A Preliminary Investigation Using Targeted LC-MS Proteomic Methods Demonstrates Unique Serum Profiles of Hospitalized SARS-CoV-2 Patients

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Introduction

With advancements in LC-MS instrumentation, proteomic productivity is moving from identifications and Venn diagrams, to establishing quantitative differences between proteins in samples from a disease state, vs. samples representing a normal or control state. Peptides of interest selected from discovery investigations, can serve as proxies for either total protein or functional protein amounts. With the introduction of targeted analysis, technical variance in LC-MS/MS analysis has dramatically improved. Nevertheless, for serum/plasma samples, small biological variances remain hard to measure robustly, mainly due to the presence of highly abundant proteins. So for validating small differences from discovery, it becomes critical to pair target peptides to sample depletion methods to best quantify and establish differentiated profiles between disease and normal states.

Serine proteases play critical roles in key processes including digestion, blood coagulation, and immunity. Most serine proteases, such as the prototypic enzymes trypsin and chymotrypsin, are secreted enzymes, while some such as Thrombin, are compartmentalized within intracellular granules, and released in response to local inflammation.

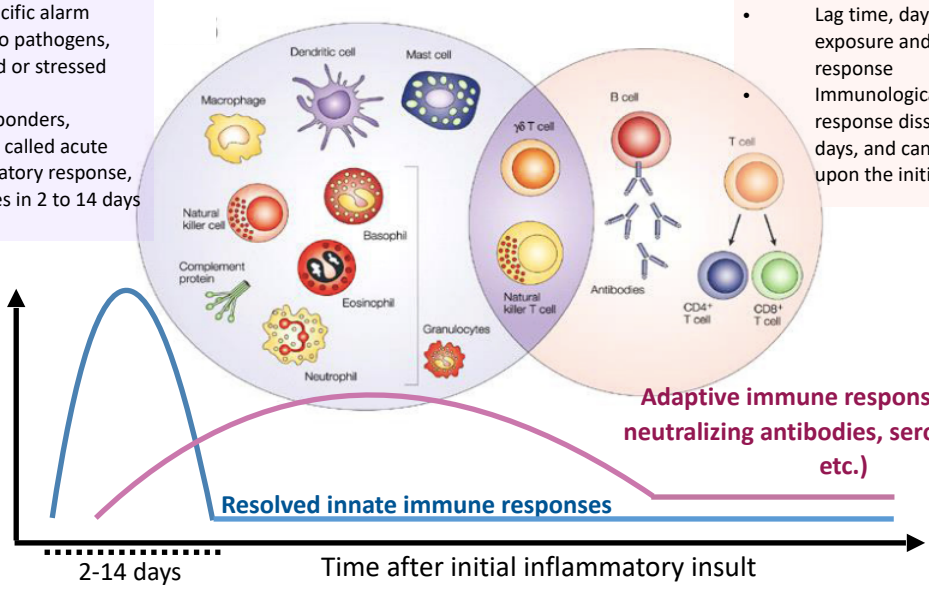
Other members of this family are cell membrane bound; the largest group being the Type II Transmembrane Serine Proteases (TTSPs). The defining features of TTSPs are an N-terminal transmembrane domain, tethered to a C-terminal extracellular serine protease domain. In SARS-CoV-2 (Covid-19), the TTSP- TMPRSS2 cleaves the viral spike (S) protein, priming its binding to the receptor domain (ACE2) on the host cell, with subsequent fusion of viral and cellular membranes. With such a crucial role, antiviral therapeutic strategies targeting TMPRSS2 are now being investigated. Nevertheless, endogenous protease inhibitors, especially the Serpin family, play a combined role of inhibiting TMPRSS2 and regulating an exuberant 'protease storm' upon viral infection. Yet Serpins pose a proteomic challenge as they have a very unique mechanism of action, one that is counter-intuitive to other inhibitory mechanisms. Because of this mechanism, there is no direct way to measure for function, using immunoassay or other binding ligand (i.e, aptamer) proteomic platforms. The same is true for another component of the innate immune response triage – the complement cascade system. For Serpin proteins along with C3 (from complement cascade), LC-MS analysis allows for unique observational windows into functional protein features, and as shown here, can be adopted to specific tryptic peptide features. This strategy allows for quantitative measurement of functional regions by LC-MS, to serve as proxies to measure and monitor functionally active Serpin and C3 sub-forms relevant to the regulation of the innate immune response. This is not possible with currently available proteomic methods.

