Received: 11 September 2022 | Revised: 15 December 2022 | Accepted: 8 January 2023

https://doi.org/10.1016/j.rpth.2023.100078

rpth research & practice in thrombosis & haemostasis

ORIGINAL ARTICLE

Inter-α-inhibitor heavy chain H4 and sepsis-related coagulation disturbances: Another link between innate immunity and coagulation

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Funding information
Faculty of Health, Aarhus University and the Health Research Fund of the Central Denmark Region.

Handling Editor: Dr Henri Spronk

Abstract

Background: The protease inhibitor inter-α-inhibitor heavy chain H4 (ITIH4) has been described as an acute-phase reactant and could potentially aid in sepsis monitoring and prognostication.

Objectives: To investigate ITIH4 plasma levels in sepsis patients compared with healthy controls and to examine the association between ITIH4 and acute-phase response markers, blood coagulation, and organ dysfunction in sepsis.

Methods: We performed a post hoc study to a prospective cohort study. Patients with septic shock (n = 39) were enrolled upon intensive care unit admission. ITIH4 was analyzed using an in-house immunoassay. Standard coagulation parameters, thrombin generation, fibrin formation and lysis, C-reactive protein, organ dysfunction markers, Sequential Organ Failure Assessment score, and disseminated intravascular coagulation (DIC) score were registered. ITIH4 levels were also investigated in a murine *Escherichia coli* sepsis model.

Results: ITIH4 did not display acute-phase behavior as mean ITIH4 levels were not increased in patients with septic shock or in *E. coli*-infected mice. However, ITIH4 exhibited large interindividual variation in patients with septic shock compared with healthy controls. Low ITIH4 was associated with sepsis-related coagulopathy, including a high DIC score (mean ITIH4: DIC, 203 μg/mL vs non-DIC, 267 μg/mL, $P = .01$), low antithrombin ($r = 0.70$, $P < .0001$) and decreased thrombin generation (mean ITIH4: first peak thrombin tertile, 210 μg/mL vs third peak thrombin tertile, 303 μg/mL, $P = .01$). ITIH4 showed moderate correlation with arterial blood lactate ($\rho = -0.50$, $P < .001$) but only weak correlations with C-reactive protein, alanine transaminase, bilirubin, and Sequential Organ Failure Assessment score (all, $\rho < 0.26$, $P > .05$).

Conclusion: ITIH4 is associated with sepsis-related coagulopathy but is not an acute-phase reactant during septic shock.

KEYWORDS
Acute-phase reaction, disseminated intravascular coagulation, ITIH4 protein, human, multiple organ failure, prognosis, sepsis

Essentials

- ITIH4 is a recently discovered protein, and not much is known about its function.
- We investigated ITIH4 in patients with sepsis (blood poisoning) and healthy blood donors.
- ITIH4 was associated with disturbed blood clotting in sepsis and thus may be a marker of more severe disease.
- A future larger study should be planned to investigate the potential of ITIH4 as a sepsis biomarker.

1 | INTRODUCTION

Sepsis is defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection [1]. Sepsis may develop rapidly, can deteriorate quickly into shock and multiorgan failure, and is associated with high mortality. It accounts for an estimated annual 10 million deaths worldwide [2], and in-hospital mortality rates for sepsis patients in the intensive care unit (ICU) have been reported to be approximately 25% in the Western world, depending on the severity of shock and the number of organs failing [3]. Furthermore, sepsis patients are at high risk of developing severe coagulation disturbances in the form of disseminated intravascular coagulation (DIC), which is associated with a nearly doubled mortality rate [4]. New sepsis biomarkers that may aid in prognostication and monitoring of treatment response are, therefore, of great interest.

Inter-α-inhibitor heavy chain H4 (ITIH4) is a potential candidate for a sepsis biomarker. ITIH4 is synthesized in the liver and circulates in plasma at concentrations of approximately 200 μg/mL [5]; thus, it is an abundant plasma protein. We have recently shown that ITIH4 acts as an inhibitor of several proteases, including mannan-binding lectin serine protease-1 (MASP-1) and MASP-2 of the lectin pathway of the complement system and plasma kallikrein [6]. Mechanistically, ITIH4 acts as bait for proteases, with cleavage of ITIH4 leading to the formation of noncovalent, inhibitory complexes between ITIH4 and the executing protease. By targeting both the innate immune system and the contact system, which are both key elements for the development of sepsis, ITIH4 represents an intriguing biomarker candidate.

Furthermore, we recently described associations between MASP-1 and sepsis-induced coagulopathy [7]; however, it is unknown if and how ITIH4 might influence this association. ITIH4 has been identified as a positive acute-phase protein in rats [8], dogs [9], and cattle [10] and has been suggested to be the primary acute-phase protein in pigs [11]. Although these studies suggest that ITIH4 is an evolutionarily conserved acute-phase protein, ITIH4 levels remain to be extensively studied during the human acute-phase response. One study found that ITIH4 levels only increased modestly in a mixed cohort of 9 patients [8], and Ma et al. [12,13] reported increased ITIH4 in patients with bacteremia compared with febrile patients without bacteremia. Therefore, the present study aimed to investigate ITIH4 plasma concentrations in critically ill patients with septic shock compared with healthy individuals and to delineate associations between ITIH4 and markers of the acute-phase response, organ dysfunction, coagulopathy, and mortality, thereby exploring ITIH4 as a marker for monitoring and prognosis in sepsis. Furthermore, to compare with an animal model, we tested the concentrations of ITIH4 in a mouse model of sepsis.

