immunochromatographic assay with an impressive 10 min turnaround has been developed for urine analyses.[44] Since renal failure has been well described to occur with SARS-CoV-2-positive patients, it is logical that proteomic analysis of urine is an area of interest. A study by Wendt et al. investigated proteomic changes in urine of 11 SARS-CoV-2 positive individuals and 33 controls by capillary electrophoresis MS. A total of 1941 urinary peptides were detected, and 166 were significantly differentially expressed between cases and controls. Combining these peptides into a statistical classifier allowed for segregation of cases and controls with an accuracy of 100% for that small cohort. Further investigation revealed 31 peptides were significantly differentially expressed in moderate and severe cases compared to fatal cases. Of these peptides, three were found to overlap with well-described markers of chronic kidney disease and one with coronary artery disease. The overlap was expected given the multiorgan dysfunction associated with COVID-19 infection. Further analysis of the data showed that 20 peptides were representative of collagen protein, indicating increased potential proteolysis of extracellular matrix, as expected in inflammation and endothelial damage. These peptides were combined into a classifier termed "COVID20" which demonstrated an AUC of 0.91 ($p < 0.0001$) for differentiation of moderate and severe SARS-CoV-2 positive cases from those critical and fatal cases. In conclusion, this report indicates that a urinary peptide-based biomarker panel may enable prognosis of COVID-19 disease course and consequently implementation of proteomics-guided personalized intervention.[44]

The Humoral Response and Seroconversion in COVID-19

The humoral immune response is critical to viral clearance through the production of targeted viral-specific antibodies. Seroconversion is defined as the duration during which these antibodies are developed and become detectable within the blood. Antibodies convey protection by neutralizing viral entry into uninfected cells. They also promote elimination of virions through opsonization, flagging infected cells for complement-mediated cell death and for viral engulfment by phagocytic cells.[45] As the antibody response matures overtime, antibodies develop a greater affinity for viral antigens through a process named "affinity maturation", which is a consequence of B-cells somatic hypermutation.[46] Finally, the antibody response is necessary for protective immunity against reinfection.[45] The duration of protective immunity can vary depending on the infecting pathogen. Research has shown that protective immunity against the coronaviruses that cause the common cold decreases after a year or two.[47] In comparison, immunity against the SARS virus decreases after three years, and antibodies produced against Middle East Respiratory Syndrome (MERS) decrease after two years.[16,48]

To date, humoral response against SARS-CoV-2 is comparable to the humoral response that can be observed in the context of other coronavirus infections and involve characteristic immunoglobulin G (IgG) and immunoglobulin M (IgM) production.[49,50] At the onset of SARS-CoV-2 infection, viral components like spike (S) and nucleocapsid (N) proteins trigger an immune response in the host to eliminate the virus. Tan Y. J. et al., who screened a panel of SARS-CoV-2 ORFs for structural proteins expressed in mammalian and bacterial cells for reactivity toward convalescent-phase patient sera, showed a higher response against the nucleoprotein (N) during the later time point (16 to 54 days), while antibodies against spike protein (S), or its receptor-binding domain (RBD) could be detected after 4−8 days from the appearance of initial symptoms.[51] Importantly,

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the dynamics of the IgM and IgG against the S, RBD, and/or N proteins of SARS-CoV-2 were detected after the onset of symptoms at different time points in infected patients. The generation of S, RBD, and N-specific IgG occurs approximately 1 week later in SARS-CoV-2-positive patients in ICU compared to non-ICU patients, while S- and RBD-specific IgG levels were 1.5-fold higher in ICU patients. The RBD-specific IgG levels were 4-fold higher in older patients than in younger patients during hospitalization. Interestingly, the S- and RBD-specific IgG levels remained elevated and were 2-fold higher in the recovered patients who were SARS-CoV-2 RNA-negative than those who were RNA-positive.[15,52] The study by Xu et al. also found that the high level of IgG at the early stage of SARS-COV-2 infection was unique, compared with other viral infections which usually use IgM as an early marker for the acute phase.[53] Furthermore, a persistent level of IgG was detected for a longer period, whereas IgM levels started to decline after 3 months.[54] Long Q.-X. et al. showed that asymptomatic individuals had a significantly weaker immune response to SARS-CoV-2 infection.[55] Of asymptomatic individuals, 81.1% (30 of 37) compared to symptomatic individual with 83.8% (31 of 37) showed positivity for IgG at approximately 3−4 weeks after exposure. The COVID-19 IgM positive was lower (62.2%, 23/37) in the asymptomatic group compared to 78.4% (29/37) for the symptomatic group. Interestingly, in the acute phase, IgG levels in the symptomatic group (median S/CO, 20.5; IQR, 5.8−38.2) were higher than those in the asymptomatic group (median S/CO, 3.4; IQR, 1.6−10.7). In the early convalescent phase, IgG levels remains higher in the symptomatic group while it decayed in the asymptomatic group.[55] However, the divergence or changes in an individual's response to different SARS-CoV-2 proteins and individual differences in the clearance of the antigen and/or the corresponding antibodies have not yet been investigated. Similarly, Mariën J. et al., reported that humoral immunity against SARS-CoV-2 may not be long-lasting in persons with mild illness where IgG antibody levels were significantly lower in the mild-infection group using N protein and RBD antigen.[56] A similar trend was shown in IgM and IgA response, where titers fell below the detection threshold in more than 20% of mild cases compared to severe SARS-CoV-2-infected individuals and 41.1% in all cases within four months of the first evaluation.[57] Seow et al. showed consistent results by describing the longitudinal decline of the IgM and IgA antibody responses in SARS-CoV-2 infection in sequential sera from SARS-CoV-2-PCR-confirmed patients collected from the onset of symptoms up to 94 days post-symptom-onset.[58] However, the IgG reactivity remained high in most individuals, even up to 94 days post-onset of symptoms. As such, the duration of immune responses remains unclear, and most likely the differences are the results of sensitivity and specificity of the available serological tests.[58]

