Protease inhibitor plasma concentrations associate with COVID-19 infection

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Abstract

Protease inhibitors influence a range of innate immunity and inflammatory pathways. We quantified plasma concentrations of key anti-inflammatory protease inhibitors in chronic haemodialysis patients with COVID-19. The samples were collected early in the disease course to determine whether plasma protease inhibitor levels associated with the presence and severity of COVID-19. We used antibody-based immunoassays to measure plasma concentrations of C1 esterase inhibitor (C1-INH), alpha2-macroglobulin (α2M), antithrombin, and inter-alpha-inhibitor heavy chain 4 (ITIH4) in 100 serial samples from 27 haemodialysis patients with COVID-19. ITIH4 was tested in two assays, one measuring intact ITIH4 and another also detecting any fragmented ITIH4 (total ITIH4). Control cohorts were 32 haemodialysis patients without COVID-19 and 32 healthy controls. We compared protease inhibitor concentration based on current and future COVID-19 severity and with CRP. Results were adjusted for repeated measures and multiple comparisons. Analysis of all available samples demonstrated lower plasma C1-INH and α2M and higher total ITIH4 in COVID-19 compared to dialysis controls. These differences were also seen in the first sample collected after COVID-19 diagnosis, a median of four days from diagnostic swab. Plasma ITIH4 levels were higher in severe than non-severe COVID-19. Serum CRP correlated positively with plasma levels of antithrombin, intact ITIH4, and total ITIH4. In conclusion, plasma protease inhibitor concentrations are altered in COVID-19.

Key Words: Protease inhibitors, innate immunity, COVID-19, coronavirus

Main Text

Introduction:

Infection by human coronavirus SARS-CoV-2 can lead to coronavirus disease 2019 (COVID-19) with clinical sequelae that range from mild symptoms to fatal pneumonitis. The immunological determinants of COVID-19 severity are not understood. Innate immune responses can influence COVID-19 susceptibility and severity by contributing to viral clearance and inflammation triggered by SARS-CoV2(1). Severe COVID-19 is characterised by inflammatory and immuno-thrombotic pathway activation, many of which are regulated by protease inhibitors.

Inhibitors of plasma proteases often target multiple, distinct proteolytic events within immuno-thrombotic cascades, making them prime candidates for influencing the disease course of COVID-19. C1 esterase inhibitor (C1-INH, SERPING1) is a protease inhibitor that influences multiple innate immunity and inflammatory pathways(2). In addition to inhibiting the complement classical and lectin pathways, C1-INH is a primary inhibitor of, for example, activated coagulation pathway factors XII and XI, activated plasma kallikrein, plasmin, tissue-type plasminogen activator, and thrombin(3). C1 esterase inhibitor circulates at serum concentrations of 210-290 µg/ml in adults. Antithrombin III (AT, SERPINC1), alpha2-macroglobulin (α2M, A2M) and alpha1-antitrypsin (SERPINA1) are protease inhibitors that circulate at similarly abundant serum concentrations of about 150 µg/ml, 140-410 µg/ml and 1000-1500 µg/ml respectively(4-6). The newly characterised Inter-alpha-inhibitor heavy chain 4 (ITIH4) has serum concentrations of about 226 µg/ml(7). When ITIH4 is cleaved by an enzyme, such as lectin complement proteases and kallikrein, the cleaved ITIH4 fragment forms a non-covalent, inhibitory complex that inhibits the enzyme(8).

Mass spectrometry-based proteomic studies have identified associations between COVID-19 and protease inhibitors, including C1-INH, ITIH4, AT, and alpha-1-antitrypsin(9-11). SARS-CoV-2 proteins are predicted to interact with C1-INH and reduce C1-INH availability, which could contribute to inflammatory and pro-coagulant states observed in severe COVID-19(12). Reduced serum AT levels have been documented in patients with bacterial septicaemia, associate with markers of disseminated intravascular coagulation(13, 14), and have been observed in cohorts of critically ill patients with COVID-19(15-17). However, accurate circulating protease inhibitor concentrations quantified with targeted antibody-based assays have not been reported in COVID-19. Finally, the potential contribution of protease inhibitors to the pathogenesis of mild and recent-onset COVID-19 is not known.