2 | METHODS

2.1 | Design and study population

This was a post hoc study to a prospective cohort study [7]. Patients with septic shock ≥18 years old were included from the ICU, Aarhus University Hospital, Aarhus, Denmark, from October 2016 to February 2018, as previously described [7]. The diagnosis of sepsis was based on a clinical suspicion of infection assessed by the admitting intensive care physician plus an increase in Sequential Organ Failure Assessment (SOFA) score ≥2 [1]. Shock was defined as need for vasopressor (noradrenaline) to maintain mean arterial pressure ≥65 mm Hg despite adequate volume resuscitation. Exclusion criteria were (1) pregnancy, (2) active cancer or chemotherapy within 3 months, (3) major trauma or surgery within 24 hours, (4) known congenital bleeding disorder or thrombophilia, and (5) plasma transfusion within the last 3 days. Patients were enrolled consecutively on weekdays if they fulfilled the diagnostic and inclusion criteria and did not check any exclusion criteria. All patients received thromboprophylaxis from low molecular weight heparin (dalteparin 5000 IU once daily) according to local institutional guidelines.

Blood samples were obtained the morning after admission (day 1), day 2, and day 3. Blood was drawn from an already placed arterial cannula into evacuated anticoagulation tubes (sodium citrate 3.2%,

Greiner Bio-One, and EDTA, BD) and centrifuged at 3000 × g for 25 minutes at room temperature to obtain platelet-poor plasma. Plasma was aliquoted and stored at -80 °C until analysis. The project was approved by the local institutional board and the Danish Data Protection Agency. According to the Danish law on ethics, the requirement for written informed consent was waived after a formal review by the regional Health Ethics Committee (file no. 1-16-02-505-16). The study was performed in accordance with the Helsinki Declaration and the Danish Health Care Act.

EDTA plasma from blood donors (n = 138), who were free from known heart, lung, liver, kidney, hematological and metabolic diseases, did not take any medication, and were randomly enrolled from the blood bank at the Department of Clinical Immunology, Aarhus University Hospital, Denmark as previously described by our group [14], were used as healthy controls for ITIH4.

2.2 | Laboratory analyses

2.2.1 | Immunoassays for human and murine ITIH4

ITIH4 concentrations in EDTA plasma from patients with septic shock and healthy individuals were analyzed at the Department of Biomedicine, Aarhus University, Aarhus, Denmark, as previously described [5]. The assay is a sandwich-type, time-resolved immunoflourometric assay. Briefly, wells of microtiter plates (FluoroNunc, Merck) were coated with a polyclonal rabbit anti-ITIH4 antibody [6]. Plasma was thawed and diluted 1:10,000 in a buffer consisting of 25 mM Tris, 1 M NaCl, 1 mg/mL human serum albumin, 100 μg/mL heat-aggregated human IgG, and 100 μg/mL rabbit IgG (7406404; Lampire), pH 7.4. The diluted samples were added to the coated microtiter wells. Each microtiter plate contained internal quality controls in 3 levels (human serum pool, in-house preparation) and diluted 1:10,000. Standards, samples, and controls were diluted and added automatically to plates using a Janus Varispan automated workstation (PerkinElmer). All samples were added in duplicate. In-house biotinylated rabbit anti-ITIH4 antibodies, europium-labeled streptavidin (PerkinElmer), and enhancement solution (Ampliqon) were added in successive steps with triple washing steps in between each step. The europium in the wells was detected with a fluorometer (Victor X5, PerkinElmer) performing time-resolved fluorometry. Intra-assay and interassay coefficients of variation were below <15%. We have previously validated the ITIH4 assay, including reference interval, serum-plasma correlations, freeze-thaw cycles, and diurnal variation [5]. The person performing the analysis (J.B.L.) was blinded to the patients' clinical information and other laboratory results at the time of analysis.

In addition, we tested the concentration of ITIH4 in samples from mice in a murine *Escherichia coli* sepsis model. The test was in many ways similar to the assay for human ITIH4 described above, although in this case, using antibodies made against mouse ITIH4. The results and details of this assay are described in Supplementary Figure 2. The person performing the analysis was blinded to the infectious status of the mice.

2.2.2 | Western blot of ITIH4

It has been suggested that ITIH4 is cleaved by enzymes during inflammatory conditions; thus, we examined whether ITIH4 was present as a nondegraded protein in the EDTA plasma samples. We thus performed western blot analysis of samples from healthy donors and from patients with sepsis. The procedure is described in detail in Supplementary Figure 1. Following separation of samples by SDS-PAGE and blotting them onto nitrocellulose membranes, ITIH4 was detected with rabbit anti-human ITIH4 antibody. We used a mixture of 2 antibodies: one targeted against the N-terminal region and another against the C-terminal region. This ensured that both N- and C-terminal fragments of ITIH4 were detected, as shown previously by Pihl et al. [6]. Similarly, we tested the degradation status of mouse ITIH4 from mice with and without sepsis by western blotting using rabbit anti-mouse ITIH4 antibody, as described in Supplementary Figure 3.

2.2.3 | Markers of the acute-phase response, innate immunity, and organ dysfunction

Total blood leukocyte count, arterial blood lactate and plasma C-reactive protein (CRP), alanine transaminase (ALT), and total bilirubin were analyzed at the Department of Clinical Biochemistry, Aarhus University Hospital according to ISO 15189:2012 accredited routine protocols as part of the original sepsis cohort study [7]. MASP-1 was analyzed at the Department of Biomedicine, Aarhus University, Aarhus, Denmark, using an in-house time-resolved immunoflourometric assay protocol as previously described [7].

2.2.4 | Coagulation markers

Blood platelet count, activated partial thromboplastin time (aPTT), international normalized ratio, and plasma fibrinogen (functional, Clauss), fibrin d-dimer, and antithrombin (functional) were analyzed at the Department of Clinical Biochemistry, Aarhus University Hospital, Denmark, according to ISO 15198-accredited routine protocols, as previously described [7]. The DIC score was calculated according to the International Society on Thrombosis and Haemostasis [15]. A DIC score of ≥5 is compatible with the presence of overt DIC. Ex vivo thrombin generation assay and turbidimetric fibrin formation and lysis (clot-lysis) assay were performed in citrated platelet-poor plasma as previously described [7,16].

