The long Pentraxin PTX3 serves as an early predictive biomarker of co-infections in COVID-19

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eBioMedicine
2024;105: 105213
Published Online 21 June 2024
https://doi.org/10.1016/j.ebiom.2024.105213

Summary

Background COVID-19 clinical course is highly variable and secondary infections contribute to COVID-19 complexity. Early detection of secondary infections is clinically relevant for patient outcome. Procalcitonin (PCT) and C-reactive protein (CRP) are the most used biomarkers of infections. Pentraxin 3 (PTX3) is an acute phase protein with promising performance as early biomarker in infections. In patients with COVID-19, PTX3 plasma concentrations at hospital admission are independent predictor of poor outcome. In this study, we assessed whether PTX3 contributes to early identification of co-infections during the course of COVID-19.

Methods We analyzed PTX3 levels in patients affected by COVID-19 with (n = 101) or without (n = 179) community or hospital-acquired fungal or bacterial secondary infections (CAIs or HAIs).

Findings PTX3 plasma concentrations at diagnosis of CAI or HAI were significantly higher than those in patients without secondary infections. Compared to PCT and CRP, the increase of PTX3 plasma levels was associated with the highest hazard ratio for CAIs and HAIs (aHR 11.68 and 24.90). In multivariable Cox regression analysis, PTX3 was also the most significant predictor of 28-days mortality or intensive care unit admission of patients with potential co-infections, faring more pronounced than CRP and PCT.

Interpretation PTX3 is a promising predictive biomarker for early identification and risk stratification of patients with COVID-19 and co-infections.

Funding Dolce & Gabbana fashion house donation; Ministero della Salute for COVID-19; EU funding within the MUR PNRR Extended Partnership initiative on Emerging Infectious Diseases (Project no. PE00000007, INF-ACT) and MUR PNRR Italian network of excellence for advanced diagnosis (Project no. PNC-E3-2022-23683266 PNC-HLS-DA); EU MSCA (project CORVOS 860044).

Keywords: PTX3; COVID-19; Biomarker; Community-acquired infections; Hospital-acquired infections

Introduction

Coronavirus infection and disease 2019 (COVID-19) has placed huge strains on health care systems globally. As of November 2023, more than 770 million cases of COVID-19 have been reported to WHO, with over 6.9 million deaths worldwide (https://covid19.who.int). Several clinical complications are reported in COVID-19. Among these, secondary infections appear to be a relevant issue for different reasons. The percentage of subjects developing secondary infections is estimated between 3 and 14%.[1–9] Secondary infections in COVID-19 are associated with adverse outcomes, such as longer

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Research in context

Evidence before this study

We conducted a search on PubMed Advanced Search Builder for the presence in the title or abstract of the terms 'biomarkers of co-infections in COVID-19', finding 80 articles published in the period 2019–2024. Among these, only 10 articles refer to biomarkers for the identification of bacterial or fungal community or hospital-acquired infections in patients with COVID-19. Six of these articles emphasize the difficulty and the limitations of the classical biomarkers of infections routinely used by clinicians, such as Procalcitonin (PCT) and C-Reactive Protein (CRP), in patients with COVID-19. The results of these studies point out the urgency of new biomarkers for the clear identification of secondary infections in the presence of preexisting viral infections. The identification of these patients can reduce the abuse of antibiotic therapy, largely used in patients with COVID-19, reducing the risk of the onset of multidrug resistant pathogens.

PTX3 in an emerging biomarker of the acute phase response. In patients with COVID-19, circulating PTX3 concentration at hospital admission is an independent predictor of 28 days mortality, positively correlating with disease severity and worse outcome. However, in this study, patients with secondary infections were excluded, to avoid confounding factors.

Added value of this study

In this single center retrospective study conducted at Humanitas Research Hospital, using plasma samples from hospitalized patients with COVID-19, enrolled from March 2020 to March 2021, we evaluated the ability of PTX3 to identify community or hospital acquired secondary infections (CAIs or HAIs) in patients with COVID-19. In details, we found that: *i)* PTX3 plasma concentrations at diagnosis of CAI or HAI are significantly higher than those in patients without secondary infections; *ii)* the increase of PTX3 plasma levels is associated with the highest hazard ratio for CAIs and HAIs compared to PCT and CRP; *iii)* in Cox regression analysis, PTX3 is also the strongest predictor of secondary infections, faring more pronounced than CRP and PCT; *iv)* in patients for whom we had serial PTX3, PCT and CRP measurements, PTX3 plasma levels almost doubled few days before HAI diagnosis, when PCT and CRP concentration were still in the range of moderate increase or normality; v) PTX3 plasma concentration has prognostic significance in patients with COVID-19 and secondary infective complications.

Implications of all the available evidence

Altogether, the results of this study suggest PTX3 is a promising early predictive biomarker of co-infections and risk stratification in COVID-19.

hospitalization time and higher intensive care unit (ICU) admission and mortality rate.[2–6] Because of the intrinsic risk of secondary infections, the prescription and use of broad-spectrum antibiotics are excessive, with 70–85% of patients with COVID-19 receiving antimicrobial therapy,[7–10] compared to the really estimated rate of co-infections. The massive use of antibiotic treatments can result in antimicrobial resistance, one of the leading causes of worldwide death,[11] in addition to unnecessary high costs, and risk of antibiotic-associated toxicity. The COVID-19 pandemic interrupted the infection surveillance and antibiotic treatment monitoring programs, exacerbating the alarming issue of the development of antimicrobial resistant pathogens.[12] Global indications concerning antimicrobial policy in the COVID-19 era and specific tools for the identification of COVID-19 co-infections are urgently required.[6–8,13] On this basis, the identification of biomarkers able to early identify secondary infections in patients with COVID-19, is of great interest to clinicians.[14,15]

Innate immunity plays a crucial role in the response to SARS-CoV-2 infection[16,17] and selected humoral innate immunity molecules act as biomarkers for COVID-19 severity.[16–18] The long Pentraxin 3 (PTX3) is an innate immunity pattern recognized molecule involved in response to pathogens, inflammation and tissue repair.[19] PTX3 expression is rapidly induced in inflammatory conditions and different types of infections and has been proposed as an early biomarker in these conditions.[19,20] In infections, including sepsis, the concentration of circulating PTX3 is increased, associating with severity, organ dysfunction and death.[21,22] A prognostic index comprising PTX3, selected cytokines and clinical parameters was shown to perform better than SOFA in predicting 90-days mortality in sepsis.[22] In addition, PTX3 concentration in bronchoalveolar lavage fluid was a reliable early biomarker of ventilator-associated pneumonia.[23] In patients with COVID-19, PTX3 is up-regulated in myelomonocytic and lung endothelial cells[18] and its circulating concentration at hospital admission is an independent predictor of 28 days mortality, positively correlating with disease severity, worse outcome and viral load.[18,24–27] PTX3 plasma concentration can also predict COVID-19 complications including intubation, thrombotic events and long COVID.[24,28]

The primary aim of this study was to evaluate whether PTX3 serves as biomarker discriminating community- and hospital-acquired secondary infections (CAIs and HAIs) in patients with COVID-19. We found that plasma PTX3 serves as an early biomarker of secondary infections in patients with COVID-19 and a prognostic tool to identify high-risk patients with this

complication, performing more pronounced than classical biomarkers, such as C-reactive protein (CRP) and procalcitonin (PCT).

Methods

Ethics

The study was approved by the Humanitas Clinical and Research Center Ethical Committee (authorization 233/20); the requirement for informed consent was waived. This is a single-center retrospective study conducted at IRCCS Humanitas Clinical and Research Center (Rozzano, Italy).

Study design

We selected 101 hospitalized patients with a COVID-19 laboratory-confirmed diagnosis[18] and a concomitant secondary infection by a fungal or bacterial pathogen and 179 patients affected by COVID-19 with no evidence of secondary infection with available plasma samples collected at admission and longitudinally during hospitalization. We included all males and non-pregnant females (self-reported by study participants), 18 years of age or older. Nasopharyngeal swab for influenza A, B and H1N1 was also routinely performed to exclude viral co-infections at admission. Patients with bacterial or fungal secondary infections were selected based on positive blood, urine or bronchoalveolar lavage or bronchoaspirate culture, concomitant clinical signs of infection (fever and increasing plasma concentration of CRP and PCT) and antibiotic treatment. In case of pneumonia, bacterial and fungal superinfection was defined by evidence of new pulmonary infiltrate at chest X rays or thorax CT scan plus positive bronchoalveolar lavage or bronchoaspirate culture.

