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Amyloid-Fibrinogen Aggregates ("Microclots") Predict Risks of Disseminated Intravascular Coagulation and Mortality

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Abstract:
Microclots have been associated with various conditions, including post-acute sequelae of SARS-CoV-2 infection. They have been postulated to be amyloid-fibrin(ogen) aggregates, but their role as a prognostic biomarker remains unclear. To examine for their possible clinical utility, blood samples were collected for the first 96 hours from critically ill patients (n=104) admitted to the intensive care unit (ICU). Detection was by staining platelet-poor plasma samples with Thioflavin T and visualized by fluorescent microscopy. Image J software was trained to identify and quantify microclots, which were detected in 44 [42.3%] patients on ICU admission but not in the remaining 60 [57.7%] or in 20 healthy controls [0.0%]. Microclots on admission to ICU were associated with a primary diagnosis of sepsis (microclots present in sepsis=23/44 [52.3%] vs microclots absent in sepsis=19/60 [31.7%], P=0.044). Multicolour immunofluorescence demonstrated that microclots consisted of amyloid-fibrinogen aggregates, which was supported by proteomic analysis. Patients with either a high number or larger-sized microclots had a higher likelihood of developing disseminated intravascular coagulation (DIC) (OR=51.4 [95% CI=6.3-6721.1], P<0.001) and had an increased probability of 28-day mortality (OR=5.3 [95% CI=2.0-15.6], P<0.001). This study concludes that microclots, as defined by amyloid-fibrin(ogen) aggregates, are potentially useful in identifying sepsis and predicting adverse coagulopathic and clinical outcomes.

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Short title: Microclots Predict DIC and Mortality

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Data Sharing Statement: The data presented in this study are available on reasonable request from the corresponding author.

Key points:

- Microclots are present in increased number and size in critically ill patients compared to healthy controls and are associated with sepsis.
- Microclot levels measured on admission to critical care predict risks of disseminated intravascular coagulation development and mortality.

Abstract

Microclots have been associated with various conditions, including post-acute sequelae of SARS-CoV-2 infection. They have been postulated to be amyloid-fibrin(ogen) aggregates, but their role as a prognostic biomarker remains unclear. To examine for their possible clinical utility, blood samples were collected for the first 96 hours from critically ill patients (n=104) admitted to the intensive care unit (ICU). Detection was by staining platelet-poor plasma samples with Thioflavin T and visualized by fluorescent microscopy. Image J software was trained to identify and quantify microclots, which were detected in 44 [42.3%] patients on ICU admission but not in the remaining 60 [57.7%] or in 20 healthy controls [0.0%]. Microclots on admission to ICU were associated with a primary diagnosis of sepsis (microclots present in sepsis=23/44 [52.3%] vs microclots absent in sepsis=19/60 [31.7%], P=0.044). Multicolour immunofluorescence demonstrated that microclots consisted of amyloid-fibrinogen aggregates, which was supported by proteomic analysis. Patients with either a high number or larger-sized microclots had a higher likelihood of developing disseminated intravascular coagulation (DIC) (OR=51.4 [95% CI=6.3-6721.1], P<0.001) and had an increased probability of 28-day mortality (OR=5.3 [95% CI=2.0-15.6], P<0.001). This study concludes that microclots, as defined by amyloid-fibrin(ogen) aggregates, are potentially useful in identifying sepsis and predicting adverse coagulopathic and clinical outcomes.

Introduction

Microclots have been reported in the post-acute sequelae of SARS-CoV-2 infection (PASC)[1] and thrombotic conditions, such as pulmonary embolism.[2] They have been detected by staining for amyloid protein, and contain fibrin(ogen) as well as other coagulation factors and molecules involved in inflammation.[3] As these amyloid fibrin(ogen)-containing aggregates are relatively resistant to fibrinolysis, they have been postulated to cause widespread microthrombosis with adverse clinical consequences.[4] However, their role as a biomarker of disease or in immunothrombotic pathogenesis remains unclear. This study aims to examine the relevance of microclots, as defined by amyloid-fibrin(ogen) aggregates, in critical illness where patients often develop coagulation and circulatory disturbances that affect their prognosis.[5]

Methods

Study design

Blood samples were collected for the first 96 hours (study duration) from patients (n=104) admitted to the intensive care unit (ICU) at the Royal Liverpool University Hospital between June 2009 and June 2013. Patients were enrolled in accordance with the protocol approved by the National Research Ethics Service Committee North West–Greater Manchester West and Liverpool Central (Reference Numbers: 07/H1009/64 and 13/NW/0089). Written informed consent or assent from next of kin was obtained. Exclusion criteria were transfers from other ICUs, ICU readmissions within 30 days or insufficient plasma preserved to perform analysis. Measurements included whole blood cell counts and coagulation parameters. Age, gender, ethnicity, admission Acute Physiology and Chronic Health Evaluation (APACHE) II scores, daily Sequential Organ Failure Assessment (SOFA) scores, and modified SOFA scores (platelet component removed to avoid bias from thrombocytopenia) were recorded together

with 28-day mortality (from ICU admission). Sepsis was originally defined using the American College of Chest Physicians/Society of Critical Care Medicine 2001 international sepsis definition[6] and revised to meet Sepsis-3 criteria.[7] DIC scoring was performed daily according to International Society for Thrombosis and Haemostasis (ISTH) criteria.[8] DIC was diagnosed with a score ≥5 from the platelet count, fibrinogen, D-dimer and prothrombin time (PT).[8] Blood samples were collected from 10 healthy controls recruited in September 2023 (Reference Number: 16/NW/0170) and 10 historical healthy controls (Reference Number: 13/NW/0089).

Platelet-poor plasma (PPP) was obtained from venous blood that had been collected into 3.2% sodium citrate tubes without stasis (1-part citrate 9-parts blood) and centrifuged at 2600g at room temperature (RT) for 10 minutes. PPP was aliquoted and stored at -80^{o}C until analysis.

Microclot detection

On the day of analysis, frozen plasma samples were thawed at 37^{o}C for 10 minutes. Microclots were detected by incubating citrated PPP with Thioflavin T (ThT) at 5μM for 30 minutes at RT, protected from light. 3μl was smeared on a slide and analysed by fluorescent microscopy (Olympus IX83, Ex:467-498nm and Em:513-556nm). Five representative fields/sample were imaged (x20 magnification) and analysed using Fiji (Image J)-Labkit plugin. Software classifier was used to analyse all samples and trained to identify and quantify microclots according to number (count/field) and size (pixels/field). Receiver operating characteristic (ROC) analysis for DIC was used to develop cut-off values to define microclot absence (number: <1.0/field, size: <200pixels/field) and microclot presence (number: >1.0/field and area: >200pixels/field) of microclots (Supplemental Figure 1).