2.3 | Clinical data

Information on age, sex, comorbidities, infection (suspected focus, positive cultures), disease severity (Simplified Acute Physiology Score [SAPS]-III score), organ dysfunction (SOFA score), blood product

transfusions, crystalloid fluid resuscitation, fluid balance, and 30-day mortality were collected prospectively from the medical records and ICU charts.

2.4 | Sample size and statistics

The present study was a post hoc study with a fixed sample size. Therefore, a sample size calculation was not performed.

Data were visually assessed with quantile-quantile plots for normal distribution. Descriptive statistics were reported as mean with SD if data followed a normal distribution or median with IQR if data did not follow a normal distribution. Comparison between groups was performed with Student's *t*-test, Mann–Whitney U-test, or one-way analysis of variance (ANOVA) for more than 2 groups. Correlation was assessed with linear regression analysis, Pearson's *r*, or Spearman's ρ. Statistical analyses were performed in Stata 14 (StataCorp). Graphs were created in GraphPad Prism version 8 (GraphPad).

2.5 | Investigations in mice

A murine model of *E. coli*-induced sepsis was used as described by Greve et al. [17]. Balb/cJRj mice (8–10 weeks, 24.5 ± 0.1 g) were anesthetized with a subcutaneous injection of ketamine (100 mg kg^{-1}) and xylazine (10 mg kg^{-1}). The mice were exposed to 33 x 10^7 uropathogenic *E. coli* (O6:K13:H1) or vehicle controls via a tail vein. The bacterial load was adjusted to achieve an ID_{50} after approximately 3 hours. Citrated plasma was isolated from the inferior vena cava as the animals were terminated immediately after injection of the bacteria/vehicle (time 0) or after 0.5, 1, 1.5, 2, and 2.5 hours following intravenous exposure. The number of mice was as follows: Baseline (0 h): n = 15; at 0.5 h: +*E. coli* n = 7, -*E. coli* n = 7; at 1 h: +*E. coli* n = 11, -*E. coli* n = 9; at 1.5 h: +*E. coli* n = 4, -*E. coli* n = 4; at 2.0 h: +*E. coli* n = 12, -*E. coli* n = 9; at 2.5 h: +*E. coli* n = 8, -*E. coli*, n = 12. Plasma concentrations of ITIH4 were measured using a sandwich-type immunoassay as described in Supplementary Figure 2. The degradation status of ITIH4 in the samples was tested by western blot analysis, as illustrated in Supplementary Figure 3. Balb/cJRj mice were purchased from Janvier Labs (Saint-Berthevin). All animals were handled according to the Danish animal welfare regulation and had free access to standard rodent diet and water. All experiments were approved by the Danish ethics committee for animal research (2020-15-0201-00422).

3 | RESULTS

3.1 | ITIH4 in patients with septic shock and healthy controls

EDTA plasma for ITIH4 analysis was available from 39 of 42 patients included in the original cohort. Demographic and clinical information for the 39 patients with septic shock and plasma concentrations of inflammatory and organ function markers are displayed in the Table.

TABLE Demographic, clinical, and laboratory characteristics of patients with septic shock.

Age, y; mean (SD)	70.2 (12.0)
Sex, male/female; n (%)	27 (69)/12 (31)
SAPS III score at admission; median (IQR)	65 (58–72)
SOFA score on day 1; median (IQR)	10 (7–13)
Comorbidities; n (%)	
Cardiovascular	21 (54)
Pulmonary	8 (21)
Hepatic	4 (10)
Renal	6 (15)
Diabetes mellitus	8 (21)
30-day mortality, nonsurvivors; n (%)	10 (26%)
Acute-phase reactants and organ dysfunction markers	
C-reactive protein, mg/L; median (IQR)	213 (107–313)
Total blood leukocyte count, × 10^9/L; median (IQR)	13.4 (9.4–20.9)
Alanine transaminase, U/L; median (IQR)	36 (23–60)
Bilirubin, umol/L;	15 (9–39)
Lactate (arterial), mmol/L; median (IQR)	1.7 (1.2–3.0)

SAPS, Simplified Acute Physiology Score; SOFA, Sequential Organ Failure Assessment.

All patients were of ethnic North European origin. Eight of the 39 patients had blood drawn on day 1 only (discharge from ICU before day 2, n = 6; death, n = 1; consent for further blood sampling withdrawn, n = 1), and an additional 6 patients had blood drawn on days 1 and 2 only (discharge from ICU before day 3, n = 3; received plasma transfusion before day 3, n = 2; consent for further blood sampling withdrawn, n = 1). All patients were followed up on day 30 through electronic medical records. The healthy controls comprised 82 male and 56 female blood donors with an average age of 35.5 years (range 18–66 years).

If ITIH4 is cleaved by an enzyme and subsequently forms a non-covalent inhibitory complex, it will appear as protein bands at 80 kDa and 40 kDa if tested by western blotting [6]. To investigate the integrity of ITIH4 in patients with sepsis, we performed western blotting analysis on samples from 7 patients with septic shock and 3 healthy controls, which showed that ITIH4 was present as an intact protein at 120 kDa in both patients and healthy controls (Supplementary Fig. 1).