The 101 patients affected by COVID-19 with bloodstream infection (BSI), pneumonia or urinary tract infections (UTI) were divided in patients with CAIs, identified within the first 72 h from hospital admission (n = 31), and patients with HAIs, identified at least 72 h after hospital admission (n = 70) (Fig. 1). As control, we selected a group of 179 patients with COVID-19 and no evidence of a concomitant secondary infection randomly selected from all patients with COVID-19 consecutively evaluated from March 2020 to March 2021 (Fig. 1). The control patients with COVID-19 were divided in two groups of 104 and 75 individuals matched with patients with CAI and HAI, respectively, based on the availability of PTX3 measurements at time-points comparable with those of patients with COVID-19 and secondary infections (e.g., within or after the first 72 h from hospital admission).

Laboratory tests

Laboratory testing at hospital admission included complete blood count, renal and liver function (transaminase, total/direct/indirect bilirubin, gamma-glutamyl transferase and alkaline phosphatase), creatinine kinase, LDH, myocardial enzymes, electrolytes and triglycerides. A panel of acute-phase proteins including PCT, CRP, serum ferritin, D-dimer, fibrinogen and Interleukin-6 (IL-6) was measured. Clinical laboratory data were extracted from the curated clinical institutional database of COVID-19 for all the enrolled patients. For each patient, we considered laboratory results collected within a range of 3 days from the day of co-infection, giving the priority to measurements performed before the infection diagnosis.

Sample collection and PTX3 measurement

Venous blood samples were obtained for PTX3 measurement. Samples were stored at 4 °C and centrifuged within 2 h of collection. EDTA plasma was then stored at −80 °C until use. PTX3 plasma concentrations were measured, as previously described,[18] by a sandwich ELISA (detection limit 0.1 ng/ml, inter-assay variability from 8 to 10%) developed in-house with validated recombinant PTX3 and antibodies, by personnel who were blinded to patient characteristics. For patients with COVID-19 and secondary infections, plasma samples were selected at the time closest to the infective event (±3 days). When more than one PTX3 values were available around the co-infection time point, the priority was given to measurements preceding the infection diagnosis.

Based on the evidence that PTX3 levels in patients with COVID-19 are significantly higher than in healthy subjects at admission and, in the absence of complications, decrease afterwards,[18,29] for the control groups we selected samples collected within the first 72 h or >72h from hospital admission, to be matched with CAI and HAI samples, respectively. In addition, PTX3 was analyzed at admission, at an intermediate time-point between admission and co-infection, and finally at the time point closest to hospital discharge or in hospital death.

PTX3 was compared with classical biomarkers of infection and inflammation (PCT, CRP). In selected patients, depending on sample availability, PTX3, CRP and PCT plasma levels were measured longitudinally, in a timeframe of 6 days before and 5 days after the microbiological diagnosis of secondary infection. Due to the lack of daily PTX3 measurements for several patients, the longitudinal evaluation was performed merging 2 days, thus generating curves with six time-points from the microbiological test (−6/−5; −4/−3; −2/−1; 0/+1; +2/+3; +4/+5). When two measurements were available, the average of the two values was used.

Data and statistical analysis

Descriptive statistics included means with standard error [SEM] and medians with interquartile ranges [IQR] for continuous variables, and frequency analyses (percentages) for categorical variables. The distribution of

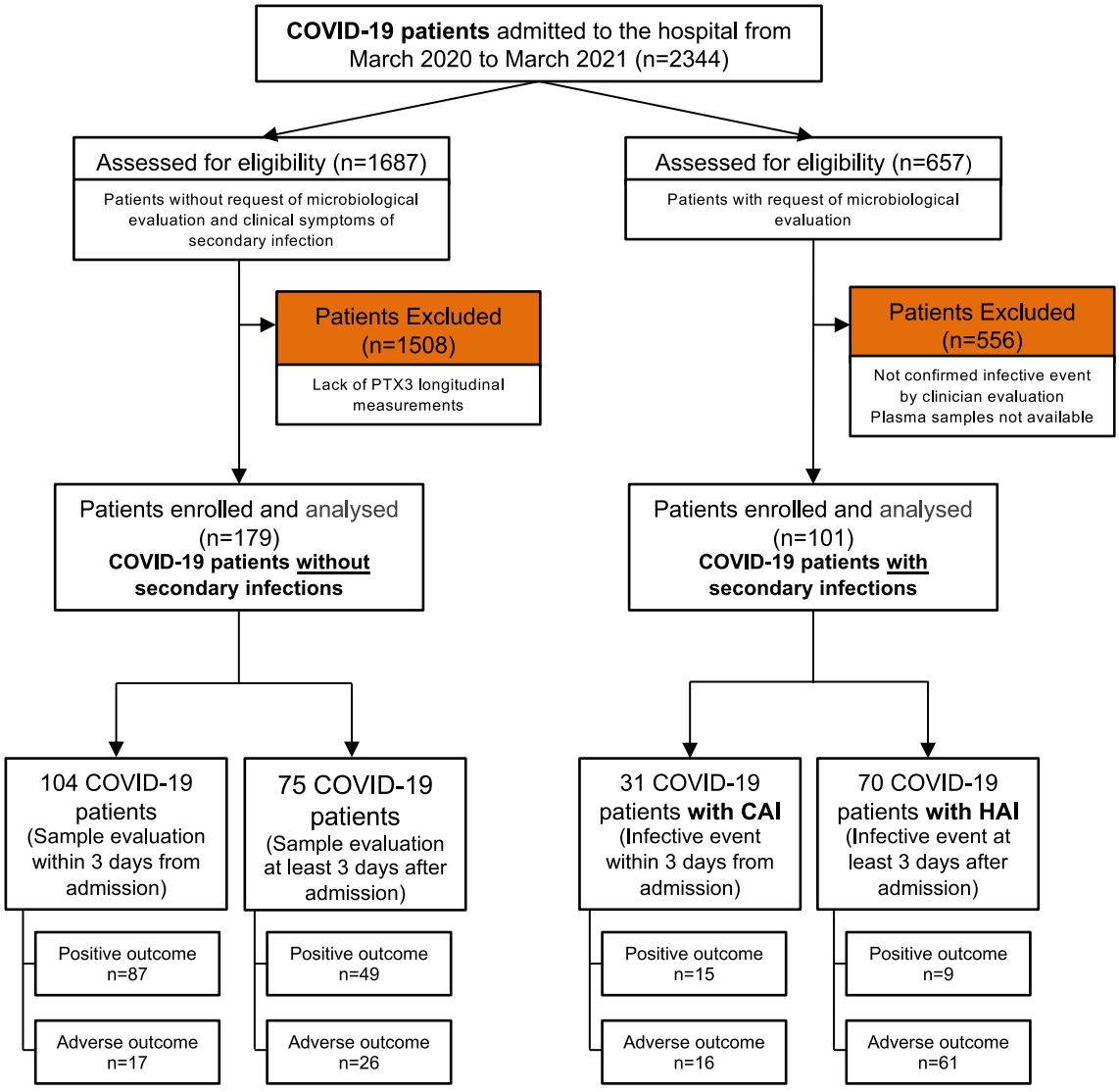

***Fig. 1:* Flow of patients in the study**. Community-acquired infection (CAI); hospital-acquired infection (HAI).

clinical and demographic variables was studied with the Kolmogorov–Smirnov test, while statistical significance was assessed through t-test (Mann–Whitney or Welch's correction) or Kruskal–Wallis multiple comparison test, as appropriate. For the correlation and association analysis, the non-parametric variables were converted in logarithmic scale. Age was stratified in 4 groups (<45, <65, <85, <100 years). The receiver operating characteristic (ROC) analysis was performed for PTX3, CRP and PCT concentration in order to evaluate the cut-off values for the identification of co-infection and adverse outcome (death/ICU admission) in patients with COVID-19.

To identify the association between PTX3, CRP and PCT concentrations and the outcome in patients with COVID-19 and secondary infections, we used time-to-event (survival) methods for censored observations. The study end point was 'hospital death or ICU admission'. Time to event was defined as the time from hospital admission until the date of event or censoring. Patients discharged before 28 days from the hospital and alive were considered event-free.[30] Kaplan–Meier estimates were used to draw the cumulative incidence curves by level of the aforementioned biomarkers defined by the cut-off value identified by ROC analysis (low and high). The biomarkers were compared with a log-rank test. Finally, multivariable Cox proportional hazards models of prognostic factors were used. The analyses were based on non-missing data (missing data not imputed). Confounders were selected according to a

review of the literature, statistical relevance and consensus opinion by an expert group of physicians and methodologists. After fitting the model, the proportional hazards assumption was examined on the basis of Schoenfeld residuals. The hazard ratios (HRs) are presented with their 95% confidence interval (CI) and the respective P values. A ratio higher than 1.0 implies a higher probability of adverse outcome compared to the reference group. Stata 15.0 software was used to analyze the data (Stata Corp., College Station, TX, USA). P-values less than 0.05 were considered statistically significant. Power analysis was not conducted to determine the sample size required to achieve 80% statistical power considering the retrospective nature of the study. All tests were two-sided. No missing data were imputed in any of the analyses.