The strong serological response to SARS-CoV-2 infection generated is of interest from a diagnostic standpoint and ELISAs have been developed to detect anti-SARS-CoV-2 antibodies.[59] Although nucleic acid amplification tests (NAATs) of nasopharyngeal swabs are the primary method for detecting the infection, they can only be used to diagnose disease during a narrow window and have been found to be associated with a concerning numbers of false negative results.[60] Thus, it has been proposed that serological assays could be used as a companion diagnostic. Indeed, it has been found that the use of NAATs and the serology test in conjunction boosts diagnostic sensitivity in comparison to using either test alone.[29] It has also been suggested that serological profiling of patients with COVID-19 allows for the interrogation of interactions between antibody isotypes and viral proteins and should help elucidate the heterogeneity of clinical presentations.[59] Furthermore, serological assays could be used to identify individuals who have been infected with SARS-CoV-2 that were asymptomatic or had mild symptoms and therefore were unlikely to have received a NAAT. Testing for antibodies can potentially provide a better understanding of how widespread the virus is within a population. Certainly, antibody testing will be necessary to run highly powered and accurate prevention trials.[59]

Despite the many advantages, serological assays for SARS-CoV-2 can generate false positive results, due to cross reactivity (nonspecific binding from high levels of pre-existing antibodies against common epitopes of analogous proteins of related viruses) particularly with patients with chronic inflammatory diseases as discussed by Kharlamova N. et al.[61] Moreover, these assays may lack the resolution to quantify changes in the immune response over time, which could help in understanding clinical progression.[48,59] Of note, there can be a high variability between individuals in the kinetics of IgM and IgG responses to SARS-CoV-2 and in the half-life of IgM and IgG. In addition, competition between IgM and IgG for the target structures likely have profound influence on the final outcome of IgG and IgM determination in blood.[62] In lieu of the stated limitations, it is clear there is need for additional biomarkers to support accurate detection of SARS-CoV-2 as well as monitor immune protection in response to treatment and vaccination.

COVID-19-Induced Autoantibodies

There is increasing evidence that there can be enhanced autoantibody production in COVID-19 patients, a situation where the host produces antibodies against their own proteins.[63] As presented, among 52 severe cases of SARS-CoV-2 infections with high levels of the inflammation marker CRP, >70% of patients showed increased production of the antinuclear or rheumatoid factor antibodies, which are classical markers for autoimmune diseases such as rheumatoid arthritis (RA), systemic sclerosis, and eosinophilic granulomatosis with polyangiitis.[64] In individuals with severe SARS-CoV-2 infections, Zhou and colleagues showed the prevalence of additional autoantibodies, including anti-52 kDa SSA/Ro antibody, anti-60 kDa SSA/Ro antibody, and antinuclear antibody (20%, 25% and 50% respectively).[65] Another study showed similar trend of the B cell responses correlated with severity of the SARS-CoV-2 infection and with the presence of the autoantibodies against multiple targets, including phospholipids and type-I interferons (IFN).[66] A study by Bastard et al. showed that antitype I IFN autoantibodies was present in approximately 10% of severe SARS-CoV-2 positive cases (N = 782 severe COVID-19) and not present in patients with mild COVID-19 (N = 443 mild or asymptomatic individuals) or in healthy controls not infected with SARS-CoV-2 (N = 1160). Importantly, they proposed that these antibodies blocked interferon action, resulting in an attack on the immune system rather of fighting the virus. Moreover, they found that the patients with harmful antibodies were men (95 of 101; 94%), who are more likely to develop severe COVID-19. This proportion of males was higher than that observed in patients with critical COVID-19 without autoantibodies (75%; Fisher

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exact test, $P = 2.5 \times 10^{-6}$), and the proportion was much higher than that in male patients in the asymptomatic or paucisymptomatic cohort (28%; Fisher exact test, $P < 10^{-6}$). The study also suggests a potential therapeutic treatment base on the removal of the neutralizing autoantibodies against type I IFNs from the blood of SARS-CoV-2 positive patients, which could ease symptoms of the disease.[67]