Here we describe plasma levels of key protease inhibitors in patients with COVID-19, including individuals with mild disease. We collected serial samples during the first wave of the COVID-19 pandemic in London, the United Kingdom, from patients established on maintenance haemodialysis renal replacement therapy. Due to pre-existing chronic kidney disease (CKD), high comorbidity burden, relatively old age, and a high proportion of non-white ethnicity, the patient population was at increased risk of severe COVID-19(15, 18-20). Furthermore, the necessity to attend outpatient haemodialysis meant individuals were screened for pyrexia and symptoms of COVID-19 and subsequently diagnosed, enrolled, and sampled at an early point in the disease course. We

quantified plasma concentrations of C1-INH, AT, α2M, and ITIH4 and identified multiple associations between protease inhibitor levels and COVID-19 severity.

Methods

All participants provided written informed consent and were enrolled in The Impact of COVID-19 on Renal and Immunosuppressed Patients study (IRAS ID 282077). The study was approved by the Health Research Authority, Research Ethics Committee (reference: 20/WA/0123) and conducted in accordance with the Declaration of Helsinki principles. We screened all individuals for symptoms and pyrexia at haemodialysis, clinic, or emergency hospital attendance and tested individuals with SARS-CoV-2 nasopharyngeal PCR swab. We diagnosed COVID-19 from the date of the first positive SARS-CoV-2 PCR swab. We screened controls for asymptomatic COVID-19 infection with negative PCR swab and IgG assay for SARS-CoV-2 antibodies, as previously described(21). Blood sampling commenced as soon as feasible after COVID-19 diagnosis.

Clinical data were collected from electronic medical records, anonymised, and stored on secure computer networks at Imperial College Healthcare Trust. We defined COVID-19 severity based on World Health Organisation (WHO) classifications (WHO clinical management of COVID-19: Interim guidance 27 May 2020. https://apps.who.int/iris/handle/10665/332196) adapted for clinical data availability. Mild was defined as COVID-19 symptoms but no evidence of pneumonia and no hypoxia. Moderate was defined as symptoms of pneumonia, but peripheral oxygen saturation (SaO2) greater than 92% on air or an oxygen requirement no greater than 4L per minute. Severe was defined as SaO2 less than 92% on air, respiratory rate more than 30 per minute, or oxygen requirement more than 4L per minute. Critical was defined as organ dysfunction, signs of systemic shock, or the need for high dependency or intensive care support, for example, non-invasive ventilation or intubation. Severity scores were charted throughout a patient's illness, including at each sampling point. For some analyses, we combined mild and moderate COVID-19 as 'non-severe' and severe and critical as 'severe.'

We measured plasma C1-INH, AT, α2M, and ITIH4 concentrations of 100 plasma samples from 27 patients with CKD and COVID-19, and 1 sample each from 32 haemodialysis control patients without COVID-19 (dialysis controls) and 32 healthy volunteers with neither kidney disease nor COVID-19 (healthy controls), providing a total of 164 samples. Samples were taken at the start of haemodialysis treatment. Blood was collected in EDTA tubes and centrifuged to obtain plasma and stored at –80°C. Protease inhibitor concentrations were analysed in EDTA plasma with 'in-house' sandwich-type, antibody based immunoassays designed and performed at Aarhus University, Denmark(8) or with the use of commercial antibody pairs (Supplemental methods). ITIH4 was tested in two assays; one detects intact ITIH4 only; the other also detects ITIH4 cleaved by enzymes (total ITIH4)(Supplemental methods). We did not have access to an assay to measure alpha1-antitrypsin. Of the 100 COVID-19 samples, 63 were collected coincidentally with clinical samples for C-reactive protein (CRP).