Role of funders

This study is independent from funding concerning the study design, data collection, experimental workflow, data analyses, interpretation and writing of the manuscript.

Results

Clinical and demographic characteristics of patients enrolled in the study

Demographic characteristics of patients enrolled in this study are shown in Supplementary Table S1. Patients with secondary infections were divided in CAIs or HAIs. Controls for patients with co-infections were consecutively hospitalized patients without clinical or laboratory evidence of secondary infection, selected based on the availability of plasma samples at time points equivalent to secondary infection diagnosis after hospital admission. In patients with COVID-19 and CAIs, the frequency of ICU admission and mortality were significantly higher, in comparison with controls (Supplementary Table S1). Patients with HAIs had a significantly longer hospitalization, higher frequency of ICU admissions and death rate when compared with controls (Supplementary Table S1).

In patients with COVID-19 and CAIs, the main infectious agents were *Enterococcus faecium* and *Escherichia coli* in BSI, *Pseudomonas aeruginosa* in pneumonia and *Enterococcus faecalis* in UTI (Supplementary Table S2). Thirteen cases without a specific microbiological isolation but with clinical and laboratory signs of co-infection were identified. Two patients with CAIs developed sepsis.

For the HAI cohort, the most reported pathogens for BSI were *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Candida albicans*, those for pneumonia were *Pseudomonas aeruginosa* and *S. aureus* (Supplementary Table S3). In this cohort, 10 patients developed UTI with varied etiologies. In 13 patients with co-infections, the agent was not isolated, but patients had clinical and laboratory signs of co-infection (i.e., fever and elevation of PCT and CRP). Three patients with HAIs developed sepsis.

The laboratory characteristics of patients with COVID-19 and secondary infections at the coinfection diagnosis and their controls are reported in Supplementary Table S4. As expected, the circulating concentration of IL-6 and the absolute counts of white blood cells and neutrophils were significantly higher in patients affected by COVID-19 with secondary infections compared to controls with COVID-19 (Supplementary Tables S4 and S5).

PTX3 as a predictive biomarker of CAIs and HAIs in patients with COVID-19

To define the potential of PTX3 to serve as biomarker of COVID-19 co-infections, we divided patients with CAIs and HAIs. As previously shown,[18] at hospital admission, patients with COVID-19 without co-infections had higher than normal PTX3 plasma concentration (median [IQR] 19.90 [13.28–57.04] ng/ml *vs* 2 ng/ml), which declined during hospitalization (median [IQR] 7.72 [4.27–14.76] ng/ml at 8 [range 6–18] days after admission) (Fig. 2a and b and Supplementary Table S5). In patients with CAIs, PTX3 concentration analyzed within 3 days from hospital admission were significantly higher in comparison to hospitalized patients without co-infections (80.44 [35.67–136.9] ng/ml in COVID-19 with CAIs *vs* 19.9 [13.28–57.04] ng/ml in patients with COVID-19; median [IQR], $P < 0.0001$ [Wilcoxon-Mann-Whitney test]) (Fig. 2a). Similarly, patients with HAIs had significantly higher PTX3 plasma concentration at diagnosis of secondary infection (12 [range 7–18] days after admission), compared to patients without a secondary infection analyzed at least 3 days after admission (8 [range 6–18] days after admission). In this cohort, PTX3 concentration was 45.32 [19.78–76.27] ng/ml in HAIs *vs* 7.72 [4.27–14.76] ng/ml (median [IQR], $P < 0.0001$ [Wilcoxon-Mann-Whitney test]) in patients without coinfections (Fig. 2b).

The same trend was observed in both patient cohorts with CAIs or HAIs for CRP and PCT, but with substantially higher overlap with the control patient cohorts with COVID-19 and lower statistical significance compared to PTX3 (Fig. 2c–f; Supplementary Table S5). For all these results, no differences were observed depending on gender.

The ROC curve analysis showed that a cut-off value of 34.35 ng/ml for PTX3, 7.39 mg/dl for CRP and 0.57 ng/ml for PCT, measured within 3 days from hospital admission, predicted CAIs with an area under the curve (AUC), sensitivity and specificity values overlapping for the three proteins. The AUC and sensitivity were higher for PTX3 compared to CRP and PCT, but specificity was lower in comparison to PCT (Fig. 2g). In contrast, a PTX3 cut-off value of 17.31 ng/ml, measured at least 3 days after hospital admission, exhibited

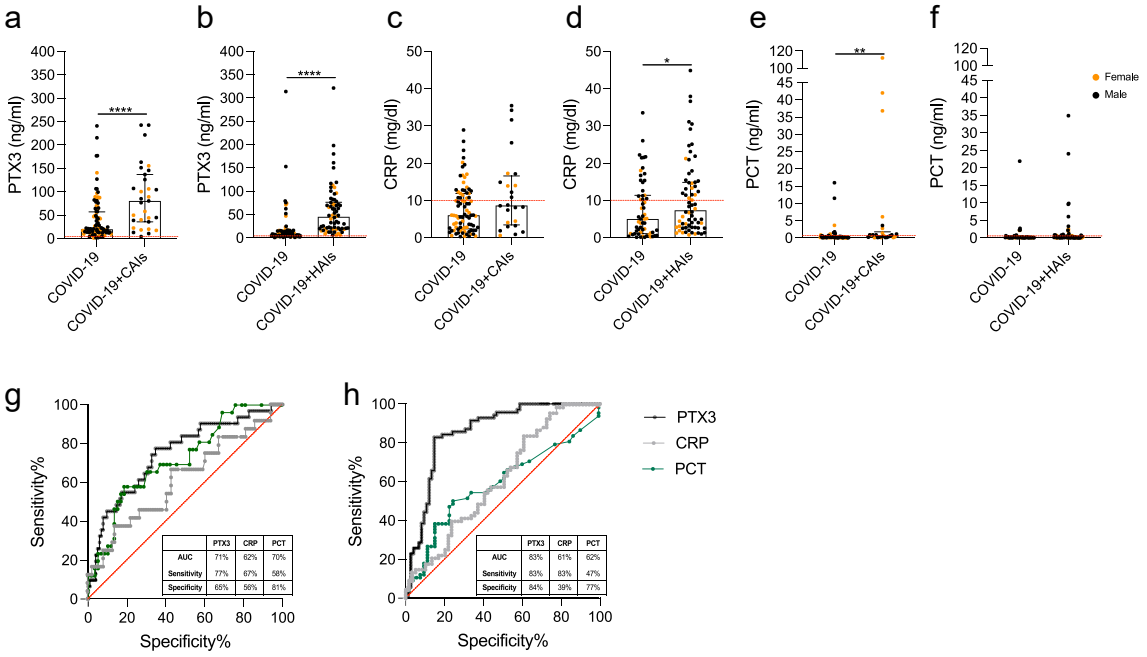

***Fig. 2:* PTX3 plasma concentration in patients affected by COVID-19 with secondary infections.** (a–f) PTX3, CRP and PCT plasma concentration at the time of co-infection diagnosis in patients affected by COVID-19 with CAIs (n = 31) (a-c-e) and HAIs (n = 70) (b-d-f) and their timing-related controls (n = 104 and 75 respectively). (g–h) ROC-analysis of PTX3, CRP and PCT for the identification of CAI (g) or HAI (h). a–f: Median with interquartile range are shown. Statistical significance was assessed with Wilcoxon-Mann-Whitney test. *: P < 0.05; **: P < 0.01; ****: P < 0.0001.

significantly higher predictive performance for HAIs compared to CRP and PCT, as evidenced by AUC, sensitivity, and specificity values of 83%, 83%, and 84%, respectively (Fig. 2h). In the HAI cohort, PTX3 retained the highest positive and negative predictive values for secondary infection compared to CRP and PCT, whereas in CAIs, PTX3 showed the highest negative predictive value for secondary infection compared to the other biomarkers (Supplementary Table S6). The multimarker analysis showed that the combination of PTX3 with CRP and PCT improved the identification of CAIs (AUC PTX3: 71%; AUC PTX3 + CRP: 77%; AUC PTX3 + CRP + PCT: 74%), but not of HAIs.

