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forward together
sonke siya phambili
saam vorentoe

# Endotheliopathy of Long COVID

Resia Pretorius and collaborators

# Collaborators and postgraduate students contributing to this presentation

- Douglas B Kell (University of Liverpool)
- Alain Thierry (INSERM: France)
- Uvi Naidoo (Austria)
- Melanie Walker (Department of Neurological Surgery (University of Washington)
- Alakendu Sekhar (Department of Neurology, The Walton Centre, Aintree University Hospital)
- Dr Chantelle Venter (Blood lab manager: Stellenbosch University)
- Justine Grixti (Postdoc: University of Liverpool)
- Massimo Nunes (PhD: 2024) Stellenbosch University
- Tom Usher, Anel Thompson, Maxine Waters, Liz Copley (MSc students: Stellenbosch University)

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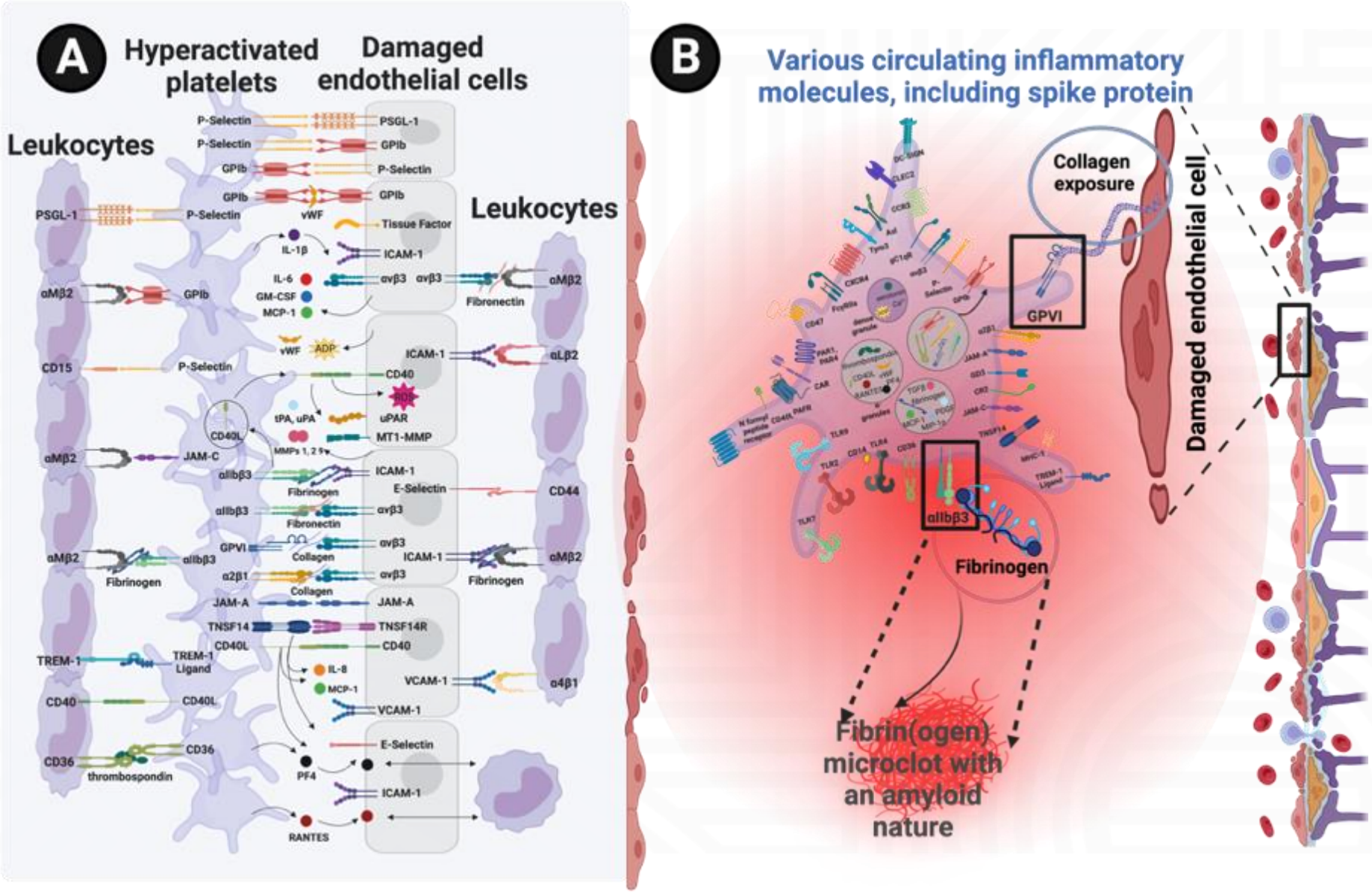
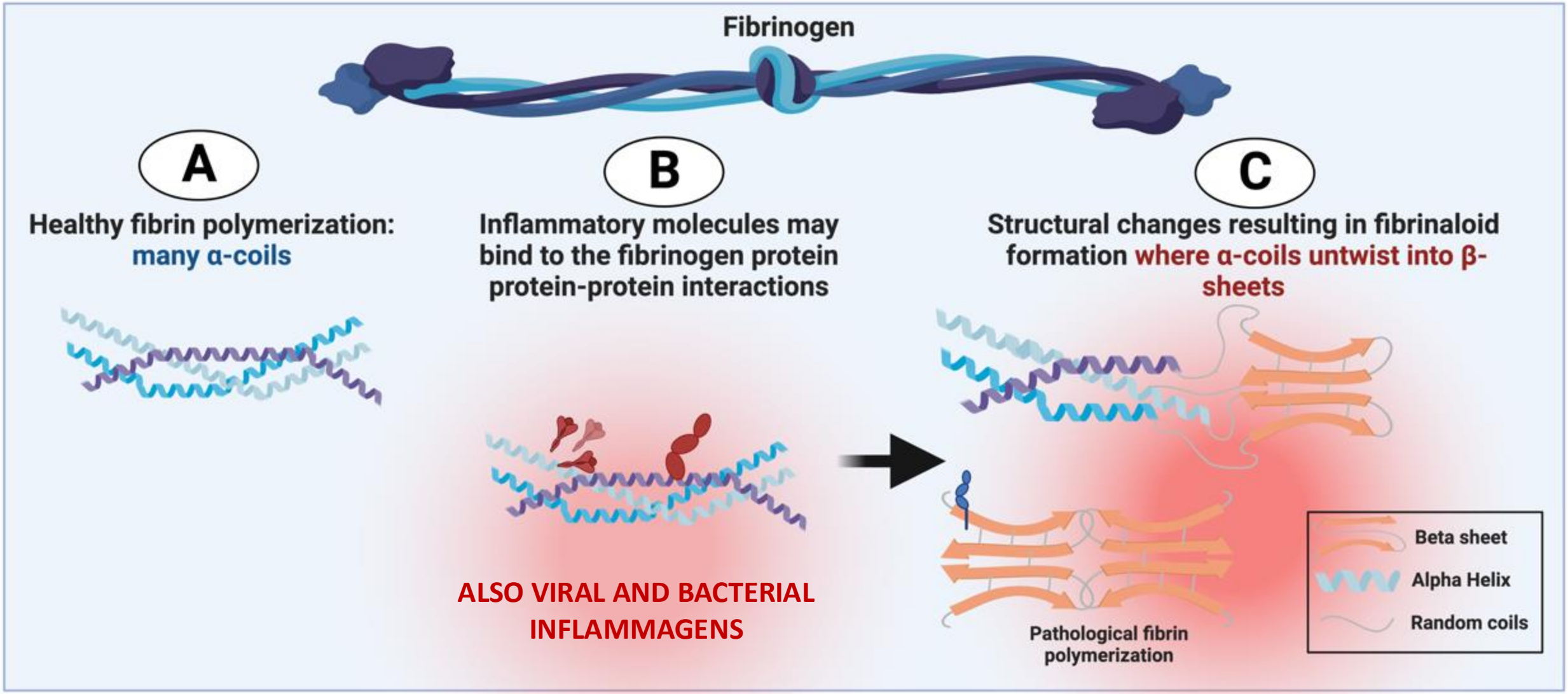
# Platelets, endothelial and fibrin(ogen) interactions

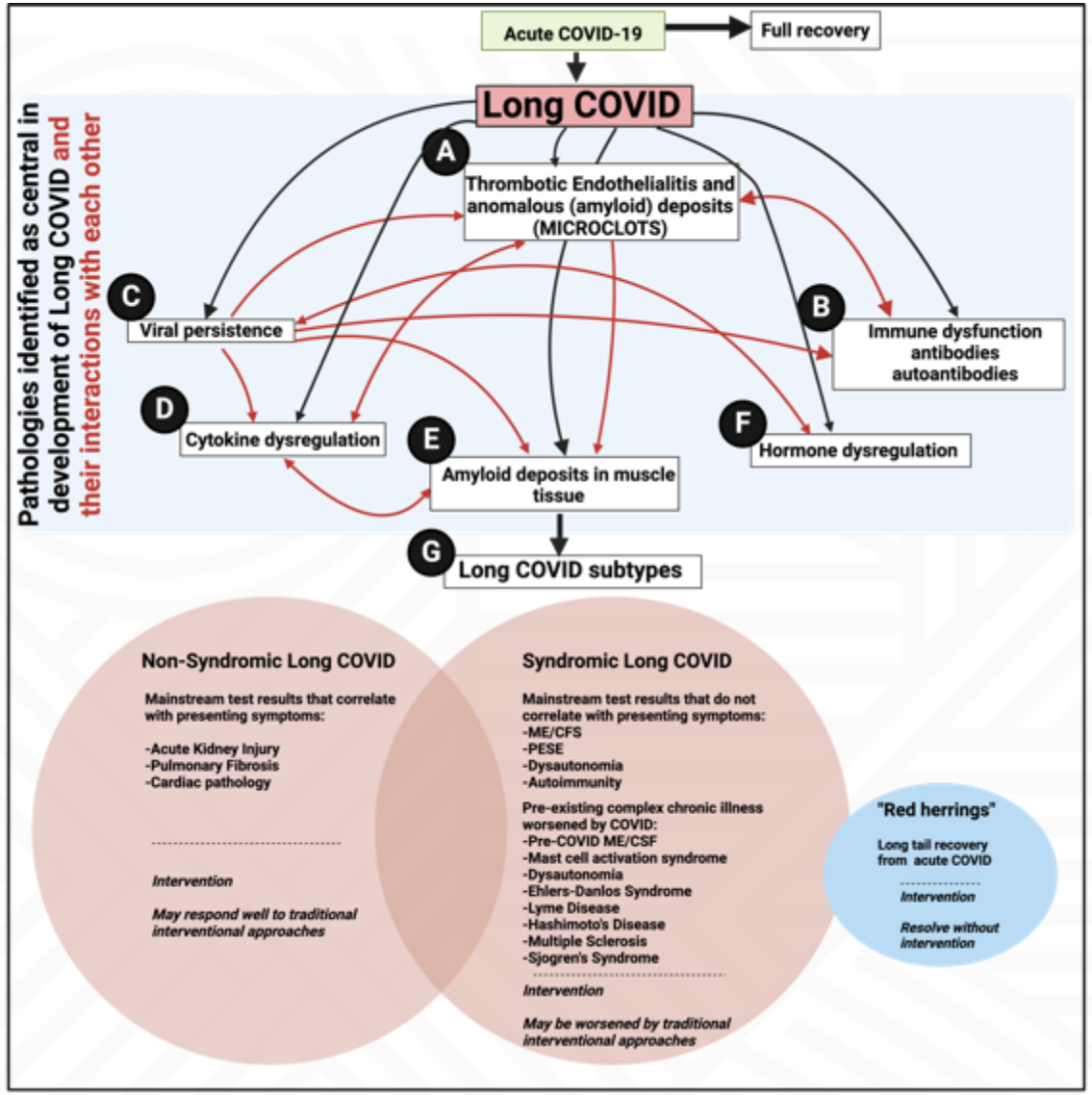


# Pathological clotting



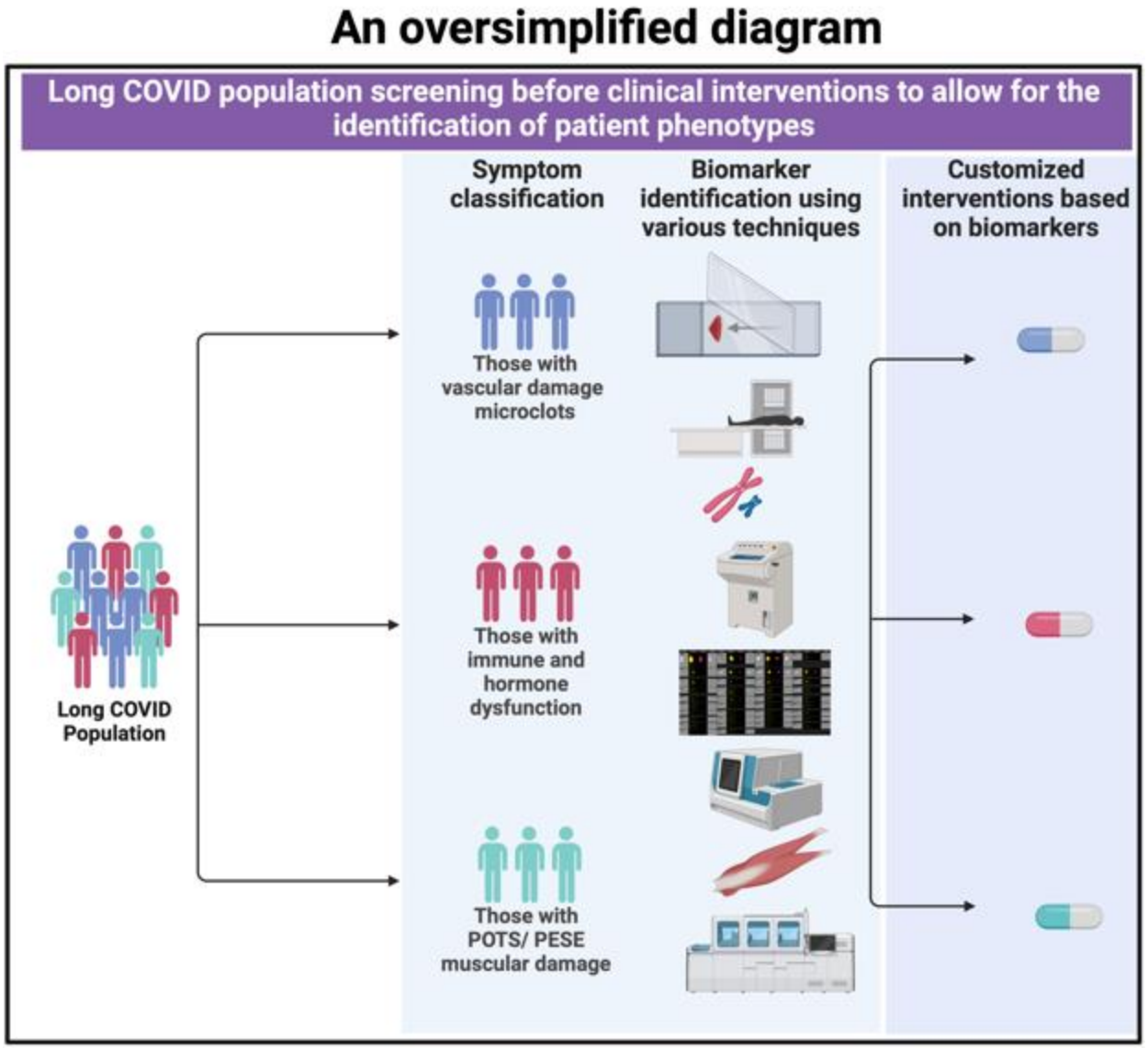

Kell DB, Pretorius E. Proteins behaving badly. Substoichiometric molecular control and amplification of the initiation and nature of amyloid fibril formation: lessons from and for blood clotting. *Progress in Biophysics and Molecular Biology* 2017; 123: 16-41.

# The current Long COVID hypotheses: what do we, and others say



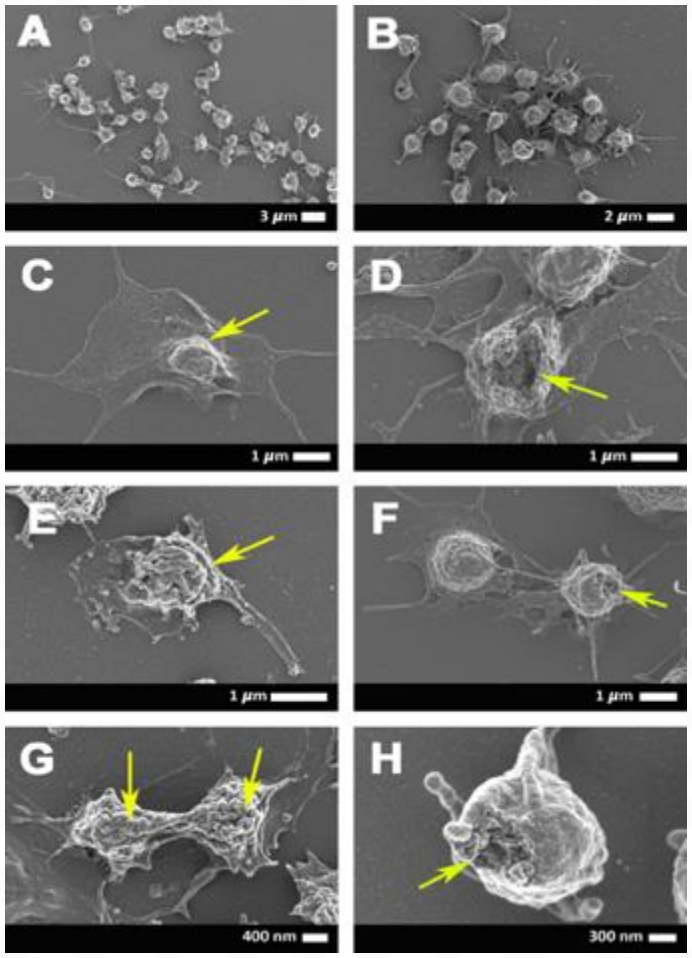

An overview figure to show the key pathologies (black arrows) involved in the long COVID, as well as their interactions with each other (red arrows). **A)** Thrombotic endothelialitis and microclots as described and reviewed by (Altmann et al., 2023, Turner et al., 2024, Okuducu et al., 2024, Kell et al., 2024, Kell et al., 2022, Kell and Pretorius, 2022, Kell and Pretorius, 2023, Kruger et al., 2022, Pretorius et al., 2021, Pretorius et al., 2022, Turner et al., 2023a, Turner et al., 2023b); **B)** Immune dysfunction and autoantibodies (Klein et al., 2023, Turner et al., 2023a, Kell and Pretorius, 2023, Saito et al., 2024); **C)** Viral persistence (Proal and VanElzakker, 2021, Kell and Pretorius, 2022, Proal et al., 2023); **D)** Cytokine dysregulation (Silva et al., 2024, Saito et al., 2024, Turner et al., 2024); **E)** Muscle involvement (Appelman et al., 2024); **F)** hormone dysregulation (Silva et al., 2024); **G)** subtypes of Long COVID as a result of the key pathologies driving the symptoms (Al-Aly et al., 2021, Turner et al., 2023a, Al-Aly and Topol, 2024).