The mean ITIH4 plasma concentration did not differ significantly between patients with septic shock on day 1 and healthy individuals. Mean (SD) was 245 (66) μg/mL in patients compared to 226 (29) μg/mL in healthy individuals, with a difference between means of 19 μg/mL (95% CI: -3 to 41 μg/mL, *P* = .09, Figure 1A). The ITIH4 concentration in individual patients was relatively consistent from day 1 to 3 (Figure 1B). However, we observed a large interindividual variation among the patients, which did not seem to depend on total fluid

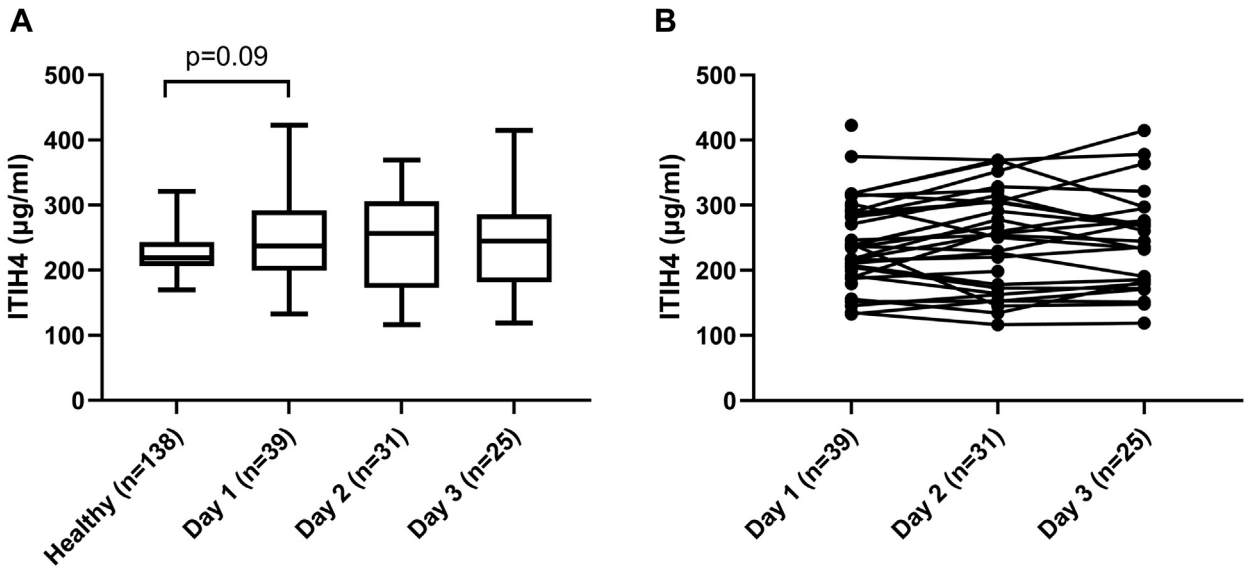

FIGURE 1 ITIH4 in patients with septic shock and healthy controls. (A) ITIH4 plasma concentrations in healthy individuals and patients with septic shock on days 1, 2, and 3 after intensive care unit admission. Median, IQR, and minimum-maximum are illustrated. *P* values were calculated by Student's *t*-test. (B) ITIH4 plasma concentrations in patients with septic shock plotted on an individual level. ITIH4, inter-α-trypsin inhibitor heavy chain H4.

balance within the last 24 hours (linear regression, β -3.41; 95% CI: -13.26–6.42, *P* = .48).

ITIH4 did not correlate with CRP (Spearman's ρ = 0.19, *P* = .23, Figure 2A). We found a moderate positive correlation with MASP-1 (Spearman's ρ = 0.48, *P* < .01, Figure 2B) and fibrinogen (Pearson's r = 0.56, *P* < .01). The plasma ITIH4 levels did not differ between patients grouped according to the disease-causing pathogens (Figure 2C). It must be noted that the subdivision into groups according to the various disease-causing pathogens critically reduced the number of observations.

3.2 | ITIH4 in mice with sepsis

Since we did not observe increased ITIH4 in patients with septic shock and ITIH4 has been suggested to be an acute-phase protein in some animals, we extended our study of ITIH4 in septic conditions to a murine *E. coli* sepsis model. In this model, the animals were terminated at various time points up to 2.5 hours after induction of sepsis. Tumor necrosis factor-α, interleukin (IL) 1β, and IL-6 at 2.5 h were significantly higher in *E. coli*-exposed mice than in control mice [17], confirming an acute-phase response. During the first 2 hours, ITIH4 concentrations were similar in mice exposed to uropathogenic *E. coli* compared to mice given saline solution (Supplementary Fig. 2D). At 2 and 2.5 hours, ITIH4 was statistically significantly lower in the group given *E. coli*, despite a large variation in ITIH4 concentrations at 2.5 hours (Supplementary Fig. 2D). Similar to our patient population, we confirmed that the murine plasma samples contained intact 120 kDa mouse ITIH4 by western blotting (Supplementary Fig. 3).

3.3 | ITIH4 and sepsis-related coagulopathy

Associations between ITIH4 and coagulation parameters were investigated in patients with septic shock after excluding patients who had received anticoagulant treatment (vitamin K antagonists or direct oral anticoagulants) within 3 days prior to ICU admission (n = 6), leaving a total of 33 patients. ITIH4 was lower in patients with overt DIC (n = 11, mean [SD] of 203 [48] µg/mL) than in patients with nonovert DIC (n = 22, mean [SD] of 267 [68] µg/mL) on day 1, with a difference between means of 63 µg/mL (95% CI: 16-110 µg/mL) (*P* = .01, Figure 3A). Moreover, the plasma levels of ITIH4 decreased with increasing DIC score (Spearman's ρ = -0.50, *P* < .01, Figure 3B), increasing aPTT (Spearman's ρ = -0.43, *P* < .01, Figure 3C), and decreasing antithrombin (Pearson's r = 0.70, *P* < .0001, Figure 3D).