Innate

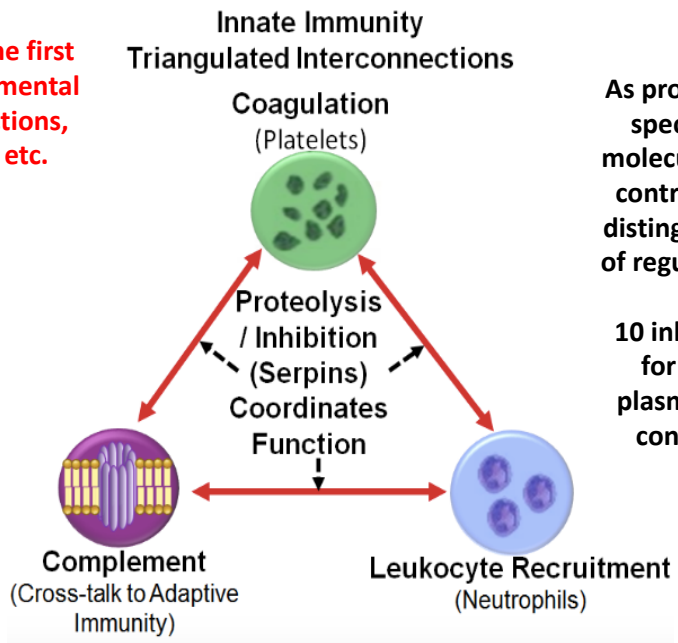
- Non-Specific alarm system to pathogens, damaged or stressed cells
- First responders, clinically called acute inflammatory response, dissipates in 2 to 14 days

Adaptive

- Lag time, days between exposure and maximal response
- Immunological memory, response dissipates in years not days, and can vary dependent upon the initial insult



These pathways are the first responders to environmental insults, wounds, infections, nascent neoplasms, etc.