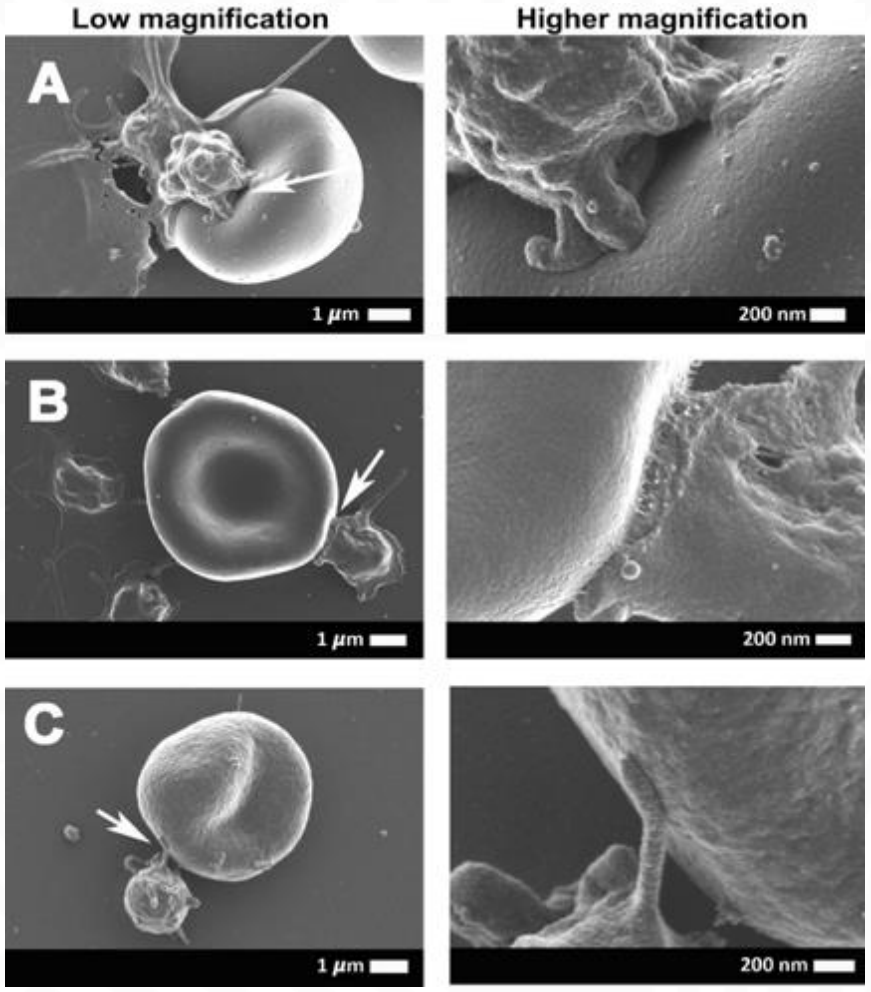
# What do we need in Long COVID research?



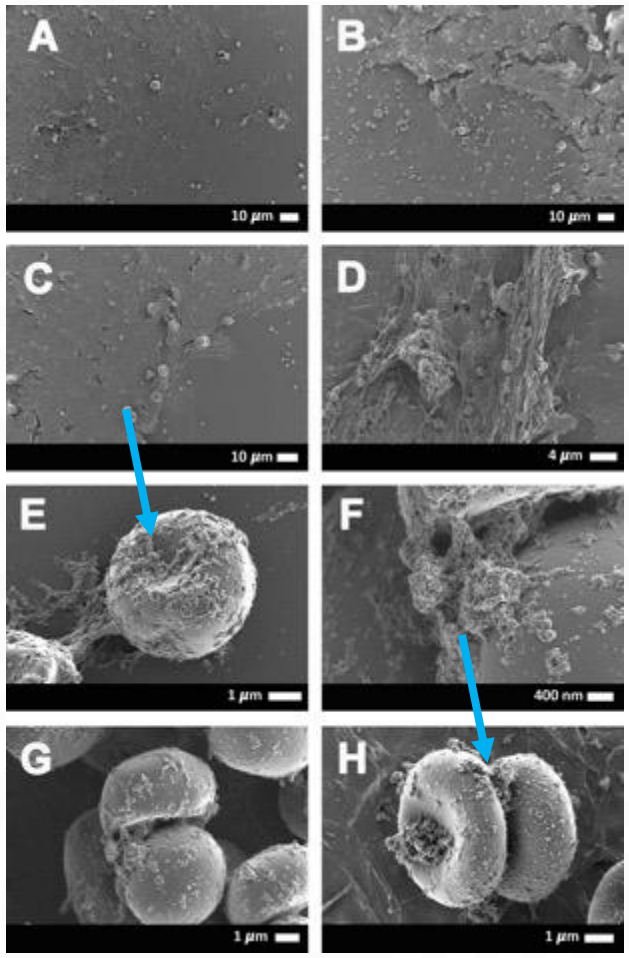
# Platelet and clotting pathologies in acute COVID-19