Statistical analyses were performed using Graphpad Prism 9.0. Protein concentrations were displayed as mean with interquartile range (IQR). Differences in clinical characteristics were calculated with the Mann-Whitney U test for continuous and Fisher Exact tests for categorical data. Because our data included serial samples and different sample numbers in each cohort, we analysed these data with a mixed model that uses a compound symmetry covariance matrix and is fitted using Restricted Maximum Likelihood (REML). We adjusted the data for non-sphericity with the Geisser-Greenhouse correction. Differences between first sample lectin pathway concentrations were calculated with Kruskal-Wallis tests, follow-up comparison of the mean rank of every column, and adjustment of P values for multiple comparisons. We calculated correlations with Spearman's rank test. We adjusted p-values for multiple comparisons using Bonferroni's multiple comparisons test.

Results

Eleven of the 27 COVID-19 patients (41%) had severe disease (Table 1). Four patients (15%) died from COVID-19. Levels of clinical biomarkers associated with COVID-19, including CRP and D-dimer, were higher in the severe compared to non-severe disease cohorts (Table 1). Of the COVID-19 patients, 37% (10 of 27) were Asian, and 22% (6 of 27) were of Black ethnicity (Table 1). Our COVID-19 patient population had a median age of 73 years (range 40-88 years), which was significantly older than the dialysis control (62 years, p=0.004) and the healthy control (48 years, p<0.0001) cohorts (Table 1).

We identified significantly greater plasma C1-INH and α2M levels in haemodialysis patients without COVID-19 than healthy controls (Supplementary table). Mean plasma C1-INH concentrations were 325 μg/ml in dialysis control compared with 127 μg/ml in healthy control cohorts (p=0.0005). Mean plasma α2M concentrations were 1500 μg/ml in dialysis control compared with 866 μg/ml in healthy control cohorts (p<0.0001). We used the dialysis control cohort for ongoing comparisons.

We first determined whether protease inhibitor levels associated with COVID-19 diagnosis and severity at the time of sampling. This approach allowed us to utilise all samples and avoided sample selection bias. We identified lower plasma concentrations of C1-INH (p=0.0003) and α2M (p=0.0002) and higher plasma concentrations of total ITIH4 (p=0.008) in COVID-19 samples (Figure 1 and Supplementary Table). Plasma intact ITIH4 levels were higher in samples from severe than non-severe COVID-19 (p=0.02, Figure 1 and Supplemental Table). We did not identify differences between sub-cohorts of white, black, and Asian ethnicity patients (data not shown).

We identified similar associations between protease inhibitor concentrations and COVID-19 at the first sample collected after COVID-19 diagnosis. Plasma C1-INH (p=0.01) and α2M (p=0.001) were lower and total ITIH4 (p=0.003) were higher in COVID-19 patients (Figure 2). Intact ITIH4 was higher in patients who developed severe COVID-19 (p=0.01, Figure 2). These samples were collected at median 4 days (Inter-quartile range (IQR) 2 to 10 days) from positive SARS-CoV2 swab and 6 days (IQR 4 to 11 days) from symptom onset. These data demonstrate protease inhibitor concentrations are altered early in COVID-19 disease course.

We next examined if plasma protease inhibitor concentrations correlated with CRP because this clinical biomarker associates with active inflammation and severity of COVID-19(22). Plasma AT, intact ITIH4, and total ITHI4 correlated with serum CRP measured on coincidentally collected samples (Figure 3A). Correlations between AT and intact ITHI4 and CRP were also significant when only the first collected samples post COVID-19 diagnosis were analysed (Figure 3B).

Discussion

We demonstrated significant differences in protease inhibitor plasma concentrations in COVID-19. We identified reduced C1-INH and α2M and raised total ITIH4 levels in COVID-19 samples. Intact ITIH4 also associated with COVID-19 severity. Additionally, AT and ITIH4 levels correlated positively with CRP, a biomarker of COVID-19 severity and inflammation(22). Notably, the associations were also detectable in samples collected early in the disease course. These findings indicate plasma protease inhibitors and inflammation interact both in early and established COVID-19. Accordingly, raised plasma ITIH4 could be developed as a biomarker of COVID-19 severity. Furthermore, the rebalancing of protease inhibitor levels, such as replenishing C1-INH or α2M, could be considered a therapeutic strategy for COVID-19. This is of particular relevance given the description of clinical improvement in four of five patients treated with human recombinant C1-INH for severe COVID-19 (23). However, further research is needed to establish whether associations between protease inhibitor changes and COIVD-19 are causatve and to delineate the mechanisms that explain these associations.