In addition, the plasma concentration of PTX3 emerged as a statistically significant predictor of these COVID-19 complications with a crude hazard ratio (HR) of 8.08; 95% CI 2.83–23.05; P < 0.0001 for CAIs and 20.76; 95% CI 7.56–57.02; P < 0.0001 for HAIs [univariable logistic regression]) (Table 1). CRP and PCT also demonstrated significant predictive value for HAIs and CAIs, respectively. In a multivariable analysis (adjusted for age, gender, CRP and PCT), PTX3 remained the predictor of secondary infections with the highest adjusted (a)HR in CAIs or the only significant in HAIs (aHR 11.68; 95% CI 2.23–61.19; P = 0.004 for CAIs and 24.90; 95% CI 7.15–86.70; P < 0.0001 for HAIs [multivariate logistic regression]) (Table 1).

These results indicate that PTX3 is a promising biomarker for the discrimination of secondary infections in COVID-19, in particular HAI, because the massive increase of PTX3 production in the early phase of COVID-19 infection may mask CAIs.

Longitudinal analysis of PTX3 plasma concentration in patients affected by COVID-19 with HAIs

We next investigated the dynamics of PTX3 and the other two classical biomarkers of infection during the hospitalization of patients with COVID-19 and co-infection. To this aim, we evaluated and compared PTX3, CRP and PCT plasma concentration at four-time points in most patients with HAIs, depending on sample availability, i.e., admission, the day of HAI diagnosis, an intermediate time point between these two, specific for each patient (9 [range 6–12] days after admission and 7 [range 5–10] before HAI diagnosis), and the day of discharge or in hospital death (Supplementary Figure S1a). As reported in Fig. 3a and Supplementary Figure S1b, PTX3 peaked at admission (median [IQR] 55.12 [27.52–101.4] ng/ml), significantly decreased at the intermediate time-point (median [IQR] 14.94 [9.68–18.92] ng/ml) (P < 0.0001 *vs* the admission [Kruskal–Wallis test with Dunn's multiple comparison test]), then significantly increased again at the co-

Parameter	CAIs						HAIs					
	Univariable model			Multivariable model			Univariable model			Multivariable model		
	HR	(95% CI)	P-value	aHR	(95% CI)	P-value	HR	(95% CI)	P-value	aHR	(95% CI)	P-value
Age (per class of age)	1.18	0.72–1.92	0.515	0.86	0.40–1.84	0.700	1.09	0.71–1.69	0.686	1.33	0.66–2.66	0.421
Gender (male *vs* female)	1.33	0.59–3.02	0.497	0.80	0.20–3.08	0.742	0.91	0.43–1.93	0.808	0.52	0.17–1.59	0.252
PTX3 (per 1 ng/ml increase)[a]	8.08	2.83–23.05	**<0.0001**	11.68	2.23–61.19	**0.004**	20.76	7.56–57.02	**<0.0001**	24.90	7.15–86.70	**<0.0001**
CRP (per 1 mg/dl increase)[a]	2.43	0.82–7.16	0.107	0.32	0.06–1.61	0.166	2.48	1.28–4.79	**0.007**	1.12	0.36–3.45	0.841
PCT (per 1 ng/ml increase)[a]	3.30	1.52–7.16	**0.003**	3.52	1.09–11.38	**0.035**	1.70	0.90–3.23	0.103	1.02	0.40–2.63	0.967

Bold denotes statistically significant P values. HR = hazard ratios; aHR = adjusted hazard ratios; CI = confidence intervals. [a]Variable expressed in logarithmic scale.

Table 1: **Predictors of CAIs and HAIs in patients with COVID-19: univariable and multivariable logistic analysis.**

infection time-point (median [IQR] 45.32 [19.33–78.95] ng/ml) (P < 0.0001 *vs* the intermediate time point [Kruskal–Wallis test with Dunn's multiple comparison test]). After HAI diagnosis, PTX3 concentration decreased again at the last time point analyzed (Fig. 3a). When patients were divided based on outcome, only in surviving patients the PTX3 concentration significantly decreased after the co-infection (P < 0.0001 vs HAI diagnosis [Kruskal–Wallis test with Dunn's multiple comparison test]), whereas in non-survivors, only a

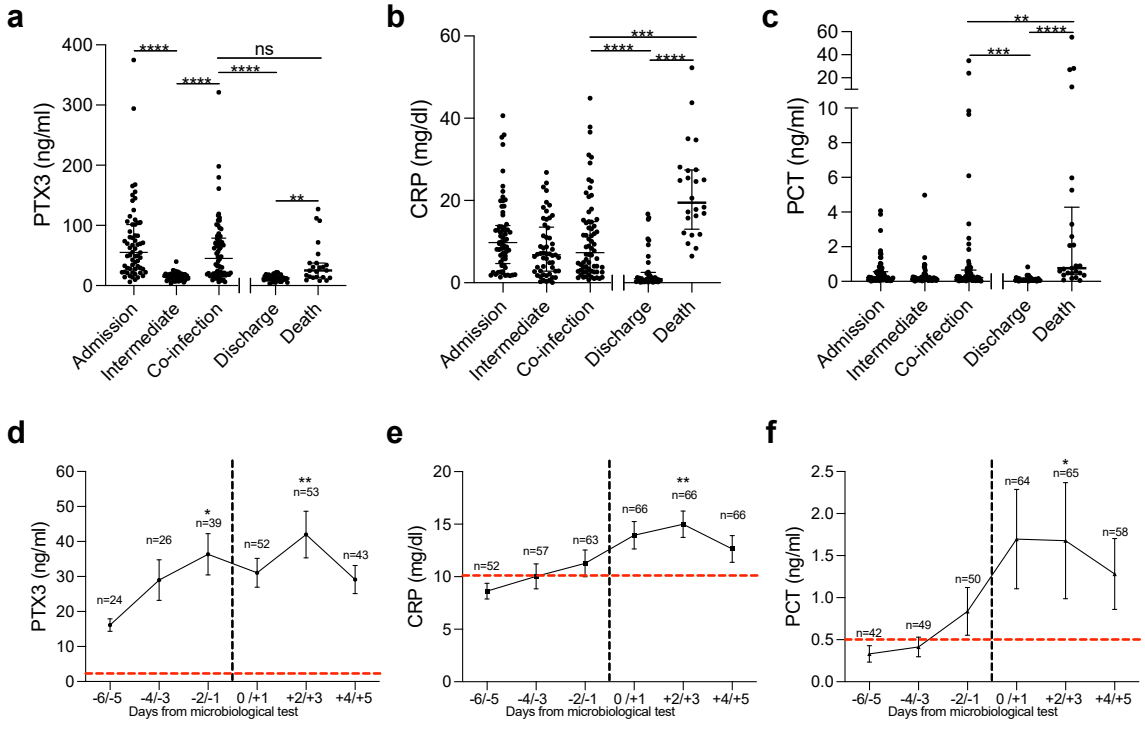

Fig. 3: **Modulation of inflammatory markers during hospitalization and at the co-infection in patients affected by COVID-19 with HAIs.** (a–c) PTX3 (a), CRP (b) and PCT (c) plasma concentration in patients with HAIs at admission, co-infection, an intermediate time point between these two, specific for each patient, and at discharge or death. Admission, n = 62-65-64; Intermediate, n = 50-54-53; Co-infection, n = 70-68-68; Discharge, n = 37-37-35; Death, n = 23-24-25, for PTX3, CRP and PCT, respectively. Median and interquartile ranges are shown. Statistical significance was assessed by Kruskal–Wallis test with Dunn's multiple comparison test. **P < 0.01; ***P < 0.001; ****P < 0.0001. (d–f) PTX3 (d), CRP (e) and PCT (f) plasma concentrations in patients with COVID-19 and HAIs in the days preceding and following the microbiologic test. Mean and standard error of the mean (SEM) are shown. The number of values for each time-point is reported in the graphs. The vertical black dotted line shows the day of the microbiological test. The red horizontal dotted lines show the normal plasma concentration of PTX3 (d), the highest concentration of the range for moderate elevation for CRP (e), and the threshold for an infection for PCT (f). Statistical significance was assessed by Kruskal–Wallis test with Dunn's multiple comparison test comparing every time-points *vs* the time-point −6/−5.

trend of decrease was observed (Fig. 3a and Supplementary Figure S1c). In agreement, the last available PTX3 measurement showed significantly higher levels in non-survivors compared to discharged patients (25.17 [13.93–37.65] ng/ml *vs* 11.97 [9.41–16.29] ng/ml; median [IQR], P = 0.0021 [Kruskal–Wallis test with Dunn's multiple comparison test]) (Fig. 3a).