## Structural changes in platelets



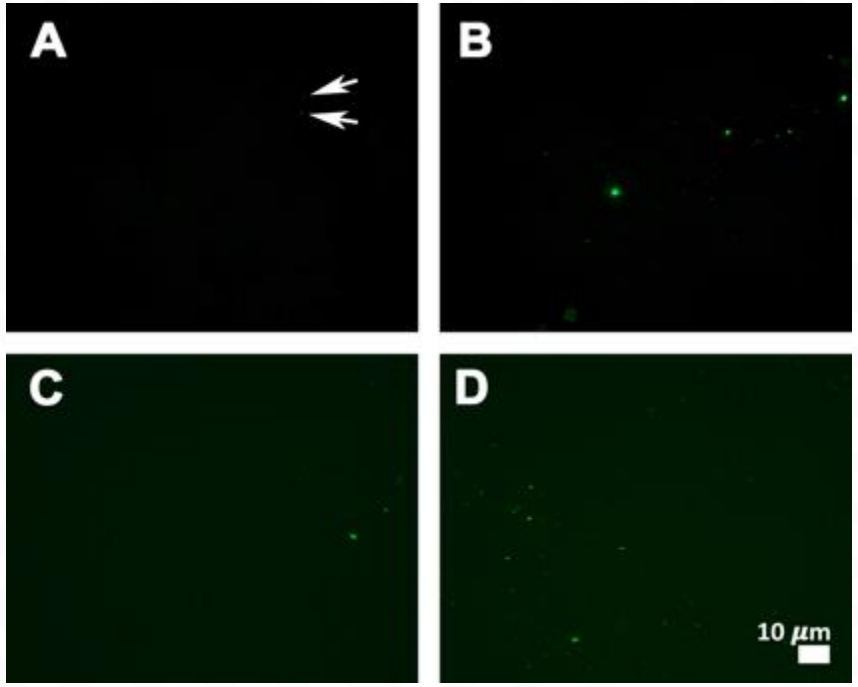




## Microclots

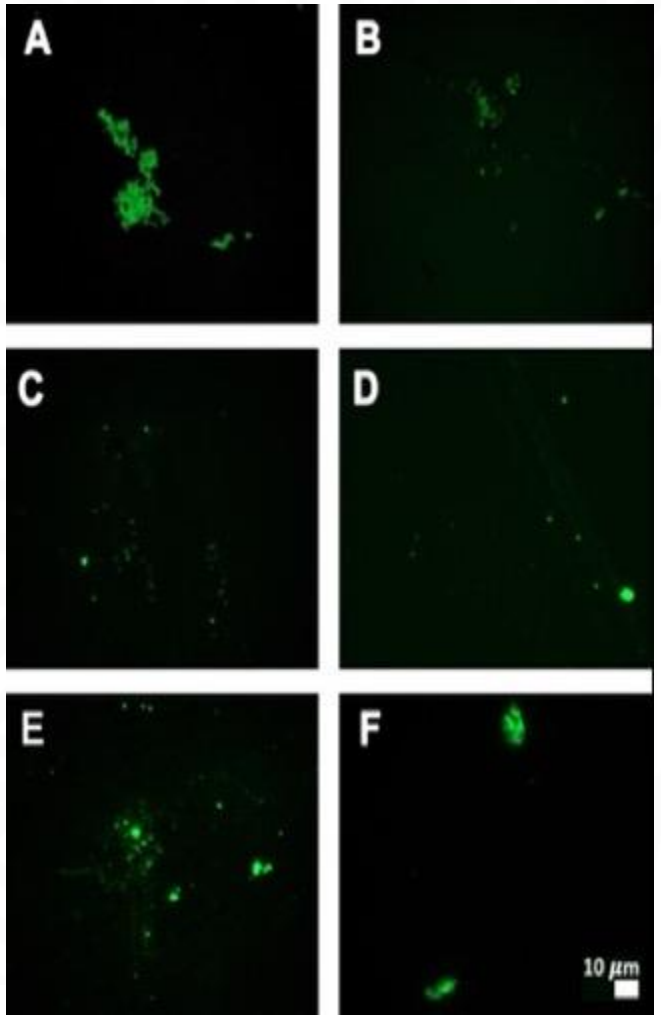


# Structural changes in fibrin(ogen)

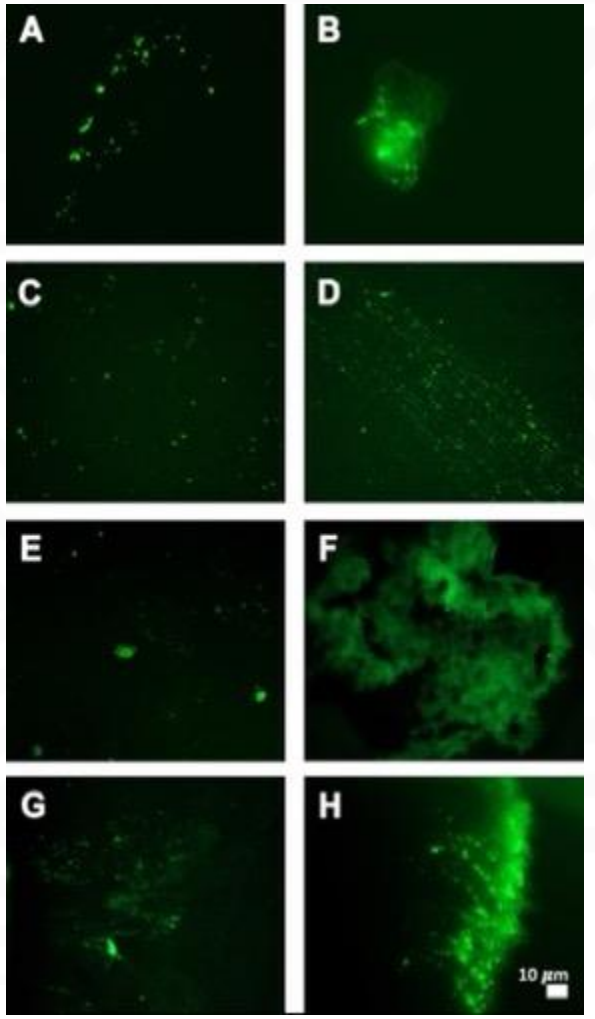
## Healthy Plasma



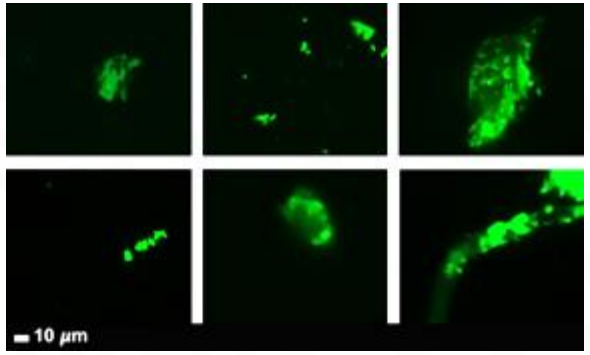

## Type 2 Diabetes Plasma

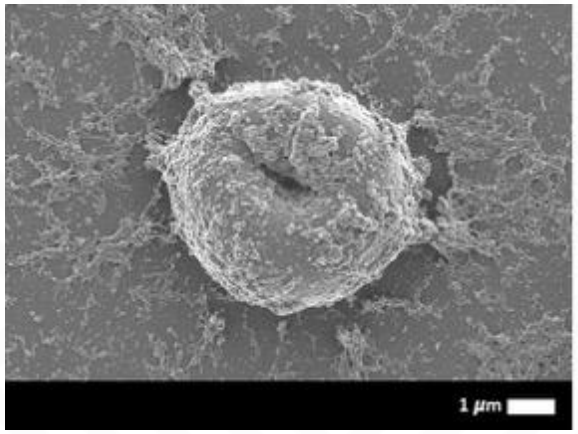


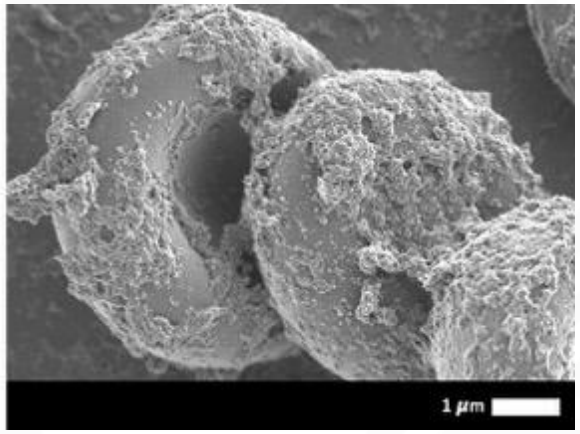

## COVID-19 Plasma

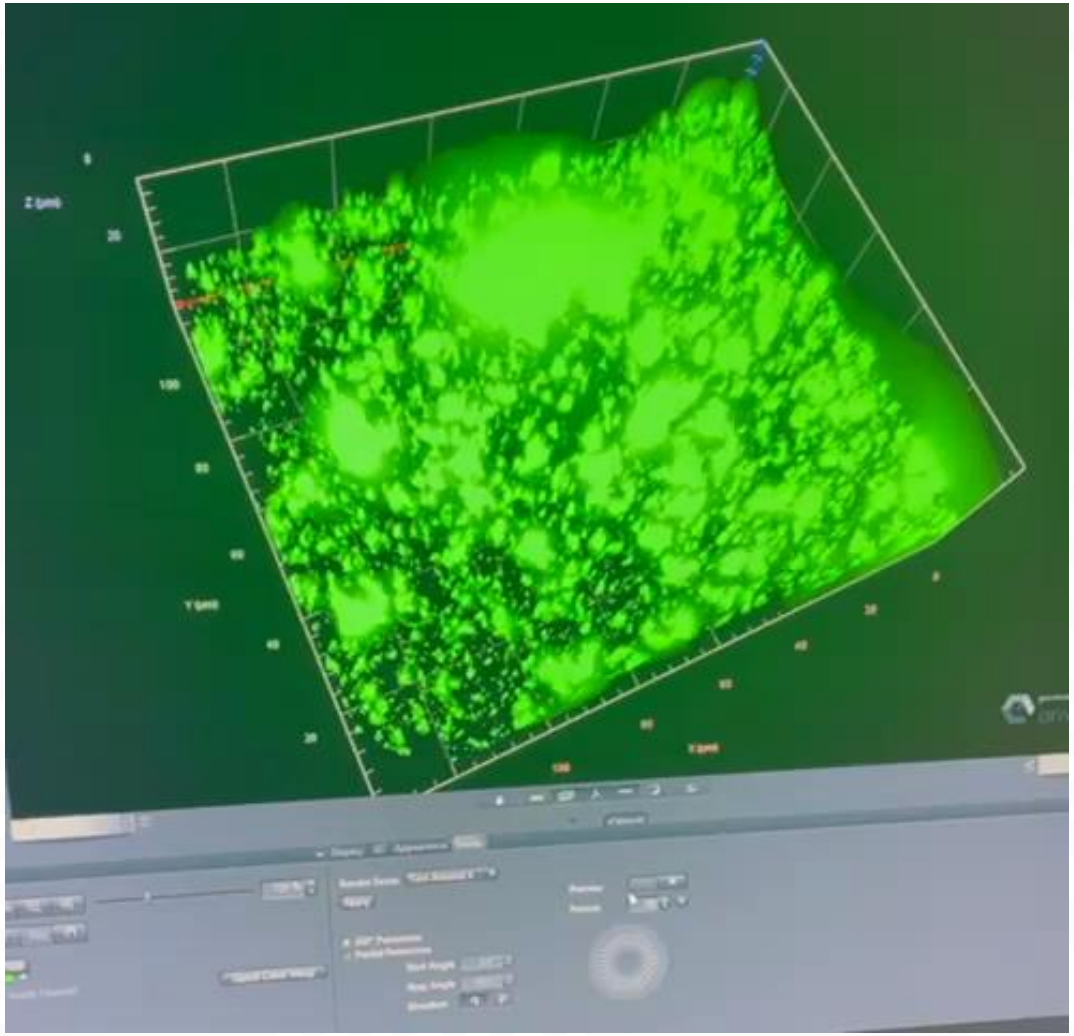


# Fluorescence microscopy and confocal microscopy in Long COVID



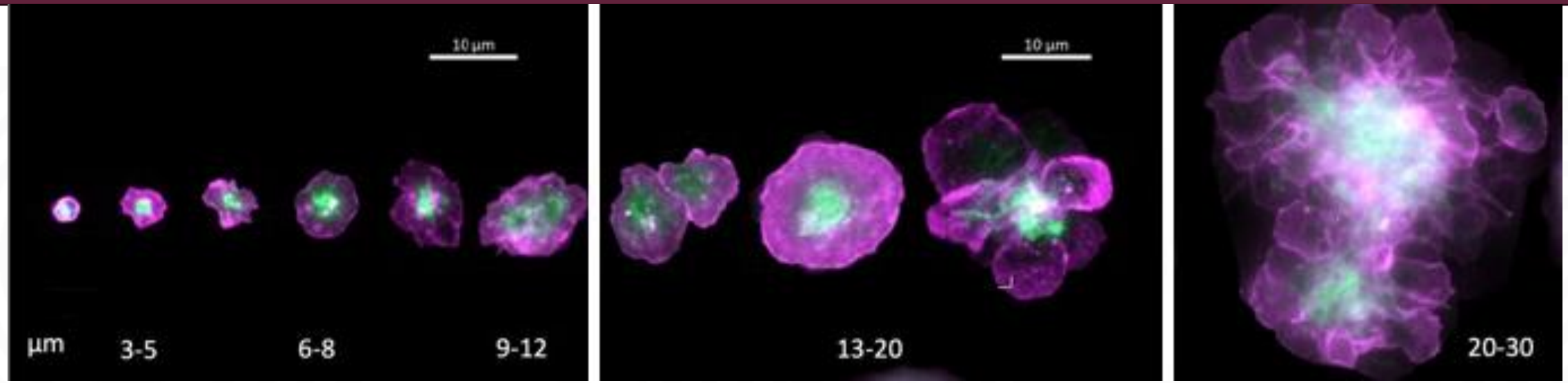



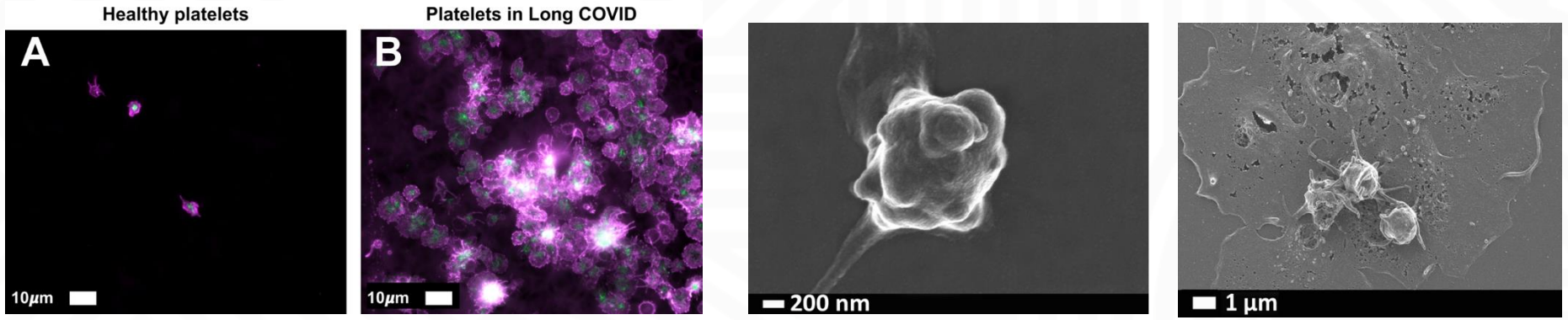
- An important diagnostic biomarker to either group patients
- An useful diagnostic marker, to include before and after treatment

# Fluorescence microscopy and confocal microscopy in Long COVID




**CD62P (PE-conjugated)** (Pinkish signal) = P-selectin

**PAC-1 (green signal)** = glycoprotein IIb/IIIa on the platelet membrane.




Laubscher GJ, Lourens PJ, Venter C, Kell DB, Pretorius E. TEG®, Microclot and Platelet Mapping for Guiding Early Management of Severe COVID-19 Coagulopathy. Journal of Clinical Medicine. 2021;10(22).

# Microclots, their content and diagnosis for hypercoagulation in Long COVID (and ME/CFS): a measurable biomarker in clinical trials

## Microclot Testing a new diagnostic frontier

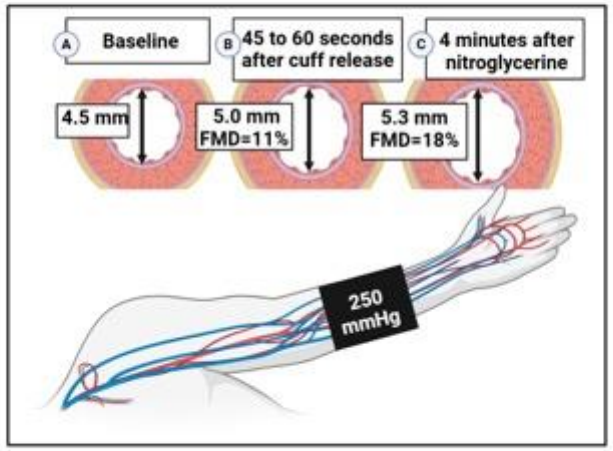
Our patented diagnostic technology detects abnormal microclots in the blood, indicating poor vascular health, inflammation, and an increased risk for thrombotic endotheliolitis. This pioneering test has been used to detect and aid in the treatment of microclots in thousands with Long Covid, a debilitating condition impacting millions worldwide and deeply affecting global health, society, and the economy.

License Microclot Technology

# Hypercoagulation (microclot formation/platelet hyperactivation) and endothelial damage

## Special investigations to consider in individuals with Long COVID:

1) **Flow Mediated Dilatation (FMD):**
Useful to confirm endothelial dysfunction.

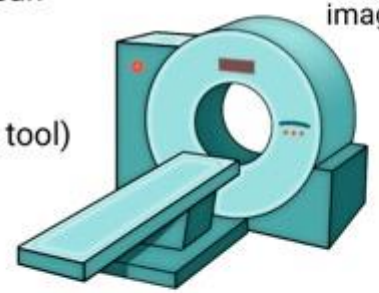



2) **Peripheral arterial tonometry and finger thermal monitoring:**
For example: The EndoPAT® device and E4-diagnose device (Polymath Company.)
Useful to confirm endothelial dysfunction.

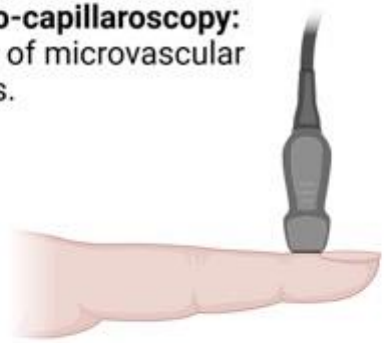
3) **Radiology:**
Useful to detect **vasculopathic** and **thrombotic phenomena.**

- SPECT / VQ scan
- DECT
- MRI (research tool)

Perfusion-based imaging



4) **Nailfold video-capillaroscopy:**
Identification of microvascular abnormalities.



Kruger A, Joffe D, Lloyd-Jones G, Khan MA, Šalamon Š, Laubscher GJ, et al. Vascular Pathogenesis in Acute and Long COVID: Current Insights and Therapeutic Outlook. Semin Thromb Hemost. 2024.

# The nature of microclots: are they indeed “clots” and can we use them as measurable diagnostic markers in clinical trials

- Are the dense amyloid deposits  indeed “microclots” or (simply) amyloid particles that are merely “present” in plasma, with no meaning or reason to worry about.

- The (perhaps philosophical questions) that we need to debate:
    - *If the amyloid deposits are present, why are they there?*
    - *If they are present in all inflammatory diseases (in higher concentrations and size ranges) than in controls: IS IT A REASON TO IGNORE THEM?*
    - ***Do we need to understand differences between microclots in different diseases?***

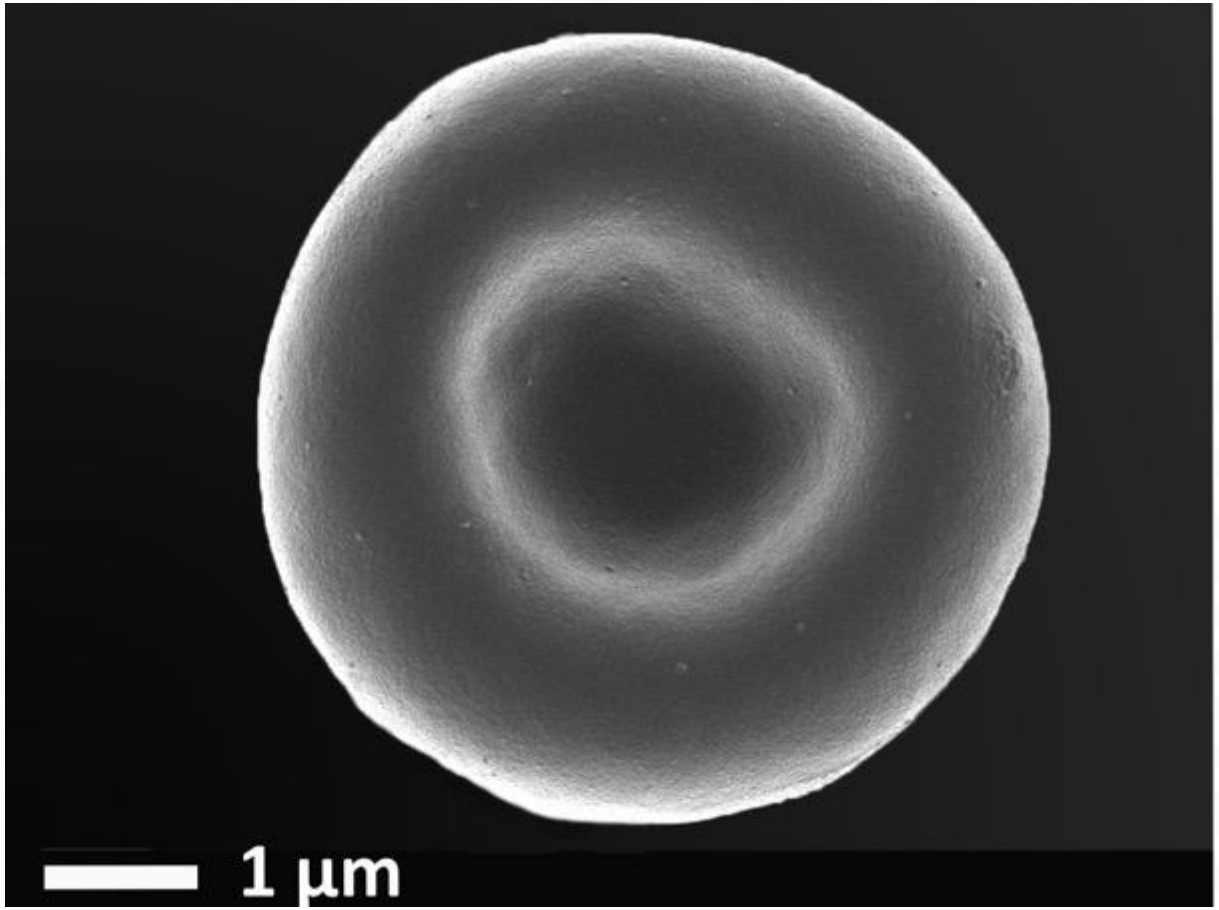
# The composition of a ”true” thrombus or blood clot

1. Platelets: Small blood cells that play a crucial role in the clotting process. They adhere to the site of a blood vessel injury, aggregate together, and form a temporary plug.
2. Fibrin: A protein formed from fibrinogen (a soluble plasma protein) during the clotting process. Fibrin strands weave through the platelet plug to stabilize and strengthen the clot.
3. Red Blood Cells (RBCs): These cells can become trapped in the fibrin mesh, contributing to the mass of the thrombus.
4. White Blood Cells (WBCs): These cells are also present in the thrombus and can play roles in inflammation and immune responses.
5. Neutrophil Extracellular Traps (NETs): In some thrombi, especially in conditions like infections and inflammation, neutrophils release NETs, which are networks of extracellular fibers that trap pathogens but can also contribute to the thrombus structure.
6. Other Plasma Proteins: Various other proteins, including clotting factors and inflammatory mediators, are involved in the formation and stability of the thrombus.

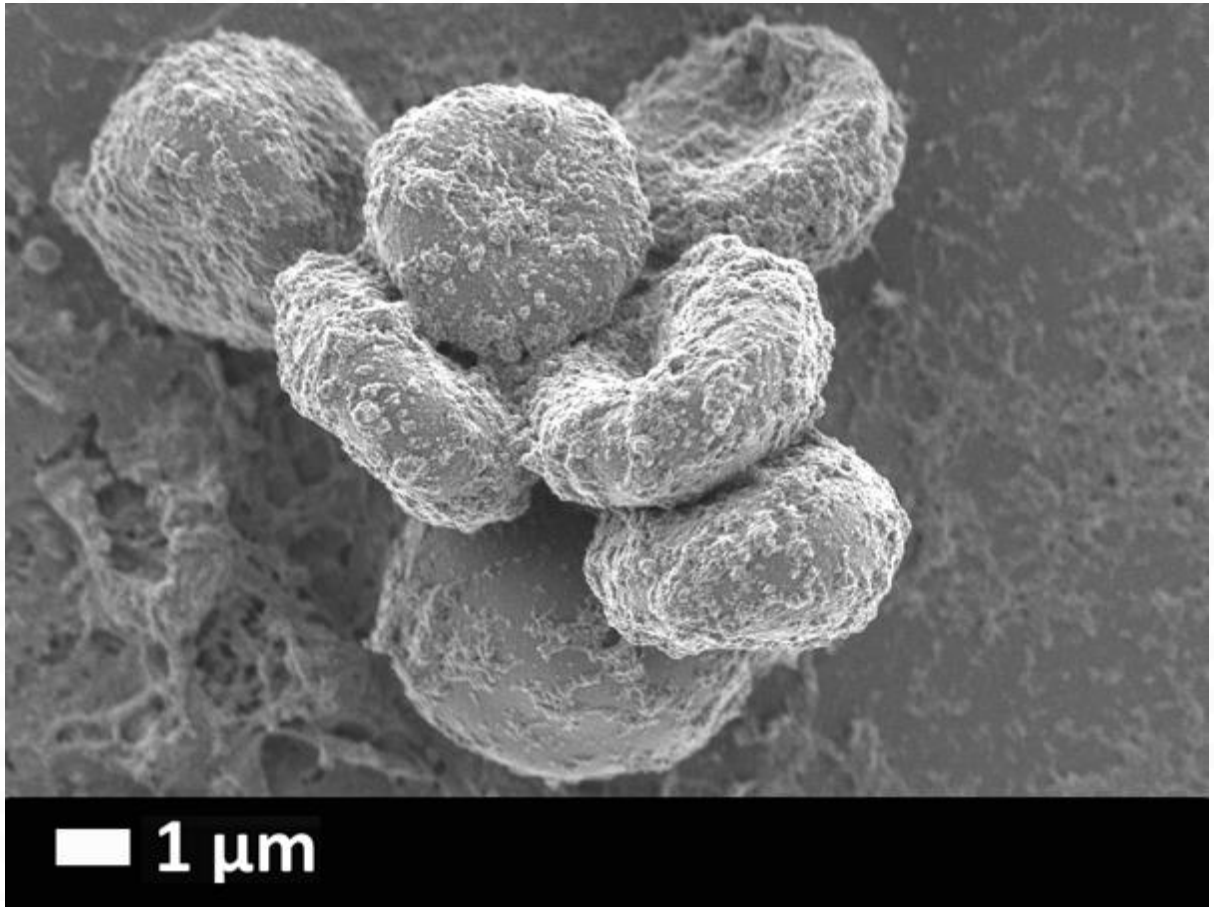
The composition and structure of a thrombus can vary depending on its location in the circulatory system and the underlying conditions that led to its formation.