Microclot proteomics

Citrated PPP was diluted with phosphate-buffered saline (PBS) (v/v) and centrifuged and washed twice at 20,000g for 30 minutes. Pellets were observed in patients with microclots but not from healthy controls or patients without microclot, and therefore microclot analysis was performed against a control plasma-derived library. The samples were digested with Trypsin/Lys-C Mix (Promega, Madison, WI) at a 25:1 protein:protease ratio (w/w) overnight at 37^{o}C. Digested microclots from 3 individual ICU patients were processed and subjected to Sequential Window Acquisition of all THeoretical fragment ion spectra (SWATH)/Data-Independent Acquisition (DIA) and Data-Dependent Acquisition (DDA) (for quality control purposes). Mass spectrometry data were acquired on a TripleTOF 6600 (Sciex) using a 120 minute gradient (bioZen 2.6 um Peptide XB-C18 nano Column, 250 x 0.075 mm, Phenomenex). The SWATH method comprised a total of 100 overlapping windows with variable isolation width spanning a m/z range of 400-1500 were acquired for 50ms. The m/z range for product ion scans was 100-1650 with an accumulation time of 33ms. The total cycle time was 3.7s. Retention time alignment and peptide/protein quantification were performed against a previously acquired control plasma-derived spectral library using version 1.8 of Data-Independent Acquisition by Neural Networks (DIA-NN), with a false discovery rate of 1% and with both match between runs and unrelated runs selected (Figure 1).

Microclot composition

To determine if the ThT-positive aggregates contained fibrinogen, plasma was mixed with AlexaFluor647-labelled anti-fibrinogen β antibody (SantaCruz) (1:100 dilution) for 60 minutes at RT, protected from light. After 60 minutes ThT was added (final concentration: 5μM) and incubated for a further 30 minutes. To exclude non-specific interactions, an AlexaFluor647-labelled isotype control was used (Thermofisher) (1:100 dilution). 3μl was smeared on a slide and analysed by fluorescent microscopy (Olympus IX83) to assess for co-localization.

Assay reliability

To assess the effect of freeze-thaw and long-term storage on the presence of microclots, samples from 10 healthy donors were analysed fresh and following storage (at -80°C) for seven days. Historical healthy control samples (stored at -80°C), recruited simultaneously with the ICU cohort, were also used.

Five microscope fields at x20 magnification were used following assessment of coefficient of variation (CV) from 1 to 10 fields, which demonstrated no statistically significant improvement on increasing the number of fields above 5 (Supplemental Figure 2).

To determine intra- and inter-assay variability, PPP from healthy controls, and ICU patients with low and high microclot levels were assessed. Each sample was analysed in triplicate and repeated on subsequent days. The overall intra-assay CV was 3.2% [range: 0.0-10.8%] for numbers and 4.5% [range: 0.0-17.9%] for size, and the inter-assay CV was 2.5% [range: 0.0-13.1%] for numbers and 6.3% [range: 0.0-22.0%] for size. Specifically, the intra and inter-assay CV was 0.0% in samples where microclots were absent (normal controls and ICU with low microclot levels). In samples with high levels, the intra-assay CV for numbers were 9.8% [range: 8.7-10.8%], and 12.4% [range: 6.8-17.9%] for size and the inter-assay CV for numbers were 9.3% [range: 5.5-13.1%], and 17.0% [range: 12.2%-22.0%] for size.

Statistical Analysis

The Kruskall-Wallis test was used for comparison of continuous variables, presented as median [interquartile range; IQR]. The Fishers Exact/Chi-squared test for comparison of categorical variables were presented as counts [percentage]. Microclots were analysed as continuous variables or categorized. Mann-Whitney U test was used to compare categorical microclot levels to continuous clinical variables. Correlation was assessed using Spearman's rank. ROC curve and multivariate regression (adjusted for age and gender) assessed

admission aggregate levels in predicting DIC and 28-day mortality. Statistical tests were performed on SPSS software (IBM, version 29). A 2-tailed P value of <0.05 was considered significant.

Results

Microclots are present in critically ill patients and are associated with sepsis

Out of 104 ICU patients, microclots were present on admission in 44 [42.3%] but absent in the remaining 60 [57.7%] and healthy controls [0.0%] (Figure 2A-D). Microclots were detected in a range of diseases requiring ICU admission and there was a significant association with sepsis (microclots present in sepsis=23/44 [52.3%] vs microclots absent in sepsis=19/60 [31.7%], P=0.044). (Figure 2E and Table 1). Microclots were also significantly associated with the development of coagulopathy (Table 1). Approximately half [n=21/44] of patients with microclots had thrombocytopenia (platelets<150x10^9/L) on admission compared to 20.0% [n=12/60] without. Platelet counts dropped in those with microclots but remained stable in those without. Their presence were also associated with prolonged PT on admission, and reduced fibrinogen levels at 72 hours post-admission. Elevated D-Dimers were observed regardless of whether microclots were present or absent. Collectively, these changes suggested the possibility of DIC and application of the ISTH score showed that patients with DIC had significantly higher microclot levels (Table 1) (number: 2.2 [1.4-4.4] and size: 582 [248-822]) than in those without (number: 0.4 [0.1-1.0] and size: 59 [0-190]; P<0.001) (Figure 2F).

Characterisation of microclots isolated from critically ill patients demonstrate amyloid-fibrin(ogen) aggregates

To assess microclot composition, proteomic analysis was performed. Centrifugation of ICU patient plasma with microclots resulted in a visible pellet, which was not observed in healthy

controls or ICU patients without microclots. A similar observation has been observed previously between patients with and without detectable microclots.[4] The pellet stained strongly when incubated with ThT, confirming the presence of microclots. Mass spectrometry analysis revealed consistent components of the microclots (Figure 3A), including a predominance of fibrinogen-α chain (Figure 3B), fibrinogen-β chain (Figure 3C), fibrinogen-γ chain (Figure 3D), inflammatory molecules and lipoproteins, when compared to a control plasma-derived library. To further confirm that microclots were amyloid-fibrinogen aggregates, a fluorescently labelled fibrinogen-β chain antibody identifying the same protein sequence obtained from proteomics was used alongside amyloid staining. Multicolour immunofluorescence microscopy confirmed co-localisation of amyloid and fibrinogen (Figure 4 and Supplemental Figure 3) which was not observed in the isotype control (Supplemental Figure 4).