We also investigated whether ITIH4 plasma concentrations correlated with ex vivo thrombin generation on day 1 (Figure 4A–B). We found that low ITIH4 was associated with reduced peak thrombin concentration (ANOVA, *P* = .01; post hoc test of 1st vs 3rd tertile: difference of means 93 µg/mL [95% CI: 27–159 µg/mL], *P* = .001) and with reduced endogenous thrombin potential (ANOVA, *P* = .04; post hoc test of 1st vs 3rd tertile: difference of means 82 µg/mL [95% CI: 11–153 µg/mL], *P* = .03). Accordingly, the data also showed that low ITIH4 plasma concentrations were associated with abnormal fibrin clot formation and lysis previously assessed in this population with our in-house clot formation and lysis assay [18]. Figure 4C shows patients divided into groups with a) no fibrin formation capacity, indicated by flat fibrin curves (n = 8), b) normal fibrin formation and lysis capacity (n = 13), and c)

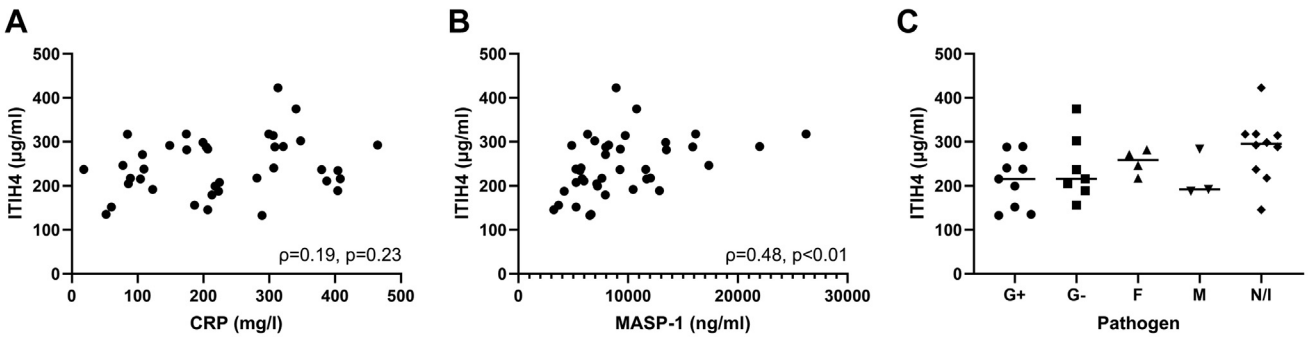

FIGURE 2 Association between ITIH4 and CRP, MASP-1, and pathogen. Day 1 after intensive care admission. (A) Correlation between CRP and ITIH4 plasma concentrations. (B) Correlation between MASP-1 and ITIH4 plasma concentrations. (C) ITIH4 plasma concentration in sepsis patients grouped according to the identified pathogen. Median, IQR, and minimum-maximum are illustrated. The ρ and *P* values were calculated by the Spearman correlation test. CRP, C-reactive protein; F, fungal; G+, gram-positive; G-, gram-negative; ITIH4, inter-α-trypsin inhibitor heavy chain H4; M, mixed; MASP-1, mannose-binding lectin-associated serine protease 1; NI, not identified.

hypofibrinolysis indicated by lysis-resistant clots (n = 11) (one patient was excluded because of high between-duplicate coefficient of variation despite repeated testing). Patients with hypocoagulability or lysis resistance had lower ITIH4 concentrations than patients with normal fibrin formation and lysis, though not statistically significant (ANOVA, *P* = .05).