# RBCs: Healthy vs Long COVID

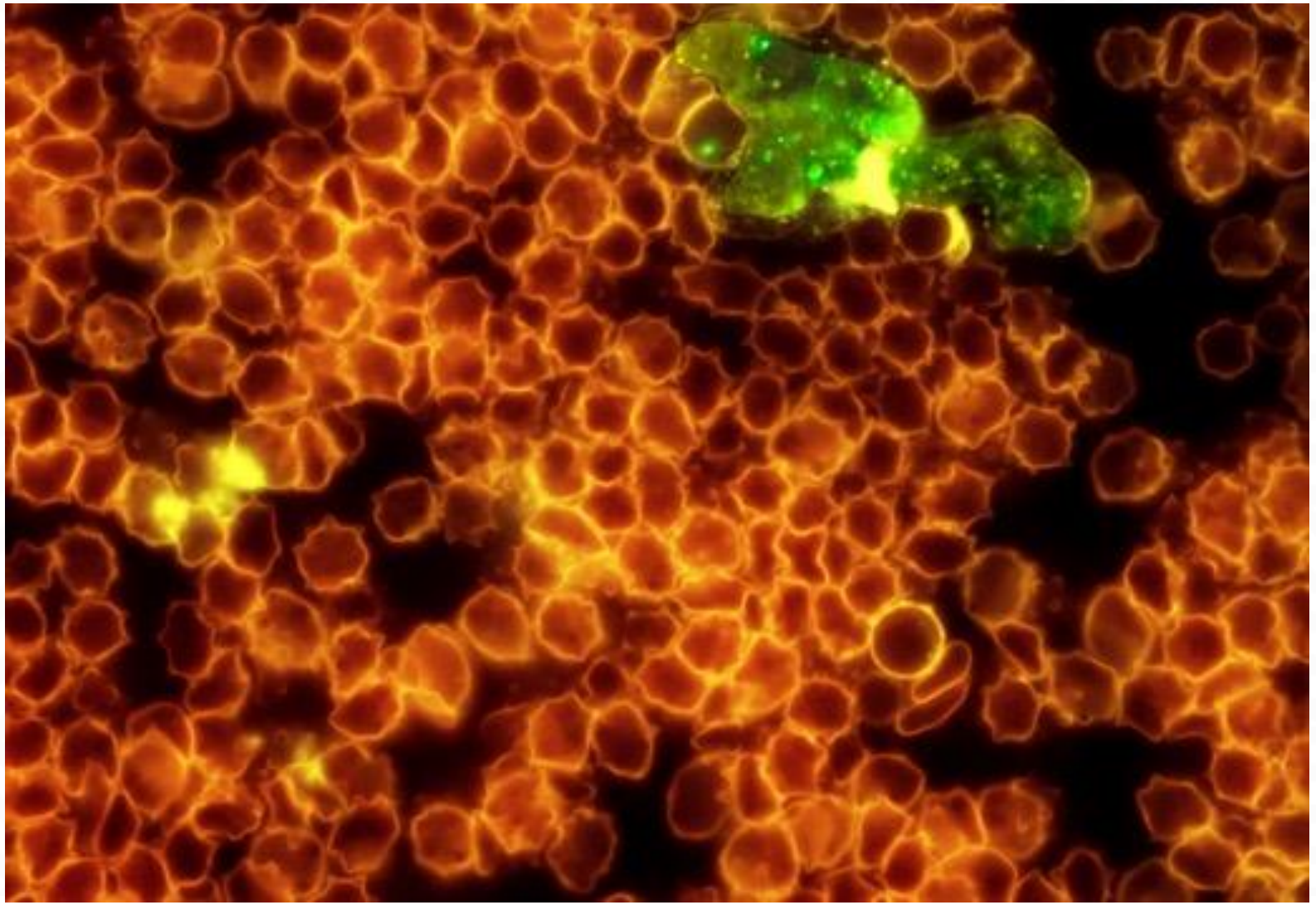
RBC from a healthy individual



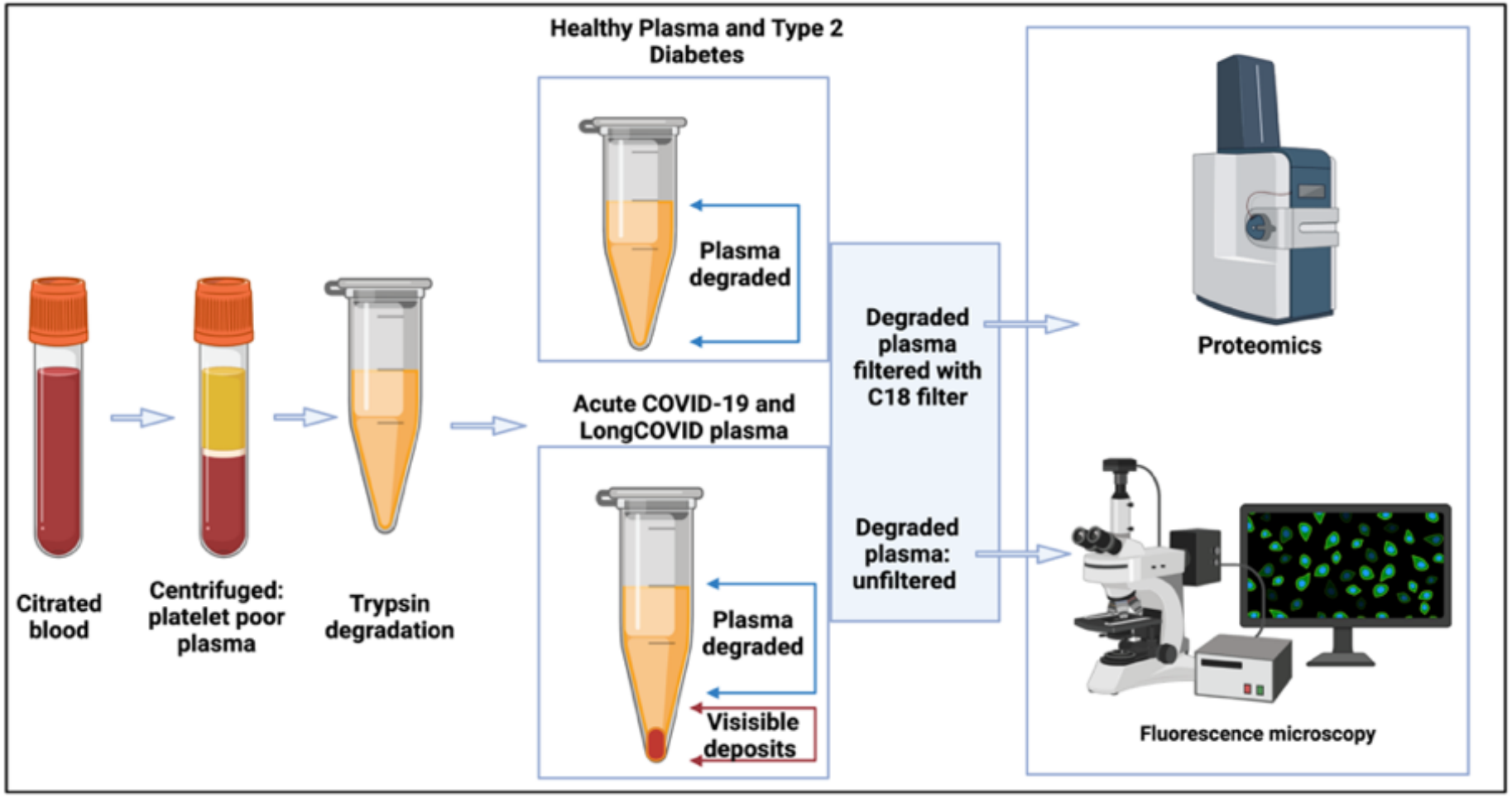

RBCs from individual with Long COVID covered with microclots



# RBCs and microclots in Long COVID



# Lysis-resistant microclots: Proteomics of plasma from healthy, diabetic, acute COVID-19 and Long COVID




Pretorius, E., Vlok, M., Venter, C., Bezuidenhout, J.A., Laubscher, G.J., Steenkamp, J., and Kell, D.B. (2021). Persistent clotting protein pathology in Long COVID/Post-Acute Sequelae of COVID-19 (PASC) is accompanied by increased levels of antiplasmin. Cardiovasc Diabetol *20*, 172.

# 2021 Proteomics Analysis: are they indeed “clots”?

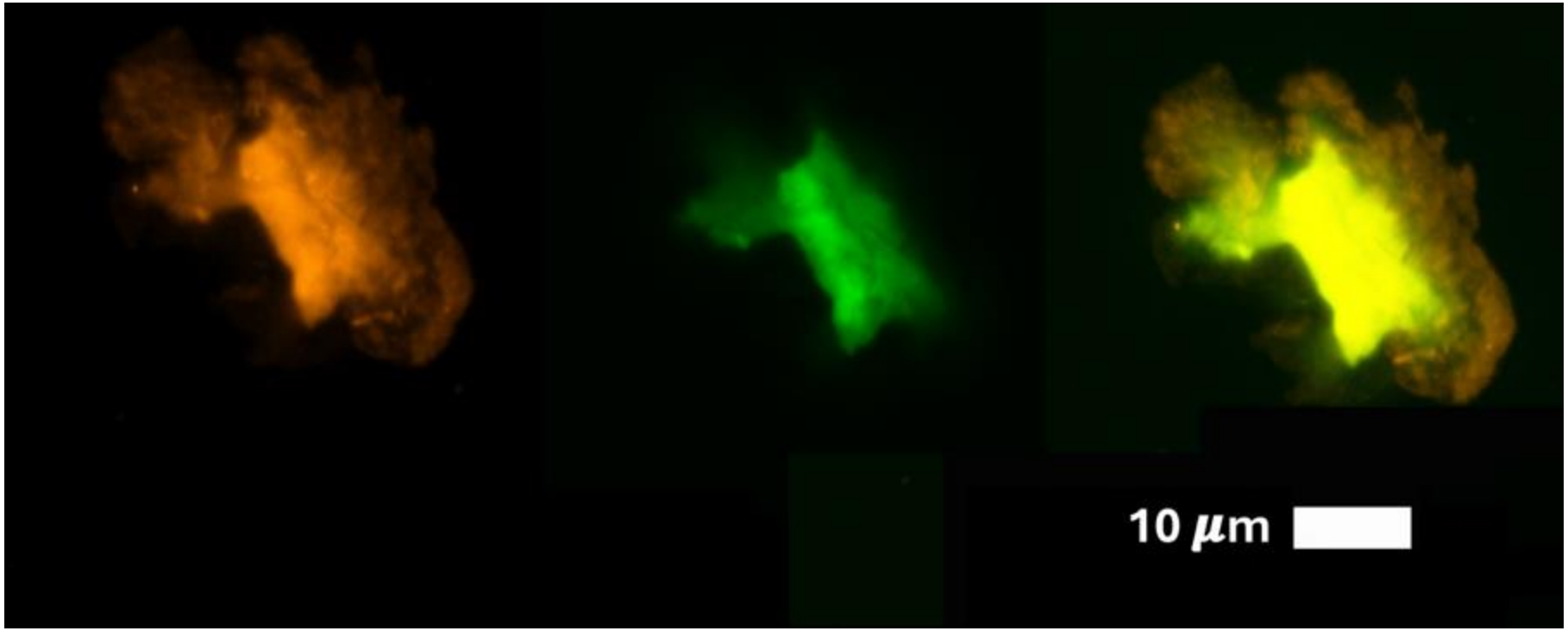
Pretorius, E., Vlok, M., Venter, C., Bezuidenhout, J.A., Laubscher, G.J., Steenkamp, J., and Kell, D.B. (2021). Persistent clotting protein pathology in Long COVID/Post-Acute Sequelae of COVID-19 (PASC) is accompanied by increased levels of antiplasmin. Cardiovasc Diabetol *20*, 172.

| | | |
|---|---|---|
| **Digested pellet deposits (microclots) from acute COVID-19 samples vs digested plasma from Control samples<br>These proteins are present in both sample types; and a fold change value more than 1 = the protein that more prevalent inside the digested pellet deposits from COVID-19 samples. These proteins were concentrated inside the digested pellet deposits.** | | |
| Protein name | Fold change | P-value |
| von Willebrand Factor | 4.5 | 0.02 |
| Complement component C4b | 4.1 | 0.05 |
| C-reactive protein | 18.7 | 0.003 |
| **Digested pellet deposits from Long COVID/PASC microclots samples vs digested plasma from Control samples<br>These proteins are present in both sample types; and a fold change value more than 1 = the protein that more prevalent inside the digested pellet deposits from Long COVID/PASC samples. These proteins were concentrated inside the digested pellet deposits.** | | |
| Coagulation factor XIII A chain | 6.9 | 0.001 |
| Plasminogen | 3 | 0.001 |
| Fibrinogen alpha chain | 4.1 | 0.0001 |
| α2 antiplasmin (α2AP) | **7.9** | **0.0002** |
| von Willebrand Factor | 10.2 | 0.001 |
| C-reactive protein | 11.2 | 0.007 |
| Serum Amyloid A (SAA4) | 17.5 | 0.01 |
| Complement component C7 | 20 | 0.0002 |
| **Digested pellet deposits from Long COVID/PASC microclots samples vs digested pellet deposits (microclots) from acute COVID-19 samples<br>These proteins are present in both sample types; and a fold change value more than 1 = the protein that more prevalent inside the digested pellet deposits from Long COVID/PASC samples. These proteins were concentrated inside the digested pellet deposits.** | | |
| Plasminogen | 2.3 | 0.0007 |
| Fibrinogen β chain | 2.8 | 0.007 |
| Coagulation factor XIII B | 2.7 | 0.01 |
| Fibrinogen α chain | 3.1 | 0.0002 |
| Complement component C6 | 7.5 | 0.01 |
| α2 antiplasmin (α2AP) | **9.2** | **0.0003** |
| Complement factor 1 | 25 | 0.0009 |

# Soluble purified fibrinogen can change to an insoluble amyloid microclot when spike protein is added

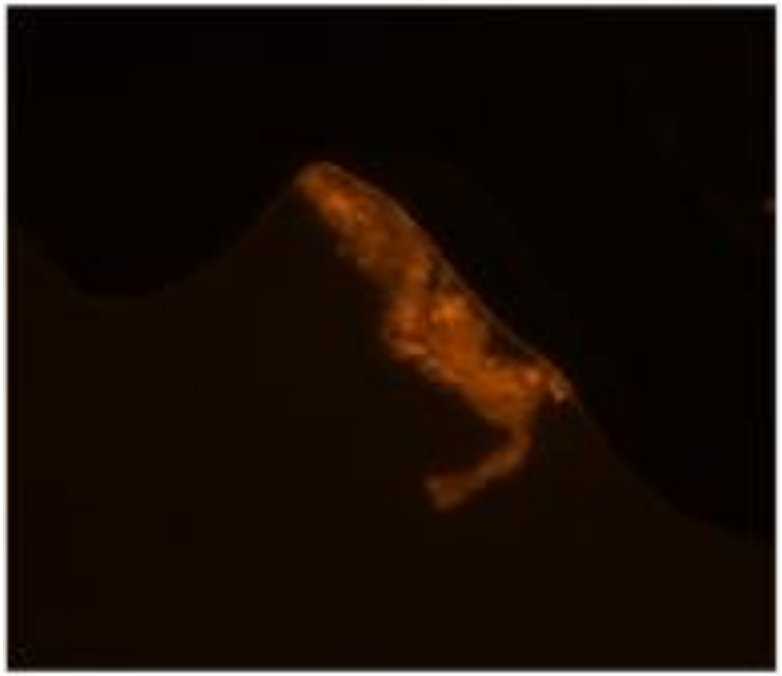
## Purified fibrinogen with spike (100 ng.ml$^{-1}$)

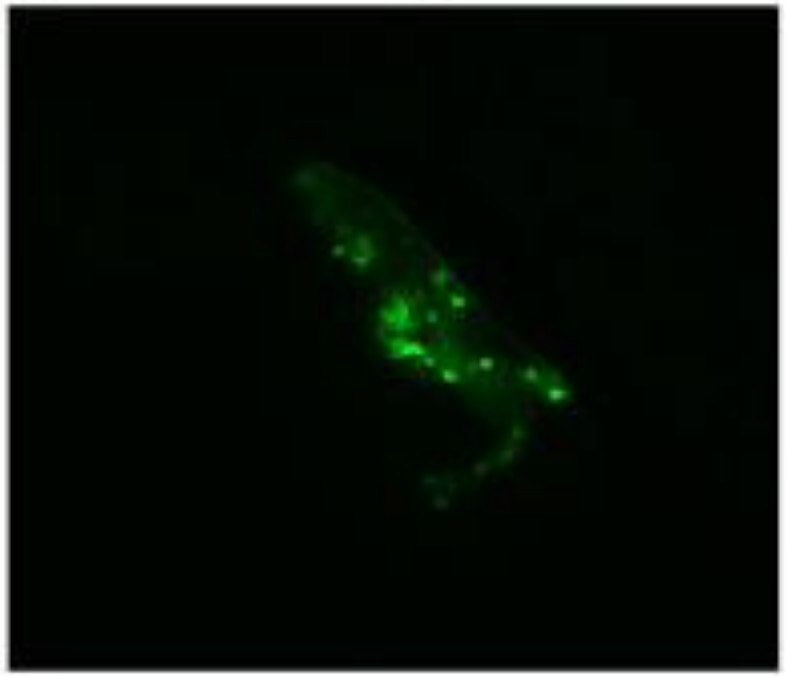
- $\alpha$-chain Fibrinogen antibody
- Thioflavin T