Microclots predict DIC development and mortality

To examine if microclot detection could predict DIC development, patients with DIC on ICU admission were excluded (7.7% [n=8/104]). Patients with microclots on ICU admission had significantly (P<0.001) increased risk of developing DIC (29.7% [n=11/37]) compared to patients without (0.0% [n=0/59]). Univariate analysis demonstrated that patients with microclots had an increased probability of developing DIC (OR=51.4 [95% CI=6.3-6721.1], P<0.001) and multivariate analysis (adjusted for age and gender) demonstrated that microclots independently predicted DIC development (Figure 5A and Supplemental Table 1).

DIC is often associated with the Multi-Organ Dysfunction (MODS) and assessment using the SOFA and modified SOFA score showed that microclots were associated with the development of organ dysfunction 24 hours post-ICU admission and remained significant throughout the study duration (Table 1). The presence of microclots also significantly

(P<0.001) increased risk of mortality (38.6% [n=17/44]) compared to those without (10% [n=6/60]). Univariate analysis demonstrated that patients with microclots had an increased probability of 28-day mortality (OR=5.3 [95% CI=2.0-15.6], P<0.001). Multivariate analysis (adjusted for age and gender) demonstrated that microclots independently predicted 28-day mortality (Figure 5B and Supplemental Table 1).

Discussion

Microclots, as defined by amyloid-fibrin(ogen) aggregates, have been detected in various conditions and their targeted removal has been proposed.[9] However, their role in disease pathogenesis remains unclear. Our study was not designed to address disease causation but aimed to objectively clarify the clinical implications of their detection. To our knowledge, our findings are the first to demonstrate their potential as a predictive biomarker in an ICU setting.

These findings of clinically relevant associations do not indicate causality between detection of microclots and development of haemostatic and organ dysfunction. Further research is needed to define their pathophysiological relevance *in vivo* and understand how and why microclots form. There are several potential pathogen- and host-related mechanisms of amyloid-fibrin(ogen) aggregate formation in critically ill patients. For example, lipopolysaccharide (LPS)[10] and the acute phase reactant, serum amyloid A,[11] can bind and alter the structure of fibrinogen to potentiate aggregate formation, which could be consistent with our findings in sepsis. The presence of amyloid and other molecules involved in inflammation, innate immune activation and coagulation could lead to better understanding of immunothrombosis and the host response to critical illness.[12]

Microclots are poorly understood in terms of their composition, mechanism of formation, relationship to blood coagulation, and clinical relevance.[12,13] Pretorius *et al*[4] demonstrated

microclots in patients with PASC, which appeared similar to the findings of Baker *et al*[2] where they demonstrated fibrin aggregates in patients with pulmonary embolism. Our findings through proteomic and immunofluorescent analyses demonstrate that microclots contain fibrin(ogen) in an ICU cohort and may provide a link to these other independent observations.

A limitation of this study is that the number of patients from a single ICU do not enable reliable information on patients without sepsis. Larger multi-centre cohort studies and in different conditions are needed. Samples have also been frozen for a period but we ensured that microclot formation was not due to freeze-thaw or prolonged storage. Assessment of paired fresh and frozen controls [n=10], along with historical controls [n=10] obtained concurrently with the ICU samples did not detect microclots (Figure 2A-D), but we cannot entirely exclude a possible effect of prolonged storage of samples from critically ill patients as opposed to healthy controls. A further but more generic limitation is the lack of universally accepted nomenclature for microclots. Other terms used include fibrinaloid microclots[14] and amyloid fibrin(ogen) particles.[15] Central to the discussion is the definition of a clot, which by strict definition, requires soluble fibrinogen to have been cleaved by thrombin into insoluble fibrin.[16] Whilst we have demonstrated amyloid-fibrin(ogen) co-localisation, there is no conclusive evidence that fibrin is always present in microclots. However, reports of relative fibrinolytic resistance[4] offer fresh insight into the biochemical nature and biophysical effects of molecular structures with potential vascular occlusive effects.

Another limitation due to the sample size is the ability to conduct multivariable analysis on the extent to which microclots, as a standalone biomarker, predict DIC and adverse outcomes independent of parameters currently used in DIC scoring, e.g. platelet count, PT, fibrinogen and D-dimer. Currently, there is no single test that can accurately diagnose DIC and no clinically available assay to predict DIC development.[17] In this current cohort, all patients

who developed DIC after ICU admission had detectable microclots on admission. Whilst a number of patients with microclots did not develop DIC, the assay has a very high negative predictive value of 100%. This could therefore serve as a rule-out test and influence triaging of care in critically ill patients. Future prospective studies in larger patient cohorts are required to extend and validate upon the findings described here and clarify the clinical utility of microclot detection in the care pathway.

In conclusion, we believe that our novel clinical observations advance the field by demonstrating the prognostic relevance of microclots in the ICU setting. There is also technological advancement through integration of computer learning to objectively calculate microclot number and size in a standardisable and, reproducible manner. This would lead to better validation of results and meaningful comparisons between studies to improve robustness of evidence generation. Overall, we believe that this is an important step forward in the potential utility of this assay as a clinically useful and practically measurable biomarker to identify sepsis, predict DIC and mortality in the ICU.

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Authorship

Contribution: J.S. performed experiments. S.T.A. and J.S. analysed the data, assisted by S.L. R.J performed mass spectrometry and analysed raw data. J.S., S.T.A, G.W. and C.-H.T. wrote, edited and reviewed the manuscript and figures; and S.T.A., G.W. and C.-H.T. designed and supervised the work.

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Table 1. Clinical and laboratory characteristics of the Intensive Care Unit patients with and without Microclots.