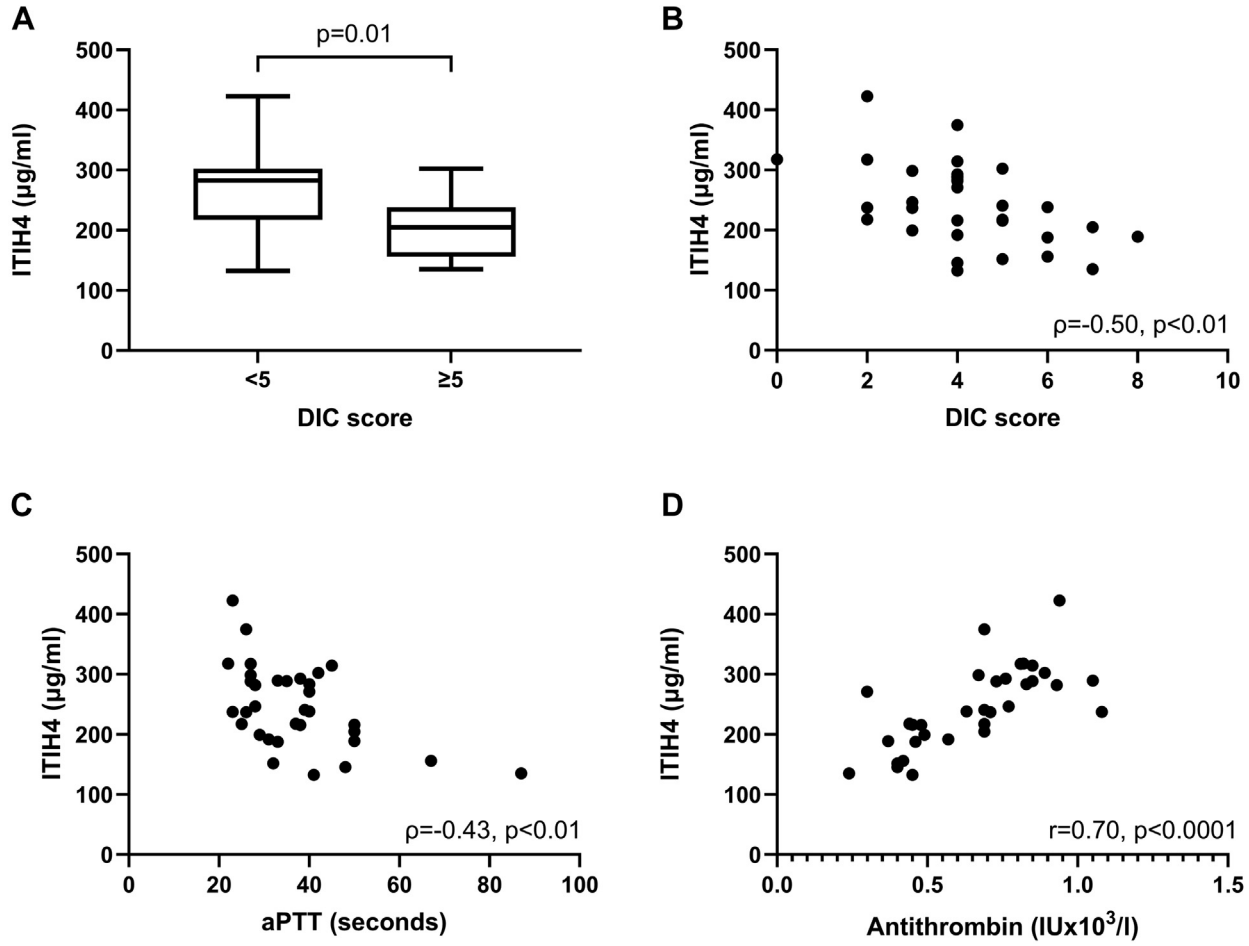

FIGURE 3 Association between ITIH4 and DIC scores, aPTT, and antithrombin. Day 1 after intensive care admission. (A) ITIH4 plasma concentrations in patients with International Society on Thrombosis and Haemostasis DIC scores of <5 and ≥5. *P* values were calculated by the Student's *t*-test. (B–D) Correlation between ITIH4 and DIC scores, aPTT, and plasma antithrombin. Correlation coefficients (r/ρ) and *P* values were calculated by Pearson and/or Spearman correlation test. Median, IQR, and minimum-maximum are shown. aPTT, activated partial thromboplastin time; DIC; disseminated intravascular coagulation; ITIH4, inter-α-trypsin inhibitor heavy chain H4.

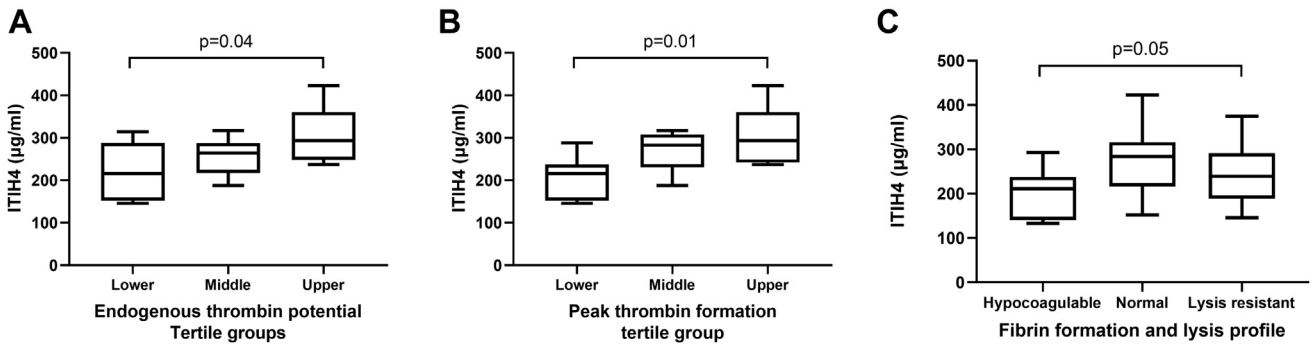

FIGURE 4 Association between ITIH4 and ex vivo thrombin generation, fibrin clot formation, and lysis. Day 1 after intensive care admission. (A) ITIH4 concentration in the lower, middle, and upper tertile of endogenous thrombin potential. (B) ITIH4 concentration in lower, middle, and upper tertile of peak thrombin formation. (C) ITIH4 concentrations in patients grouped by their fibrin clot formation and lysis profile. *P* values were calculated by ANOVA. Median, IQR, and minimum-maximum are illustrated. ITIH4, inter-α-trypsin inhibitor heavy chain H4.

3.4 | IHIT4 and markers of organ dysfunction

We observed a moderately strong negative correlation between ITIH4 and arterial lactate (Spearman's ρ = -0.50, *P* = .001, Figure 5A), indicating that lower ITIH4 concentrations were associated with decreased tissue perfusion. However, we observed only weak correlations between ITIH4 and SOFA score (Spearman's ρ = -0.26, *P* = .11) and between ITIH4 and markers of liver function (ALT, Spearman's ρ =