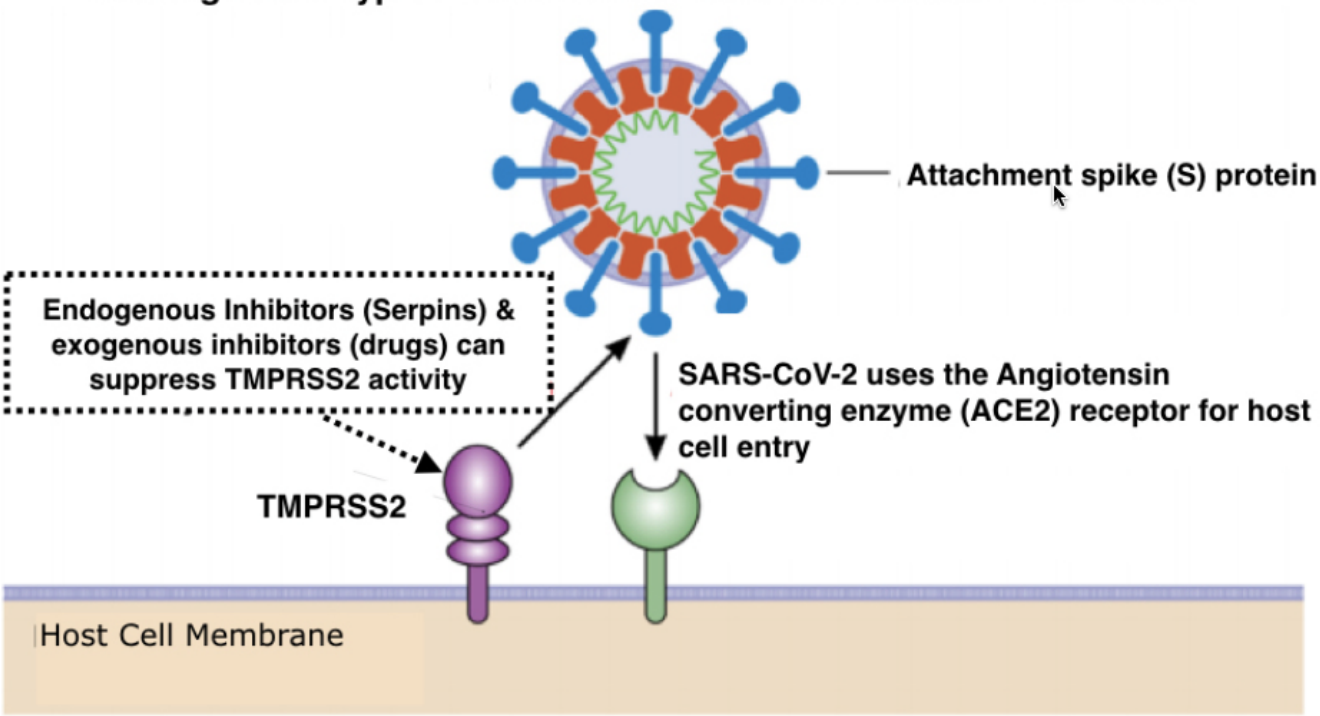


As proteolysis is <u>irreversible</u>, all species of life have evolved molecular regulatory systems to control aberrancies. The most distinguished is a protein family of regulators known as SERPINs.

10 inhibitory SERPINs account for 5-10% protein mass in plasma, and provide a central control function for innate immunity

Innate Immunity - a triangulated <u>interconnected</u> system of : Coagulation, Complement and Leukocyte pathways; regulated through a complex serine protease system

The spike (S) protein of SARS-CoV-2 is activated or primed by proteolytic cleavage from Type II Transmembrane Serine Protease - TMPRSS2

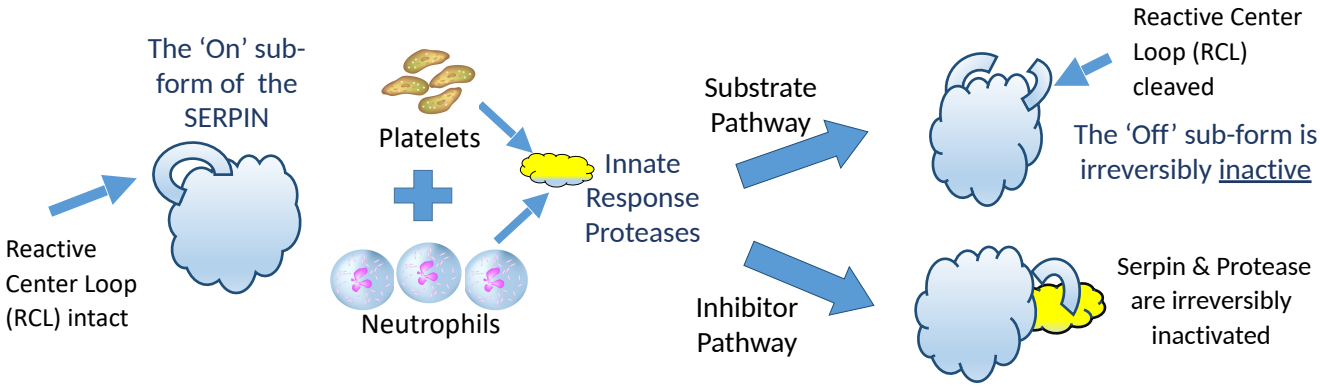


Clinical trials have started for Camostat mesilate, an orally administered serine protease inhibitor to suppress TMPRSS2 activity.