		Microclots				
	Total	**Number R value**	**Size R value**	**Absent**	**Present**	**P value**
Total number (n)	104	104	104	60	44	
Age (years), Median [IQR]	62.0 [49.0-74.0]	-0.423	-0.410	63.0 [49.0-74.8]	57.5 [47.5-71.8]	0.233
Male (n) [%]	52 [50%]	-	-	32 [53.3%]	20 [45.5%]	0.552
White ethnicity (n) [%]	95 [91.3%]	-	-	54 [90%]	41 [93.2%]	0.730
APACHE II score, Median [IQR]	19.0 [14.5-24.0]	0.279	0.249	19.0 [14.0-23.8]	19.5 [16.0-25.5.0]	0.566
Admission diagnosis (n) [%]						
Sepsis	42 [40.4%]	-	-	19 [31.7%]	23 [52.3%]	0.044
Trauma	16 [15.4%]	-	-	10 [16.7%]	6 [13.6%]	0.787
Cardiovascular	12 [11.5%]	-	-	9 [15.0%]	3 [6.8%]	0.231
Respiratory	16 [15.4%]	-	-	10 [18.3%]	6 [13.6%]	0.415
Gastro-intestinal	12 [11.5%]	-	-	8 [16.7%]	4 [9.1%]	0.553
Renal	3 [2.9%]	-	-	2 [3.3%]	1 [2.3%]	1.000
Central nervous system	3 [2.9%]	-	-	2 [3.3%]	1 [2.3%]	1.000
Coagulation parameters						
Platelets ($x10^9$/L), Median [IQR]						
Admission	194.0 [129.0-294.3]	-0.526	-0.527	202.0 [156.3-302.3]	156.0 [65.5-256.8]	0.005
24 hrs post admission	206.0 [105.0-260.0]	-0.484	-0.486	214.0 [152.0-270.0]	162.0 [51.8-249.8]	0.012
48 hrs post admission	181.0 [102.3-243.3]	-0.517	-0.444	190.0 [128.5-244.0]	107.0 [44.0-244.0]	0.014
72 hrs post admission	183.0 [99.0-234.0]	-0.539	-0.515	191.0 [127.0-277.0]	115.5 [50.5-199.5]	0.006
PT (seconds), Median [IQR]						
Admission	14.6 [13.1-17.0]	0.597	0.542	13.7 [12.8-15.6]	16.2 [13.3-20.8]	0.002
24 hrs post admission	14.5 [12.8-16.3]	0.530	0.454	13.9 [12.7-15.6]	15.6 [13.3-20.3]	0.007
48 hrs post admission	14.0 [12.2-16.4]	0.560	0.508	13.1 [11.9-15.0]	15.7 [13.0-20.3]	0.004
72 hrs post admission	13.6 [12.1-15.7]	0.523	0.469	13.4 [11.8-14.4]	15.2 [12.8-19.3]	0.006
aPTT (seconds), Median [IQR]						
Admission	32.8 [29.1-40.2]	0.458	0.483	30.8 [28.4-37.0]	35.1 [30.1-44.0]	0.038
24 hrs post admission	33.5 [29.1-40.6]	0.338	0.366	32.3 [28.3-38.3]	35.7 [30.1-43.4]	0.087
48 hrs post admission	32.2 [28.8-37.9]	0.387	0.366	30.8 [28.5-36.0]	34.9 [29.1-45.8]	0.068
72 hrs post admission	31.7 [28.5-37.2]	0.476	0.425	30.7 [28.2-36.1]	34.8 [29.8-42.0]	0.042
Fibrinogen (g/L), Median [IQR]						
Admission	3.7 [2.3-4.9]	-0.332	-0.164	3.7 [2.5-4.8]	3.8 [1.8-5.4]	0.903
24 hrs post admission	4.1 [2.8-5.1]	-0.390	-0.286	4.2 [2.8-5.0]	3.8 [2.2-5.6]	0.512
48 hrs post admission	4.4 [3.2-5.2]	-0.518	-0.456	4.5 [3.6-5.2]	3.9 [2.9-5.2]	0.173
72 hrs post admission	4.4 [3.4-5.4]	-0.565	-0.514	4.8 [3.6-5.5]	3.7 [2.8-5.0]	0.033
D-dimer (ng/ml), Median [IQR]						
Admission	3969 [1497-9328]	0.348	0.329	3490 [1393-7798]	5341 [2015-13456]	0.800
24 hrs post admission	4587 [1302-6492]	0.272	0.255	3492 [1149-5549]	5044 [2455-7395]	0.321
48 hrs post admission	3849 [2540-5974]	0.418	0.445	3458 [1950-6887]	4221 [2732-6567]	0.528
72 hrs post admission	3762 [2348-7084]	0.569	0.664	3488 [1972-5636]	6036 [2692-13734]	0.084
Total DIC (n) [%]	-	-	-	1 [1.7%]	18 [40.9%]	<0.001
Time to develop DIC (n) [%]						
Admission	8 [7.7%]	-	-	1 [1.7%]	7 [15.9%]	0.010
24 hrs post admission	4 [3.8%]	-	-	0 [0.0%]	4 [9.1%]	0.030
48 hrs post admission	3 [2.9%]	-	-	0 [0.0%]	3 [6.8%]	0.073
72 hrs post admission	4 [3.8%]	-	-	0 [0.0%]	4 [9.1%]	0.030
Developed DIC ≥24 hrs post admission (n) [%]	11 [10.6%]	-	-	0 [0.0%]	11 [29.7%]	<0.001
Organ injury						
SOFA score, Median [IQR]						
Admission	7.0 [4.0-10.0]	0.293	0.330	7.0 [4.0-8.0]	8.0 [5.0-10.0]	0.076
24 hrs post admission	8.0 [5.0-10.0]	0.437	0.448	7.0 [4.0-9.0]	8.0 [7.0-12.0]	0.003
48 hrs post admission	7.0 [5.0-9.5]	0.574	0.543	6.0 [4.0-8.3]	9.0 [7.0-13.0]	<0.001
72 hrs post admission	7.0 [4.0-10.0]	0.642	0.607	5.0 [3.0-8.0]	10.0 [7.0-12.0]	<0.001
Modified SOFA score, Median [IQR]						
Admission	6.0 [4.0-9.0]	-0.105	0.084	6.5 [3.8-8.0]	6.0 [4.0-9.0]	0.393
24 hrs post admission	7.0 [5.0-10.0]	0.324	0.338	6.0 [4.0-9.0]	8.0 [6.0-10.0]	0.031
48 hrs post admission	6.0 [4.0-8.0]	0.486	0.449	6.0 [4.0-8.0]	7.0 [6.0-10.0]	0.002
72 hrs post admission	6.0 [4.0-8.0]	0.576	0.536	5.0 [3.0-7.0]	7.0 [5.5-10.0]	0.001
Mortality (n) [%]	23 [22.1%]	-	-	6 [10.0%]	17 [38.6%]	<0.001

Definition of abbreviations: Microclot absent = (number: <1.0/field, and size: <200pixels/field); Microclot present = (number: >1.0/field and/or area: >200pixels/field); IQR = interquartile range; APACHE II = Acute Physiology and Chronic Health Evaluation II; PT = prothrombin time; aPTT = activated partial thromboplastin time; DIC = disseminated intravascular coagulation; SOFA = Sequential Organ Failure Assessment. R value = correlation with aggregate presence or absence performed using Spearman's rank correlation. P value = comparison of patients with absent versus presence of amyloid fibrin(ogen) aggregates, calculated using Mann-Whitney U test for continuous variables and $\chi 2$ test for categorical variables.