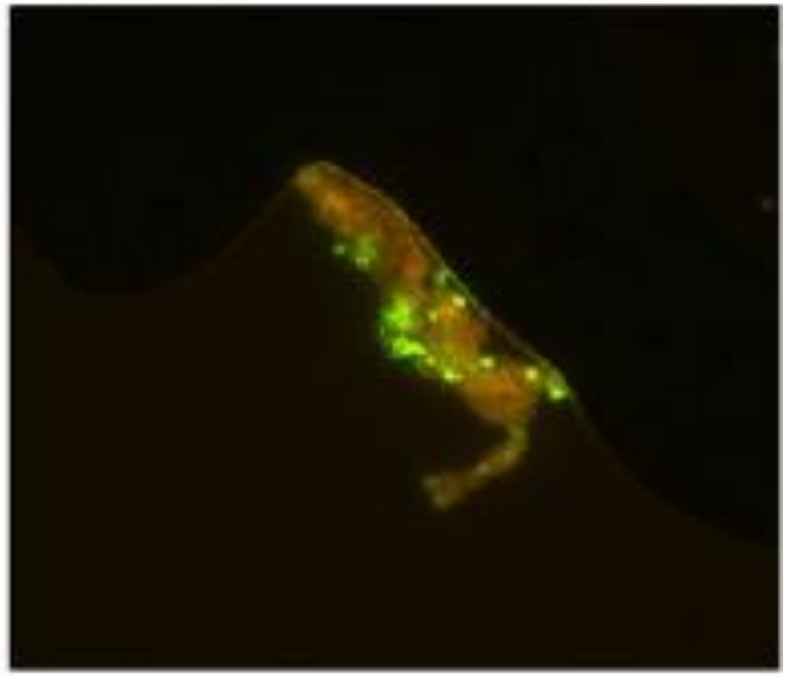


# Soluble Purified Fibrinogen can change to an insoluble amyloid microclot when LPS (50 ng.L$^{-1}$) is added

- $\alpha$-chain Fibrinogen antibody
- Thioflavin T







# More confirmation of pathological fibrin and a possible monoclonal

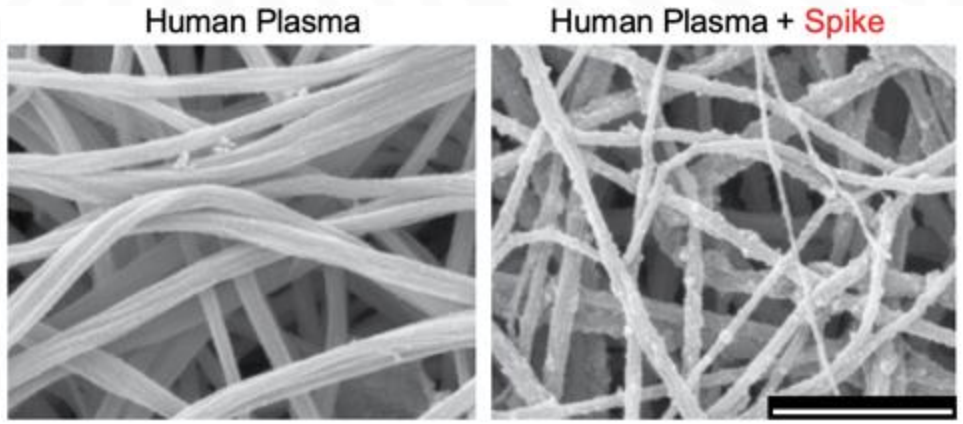
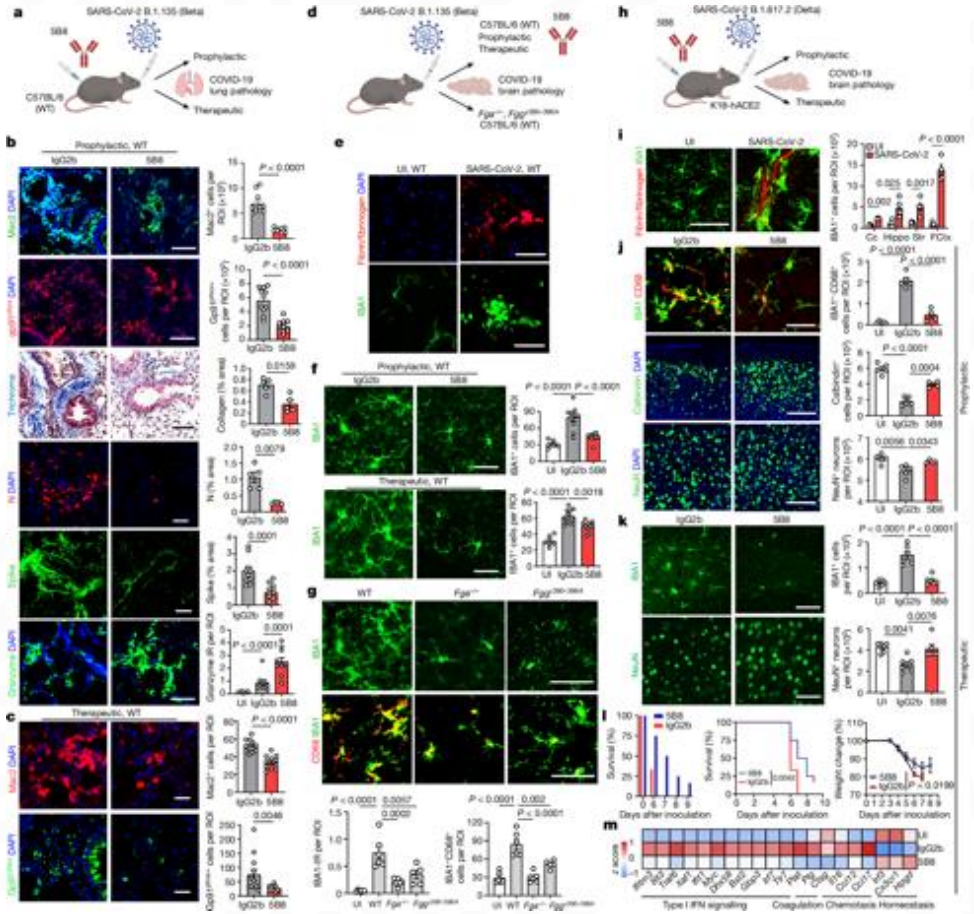



Fig. 5: Anti-fibrin antibody provides protection against SARS-CoV-2



J. K. Ryu, Z. Yan, M. Montano, E. G. Sozmen, K. Dixit, R. K. Suryawanshi, et al. **Fibrin drives thromboinflammation and neuropathology in COVID-19** Nature 2024 https://doi.org/10.1038/s41586-024-07873-4

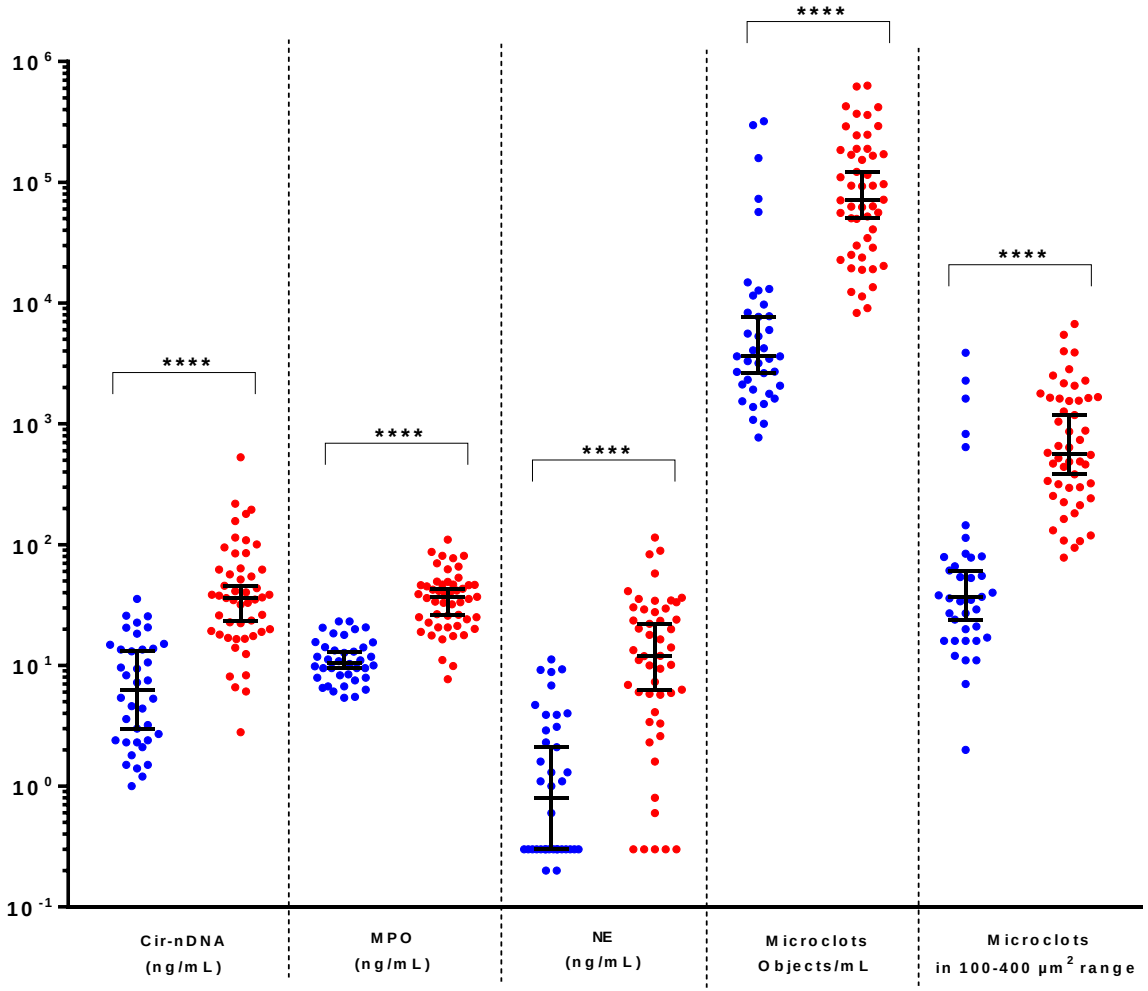
# INSERM net study with Alain Thierry

MPO

Neutrophil Elastase

CirDNA

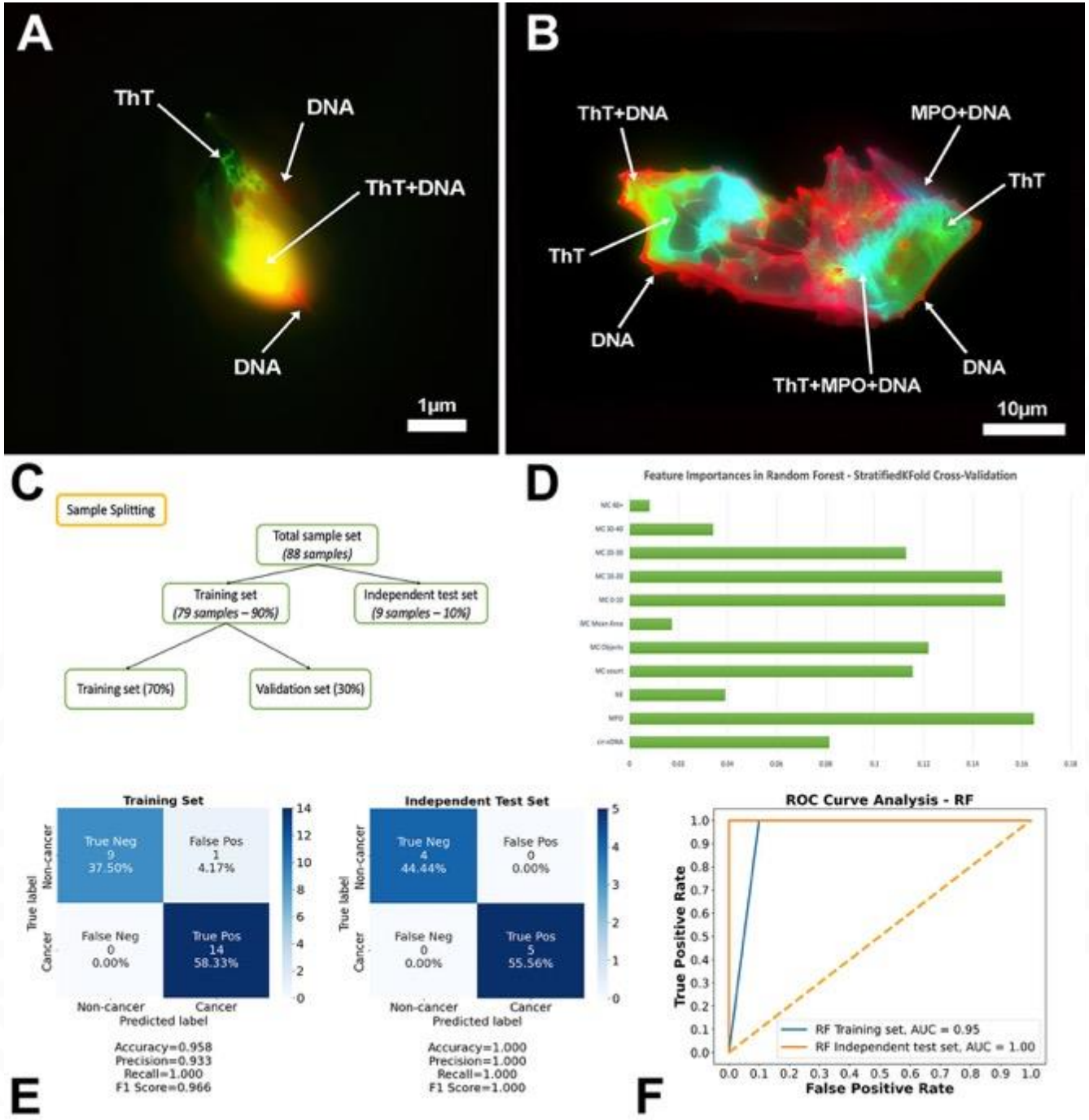
Microclots



# NETs and microclots are structurally associated: another measurable biomarker

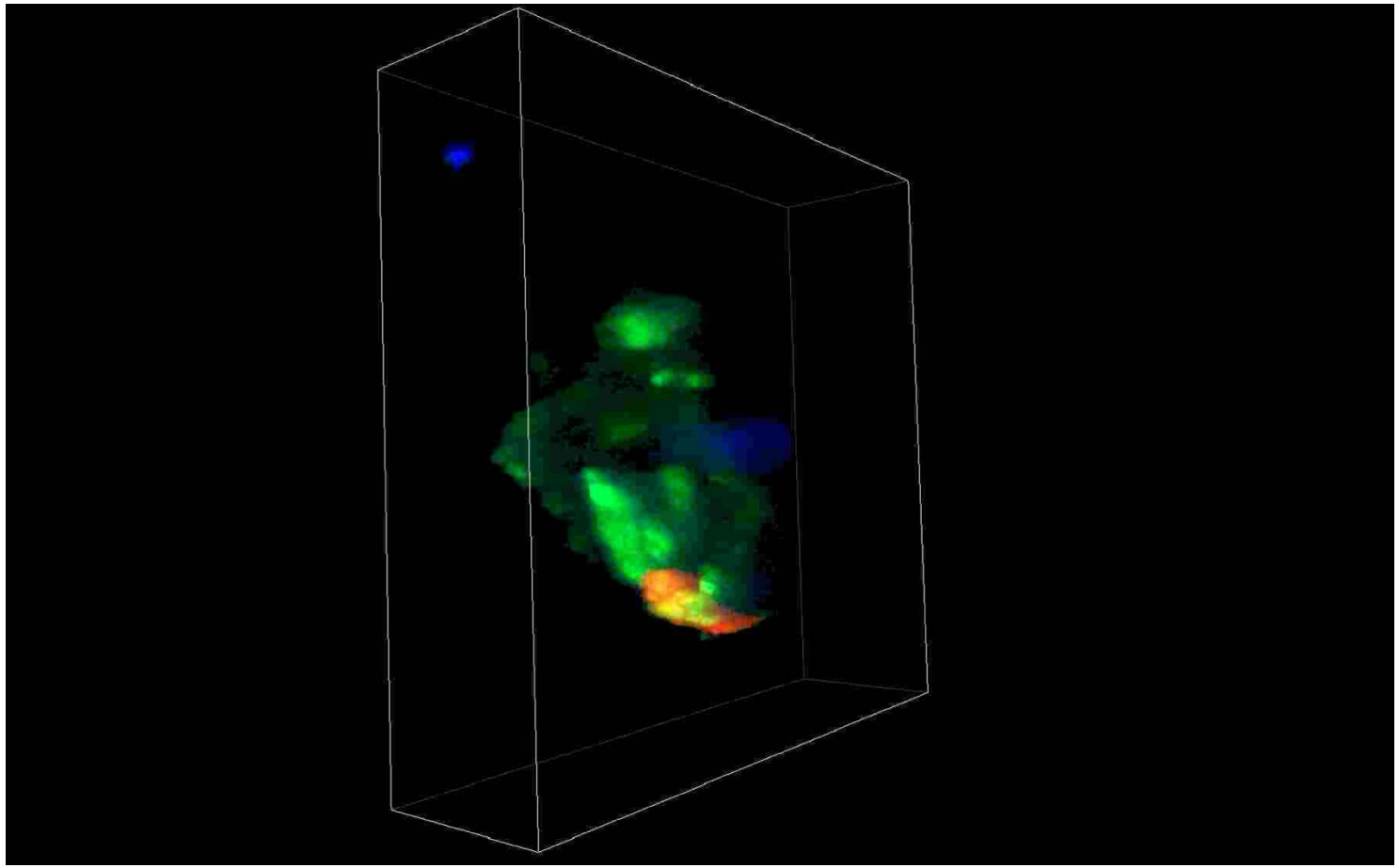
## A collaboration with the Alain Thierry group: INSERM

- Hoechst (Blue) (*or as here SYTO (red)*) stains for DNA
- ThT (green) Amyloid Microclots
- MPO (blue)



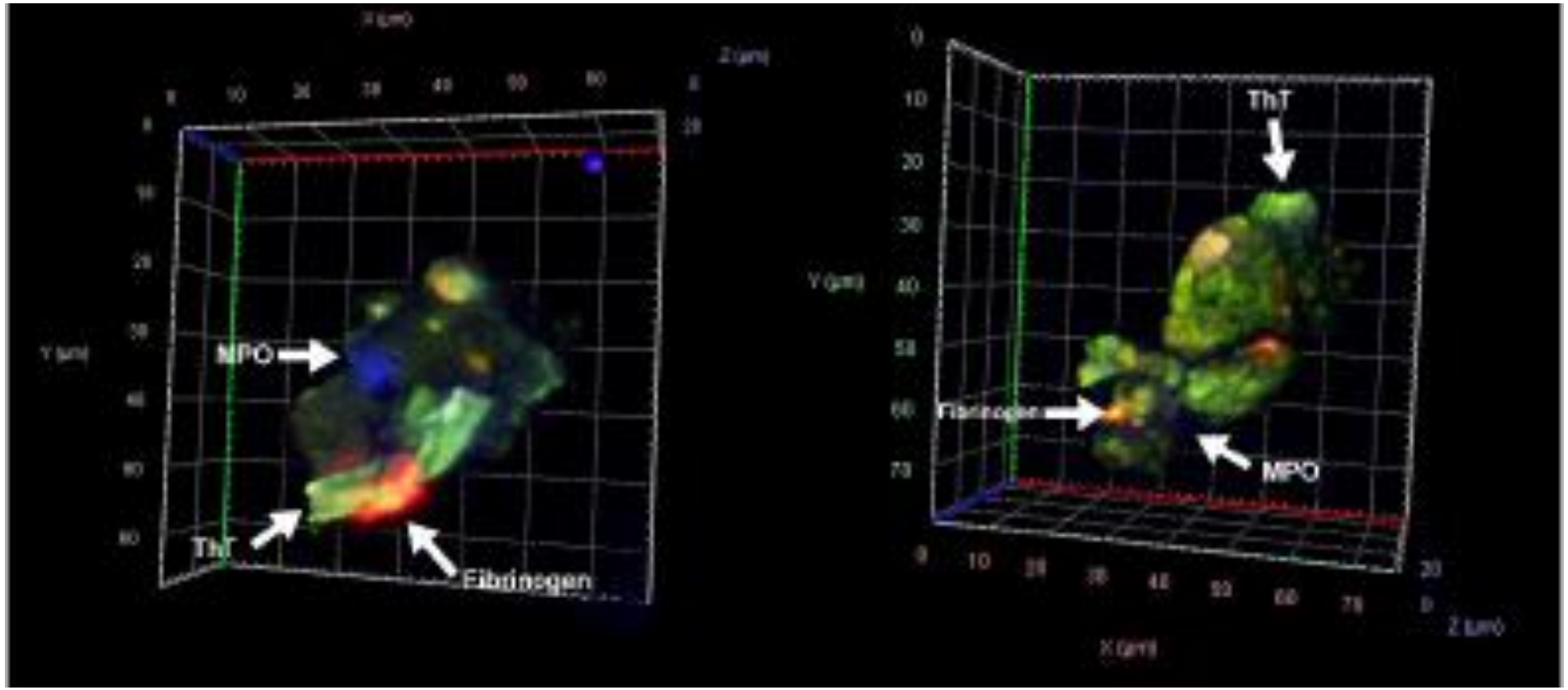
# A measurable biomarker: Long COVID microclot composition