Experimental Design

We did a preliminary investigation of the methods described here, to determine whether our selection of innate response proteins may be differentially regulated by Covid-19 infection, and whether the severity of disease may have an additional impact. For this, we chose 5 age and sex matched normal/healthy controls testing negative for Covid-19, and compared them to 5 Covid-19 hospitalized patients that had mild disease not admitted to the ICU, and Covid-19 patients with severe disease and treated in the ICU. Three Serpins: A1 (common name Alpha-1-Antitrypsin), A3 (common name Alpha-1-Antichymotrypsin) and D1 (common name Heparin Cofactor II), along with Complement C3 were monitored by targeted LC-MS proteomics to investigate the innate response parameters of the 3 patient cohorts. Innate Immunity - a triangulated interconnected system of : Coagulation, Complement and Leukocyte pathways; regulated through a complex serine protease system These pathways are the first responders to environmental insults, wounds, infections, nascent neoplasms, etc. As proteolysis is irreversible, all species of life have evolved molecular regulatory systems to control aberrancies. The most distinguished is a protein family of regulators known as SERPINs. 10 inhibitory SERPINs account for 5-10% protein mass in plasma, and provide a central control function for innate immunity.

However, unlike other endogenous inhibitors (i.e., Kunitz-type), SERPINs do not function in a strictly competitive concentration-dependent manner. Because of their "suicidal" bifurcated mechanism as shown, three functionally distinct sub-forms exist. As platelets and neutrophils continuously release proteases, the 'On' sub-form must be sufficiently generated to resolve the innate response. Yet because 'Off" sub-forms are also produced, it can obscure proteomic analysis; the assumption of counting one Serpin protein as one function is incorrect. More accurately assessing the abundance of the 'On' sub-forms is very important. This is because if and when the 'On' sub-forms are insufficient, the 'protease storm' from innate immunity continues unabated, and the systemic regulation of innate immunity becomes unbalanced. When this happens, the adaptive response can become delayed or paralyzed, further complicating prognosis and clinical management.



Inhibitory Serine Protease Inhibitors (SERPINs) circulate as three functionally distinct sub-forms. Current methods do not distinguish these sub-forms. Our methods using LC-MS analysis, can now uniquely monitor the most important sub-form for monitoring the resolution of the innate immune response – that is the functionally active sub-form. This is done by targeting tryptic peptides that span the RCL cleavage region.

For Serpin A1 (also known as Alpha-1-Antitrypsin)
RCL spanning tryptic peptide: GTEAAGAMFLEAIPM SIPPEVK
RCL Cleavage site|

For Serpin A3 (also known as Alpha-1-Antichymotrypsin)
RCL spanning tryptic peptide: ITLL SALVETR
RCL Cleavage site|

For Serpin D1 (also known as Heparin Cofactor II)
RCL spanning tryptic peptide: HQGTITVNEEGTQATTVTTVGFMPL STQVR
RCL Cleavage site|

AlbuSorb™ PLUS sample prep for Albumin & IgG depletion:

60 mg of AlbuSorb™ PLUS powder was conditioned with 400 ul of Binding buffer (BB1) and filtered on a spinfilter by centrifuge for 2 min at 1000g twice. 20 ul of serum was diluted with 250 ul BB1 buffer, and mixed cleared by passing the spin filter at 9000g for 1 min. The clarified serum was loaded onto the conditioned AlbuSorb™ PLUS beads and incubated on a rotator at room temperature for 15 min and centrifugaed for 4 min at 9,000 to obrain serum protein depleted of albumin and immunoglobulins.