Figure 1. Microclot analysis methodology. (A) Microclot Quantification: venous blood samples from critically ill patients and healthy controls were collected into sodium citrate tubes. Platelet-poor plasma (PPP) was isolated through centrifugation. PPP was incubated with Thioflavin T (ThT) and the presence of microclots assessed using fluorescence microscopy. Software was trained to quantify microclots. (B) Microclot Composition: Following centrifugation of PPP, those from critically ill patients with microclots contained visible pellets that were not observed in patients without microclots or in healthy controls. The pellet was digested with trypsin and analysed by mass spectrometry. (C) Amyloid Fibrin(ogen) Co-Localisation: PPP was incubated with fluorescently labelled anti-fibrinogen antibody and ThT. Co-localisation was assessed by fluorescence microscopy.

Figure 2. Microclots are associated with critical illness. (A) Microclots were characterized according to number of microclots per field (count/field). Typical images are presented. (B) The number of microclots (count/field) were compared between patients on admission to the intensive care unit (ICU) (n=104) and paired fresh and frozen (n=10), and historical (n=10) normal healthy controls. (C) Microclots were characterized according to size (pixels/field). Typical images are presented. (D) The size of microclots (pixels/field) were compared between patients on admission to the ICU (n=104) and paired fresh and frozen (n=10), and historical (n=10) normal healthy controls. (E) Microclot number and size were compered between non-sepsis (n=62) and sepsis (n=42) ICU patients. (F) Microclot number and size were compared between patients diagnosed with disseminated intravascular coagulation (DIC; n=19) and Non-DIC (n=85) patients. Continuous data presented in Figure 2B, D, E and F was analysed using by Mann Whitney U test.

Figure 3. Proteomic analysis of microclots from critically ill patients. (A) Trypsin-digested microclots from 3 individual ICU patients were analysed by Sequential Window Acquisition of all THeoretical fragment ion spectra – Mass Spectrometry (SWATH-MS). The 20 proteins with the highest relative SWATH-MS signal intensities across all three patients (total) were identified and represented for each individual critically ill patient and compared against a control plasma-derived library. Representative MS/MS spectra of peptides identified by both DDA and SWATH are shown for fibrinogen-α chain (B), -β chain (C) and -γ chain (D). Abbreviations: FGB = Fibrinogen beta chain, FGA = Fibrinogen alpha chain, FGG = Fibrinogen gamma chain, APOA1 = Apolipoprotein A-I, KIF4A = Chromosome-associated kinesin KIF4A, ALB = Albumin, ILF3 = Interleukin enhancer-binding factor 3, TGFBI = Transforming growth factor-beta-induced protein ig-h3, CILP2 =Cartilage

intermediate layer protein 2, SAA2 = Serum amyloid A-2 protein, APOB = Apolipoprotein B-100, APOA2 = Apolipoprotein A-II, POSTN = Periostin, SERPINA1 = Alpha-1-antitrypsin, FSIP2 = Fibrous sheath-interacting protein 2, IGHA1 = Immunoglobulin heavy constant alpha 1, HRG = Histidine-rich glycoprotein, GSTP1 = Glutathione S-transferase P, MSN = Moesin, C5 = Complement C5.

Figure 4. Amyloid aggregates co-localise with fibrin(ogen). Multicolour immunofluorescence microscopy of platelet-poor plasma from a healthy control and critically patients (n=3) co-stained with thioflavin T (left) and anti-fibrinogen-AlexaFluor647 antibody (middle). Co-localization is demonstrated in the merged image (right). 60x magnification.

Figure 5. Presence of microclots are an independent predictor of disseminated intravascular coagulation (DIC) and mortality in critically ill patients. (A) Multivariate analysis of crude and adjusted odds ratios (with patients adjusted for age and gender). The presence of microclots, as defined by microclot number (>1/field) or size (>200pixels/field), were independently associated with disseminated intravascular coagulation (DIC) development. (B) Multivariate analysis of crude and adjusted odds ratios (with patients adjusted for age and gender). The presence of microclots were independently associated with 28-day mortality.