Our data are consistent with data from mass spectrometry-based proteomic analyses of plasma and sera samples from COVID-19 patients. C1-INH was the protein with the most significantly reduced levels in samples from 31 COVID-19 patients compared to 262 controls(24). From the same study, levels of ITIH4 were increased at first sampling in COVID-19 patients and inpatients who died from COVID-19(24). These results replicate associations between COVID-19 severity and C1-INH and ITIH4 identified from an isotope-labelled, targeted proteomic strategy applied to sera from 46 COVID-19 patients and 53 controls(11). Increased ITIH4 levels were also demonstrated in a high throughput, mass spectrometry-based analysis of plasma and serum from 31 COVID-19 patients. This study also found increased C1r and C1s, the proteases inhibited by C1-INH, in COVID-19(9). Although these results from high protein coverage proteomic techniques are relevant, the accuracy of protein concentration measurements from proteomic approaches is limited(25). In contrast, our assays are derived from targeted antibody-based techniques and provide robust quantification of protease inhibitor plasma concentrations. Furthermore, we tested ITIH4 with two assays, one that gives a signal from intact ITIH4 only and one that, in addition, provides a signal from cleaved ITIH4 (total ITIH4). Both assays detected differences between the dialysis controls and COVID-19 cohorts, whereas only the intact ITIH4 assay identified differences between non-severe and severe COVID-19.

We observed markedly increased plasma C1-INH and α2M levels in uninfected haemodialysis patients compared to healthy controls. Associations have been identified between elevated serum α2M and dialysis

related amyloidosis(26, 27). To our knowledge, circulating C1-INH has not been measured in haemodialysis patients previously. We do not know why plasma C1-INH is increased compared to healthy controls. Similar to α2M, these proteins could accumulate as a result of incomplete removal across semi-permeable dialysis membranes(27). However, whether this is due to molecule size, charge, solubility or other factors requires further investigation. Additionally, assessment of the functional activity of C1-INH, which we did not measure in our cohort, is needed in haemodialysis patients.

Because these proteins influence multiple homeostatic mechanisms, altered concentrations could contribute to the complex, inflammation-associated morbidity associated with chronic haemodialysis(28). Furthermore, although these findings require further investigation, they are immediately relevant to research of C1-INH and α2M in individuals with kidney impairment, including patients with COVID-19. Recently, associations were identified between increased plasma C1-INH and COVID-19 RNAemia in samples from 123 hospitalised COVID-19 patients, 78 of whom required intensive care unit support(29). Based on our data, these findings could be confounded by an increased burden of kidney impairment and renal replacement therapy in patients with high RNAemia and severe COVID-19.

In conclusion, we identified multiple associations between plasma levels of highly abundant protease inhibitors and COVID-19. Protease inhibitor plasma concentrations reflect and may influence COVID-19 pathology. Further research into the mechanistic interplay between protease inhibitor levels and COVID-19 pathogenesis is warranted to establish whether protease inhibitors are useful biomarkers and therapeutic targets for COVID-19.

Author Contributions:

NMT, AT and ST conceived and designed the research. NMT acquired samples and data, analyzed data and wrote the manuscript. AT, AGH and ST conducted protein quantification and reviewed the manuscript. RP and YP contributed to assay design and optimisation. CLC, JEP, DCT, MW, MB, and MCP collected samples and reviewed the manuscript.