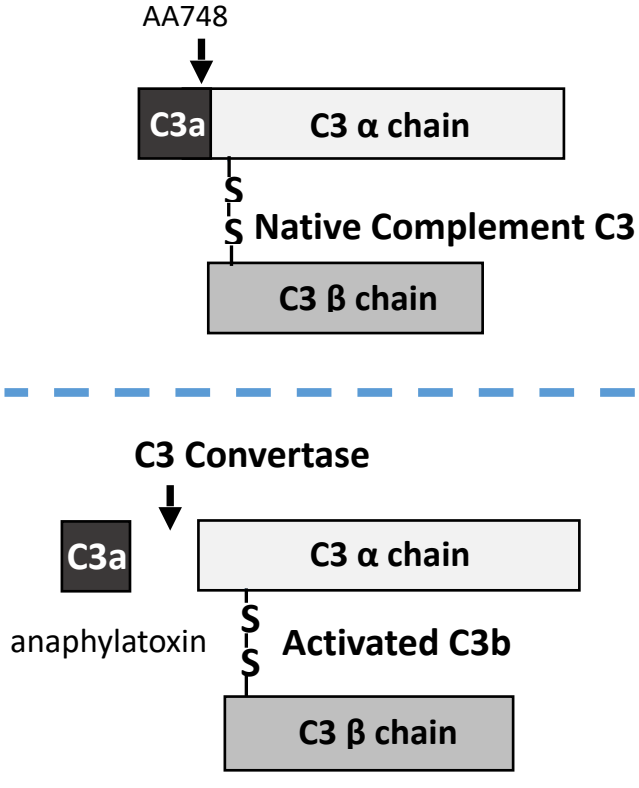
LC-MS/MS analysis was performed at the Rutgers University Proteomics core lab

Reduction & Digestion

After AlbuSorb™ PLUS depletion, an equal volume of serum eluate (~20 µg) was loaded onto SDS-PAGE as gel plug, and in-gel digested with a standard protocol; proteins in the gel bands were reduced with 10mM DTT for 30 min at 60C, alkylated with 20mM iodoacetamide for 45min at room temperature in the dark, and digested overnight with 0.4 µg of trypsin (Pierce MS Grade (ThermoFisher) at 37C. Peptides were extracted twice with 5% formic acid, 60% acetonitrile and dried under vacuum.