Figure 1

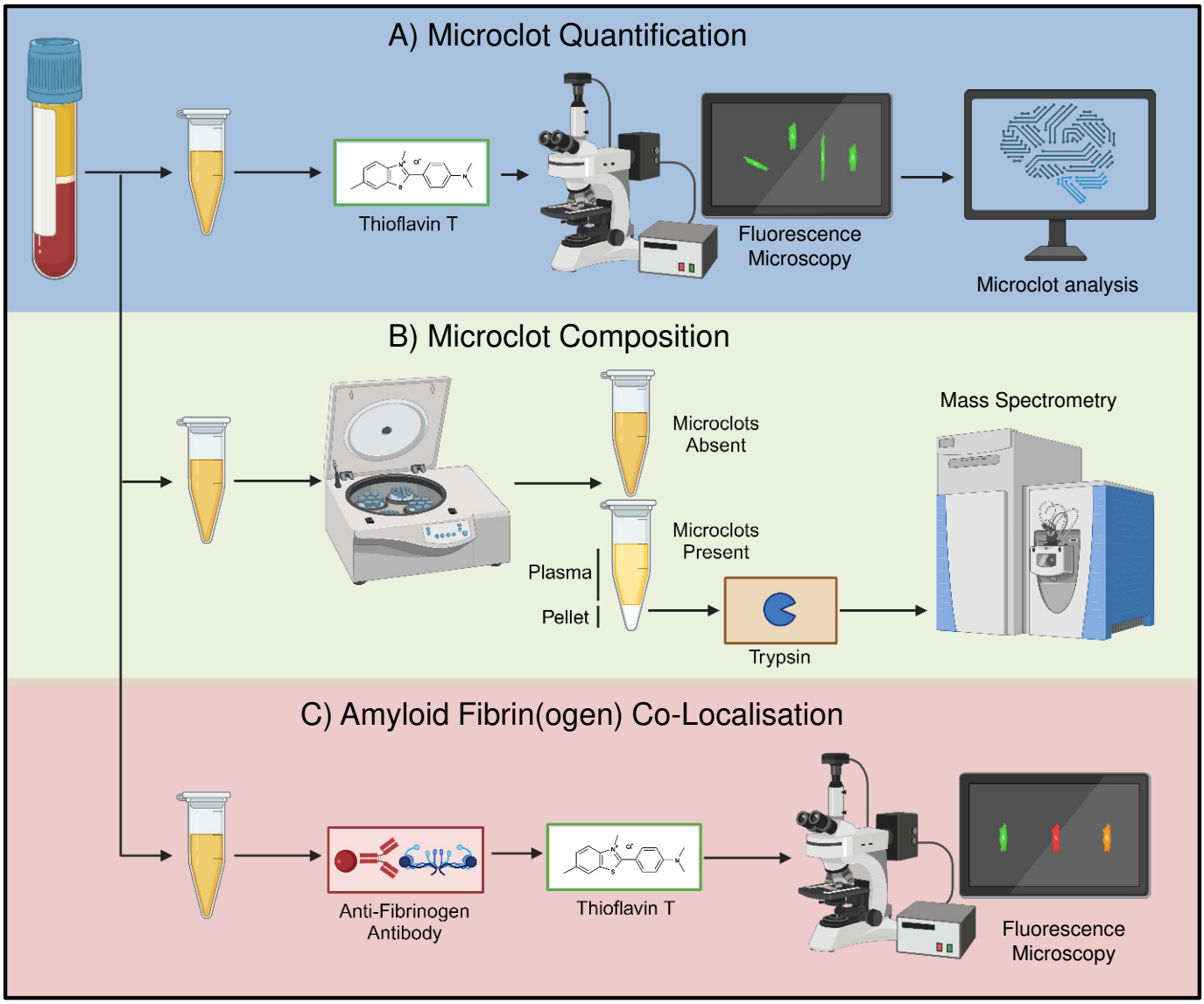


Figure 2

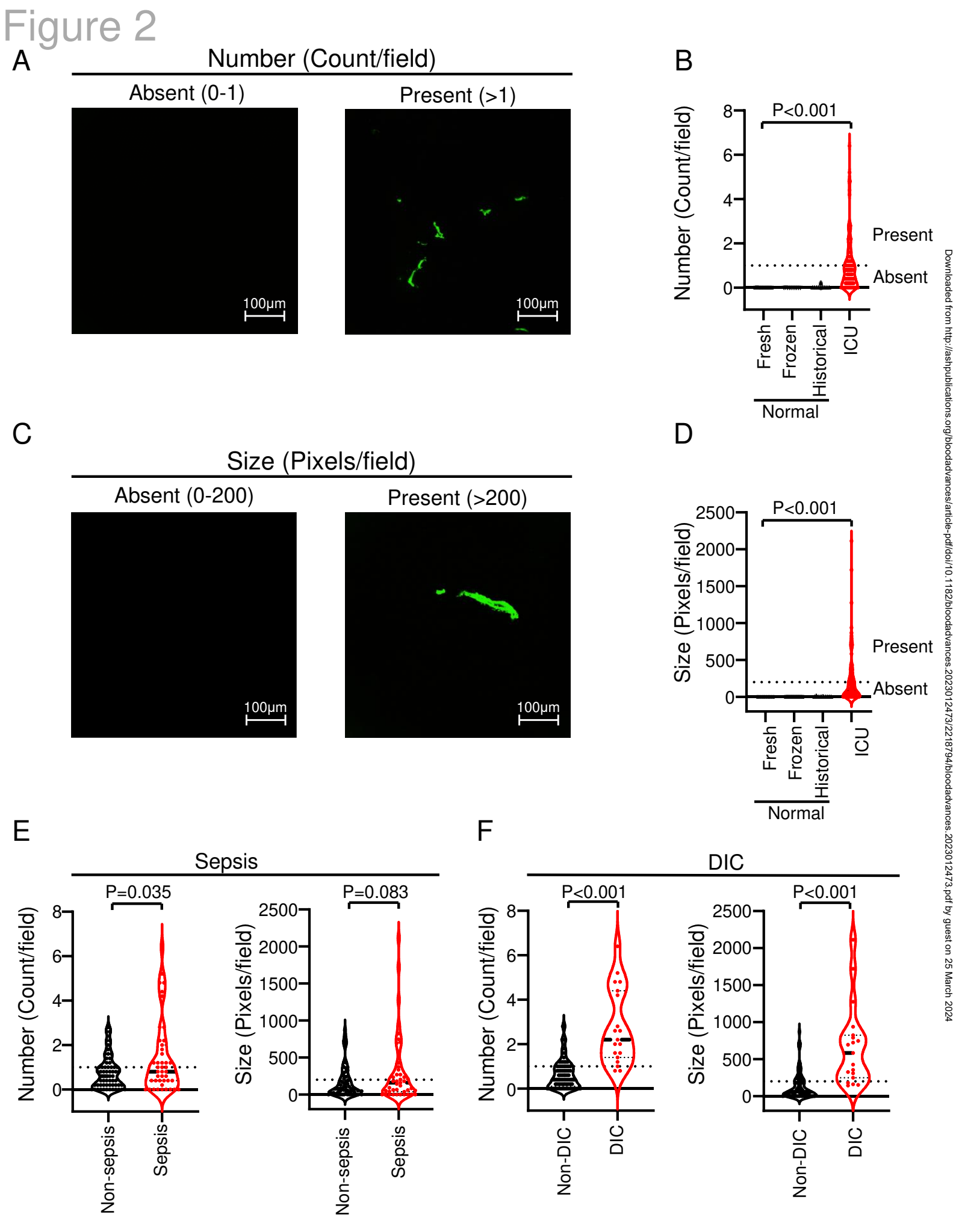