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The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The authors have no competing, conflicting, or related interests to declare. The study was approved by the Health Research Authority, Research Ethics Committee (reference: 20/WA/0123) and conducted in accordance with the Declaration of Helsinki principles.The authors thank the patients who volunteered for this study and the Imperial College Healthcare NHS Trust staff. We also thank the clinical and research staff at the Renal and Transplant Centre, Imperial College Healthcare NHS Trust, and the Centre for Inflammatory Disease, Imperial College London (Supplemental Appendix).

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Data Availability:

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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		COVID-19	Dialysis controls	Healthy controls	Severe COVID-19	Non-severe COVID-19	Difference	95% CI	p
	Number	27	32	32	11	16			
	Age, years.	73 (range 40-88)	62 (range 19-86) *				11	4-19	0.004
				48 (range 28-63) *			24	18-30	<0.0001
					66 (44-88)	74 (40-84)			
	Male	17 (63)	19 (59)	17 (53)	7 (64)	10 (63)			
Ethnicity	BAME	18 (67)	24 (75)	20 (63)	6 (54)	12 (75)			
	- Black	6 (22)	3 (9)	6 (19)	3 (27)	3 (18)			
	- Asian	10 (37)	14 (44)	14 (44)	3 (27)	7 (47)			
	- White	9 (33)	8 (25)	12 (37)	5 (46)	4 (24)			
	- Other	2 (7)	7 (22)	0 (0)	0 (6)	2 (12)			
Kidney disease	Diabetic nephropathy	11 (41)	13 (41)		7 (44)	6 (35)			
	Hypertension	2 (7)	0 (0)		1 (6)	2 (12)			
	Glomerulonephritis	4 (14)	8 (25)		1 (6)	3 (18)			
	Genetic	1 (4)	1 (3)		1 (6)	1 (6)			
	Unknown	3 (11)	9 (28)		3 (19)	2 (12)			
	Other	6 (22)	1 (3)		3 (19)	3 (18)			
Co-morbidities	Ischaemic heart disease	14 (52)	15 (47)		7 (44)	10 (59)			
	Current smoking	0 (0)	2 (6)		0 (0)	0(0)			
	Ex-smoker	19 (70)	24 (75)		11 (69)	11 (65)			
	Type 2 diabetes mellitus	12 (44)	15 (47)		8 (50)	7 (41)			
	Antihypertensive medications	22 (81)	23 (72)		13 (81)	15 (88)			
	Current immunosuppression	5 (19)	2 (6)		4 (25)	4 (24)			
COVID-19 progression	Required hospitalisation	11 (41)			16 (100)	1 (6) **			<0.0001
	Died from COVID-19	3 (11)			4 (25)	0 (0) **			0.04
Clinical biomarker at diagnostic swab	C-reactive protein. NR<5 mg/L	43 (IQR 16-93)			60 (IQR 24-138)	29 (IQR 6-77)	31	-79 to 11	0.1
	D-dimer. NR <500 ng/ml	1818 (IQR 1087-2475)			1887 (IQR 1700-2973)	1479 (IQR 958-2064)	408	-1567 to 323	0.2
	Serum troponin. NR <34 ng/L	58 (IQR 27-104)			146 (IQR 63-168)	35 (IQR 22-65) **	111	15 to 134	0.01
	Serum ferritin. NR 20-300 ug/L	841 (IQR 445-1531)			1938 (IQR 1241-2294)	520 (IQR 330-878) **	1418	529 to 1772	0.0009
	White cell count. NR 4-11 x10^9/L	5.5 (IQR 3.8-6.2)			4.3 (IQR 2.9-6.0)	5.8 (IQR 4.3-6.6)	1.5	-0.6 to 2.7	0.2
	Lymphocyte count. NR 1-4 x10^9/L	0.9 (IQR 0.5-1.1)			0.5 (IQR 0.4-0.9)	1 (IQR 0.7-1.3) **	-0.5	-0.7 to -0.1	0.03
Peak level of clinical biomarker	C-reactive protein. NR<5 mg/L	124 (IQR 37-168)			171 (IQR 140-228)	40 (IQR 24-95) **	131	91 to 192	<0.0001
	D-dimer. NR <500 ng/ml	1986 (IQR 1450-3552)			3464 (IQR 1864-4334)	1927 (IQR 1317-3005) **	1537	30 to 2844	0.049
	Serum troponin. NR <34 ng/L	69 (IQR 30-114)			152 (IQR 105-232)	40 (IQR 22-69) **	112	44 to 171	0.0004
	Serum ferritin. NR 20-300 ug/L	992 (IQR 639-2206)			2835 (IQR 1637-3408)	666 (IQR 543-938) **	2169	684 to 2646	0.0006
	White cell count. NR 4-11 x10^9/L	7.4 (IQR 5.9-9.4)			9.8 (IQR 7.7-11.1)	6.7 (IQR 5.6-7.5) **	3.1	0.9 to 5.1	0.006
	Lymphocyte count, nadir	0.7 (IQR 0.4-1.0)			0.4 (IQR 0.3-0.6)	0.9 (IQR 0.7-1.1) **	-0.5	-0.7 to -0.2	0.002