- Thioflavin T: amyloid protein
- Fibrinogen $\alpha$-chain antibody
- MPO (associated with NETs)

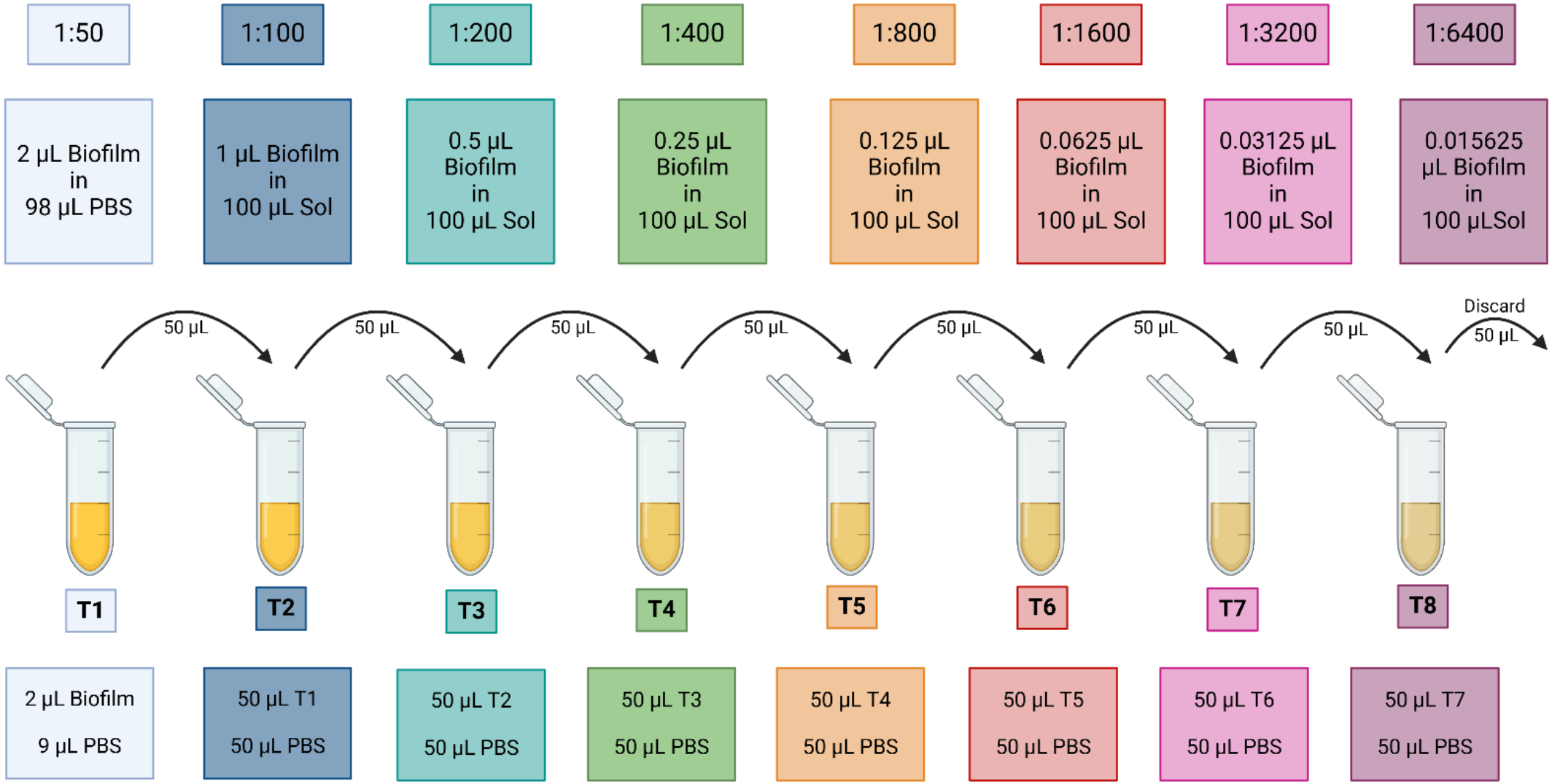


# Long COVID microclot (MPO: blue) (Fibrinogen antibody: red) (ThT green)

- Thioflavin T: amyloid protein
- Fibrinogen $\alpha$-chain antibody
- MPO (associated with NETs)

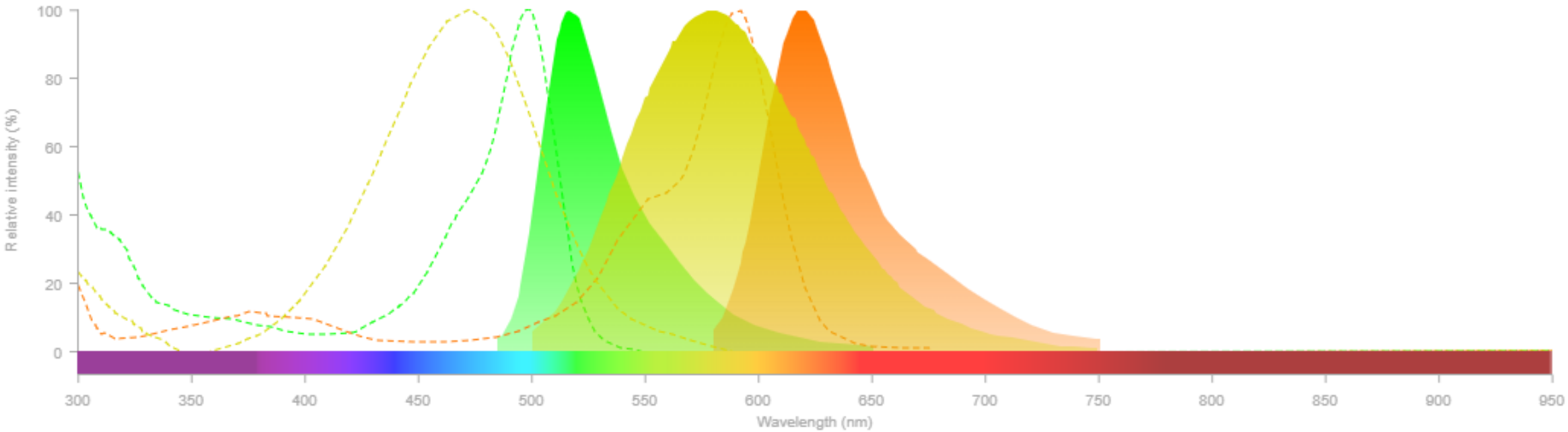


# Biofilm marker: optimizing dilution series for titrating FilmTracer™ FM® 1-43 Green Biofilm marker



# SpectraViewer: Biofilm

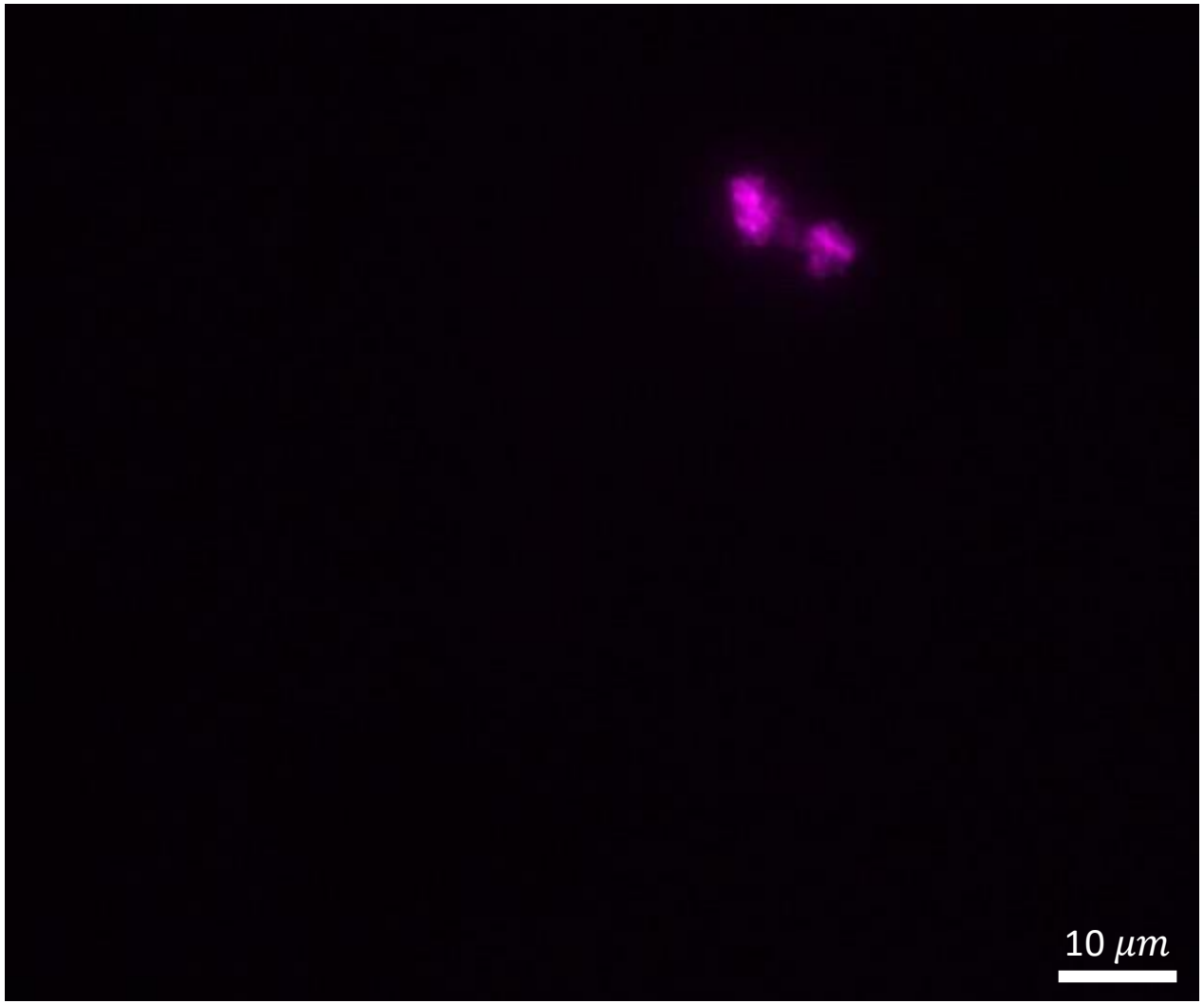
- FM1-43 stain: Biofilm
- Thioflavin T (ThT) dye
- Alexa-Fluor 594 antibody: against Fibrinogen $\alpha$-chain




ThermoFisher SpectraViewer provided a good overview of the spill over between fluorophores and, thus, the ability to view these fluorophores separately and in combination with ones that we would liek to use together. It thus allowed us to prevent spill-over.

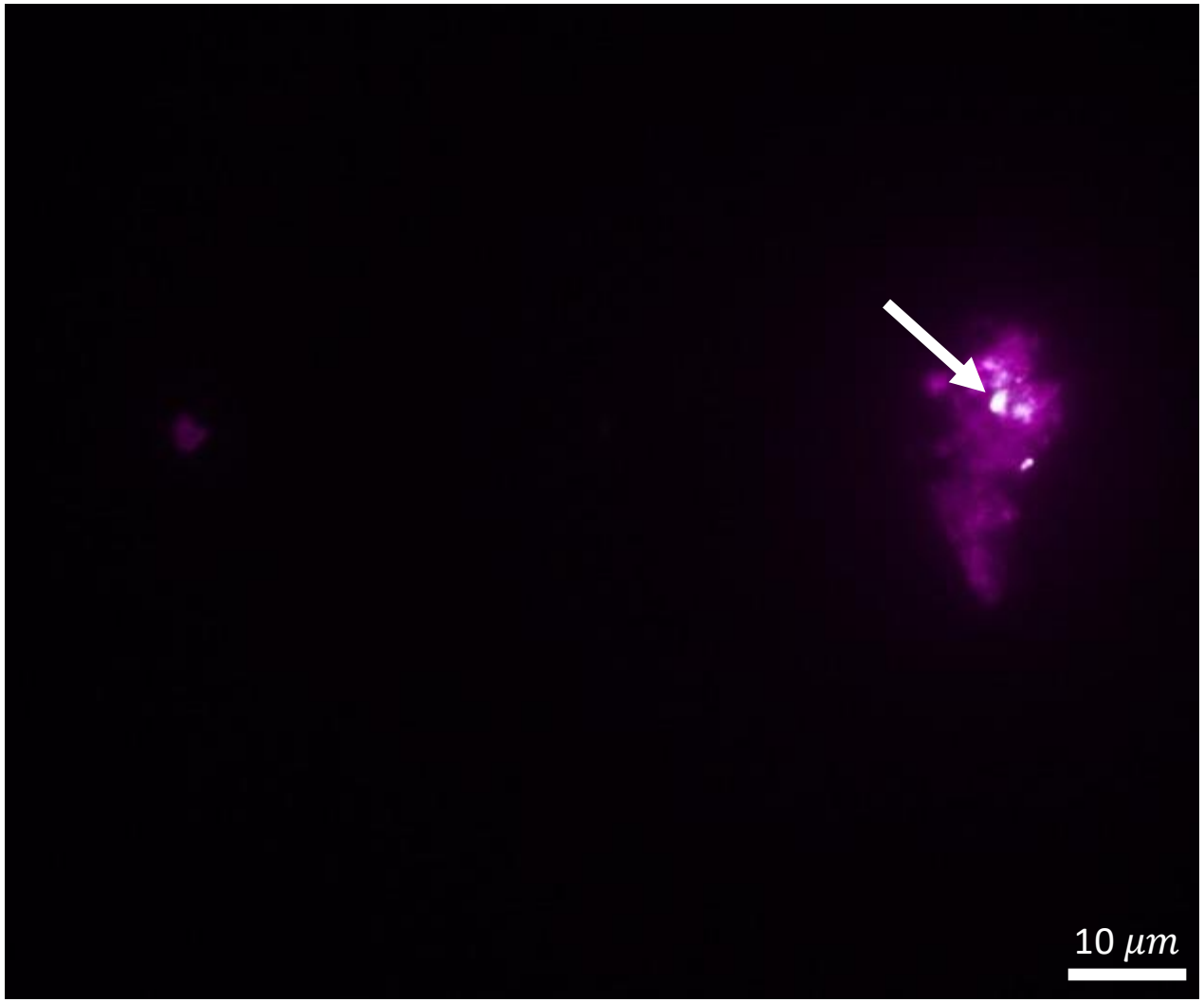
# Biofilm matrices in platelet poor plasma associated with microclots in Long COVID

- FM1-43 Biofilm stain



# Biofilm & Microclot Co-localisation in Long COVID

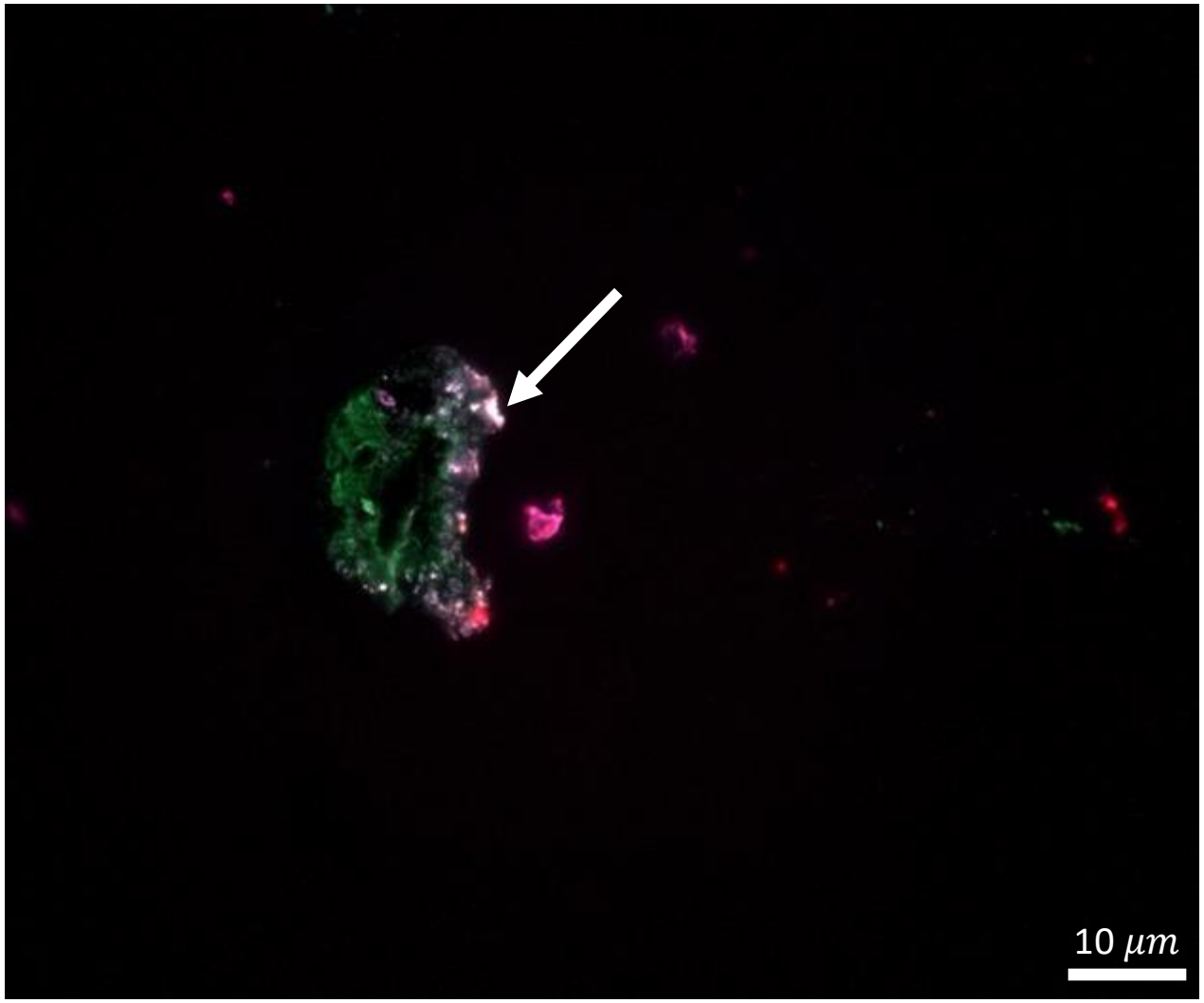
- FM1-43 stain: Biofilm
- Thioflavin T (ThT) dye