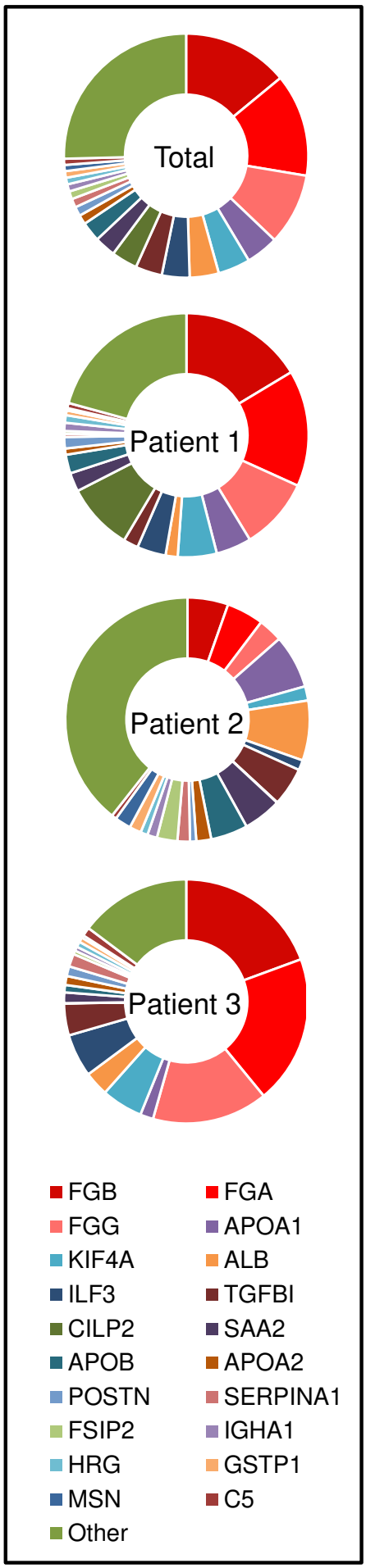
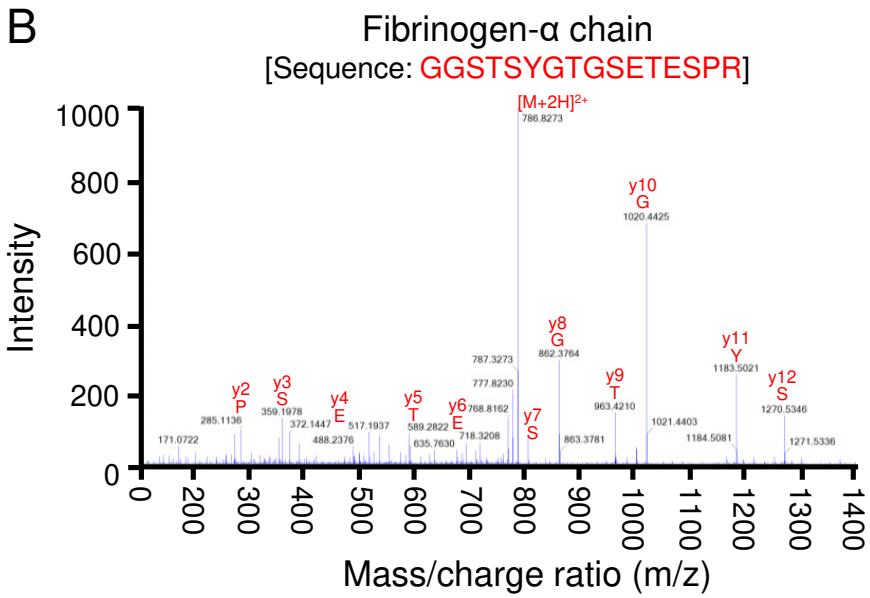
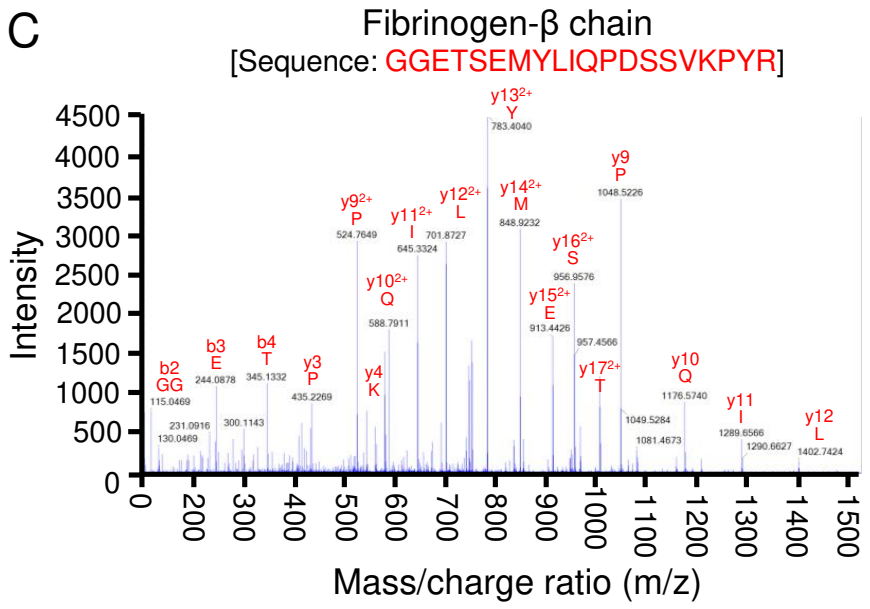