Table 1: Characteristics of haemodialysis patients with COVID-19 and control cohorts.

Data are numbers (%), median (range), or median (inter-quartile range (IQR)). * mark statistically significant differences between COVID-19 and dialysis control or healthy control cohorts. ** mark statistically significant differences between patients with severe and non-severe peak COVID-19 clinical severity. Differences calculated with the Mann-Whitney U test for continuous and Fisher Exact tests for categorical data.

Figure legends

Figure 1: COVID-19 infection associates with reduced plasma C1-inhibitor, alfa2-macroglobulin, and increased ITIH4 levels.

Plasma protease inhibitor levels in 100 samples from 27 haemodialysis patients with COVID-19. 31 samples were from patients with severe (red triangles), and 69 samples were from patients with non-severe (blue triangles) COVID-19 at sampling. Controls are 32 haemodialysis patients without COVID-19 (grey squares). Line and whiskers show the mean and standard deviation of the mean. Levels are shown in Supplemental Table 1.

We analysed differences in protease inhibitor levels by fitting a mixed model in GraphPad Prism 8.0. This mixed model uses a compound symmetry covariance matrix and is fitted using Restricted Maximum Likelihood (REML). In the absence of missing values, this method gives the same P values and multiple comparisons tests as repeated measures ANOVA. In the presence of missing values, the results can be interpreted like repeated measures ANOVA. We adjusted the data for non-sphericity with the Geisser-Greenhouse correction. All p-values are adjusted with Bonferonni's multiple comparisons tests.

C1 esterase inhibitor, C1-INH. Alpha2-macroglobulin, α2M. Antithrombin, AT. Inter-alpha-inhibitor heavy chain 4, ITIH4. ITIH4 was tested in two assays, one measuring intact ITIH4 only and another that in addition also detects any fragmented ITIH4 (total ITIH4).

Figure 2: COVID-19 infection associates with reduced plasma C1-inhibitor, alfa2-macroglobulin, and increased ITIH4 at first sampling point.