In contrast to PTX3, CRP plasma concentrations did not significantly differ at the four time points analyzed (Fig. 3b and Supplementary Figure S1d), possibly for its slow increase after an inflammatory stimulus and its long half-life.[31] When patients were divided based on outcome, CRP concentrations were significantly lower at discharge compared to the co-infection time point in survivors, whereas it remained high in non-survivors (Fig. 3b and Supplementary Figure S1e). At the last available time point, discharged patients had significantly lower CRP concentration compared to non-survivors (P < 0.0001 [Kruskal–Wallis test with Dunn's multiple comparison test]) (Fig. 3b).

Only in a limited number of cases, PCT concentration increased at the co-infection compared to the intermediate time-point (Fig. 3c and Supplementary Figure S1f), and declined after co-infection in survivors but not in deceased patients (Fig. 3c and Supplementary Figure S1g). As previously reported,[32] at admission PCT median concentration was in the range of normality (median [IQR] 0.22 [0.10–0.56] ng/ml) (Fig. 3c).

We next focused the longitudinal analysis of the behavior of PTX3 compared to CRP and PCT on the period of HAI diagnosis. In selected patients, for whom we had PTX3 measurements in a time period of 6 days before and 5 days after HAI diagnosis, we performed a longitudinal analysis comparing the three biomarkers. Although we did not have access to all time-point samples or measurements for all the patients enrolled, as shown in Fig. 3d, in this subgroup of patients, PTX3 plasma levels almost doubled between day −6/−5 (16.14 ng/ml) and −4/−3 (29.00 ng/ml) before the microbiological diagnosis of HAI. In contrast, CRP increased very slowly, remaining in the range of "moderate elevation" (1–10 mg/dl) till HAI diagnosis (Fig. 3e), and PCT reached the reference thresholds for an infection (0.5 ng/ml) only at day −2/−1 before the diagnosis (Fig. 3f). Both of them markedly increased only after the microbiological test.

Collectively, these results indicate that PTX3 acts as an early biomarker of secondary infections in patients affected by COVID-19 with both CAIs and HAIs. In HAIs, PTX3 performed more pronounced than the classical biomarkers PCT and CRP. After a HAI, the behavior of PTX3 and the other two biomarkers discriminated patients who survive or not to this COVID-19-associated complication.

The predictive potential of PTX3 in COVID-19 co-infections is not affected by the site or type of infection

To better dissect the role of PTX3 as biomarker of co-infections in COVID-19, we divided the cohort in three groups depending on the site of infection, BSI, UTI and pneumonia, further subdivided in CAIs and HAIs. As shown in Fig. 4a, irrespectively of the site of infection, PTX3 plasma concentrations were significantly higher in patients with COVID-19 and coinfection compared to patients without coinfections. Specifically, PTX3 concentration were 35.67 [13.38–94.16] ng/ml (median [IQR]) in BSI (n = 35), 46.49 [20.41–69.32] ng/ml (median [IQR]) in UTI (n = 14), 65.41 [25.46–106.10] ng/ml (median [IQR]) in pneumonia (n = 51), *vs* 15.05 [6.93–37.75] ng/ml (median [IQR]) in patients without coinfections (n = 179); P = 0.0023, 0.0155 and < 0.0001 [Kruskal–Wallis test with Dunn's multiple comparison test], respectively. When patients were divided in CAIs and HAIs, PTX3 retained its prognostic value in any condition in HAIs. In community acquired BSI and UTI, the concentration was higher than in patients without coinfections, but the number of cases was too low to reach the statistical significance (Fig. 4b).

Finally, as shown in Fig. 4c, PTX3 plasma concentration was significantly higher in patients compared to patients affected by COVID-19 without coinfection, irrespectively of the type of bacteria (Gram- or Gram + bacteria). PTX3 plasma concentration in fungal infections was higher than in patients without coinfection, but did not reach the statistical significance, probably due to the low number of cases (n = 5).

Prognostic potential of PTX3 for adverse outcome in patients with COVID-19

In line with previous studies,[18,24–27] when we stratified by outcome all patients enrolled in this study with sample availability within 72 h from admission, we observed that PTX3 plasma concentration within 3 days from hospital admission was significantly higher in patients with adverse outcome (non survivors/ICU admission) (n = 86) compared with patients with positive outcome (survivors/non ICU admission) (n = 108) (56.77 [24.58–102.3] ng/ml *vs* 19.90 [13.58–57.04] ng/ml; median [IQR], P < 0.0001 [Wilcoxon-Mann-Whitney test]) (Fig. 5a), confirming that PTX3 is a prognostic biomarker of COVID-19-associated adverse outcome, independently of this complication. Correlation with adverse outcome was also observed in the control group of CAIs, where PTX3 plasma concentration evaluated within 3 days from hospital admission was significantly higher in patients affected by COVID-19 with adverse outcome (n = 17) compared with patients with positive outcome (n = 87) (48.47 [26.68–88.94]ng/ml *vs* 18.78 [12.85–42.48] ng/ml; median [IQR], P = 0.0185 [Kruskal–Wallis test with Dunn's multiple comparison test]) (Fig. 5b). In non-coinfected COVID-19 controls for

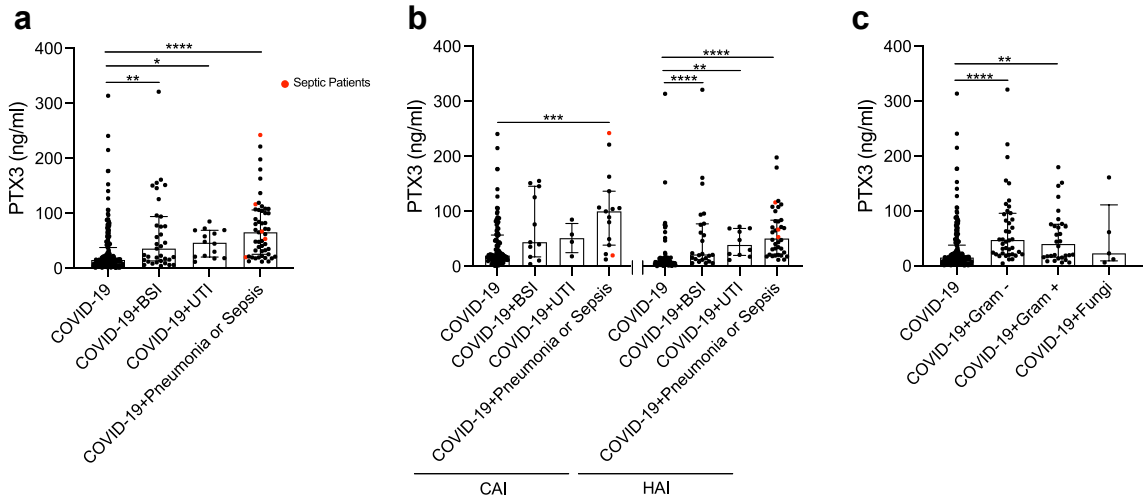

***Fig. 4:* PTX3 plasma concentration in patients with COVID-19 divided by the site and type of secondary infection.** (a) PTX3 plasma concentration in patients affected by COVID-19 with secondary infections at the time of co-infection and divided by site of infections (BSI, n = 35; UTI, n = 14; Pneumonia or Sepsis, n = 51) and their related controls (n = 179). (b) PTX3 plasma concentrations shown in a, analyzed separately in CAIs and HAIs (CAI: BSI, n = 11; UTI, n = 4; Pneumonia or Sepsis, n = 15; COVID-19 controls n = 104; and HAI: BSI, n = 24; UTI, n = 10; Pneumonia or Sepsis, n = 36; COVID-19 controls, n = 75). Patients with sepsis are shown in red. (c) PTX3 plasma concentration in patients with COVID-19 and secondary infections divided by type of pathogen (Gram-bacteria, n = 40; Gram + bacteria, n = 30, fungi, n = 5) and their related controls (n = 179). Median and interquartile ranges are shown. Statistical significance was assessed by Kruskal–Wallis test with Dunn's multiple comparison test. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

HAIs, where PTX3 was measured after 72h from admission (at 8 [range 6–18] days after admission), patients with adverse outcome had significantly higher PTX3 concentration compared to patients with positive outcome as well (Fig. 5c), suggesting that the prognostic value of PTX3 persists beyond admission. In patients with co-infection, a statistically significant increase was observed in patients with HAI and adverse outcome compared to patients with positive outcome (Fig. 5 b-c).