Co-localisation indicated by white arrow

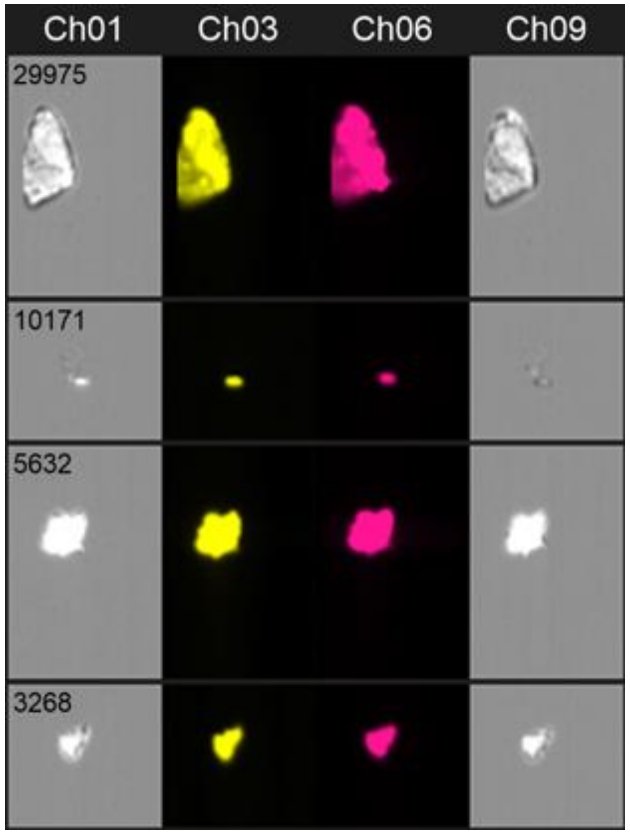
# Biofilm, Microclots & Fibrinogen in Long COVID

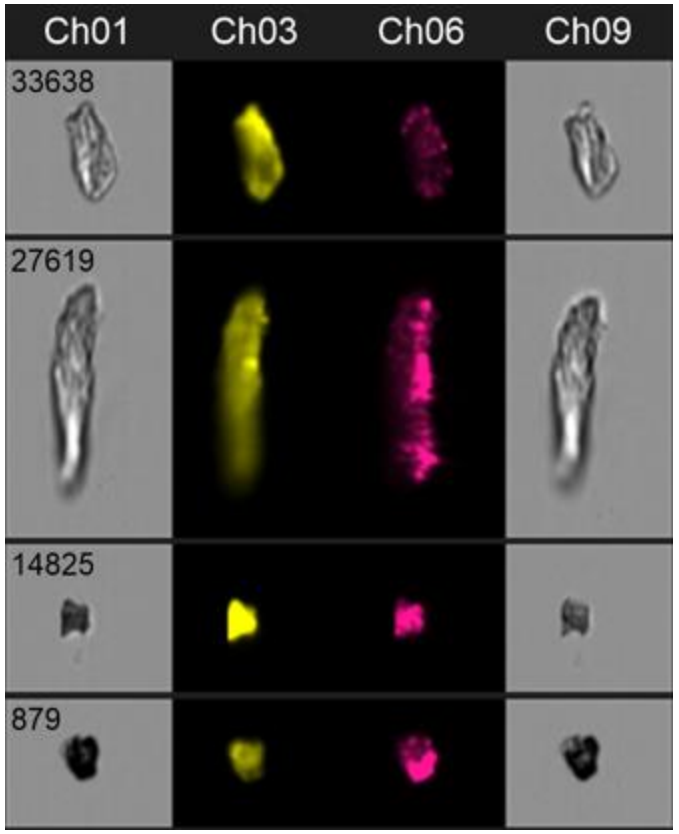
- **FM1-43 stain: Biofilm**
- **Thioflavin T (ThT) dye**
- **Fibrinogen $\alpha$-chain antibody**




Co-localisation indicated by white arrow

# Biofilm associating with Long COVID Microclots



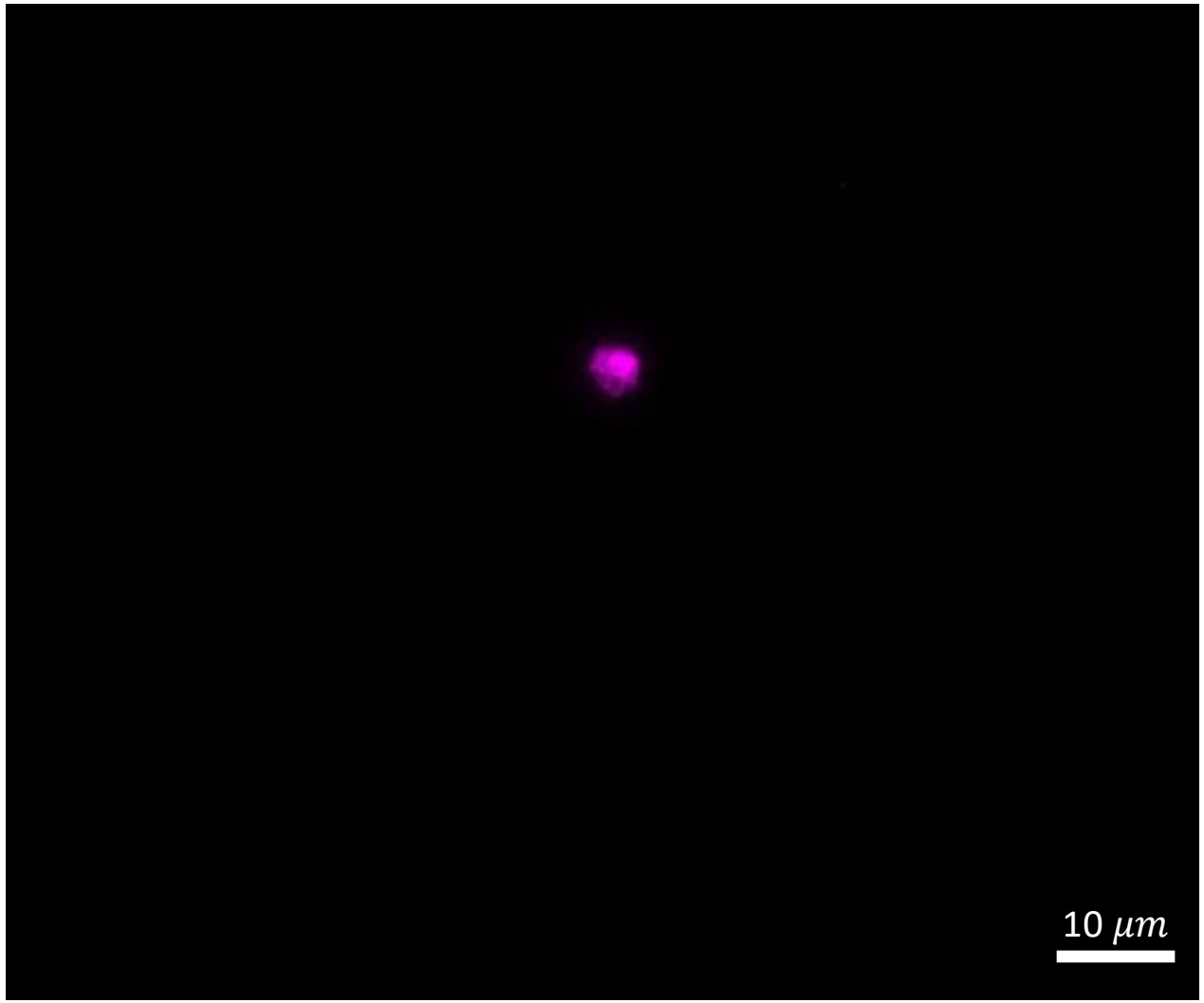

**Channel 1 and 9** is the brightfield view of the microclots

**Channel 3:** is the fluorescence intensity of the biofilm signal - we gate on channel 3 for optimal emission signal of the biofilm

**Channel 6** is side scatter (it represents complexity or granularity of the sample)

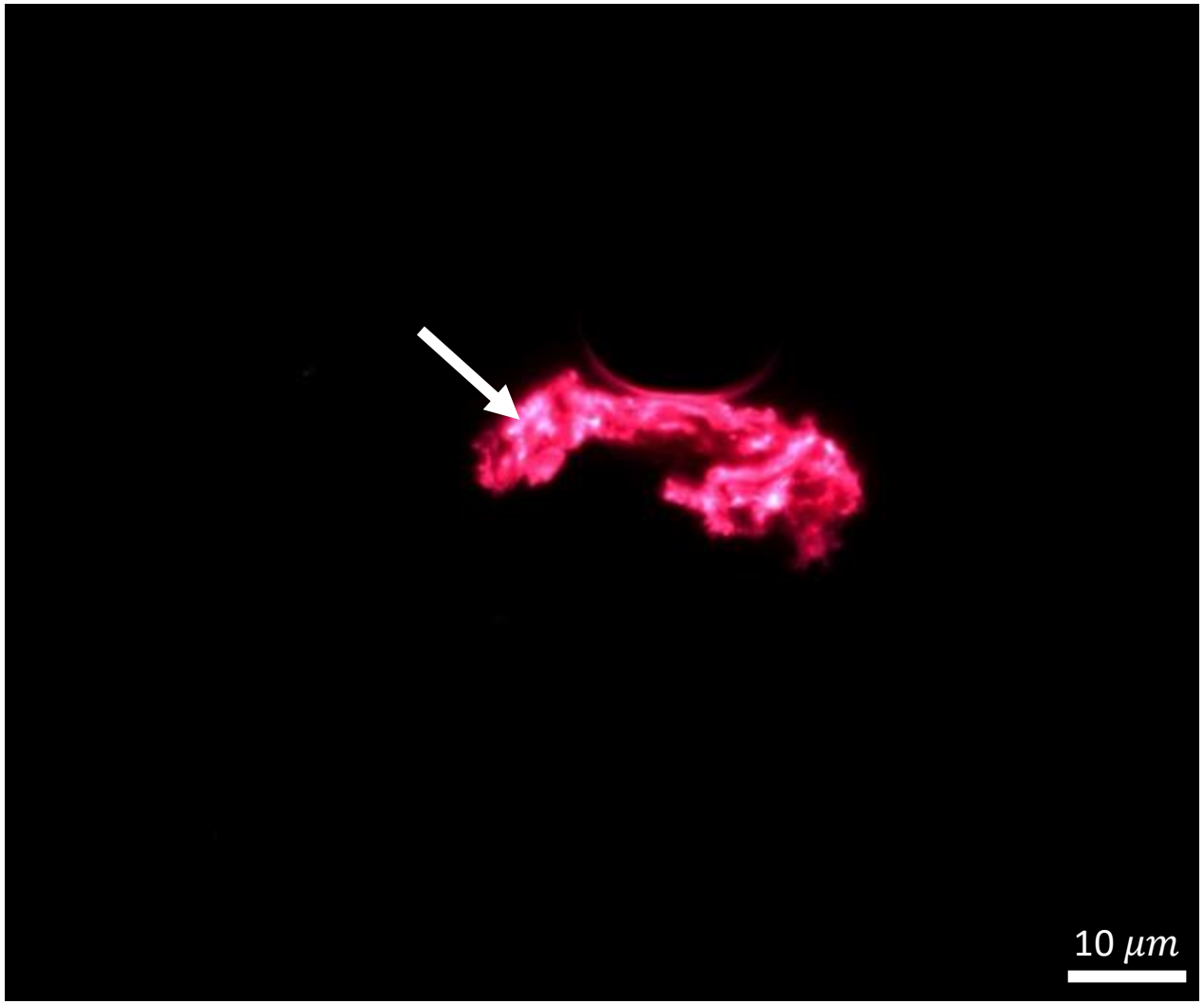
# Syndecan-1: a possible marker for endothelial damage

- CD138 (PE conjugated) antibody only for Syndecan



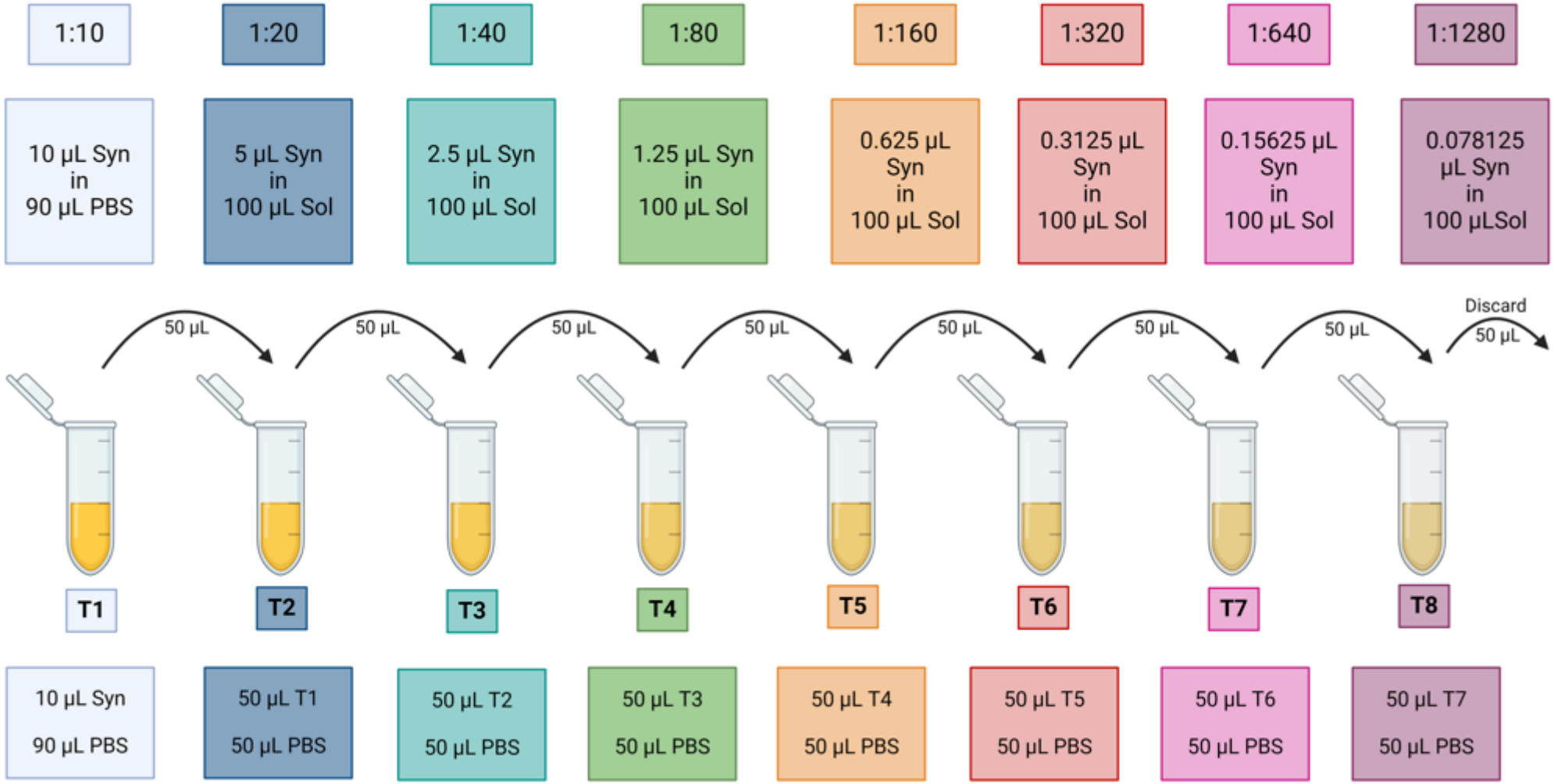
# Endothelial damage, microclots & fibrinogen

- CD138 (PE conjugated) antibody: Syndecan-1
- Thioflavin T (ThT) dye
- Alexa-Fluor 594 antibody: Fibrinogen $\alpha$-chain antibody