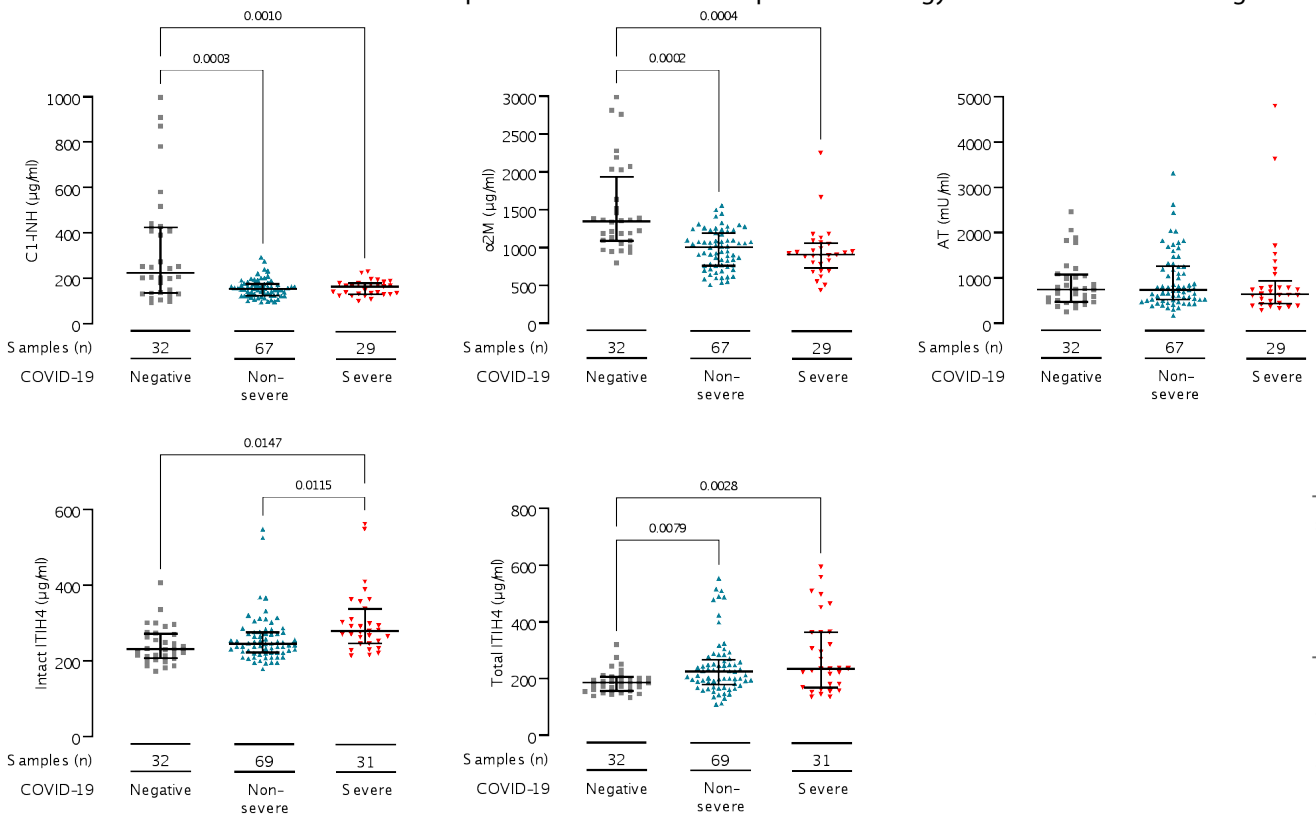
Plasma protease inhibitor levels in first samples collected after COVID-19 diagnosis from 27 haemodialysis patients. Seventeen samples were from patients who developed severe disease (red triangles) and 9 samples were from patients with non-severe (blue triangles) COVID-19 only. Samples were collected at median 4 days (Inter-quartile range (IQR) 2 to 10 days) from positive SARS-CoV2 swab and 6 days (IQR 4 to 11 days) from symptom onset. Controls are 32 dialysis patients without COVID-19 (dialysis control cohort, grey squares). Line and whiskers show the mean and standard deviations.

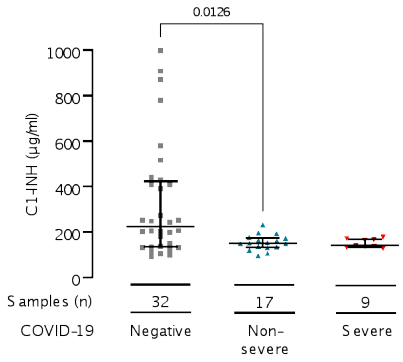
Differences in protease inhibitor levels were calculated by one-way ANOVA. All p-values are adjusted with Bonferonni's multiple comparisons tests.