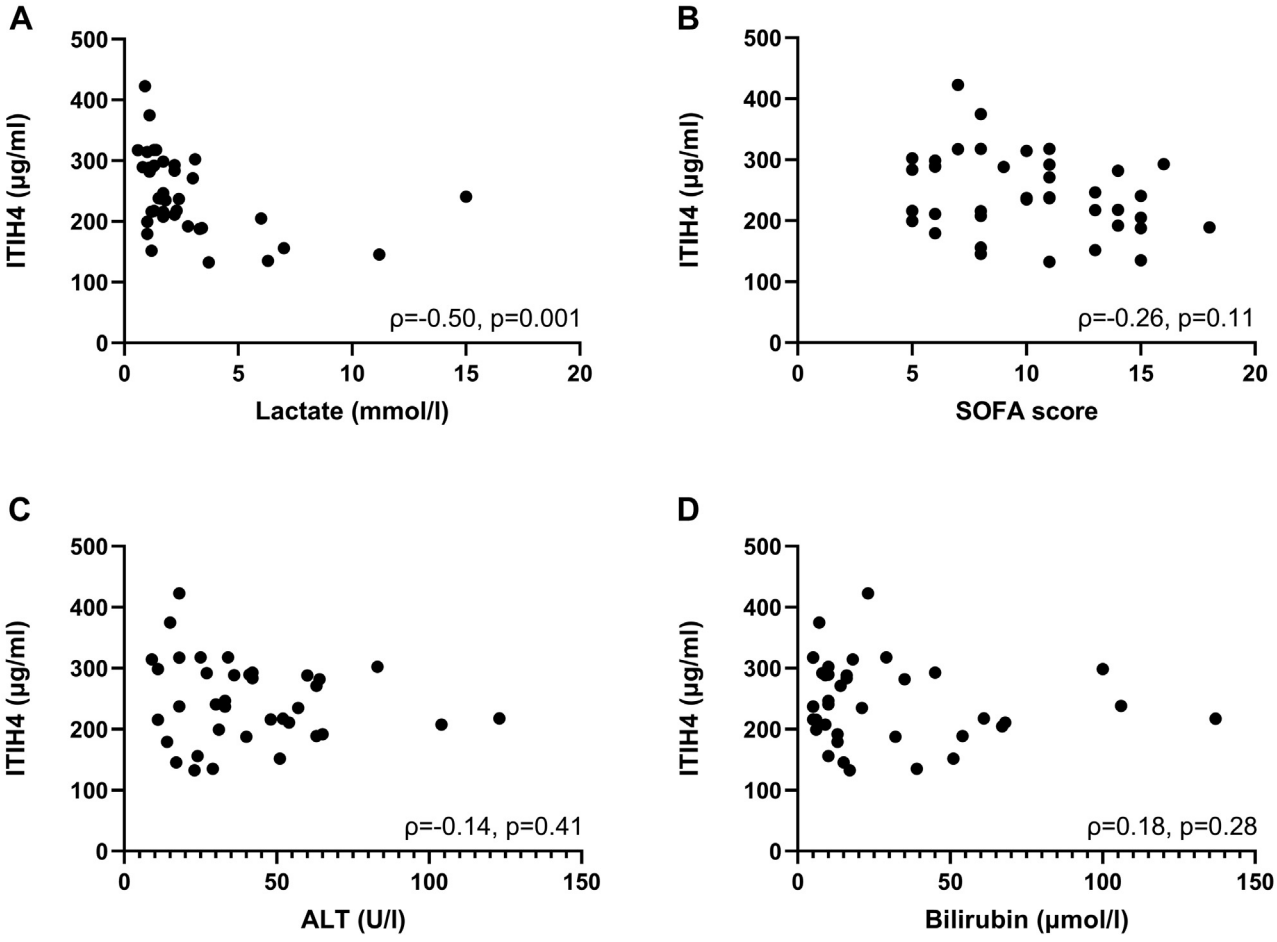

FIGURE 5 Correlation between ITIH4 and markers of organ dysfunction. Day 1 after intensive care admission. Correlation between ITIH4 plasma concentration and (A) arterial lactate, (B) SOFA score, (C) plasma ALT, and (D) plasma bilirubin. Median, IQR, and minimum-maximum are illustrated. ρ and *P* values were calculated by the Spearman correlation test. ALT, alanine transaminase; ITIH4, inter-α-trypsin inhibitor heavy chain H4; SOFA, Sequential Organ Failure Assessment.

-0.14, P = .41; bilirubin, Spearman's ρ = 0.18, P = .28) (Figure 5B–D). ITIH4 did not differ between survivors and nonsurvivors (mean (SD) 247 (13) μg/mL vs 238 (19) μg/mL, P = .70). When comparing patients who were discharged from the ICU before day 2 or 3 (n = 9) with patients who had available blood samples for all 3 days (n = 25), they were of similar age (mean 69 vs 71 years) but had slightly lower SOFA scores (median, 8 [range, 6–11] vs 10 [range, 8–13], P = .50, Mann–Whitney) and a lower female/male ratio (1/8 vs 9/16); however, mean ITIH4 did not differ between them (mean (SD), 267 (75) μg/mL vs 234 (63) μg/mL, difference in means of 32 μg/mL [95% CI, -21 to 84 μg/mL], P = .22).

4 | DISCUSSION

Surprisingly little is known about the interaction of ITHI4 with the various enzyme cascades in plasma or whether the body responds with up or downregulation of ITIH4 levels due to, eg, injuries or sepsis. ITIH4 is an abundant plasma protein that has been suggested to act as a positive acute-phase reactant [8–11]. Still, ITIH4 has garnered little interest as a biomarker for sepsis, presumably due to the previous lack of knowledge of its biological function. We recently showed that ITIH4 is a protease inhibitor that functionally lies at the intersection between innate immunity and coagulation [6], making it a prime candidate for being involved in sepsis. In the present study, we investigated ITIH4 levels in a cohort of patients with septic shock. We extended the study to also include an examination of ITIH4 levels in a mouse sepsis model.

We found that low ITIH4 plasma concentration was associated with signs of sepsis-related coagulopathy indicated by higher DIC scores, low plasma antithrombin, decreased ex vivo thrombin generation, and disturbed fibrin clot formation and lysis. Interestingly, other authors have reported associations between ITIH4 and cardiovascular disease in elderly patients [19]. However, the mechanism behind the association between ITIH4 and markers of coagulation is not clear. ITIH4 was demonstrated to be cleaved by thrombin, as well as kallikrein and plasmin [6]. Thus, low ITIH4 plasma concentrations may indicate ongoing in vivo thrombin formation and ITIH4 consumption or incorporation into the fibrin clot.

Additionally, MASP-1 has been found to activate thrombin and induce fibrin formation in vitro [20,21], and ITIH4 may modulate this process through MASP-1 inhibition [6]. It is also possible that ITIH4 may inhibit thrombin or other serine proteases of the coagulation system directly, though this has not been demonstrated. We previously found that low MASP-1 concentrations were associated with disturbed coagulation in our septic shock cohort [7], an association that was recently confirmed by Wang et al. [22]. Since ITIH4 correlated with MASP-1 plasma concentrations in the present study, the association between ITIH4 and coagulation that we describe here might also be influenced by the correlation between MASP-1 and ITIH4. Nonetheless, the findings support our previous results and underline the potential role of the innate immune system in sepsis-related coagulation disturbances.