As of today, what is missing is a broader screen of potential autoantigens in SARS-CoV-2-positive individuals and linking the specific autoantigens to clinical symptoms. There are many challenges involved in these types of studies.[68] However, researchers around the world are working earnestly to achieve a broad range of objectives, including creating physiologically relevant infection models, studying immune responses to viral infection, and developing vaccines, therapeutics, and diagnostic tests. The compelling reason to carry out this type of investigation is that COVID-19 long-term health sequelae may, at least in part, be due to autoantibodies. Identifying what are the autoantigens will provide diagnostic tools to identify those with autoantibodies induced by SARS-CoV-2 and provide hints into target or susceptible organs/cell types, and potential therapeutic routes. One key approach is to use more unbiased proteomic analysis to be carried out. Wang et al. used immunoaffinity and mass spectrometry to identify 348 proteins from human lung A549 cells that were alerted after SARS-CoV-2 infections. 198 of the identified proteins were known as targets of autoantibodies with 191 being known as autoantigens.[69] The list also includes autoantigens associated with viral replication, trafficking processes, and apoptosis, as well as new targets for further investigations, e.g., Phospholipase D Family Member 3 (PLD3), Phosphoserine Aminotransferase 1 (PSAT1), among others.[69] Overall, the study confirmed that SARS-CoV-2 causes extensive alterations of host cellular proteins and produces many potential autoantigens. Whether there is a correlation between described antigens and severity or mortality rate in COVID-19 patients must be established.

CONCLUSION

There have been numerous proteomic biomarker studies around COVID-19, diagnosis of the virus itself using targeted MS-based MRM assay, profiling of plasma/serum and urine proteomics to identify disease pathways, and proteins of interest that are altered in patients with mild or severe SARS-CoV-2 infections. The production of autoantibodies against the known autoantigens as well as cytokine signaling in a subset of individuals with severe COVID-19 could play havoc on the patient in the short and long-term. The emergence of long hauler individuals who had COVID-19, even if asymptomatic, who now have neurological and cardiovascular complications, like encephalopathy,[70] ischemic stroke,[71] Guillain-Barré syndrome (GBS),[72] and stress cardiomyopathy, myocarditis, arrhythmia, or acute plaque rupture,[73] suggests that the changes induced by the virus can have long lasting impacts. Whether that is due to changes in coagulation or cytokine storm and/or autoantibodies is not yet known, but monitoring and quantification of plasma proteins including autoantibodies should be able to provide that insight.

AUTHOR INFORMATION

Corresponding Author

Jennifer E. Van Eyk − *Advanced Clinical Biosystems Institute and the Department of Biomedical Sciences and Department of Cardiology, Smidt Heart Institute, Cedars-Sinai Medical Center, Los Angeles, California 90048, United States;* orcid.org/0000-0001-9050-148X; Phone: +1-310-423-0704; Email: jennifer.vaneyk@cshs.org; Fax: +1-424-315-2853

Authors

Angela McArdle − *Advanced Clinical Biosystems Institute and the Department of Biomedical Sciences, Cedars-Sinai Medical Center, Los Angeles, California 90048, United States*

Kirstin E. Washington − *Advanced Clinical Biosystems Institute and the Department of Biomedical Sciences, Cedars-Sinai Medical Center, Los Angeles, California 90048, United States*

Blandine Chazarin Orgel − *Advanced Clinical Biosystems Institute and the Department of Biomedical Sciences, Cedars-Sinai Medical Center, Los Angeles, California 90048, United States*

Aleksandra Binek − *Advanced Clinical Biosystems Institute and the Department of Biomedical Sciences, Cedars-Sinai Medical Center, Los Angeles, California 90048, United States*

Danica-Mae Manalo − *Advanced Clinical Biosystems Institute and the Department of Biomedical Sciences, Cedars-Sinai Medical Center, Los Angeles, California 90048, United States*

Alejandro Rivas − *Advanced Clinical Biosystems Institute and the Department of Biomedical Sciences, Cedars-Sinai Medical Center, Los Angeles, California 90048, United States*

Matthew Ayres − *Advanced Clinical Biosystems Institute and the Department of Biomedical Sciences, Cedars-Sinai Medical Center, Los Angeles, California 90048, United States*

Rakhi Pandey − *Advanced Clinical Biosystems Institute and the Department of Biomedical Sciences, Cedars-Sinai Medical Center, Los Angeles, California 90048, United States*

Connor Phebus − *Advanced Clinical Biosystems Institute and the Department of Biomedical Sciences, Cedars-Sinai Medical Center, Los Angeles, California 90048, United States*

Koen Raedschelders − *Advanced Clinical Biosystems Institute and the Department of Biomedical Sciences, Cedars-Sinai Medical Center, Los Angeles, California 90048, United States;* orcid.org/0000-0003-0835-4762

Justyna Fert-Bober − *Advanced Clinical Biosystems Institute and the Department of Biomedical Sciences and Department of Cardiology, Smidt Heart Institute, Cedars-Sinai Medical Center, Los Angeles, California 90048, United States;* orcid.org/0000-0002-2824-5056

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jproteome.1c00475

Author Contributions

§JFB and JEVE contributed equally.

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