We further investigated the prognostic potential of PTX3 in this COVID-19 cohort by applying a cut-off value identified by ROC analysis. The ROC curve analysis of the CAI cohort (patients with COVID-19 and CAIs and their controls) showed that, at hospital admission, cut-off values of 36.71 ng/ml for PTX3, 4.27 mg/dl for CRP and 0.31 ng/ml for PCT predicted an adverse outcome (death/ICU admission) in patients affected by COVID-19 with AUC, sensitivity and specificity overlapping for the three biomarkers (Fig. 5d). For the HAI cohort (patients with COVID-19 and HAIs and their controls), a PTX3 cut-off value of 16.5 ng/ml, measured at least 3 days after hospital admission, predicted an adverse outcome, with AUC, sensitivity and specificity values of 78%, 71% and 85%, respectively (Fig. 5e). In these patients, we identified cut-off values for CRP and PCT of 6.04 mg/dl and 0.22 ng/ml with lower AUC, sensitivity and specificity values (AUC: 65 and 65%; sensitivity: 59 and 51%; specificity: 70 and 78%, respectively) (Fig. 5e). In HAIs, PTX3 retained the highest positive and negative predictive values for adverse outcome compared to CRP and PCT, whereas in CAIs, PTX3 showed the highest negative predictive value (Supplementary Table S6).

We next evaluated the prognostic potential for ICU admission or mortality of PTX3 in patients with COVID-19 by using the ROC cut-off value (36.71 ng/ml) and stratifying patients in low- and high-PTX3 level groups. In the control group for CAIs, the Kaplan–Meier analysis showed an overall 28-days event-free survival of 0.96 and 0.67 in the low- and high-PTX3 level groups, respectively (Fig. 6a), in line with previous studies. In patients with CAIs, the overall 28-days event-free survival was 0.63 and 0.48 in the low- and high-PTX3 level groups, respectively (log-rank test, Chi-Squared = 29.86; P < 0.00001) (Fig. 6a; Table 2). The Kaplan–Meier analysis of CRP and PCT showed that these two biomarkers discriminate the outcome in patients with co-infection but not in patients affected by COVID-19 without secondary infections (Fig. 6b and c; Table 2).

We finally applied univariable and multivariable Cox regression analysis, to validate the association between PTX3 and outcome in the CAI cohort. On univariable analysis, PTX3 was a predictive factor of mortality or ICU admission in the CAI cohort (crude HR 5.57; 95% CI 2.40–12.95; P < 0.0001 [univariable cox regression]). CRP and PCT were also associated with adverse outcome (Table 2). After adjusting for potential confounding factors (age, gender, co-infective event, CRP and PCT), only PTX3 was confirmed as a predictor of outcome in the CAI cohort (aHR 4.80; 95% CI

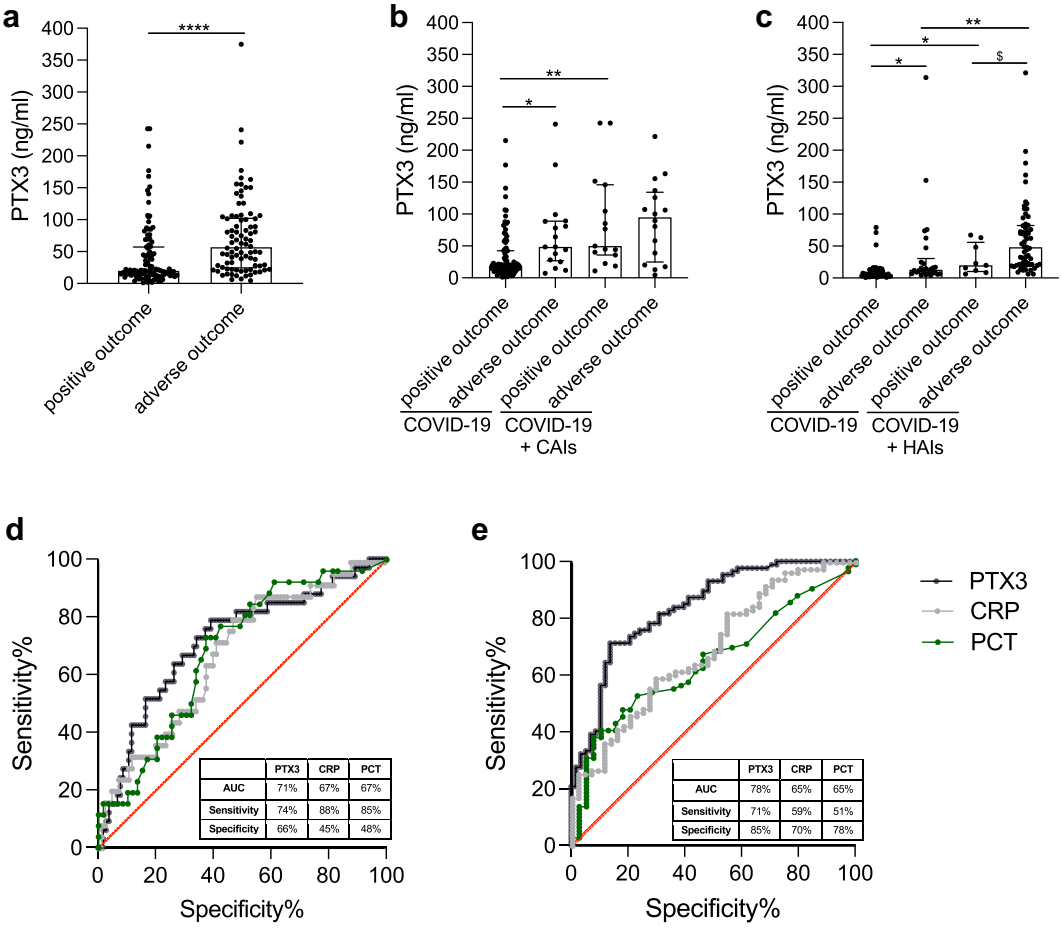

***Fig. 5:* Prognostic value of PTX3 in patients with COVID-19 and secondary infections**. (a) PTX3 plasma concentration at hospital admission in the total cohort of patients stratified according to the outcome (survivors/non-ICU admission [positive outcome] n = 108; non survivors/ICU admission [adverse outcome] n = 86). Median with interquartile range is shown. Statistical significance was assessed with Wilcoxon-Mann-Whitney test. ****: P < 0.0001. (b-c) PTX3 plasma concentration at hospital admission in patients with COVID-19 and CAIs divided by adverse outcome (COVID-19: positive outcome n = 87; adverse outcome n = 17; COVID-19 + CAIs: positive outcome n = 15; adverse outcome n = 16) (b); and HAIs at co-infection diagnosis (COVID-19: positive outcome n = 49; adverse outcome n = 26; COVID-19 + HAIs: positive outcome n = 9; adverse outcome n = 61) (c). b, c: Median with interquartile range are shown. Statistical significance was assessed with Kruskal–Wallis test (*P < 0.05; **P < 0.01) and for patients with HAI by Mann–Whitney test ($ P < 0.05). (d, e) ROC-analysis of PTX3, CRP and PCT for the identification of adverse outcome (Death or ICU admission) in patients with COVID-19 and CAI (d) or HAI (e) and their related controls.

1.24–18.62; P = 0.023 [multivariable cox regression]; Table 2). The proportional hazard assumption was not violated (P = 0.059).

In HAIs, the Kaplan–Meier analysis showed an overall 28-d event-free survival of 0.74 in the low-PTX3 level group and 0.39 in the high-PTX3 level group (log-rank test, Chi-Squared = 15.91; P = 0.0001) (Fig. 6d; Table 2). The overall 28-d event-free survival was 0.50 for high-CRP and PCT level groups, and 0.76 and 0.64 for low-CRP and PCT level groups, respectively (Fig. 6e and f; Table 2). In the univariable Cox regression analysis, PTX3 was a predictor of mortality and ICU admission in the HAI cohort (HR 2.72; 95% CI 1.67–4.41; P < 0.0001 [univariable cox regression]), behaving similarly to CRP and PCT (Table 2). However, after the adjustment for potential confounding factors, excluding the co-infective event because of the correlation between co-infection and PTX3 (Table 1), only PTX3 was confirmed as a predictor of 28-d adverse event in the HAI cohort (aHR 1.86; 95% CI 1.05–3.31; P = 0.034 [multivariable cox regression]; Table 2). The proportional hazard assumption was not violated (P = 0.383).