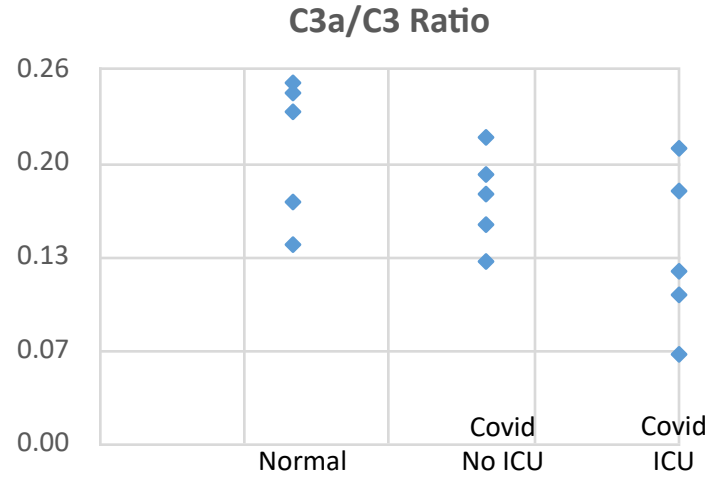
LC-MS/MS Analysis

0.2µg of system-independent retention time (iRT) peptides (Biognosys) were added to each digested samples and 1% of the sample was analyzed by LC-MSMS using Dionex nanoRSLC coupled to Orbitrap Eclipse Tribrid (ThermoFisher). The peptides were loaded on to a fused silica trap column (Acclaim PepMap 100, 75umx2cm, ThermoFisher). After washing for 5 min at 5 µl/min with 0.1% TFA, the trap column was brought in-line with an analytical column (Nanoease MZ peptide BEH C18, 130A, 1.7um, 75umx250mm, Waters) for LC-MS/MS. Peptides were fractionated at 300 nL/min using a segmented linear gradient 4-15% B in 5 min (where A: 0.2% formic acid, and B: 0.16% formic acid, 80% acetonitrile), 15-50%B in 50min, and 50-90%B in 15min. RPM method template was used to target selected ions on a target list thorugh out the run (10-60 min). The isolation width was set at 1.6 Dalton. AGC was set at 2E5 and max ion time set at 150 ms. Ions was fragmented in the HCD using 30% relative collision energy and scanned in the Orbitrap with resolution of 30,000. The data was analyzed using most up to date SKYLINEdaily (beta) with a spectral library generated in the past from the lab. Only the peptide that has the dotp>0.8 were accepted. Final spectral intensity data was normalized to the average value of the iRT peptides for each individual run.



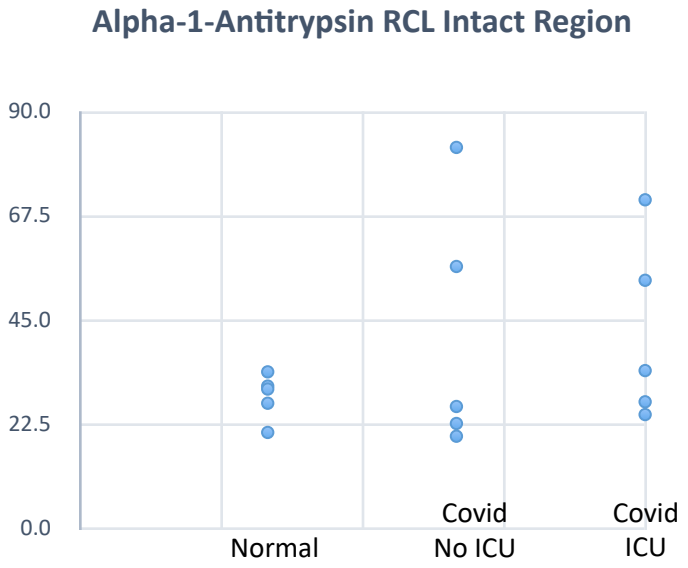
Complement Cascade

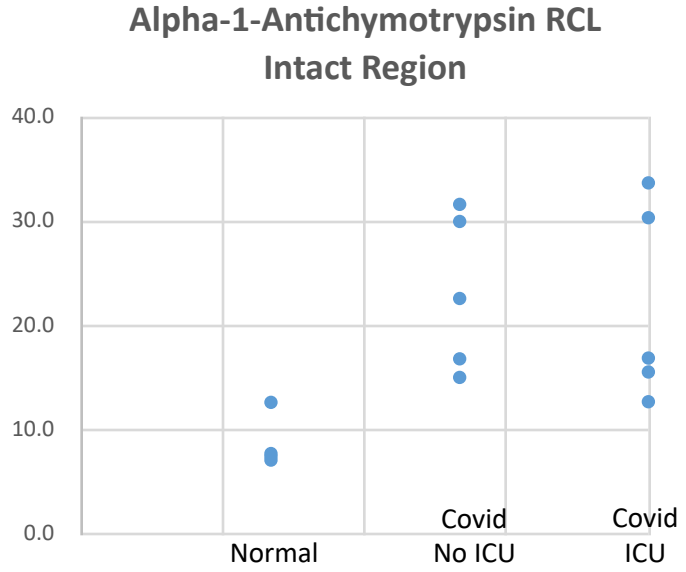
Proteins in the Complement cascade exist in the circulation in a variety of functional sub-forms generated from proteolytically derived split products. The central Complement protein – Native C3, is activated to C3b upon cleavage of the C3a anaphylatoxin region. Using our methods, we can monitor the relative abundance of the Activated C3b form by LC-MS analysis of a tryptic peptide from the C3a region as a ratio of tryptic peptides from other regions. Lower ratios of C3a/C3 would infer higher amounts of Activated C3b sub-forms.