We found that the mean ITIH4 plasma concentration was not higher in patients with septic shock than in healthy individuals. However, we observed a considerable interindividual variation in ITIH4 concentrations in patients with septic shock compared with healthy individuals, which pointed to a possible role of ITIH4 as a prognosticator within the septic shock group. We proceeded to investigate correlations between ITIH4 and well-established acute-phase reactants and found that ITIH4 correlated poorly with CRP. In contrast to our findings, Ma et al. [12,13] reported that ITIH4 serum concentrations were higher in patients with bacteremia than in febrile patients without bacteremia. However, their patient population was younger than ours and may have differed in comorbidities and illness severity. Furthermore, the authors reported ITIH4 serum concentrations of only a few ng/mL in their patient population, which is >10,000 fold lower than previously reported in healthy individuals [5] and what we here confirm in our septic shock population.

The analysis of samples from mice undergoing sepsis indicated that ITIH4 might actually be a weak negative acute-phase reactant in mice, but only after prolonged sepsis. Thus, our results indicate that ITIH4 does not display any clear acute-phase behavior in humans and mice. Interestingly, the drop in ITIH4 was also observed during the period where an increase in intravascular coagulation in the animals occurred, as indicated by high thrombin-antithrombin complexes [23]. In contrast, Ma et al. [13] found increased ITIH4 in an *E. coli* sepsis mouse model. The mouse ITIH4 levels reported by Ma et al. [13] were in the range of 100 ng/mL, ie, 1000 fold lower than the concentrations reported here. Further, we have not found information on the commercial ELISA kit used by the authors [13]. Thus, the role of ITIH4 in the murine sepsis response is not quite clear, but we did not find an increase in ITIH4 levels in the *E. coli* mouse model.

Finally, we investigated associations between ITIH4 and markers of organ dysfunction. Low ITIH4 was associated with high arterial blood lactate, which indicates decreased tissue perfusion and organ ischemia, but we found only weak correlations between ITIH4 and ALT or SOFA score and no association with 30-day mortality. Based on this, ITIH4 may not be useful as a prognostic marker in septic shock. However, as our sample size was small, this should be explored in a larger cohort. Furthermore, ITIH4 is currently analyzed in the research laboratory only and is not available on any automated platform, nor has any standardization work been performed. The diagnostic and prognostic value of ITIH4 in various conditions should be explored on a larger scale before it can be decided whether it is reasonable to implement ITIH4 in the routine laboratory.

The strengths of the present study are the prospective and longitudinal design and a well-characterized septic shock cohort with extensive clinical and laboratory data available. We performed detailed, dynamic analyses of the coagulation and fibrinolytic system. However, some limitations must be mentioned. This was a post hoc study to a previous study; thus, the sample size was fixed and relatively small, which limits our ability to perform additional stratification. The patients were heterogeneous regarding age, the underlying cause of sepsis, and comorbidities, and the underlying pathogen could not be identified in all cases. However, this reflects well a real-world septic shock population in the ICU and strengthens the external validity of our results. We did not have access to arterial lactate levels at

the time of ICU admission but only at study enrolment. Thus, we could not consider arterial lactate at admission in the diagnosis of shock. Plasma protein concentrations of ITIH4 and other markers may be influenced by a variety of factors in critically ill patients, including fluid resuscitation and increased vascular permeability; however, we found no correlation between net fluid balance and ITIH4 concentrations.

Both the coagulation system activation and the innate immune response are immediate and local reactions to damaged host cells and invading pathogens. Our findings add to the reported cross-talk between coagulation and innate immunity, such as the contact pathway as a 2-way link and promoter of thromboinflammation [24], interactions between serine proteases of the lectin pathway and the coagulation cascade in thrombotic disorders [25], and the involvement of proteins of the complement system in hematopoietic stem cell transplantation-associated thrombotic microangiopathy [26].

To conclude, ITIH4 was associated with sepsis-related coagulopathy, and the mechanism(s) behind this interesting association should be explored in future experimental studies and larger patient cohorts. ITIH4 plasma concentrations displayed wide variations in patients with septic shock, but ITIH4 did not behave as an acute-phase protein in this patient group nor a murine model of sepsis.

ACKNOWLEDGMENTS

The authors thank Alexander Grevsen, Mai Stenulm Veirup, Vivi Mogensen, and Annette Hansen for their help with patient inclusion and laboratory assistance.

FUNDING

The study received financial support from the Faculty of Health, Aarhus University, and the Health Research Fund of the Central Denmark Region.

AUTHOR CONTRIBUTIONS

J.B.L., C.L.H., H.P., A.-M.H., and S.T. contributed to the design and planning of the study. J.B.L., M.A.A., and K.M.L. were responsible for patient inclusion and sample handling. R.P. and S.T. developed the ITIH4 assay. J.B.L. performed ITIH4 analysis in sepsis patients, and R.P. performed ITIH4 analysis in mice. N.J., M.C., and H.P. were responsible for animal handling and sample processing. J.B.L. performed statistical analyses and manuscript drafting. All authors contributed to the intellectual content of the manuscript.

RELATIONSHIP DISCLOSURE

R.P., M.A.A., K.M.L., C.L.H., N.J., M.G.C., H.P., and S.T. have no conflicts of interest to disclose. J.B.L. and A.-M.H. have no interest to disclose regarding the present paper but have the following general conflicts of interest: J.B.L. has received travel support from Bayer and speaker fees from Bristol-Myers Squibb. A.-M.H. has received speaker fees from CSL Behring, Bayer, Boehringer-Ingelheim, Bristol-Myers Squibb, and Astellas and unrestricted research support from CSL Behring and Octapharma.

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SUPPLEMENTARY MATERIAL

The online version contains supplementary material available at https://doi.org/10.1016/j.rpth.2023.100078