Discussion

The clinical course of COVID-19 is highly variable and secondary infections may contribute to the complexity of

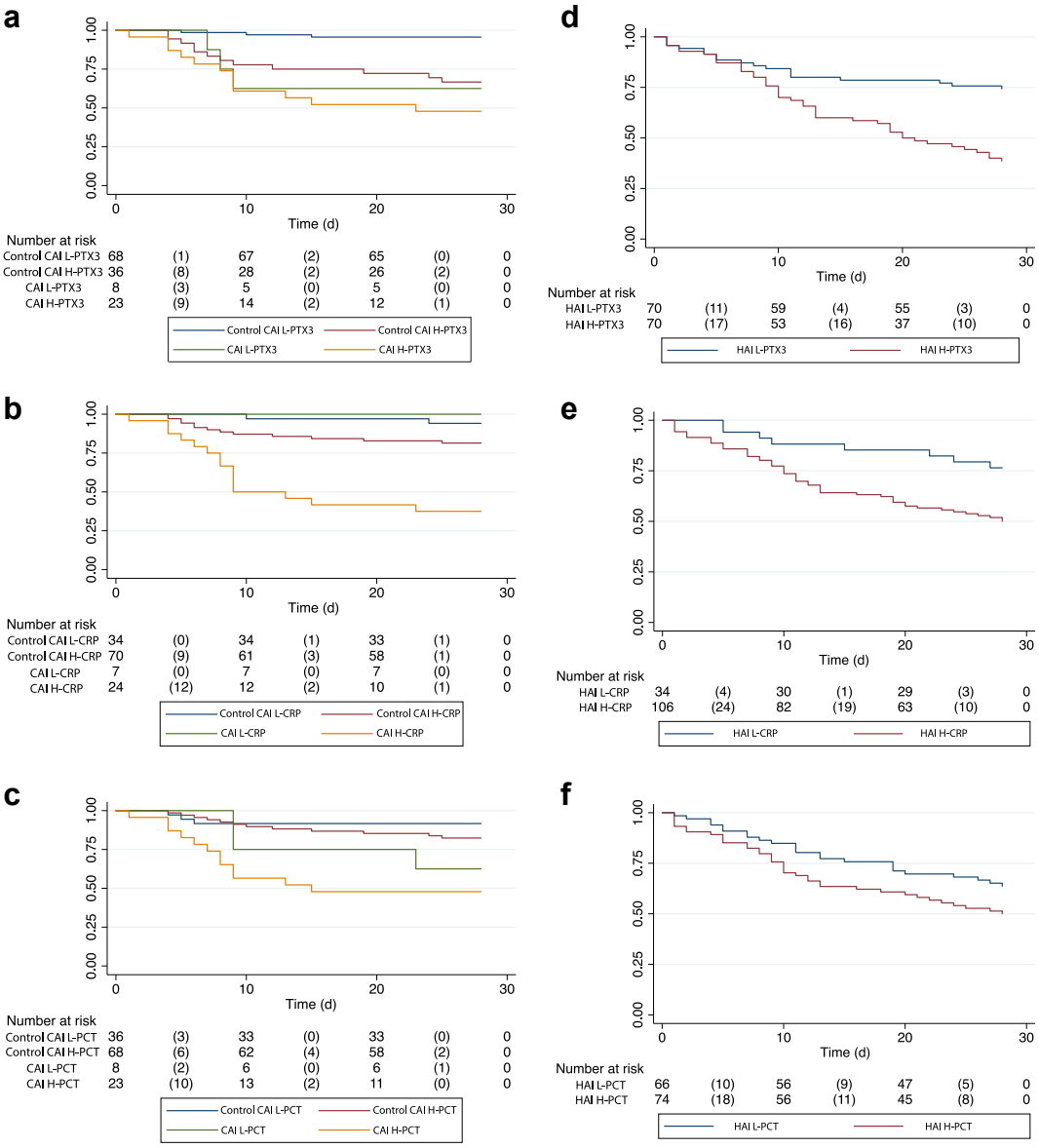

***Fig. 6:* Kaplan-Meir analysis of adverse events in patients with COVID-19 and secondary infections.** (a, b, c) Kaplan-Meir curves by levels of PTX3 (a), CRP (b) and PCT (c) (high, H or low, L) (cut-off based on the ROC analysis reported in Fig. 5d) in patients with COVID-19 and CAIs and in their control patients with COVID-19. (d, e, f) Kaplan-Meir curves by levels of PTX3 (d), CRP (e) and PCT (f) (cut-off based on the ROC analysis reported in Fig. 5e) in patients with COVID-19 and HAIs. The numbers below the plot indicate patients at risk in time in the two or four groups.

this condition. The early detection of secondary infection is clinically relevant for patient outcome.[14,15] In this study, we validated the hypothesis that PTX3 serves as early biomarker for community- and hospital-acquired secondary infections in patients with COVID-19, irrespectively of the type of organ involved and causing agent, faring more pronounced than the classical markers of infection, PCT and CRP. In addition, in both univariable and multivariable Cox regression analysis, PTX3 was a predictor of 28-days mortality or intensive care unit admission of patients with potential co-infections.

We previously demonstrated that PTX3 plasma concentration increased in patients with COVID-19 at hospital admission and served as a prognostic factor for 28-days mortality.[18] In that study, patients with secondary infections were excluded, to avoid confounding factors. In the present study, we selected patients affected by COVID-19 with CAIs and HAIs from the institutional database and analyzed PTX3 plasma

Parameter	CAI cohort							HAI cohort						
	Log-rank test		Univariable cox PH model			Multivariable cox PH Model		Log-rank test		Univariable cox PH model			Multivariable cox PH model	
	Chi-squared	P-value	HR	(95% CI)	P-value	aHR	(95% CI)	P-value	Chi-squared	P-value	HR	(95% CI)	P-value	aHR	(95% CI)	P-value
Age (per class of age)			1.05	1.02–1.08	**0.001**	2.29	1.11–4.72	**0.025**			0.99	0.97–1.00	0.124	0.72	0.45–1.16	0.173
Gender (male *vs* female)			1.37	0.65–2.87	0.411	1.09	0.37–3.23	0.880			1.19	0.66–2.13	0.564	0.88	0.46–1.71	0.714
PTX3 (per 1 ng/ml increase)[a]	29.86	**<0.00001**	5.57	2.40–12.95	**<0.0001**	4.80	1.24–18.62	**0.023**	15.91	**0.0001**	2.72	1.67–4.41	**<0.0001**	1.86	1.05–3.31	**0.034**
CRP (per 1 mg/dl increase)[a]	36.74	**<0.00001**	7.13	2.15–23.69	**0.001**	2.03	0.49–8.49	0.332	6.98	**0.0082**	2.73	1.57–4.77	**<0.0001**	2.07	0.98–4.37	0.056
PCT (per 1 ng/ml increase)[a]	21.19	**0.0001**	2.72	1.61–4.61	**<0.0001**	1.33	0.57–3.13	0.513	2.99	0.084	1.77	1.20–2.60	**0.004**	1.21	0.72–2.04	0.463
Co-infection (CAI/HAI *vs* Control)			4.16	2.03–8.53	**<0.0001**	1.14	0.39–3.33	0.815			2.16	1.27–3.67	**0.004**			
Hospital stay duration (per days increase)			0.96	0.91–1.01	0.120						0.98	0.96–0.99	**0.002**			

Clinical Deterioration and Disease Severity were considered as "ICU admission or Death": Results of Time-to-Event Analysis of the CAI cohort (n = 135) and HAI cohort (n = 145). Bold denotes statistically significant P values. PH, proportional hazards; HR, hazard ratios; aHR, adjusted hazard ratios; CI, confidence intervals. [a]Variable expressed in logarithmic scale.

Table 2: **Predictors of clinical deterioration and disease severity in patients with COVID-19 of the CAI and HAI cohort.**

concentration, showing that secondary infections induce a further increase of PTX3 plasma concentration, which may serve discriminating this complication, before the microbiological diagnosis is available. In our previous study, PTX3 was analyzed as a continuous predictor without defining a threshold value, because of the limited dimension of the cohort. Here, through a ROC curve analysis, we set 34.35 ng/ml the cut-off value discriminating CAIs in patients with COVID-19 at hospital admission and 17.31 ng/ml the cut-off value discriminating HAIs in patients with COVID-19 during hospitalization. These values have to be taken with caution, since they are influenced by several factors, including COVID-19 severity, secondary microbial agent involved, underlying therapies. Nevertheless, PTX3 was a predictor of these complications (crude HR 8.08 for CAIs and 20.76 for HAIs) and remained the only inflammatory marker persisting in multivariable analysis (including age, gender, PTX3, CRP and PCT) in HAIs and the best in CAIs (adjusted HR 11.68 for CAIs and 24.90 for HAIs).