Figure 3







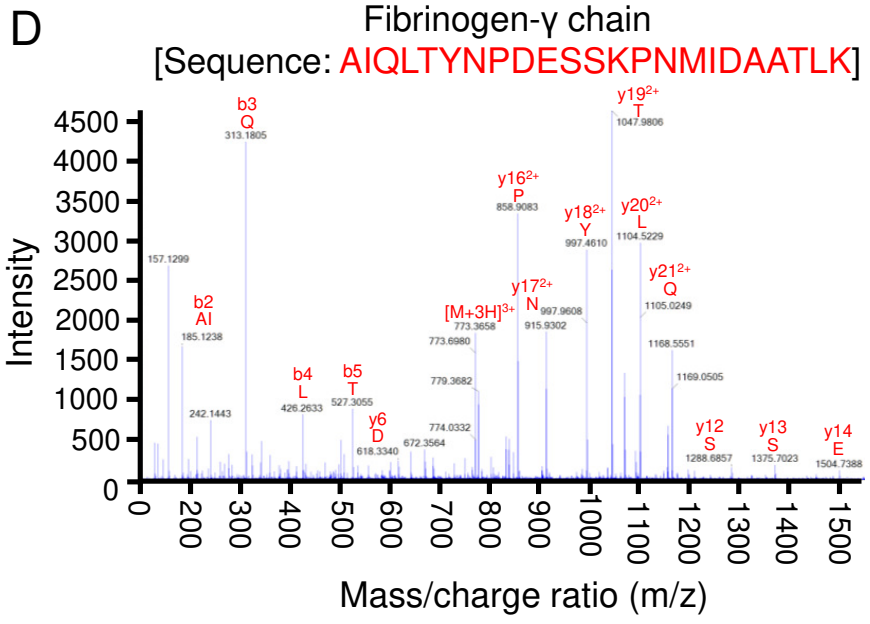


Figure 4

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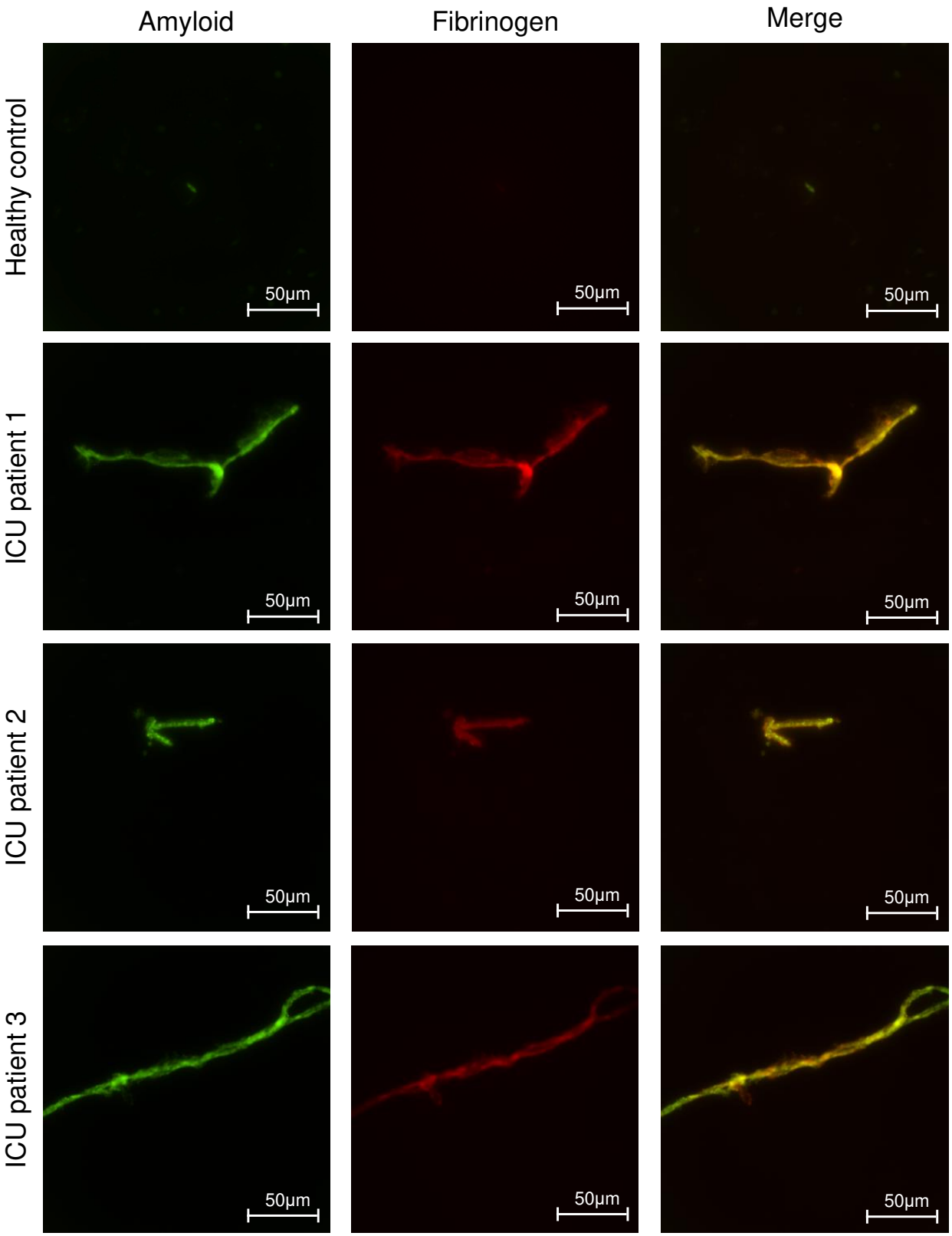
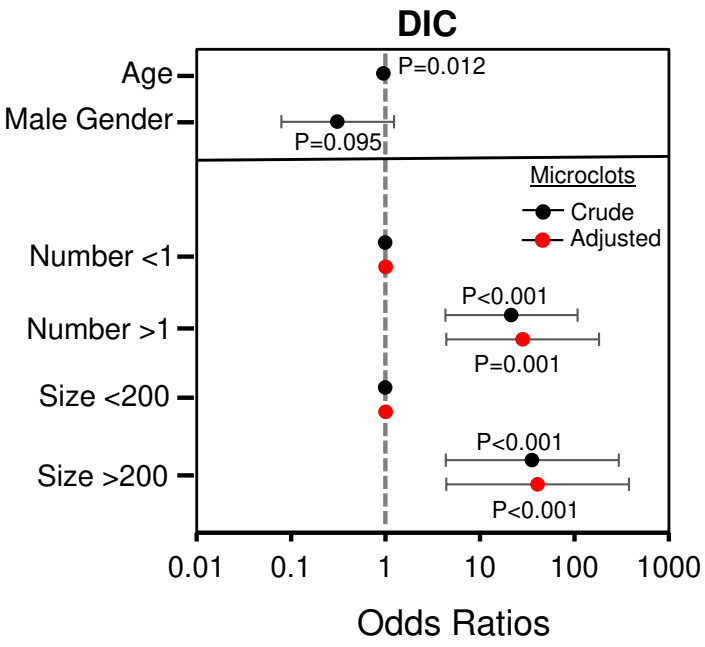


Figure 5

A



B