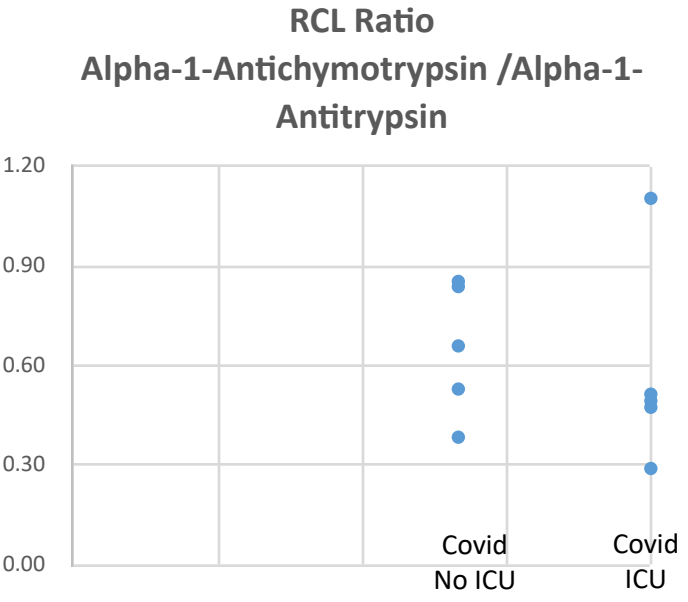


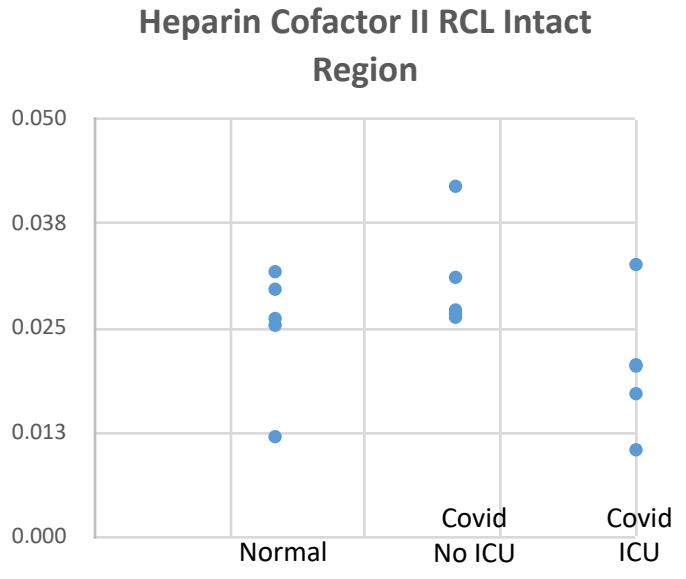
All peptides are normalized to spiked reference iRT peptides (system level signal)