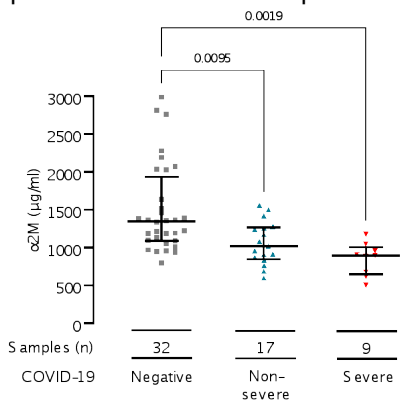
C1 esterase inhibitor, C1-INH. Alpha2-macroglobulin, α2M. Antithrombin, AT. Inter-alpha-inhibitor heavy chain 4, ITIH4. ITIH4 was tested in two assays, one measuring intact ITIH4 only and another that in addition also detects any fragmented ITIH4 (total ITIH4).

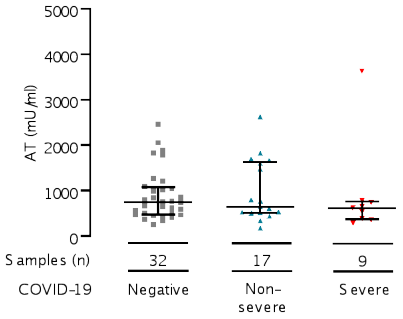
Figure 3: Associations between plasma protease inhibitor levels and CRP in COVID-19.

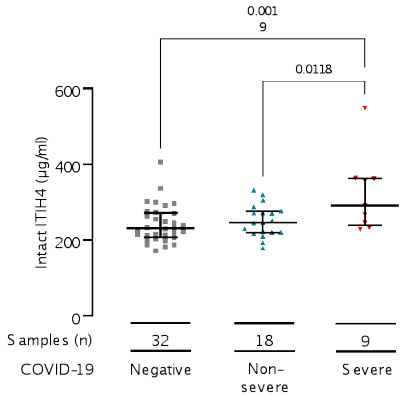
Plasma protease inhibitor concentrations and serum C-reactive protein (CRP) in samples from haemodialysis patients with COVID-19. (A) shows all available samples (63 pairs) and (B) shows the first samples after COVID-19 diagnosis (22 pairs). Correlations (r) calculated with Spearman test. Solid lines show simple linear regression, and dotted lines show the 95% confidence intervals. Significant correlations were not detected between CRP and either C1 esterase inhibitor or alfa2-macroglobulin (data not shown). Antithrombin, AT. Inter-alpha-inhibitor heavy chain 4, ITIH4. ITIH4 was tested in two assays, one measuring intact ITIH4 only and another that in addition also detects any fragmented ITIH4 (total ITIH4).

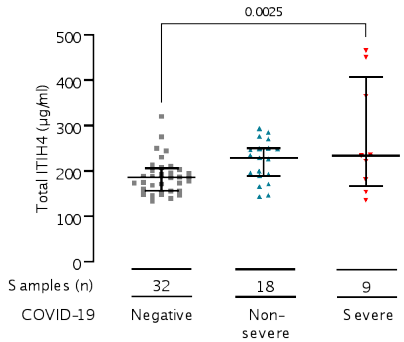












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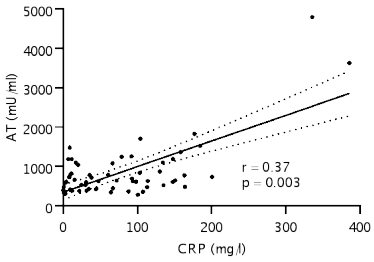

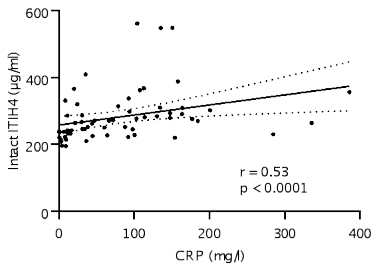

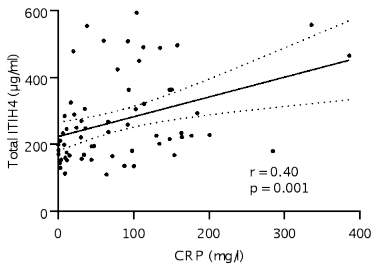

B