During hospitalization, PTX3 plasma levels remained above the normal value (2 ng/ml), however they were significantly lower than those observed in the period of hospital entrance. In patients developing HAIs, PTX3 plasma concentration rapidly increased again, with a kinetics which preceded the increase of CRP or PCT by few days, and with higher AUC, sensitivity and specificity, better discriminating HAIs than CRP and PCT. The better performance of PTX3 as biomarker in this condition reasonably depends on its transcription as an immediate-early gene in several cell types and tissues, release as a ready-made protein by neutrophils in infectious conditions and its short half-life.[20,33,34] Thus, PTX3 clearly emerges as a promising predictive marker of CAIs and HAIs developing in COVID-19, faring more pronounced than CRP and PCT in supporting the suspicion of a secondary infection complicating COVID-19, thus guiding antimicrobial therapy and identifying high-risk patients.

We also validated the hypothesis that PTX3 is a predictor of adverse outcome, defined as mortality or ICU admission, in patients with COVID-19 of the CAI (HR 5.57) and HAI cohorts (HR 2.72), and was the only biomarker with statistical significance in multivariable analysis both in CAIs (aHR 4.80) and HAIs (aHR 1.86). These results are in line with previous studies showing that PTX3 is a relevant early biomarker for poor outcomes and disease severity in patients with BSIs or sepsis.[22,35–38]

PTX3 is not a specific marker of infection, since its transcription and release are induced by several inflammatory stimuli. In infectious conditions, PTX3 emerged as marker of severity and prognosis (e.g., [21–23]), however at present PTX3 has not been reported to discriminate viral and bacterial infections. As a reference, PTX3 plasma concentration in septic conditions

was reported to range from about 35 ng/ml in severely infected non-septic patients to 210 ng/ml in septic shock,[22] in comparison with COVID-19 without secondary infections, where PTX3 plasma concentration at hospital admission ranged between 13.28 and 57.04 ng/ml [IQR] (the present study). PTX3 has been associated with response to therapy in Cytomegalovirus infection,[39] immune recovery in HIV infected patients treated with cART,[40] and liver fibrosis in patients with chronic viral hepatitis C.[41] To our knowledge, this is the first study reporting the value of PTX3 as biomarker of secondary infections associated to viral diseases. However, it is reasonable that our results can be extrapolated to other viral infections. In addition to infections, PTX3 plasma concentration is increased in different types of vascular diseases[42–44] and in COVID-19 it has been shown to correlate with D-dimer and troponin-I, suggesting an association with thrombotic complications and myocardial damage.[18] A study focused on cardiovascular complications associated with COVID-19 will be necessary to assess PTX3 plasma increase in these conditions; however, previous studies showed that PTX3 plasma concentrations are in the range of 50–250 ng/ml in microbial infections,[38,45] whereas they are in the range of 5–15 ng/ml in acute cardiovascular conditions.[46]

To date, PCT and CRP are the most widely used biomarkers in the clinical practice for secondary infection identification in patients with COVID-19.[47,48] However, their plasma levels increase slowly, CRP in particular,[20,31,49] in the presence of symptoms and when positive cultures are detected,[15] suggesting the necessity of earlier biomarkers. In addition, like PTX3, they lack of specificity. CRP is a marker of nonspecific response to inflammation and tissue damage.[49] Indeed, CRP plasma concentration reflects the severity of COVID-19, including the presence of venous thrombo-embolism, acute kidney injury and critical illness, respiratory distress syndrome and myocardial injury, predicting adverse outcome.[50–53] During viral infections and concomitant bacterial co-infections, PCT levels are influenced by multiple organ failure and cytokine storms, more than bacterial co-infections.[31,54] PCT plasma concentrations have been proposed to positively associate with COVID-19 severity and poor patient outcomes.[32,55,56] This finding was not supported by other studies,[32,50] including ours, in line with its negative regulation by IFNγ produced in viral infections.[31] PCT was proposed as a predictor of bacterial co-infections in patients admitted to the ICU,[57] but it was unable to identify CAIs,[58] and it is a poor biomarker in fungal infections. Several studies failed to demonstrate that high PCT or CRP levels are good predictors of bacterial co-infection in patients with SARS-CoV-2 pneumonia, however, negative predictive values have been successfully used to rule out bacterial respiratory co-infection.[54,58–61] Indeed, for both CRP and PCT, negative predictive values to exclude co-infections have been shown to be more reliable than positive predictive values.[31,62] In our study, PTX3 showed the highest positive and negative predictive values for secondary infection in HAIs and the highest negative predictive value in CAIs, compared to CRP and PCT. Finally, the predictive potential of CRP and PCR may be impaired by immunomodulatory treatments used in COVID-19 (e.g., dexamethasone or tocilixumab), which suppress the expression of these proteins irrespectively of clinical benefit,[63] and even in the presence of secondary infections.[64,65] PTX3 expression is also modulated by corticosteroids and its plasma concentration are induced by dexamethasone,[66] potentially reducing the specificity of this biomarker in infections treated with steroids. In our cohort of patients with CAI and HAI, PTX3 concentration was not affected by corticosteroids (data not shown). Although PTX3 reflects the efficacy of IL-6 inhibition,[63] PTX3 expression is not directly modulated by IL-6,[19,20] in contrast with CRP and PCT expression.

The major limitation of this study is its retrospective design based on non-randomized comparisons, and the lack of measurements of PTX3, CRP and PCT in the same days. In particular, for PTX3 analysis, samples were not collected daily, thus it was impossible to perform a precise analysis comparing the three biomarkers before the HAI diagnosis and after the antibiotic therapy. Future prospective and confirmatory studies will be necessary to assess the profile of PTX3 as biomarker for monitoring the clinical response to therapeutic interventions in patients with COVID-19 or other severe viral infections, associated with secondary CAIs and HAIs.

Collectively, PTX3 emerges as an early low-cost/low-tech biomarker for secondary bacterial or fungal infections in patients with COVID-19, which shows advantages compared to the classical biomarkers, including an early, rapid and massive increase in the circulation preceding the clinical signs of HAIs, its ability to identify high-risk patients with potential CAIs or HAIs, guide patient management and assessment of response to antimicrobial therapy, as well as its prognostic potential for adverse outcome.

Contributors

The members of the Humanitas COVID-19 Task Force were responsible for COVID-19 patients care or generation of the COVID-19 biobank. FS, CG contributed to the experimental design, collected patient data, performed data interpretation and drafted the manuscript. EB, SNM, performed the bioinformatic and statistical analysis. FS, EN, IDGM, MarS, RL, DS, SC, HZ, EM, MS collected specimens or conducted experiments. SS, GA, AP, AS, EC, VS collected patient data. AV, BB, PM, FT contributed to the experimental design and supervision of the study. MC, MB, AM contributed drafting the manuscript. CG conceived the study and finalized the manuscript. All authors read and approved the final manuscript. FS, EB, and CG have verified the underlying data. All authors had full access to all the data in the study and accept responsibility to submit the paper for publication.

Data sharing statement

The data are stored at the Humanitas Research Hospital in Rozzano, Italy. The clinical data that support the findings of this study and informed consent forms will be made available upon formal request to

the corresponding author and consequent approval of the proposal by the Humanitas Hospital Committee. Requests should be sent to cecilia.garlanda@humanitasresearch.it.

Declaration of interests

A.M., B.B. and C.G. are inventors of a patent (EP20182181) on PTX3 and obtain royalties on related reagents. The other authors declare no competing interests.

Acknowledgements

This work was conducted in the framework of, and made possible by, the collective effort of the Humanitas COVID-19 Task Force, supported by institutional funds. The authors thank Dr. Accornero Stefano for the contribution on data extraction. This work was supported by institutional funds, a donation from the Dolce & Gabbana fashion house (to AM and CG), a grant from the Italian Ministry of Health for COVID-19 (to AM and CG), EU funding within the MUR PNRR Extended Partnership initiative on Emerging Infectious Diseases (Project no. PE00000007, INF-ACT), MUR PNRR Italian network of excellence for advanced diagnosis (Project no. PNC-E3-2022-23683266 PNC-HLS-DA) and EU MSCA (project CORVOS 860044 to HZ). DS and SC were supported by Associazione Italiana per la Ricerca sul Cancro (AIRC, project code: 28487 and 29771 for DS and 26876 for SC).

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2024.105213.

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