Sum of Area		Samples from Hospilized Covid-19 patients Day 2 not in ICU					Samples from Hospilized ICU Covid-19 patients Day 2					Neg Covid-19, Normal/ healthy controls				
Protein	**Peptide Sequence**	CM009-LB	CM010-CC	CM018-WB	CM021-EP	CM024-RG	CO63-MH	CO64-DJ	CO71-IM	CO72-IAM	CO73-JB	TRC01004	TRC01172	TRC01231	TRC01256	TRC01283
Heparin Cofactor II RCL Intact Region	HQGTITVNEEGTQATTVTTVGFMPLSTQVR	0.042	0.027	0.027	0.031	0.026	0.017	0.020	0.010	0.021	0.033	0.026	0.025	0.032	0.030	0.012
Complement C3a anaphylatoxin	FISLGEAC[+57]K	0.141	0.073	0.069	0.259	0.038	0.024	0.045	0.022	0.071	0.047	0.138	0.079	0.111	0.082	0.194
Complement C3 beta chain region	FVTVQATFGTQVVEK	0.523	0.295	0.299	0.542	0.098	0.089	0.376	0.120	0.189	0.136	0.282	0.313	0.255	0.310	0.380
Complement C3 beta chain region	IPIEDGSGEVVLSR	0.983	0.657	0.781	1.886	0.344	0.305	1.048	0.311	0.617	0.325	0.915	0.819	0.657	0.661	1.166
Complement C3 beta (average of 2 peptides)		0.75	0.48	0.54	1.21	0.22	0.20	0.71	0.22	0.40	0.23	0.60	0.57	0.46	0.49	0.77
Ratio C3a/C3 beta		0.19	0.15	0.13	0.21	0.17	0.12	0.06	0.10	0.18	0.21	0.23	0.14	0.24	0.17	0.25
Alpha-1-Antitrypsin RCL Intact Region	GTEAAGAMFLEAIPMSIPPEVK	56.7	20.1	22.8	82.3	26.5	27.5	24.7	34.2	71.0	53.7	30.9	30.2	27.2	33.9	20.9
Alpha-1-Antichymotrypsin RCL Intact Region	ITLLSALVETR	30.0	16.8	15.0	31.6	22.6	30.3	12.7	16.9	33.7	15.6	7.7	7.8	12.6	7.5	7.1









Discussion

This data is very preliminary as the sample size was small. However, some interesting observations should be noted.

- In some hospitalized patients, functional Serpins A1 (Alpha-1-Antitrypsin) & A3 (Alpha-1-Antichymotryps) go up, in some cases severely
- Alpha-1-Antichymotrypsin more so than Alpha-1-Antitrypsin might have a protective effect as indicated by ratios of mild to severe, so exogenous therapies that can mimic the function of endogenous Alpha-1-Antichymotrypsin might therefore be effective
- Heparin Cofactor II needs better methods of observation as the signal strength was weak, but depletion was observed which may be an indicator of coagulation dysfunction; noteworthy is that this Serpin D1 is one of two, the other being Serpin C1 (Antithrombin III) that require activation by Heparin cofactors. Serpin C1 was not included in this analysis as its RCL cleavage site is the same as Trypsin so alternative digest methods must be developed to monitor its RCL intact region.
- Complement activation trends towards Covid cases, anti-Complement therapies seem warranted.

An efficient coordination of the immunological response to SARS-CoV-2 infection is fundamental to clinical resolution. If the 'protease storm' generated from the innate immune response coming from platelet and neutrophil degranulation, is insufficiently regulated, the adaptive response can be delayed or paralyzed. This can complicate prognosis and clinical management. The methods described here will help characterize the functionality of the innate immune response more precisely than current methods and through that, offer new therapeutic strategies. Most importantly, there is potential to have clinical biomarkers that report functionality of Serpins and Complement, as this cannot be achieved by ligand binding based proteomic platforms (i.e., immunoassay, aptamer). As such, these new methods that adopt the Albumin & IgG depletion product - AlbuSorb™ PLUS in targeted LC-MS workflows present a unique way to monitor biomarkers for precision medical decisions. In this regard, we plan to publish additional studies using larger sample sizes and Covid-19 cohorts that represent longer times in the ICU. Our preliminary investigations suggest similar patterns and trends to those presented here.

More information

Biotech Support Group & Lawson Health Research Institute Enter Collaborative Research Agreement to Monitor Protease Inhibitor Function During Covid-19 Infections https://www.biotechsupportgroup.com/Articles.asp?ID=612

To learn more about Lawson Health Research Institute, visit www.lawsonresearch.ca

Functional Proteomics Book Highlights Biotech Support Group's Contributions to Serpin Proteomics
https://www.biotechsupportgroup.com/Articles.asp?ID=549

A Full Range of Albumin & IgG Removal Products to Overcome the Challenges of Clinical Proteomics
https://www.biotechsupportgroup.com/Albumin-Removal-s/307.htm