Co-localisation indicated by white arrow

# Syndecan-1 titration series




https://www.biorender.com/

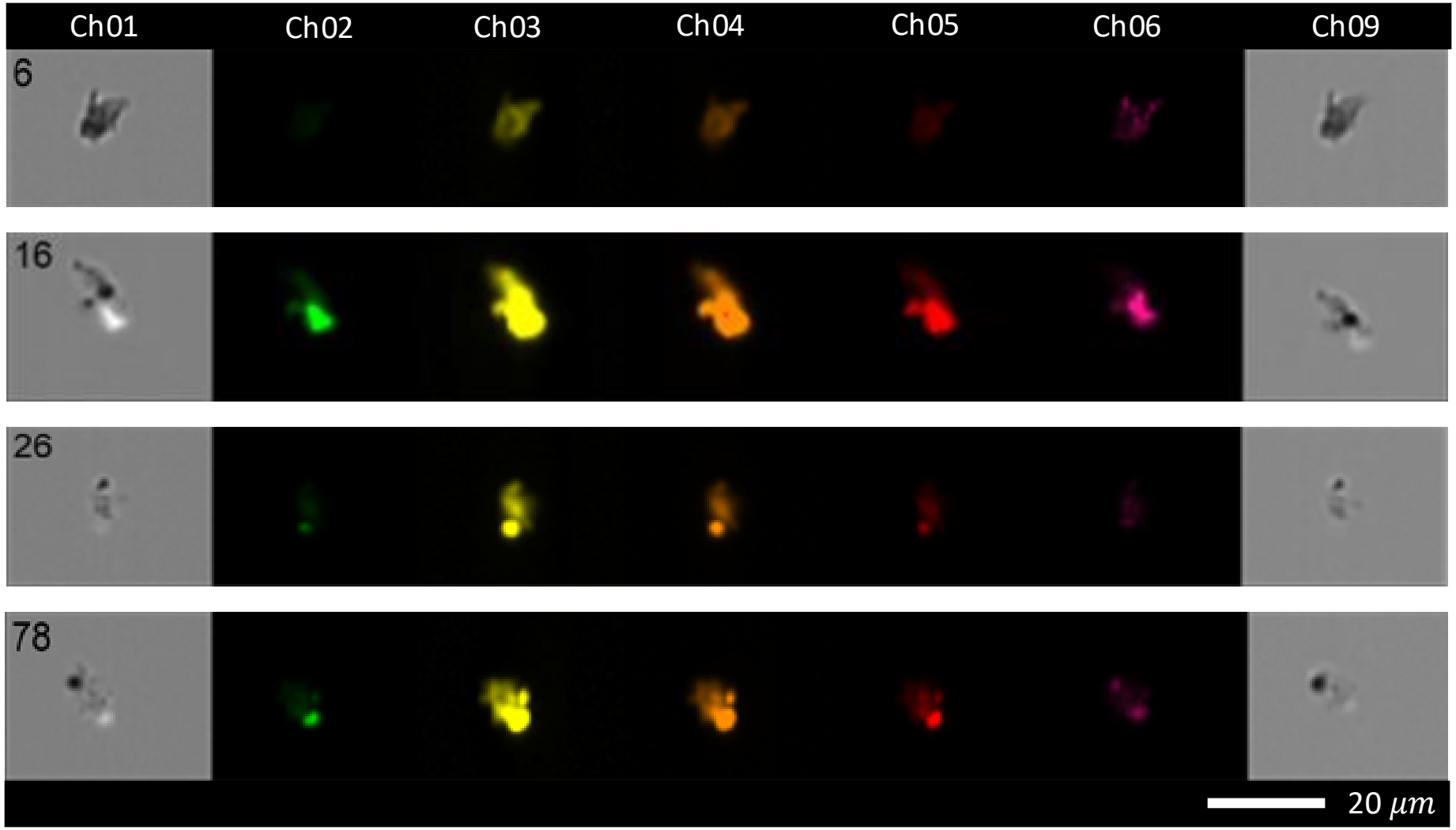
# Quantifying endothelial damage in Long COVID

**Ch01 & Ch09 = brightfield**

**Ch03 = peak PE fluorescent signal**

**Ch02-Ch05 = fluorescent signal**

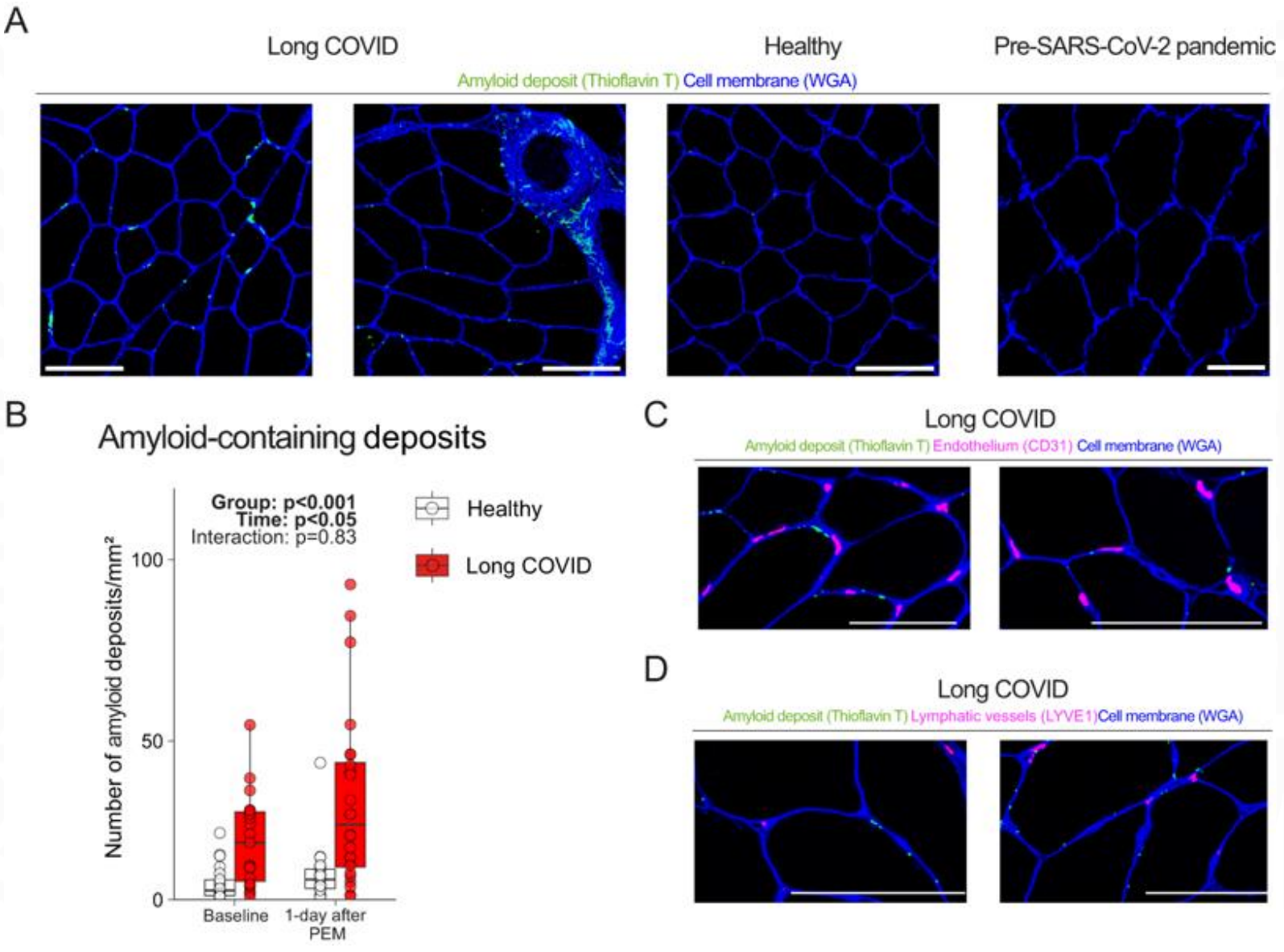
**Ch06 = side scatter**



# Microclots inside muscle biopsies

## Microclots in Long COVID

Appelman, B., Charlton, B.T., Goulding, R.P., Kerkhoff, T.J., Breedveld, E.A., Noort, W., Offringa, C., Bloemers, F.W., van Weeghel, M., Schomakers, B.V., *et al.* (2024). Muscle abnormalities worsen after post-exertional malaise in long COVID. Nature Communications *15*, 17.



# Microclots in paediatric patients



## Evidence Circulating Microclots and Activated Platelets Contribute to Hyperinflammation Within Pediatric Post Acute Sequala of COVID

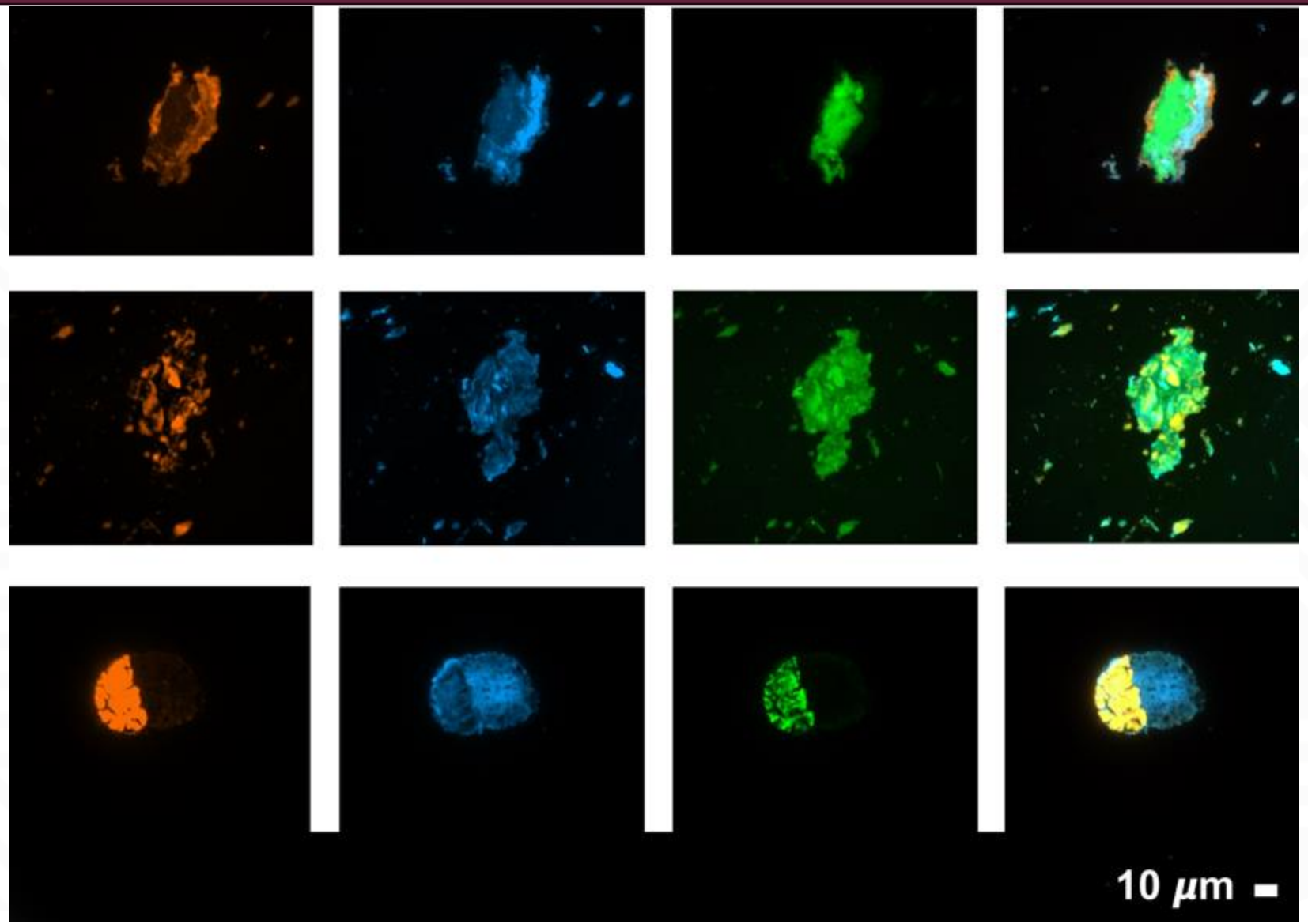
Y. K. Okuducu[1], B. Boribong[2], F. Ellett[2], S. Hajizadeh[2], M. VanElzakker[2], W. Haas[2], S. Pillai[2], A. Fasano[3], D. Irimia[2], L. Yonker[4]; [1]Department of Pediatric Pulmonary, Massachusetts General Hospital, Boston, MA, United States, [2]Massachusetts General Hospital, Boston, MA, United States, [3]Department of Pediatric Gastroenterology, Massachusetts General Hospital, Boston, MA, United States, [4]Department of Pediatric Pulmonology, MGH, Boston, MA, United States.

**Corresponding author's email: kaan.okuducu@icloud.com**

Rationale: Individuals infected with SARS-CoV-2 are at increased risk of cardiovascular events for weeks to months after acute infection. The most severe post-acute sequala of COVID (PASC) is multisystem inflammatory syndrome in Children (MIS-C). Multisystem inflammatory syndrome in children (MIS-C), can present with coronary artery dilation, myocarditis, or ventricular failure. Long COVID appears to be driven by the same pathomechanism, albeit with more indolent presentation. Microclots have been identified in individuals with Long COVID, potentially contributing to vascular inflammation and end-organ injury. As MIS-C shares multiple pathologic features with Long COVID, we hypothesized that microclots also play a vital role in the pathogenicity of PASC including MIS-C. Methods: We used microscopy and proteomic analysis to test whether microclots were present in the blood of children with MIS-C and compare this presence with acute pediatric COVID-19 and Long COVID in children. Results: We showed that children with MIS-C and Long COVID contained many microclots within their plasma, a finding absent in healthy children. Additionally, activated platelets were seen in both PASC syndromes, contributing to hyperinflammation, and when visualized, microclots became ensnared in neutrophil extracellular traps. Proteomic analysis corroborated elevated levels of clotting-related proteins in MIS-C. Steroids and intravenous immune globulin (IVIG) dramatically reduced microclot formation in MIS-C. Conclusion: We discovered evidence that children with PASC generate circulating microclots, activated platelets, and increased levels of clotting-related proteins. These results can give us insight into the causes of hyperinflammation within PASC including MIS-C and Long COVID and lead us toward more targeted and effective therapies.

# Microclots in paediatric patients (Dr Uvi Naidoo and Liz Copley)

- Fibrinogen $\alpha$-chain antibody
- Thioflavin T: amyloid protein
- Hoechst (NETs)
- Overlay of all three yellow/white



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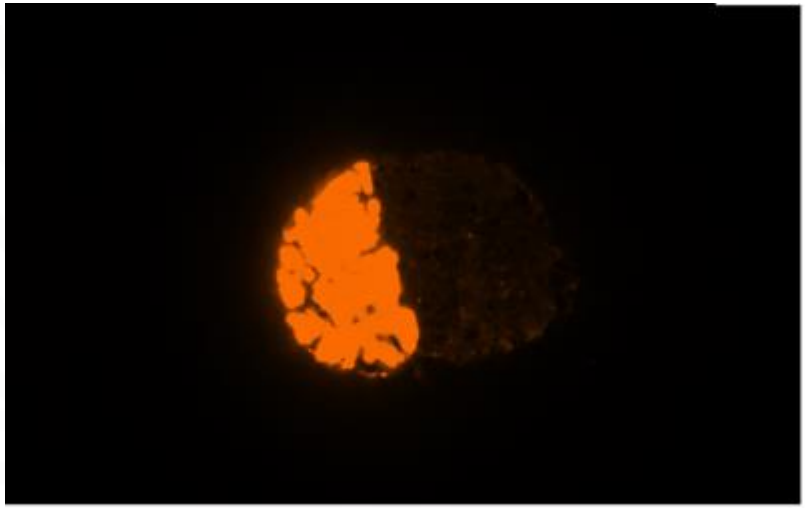
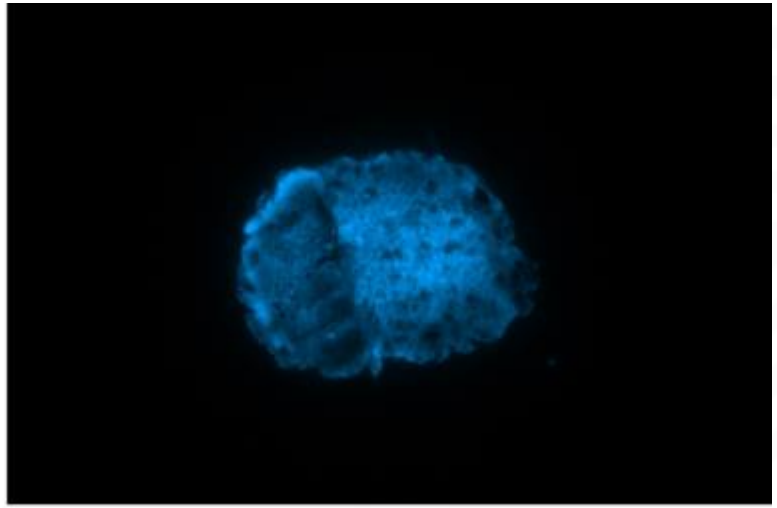
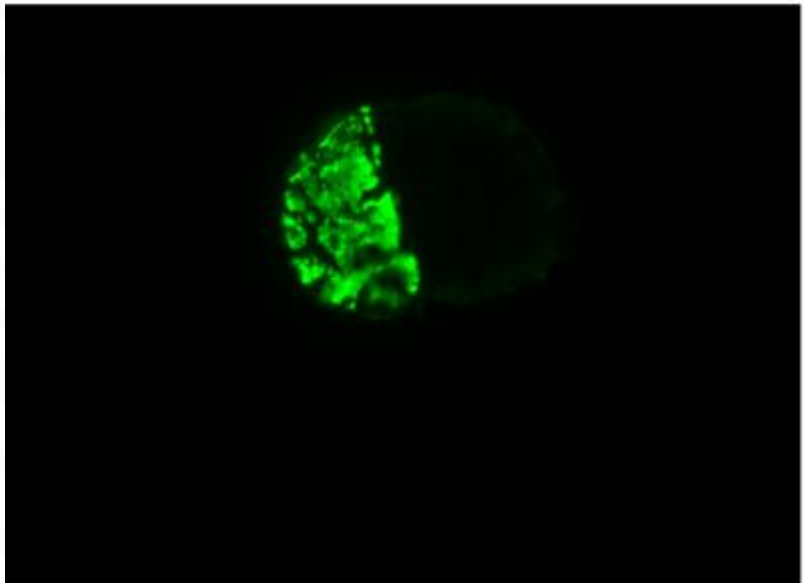
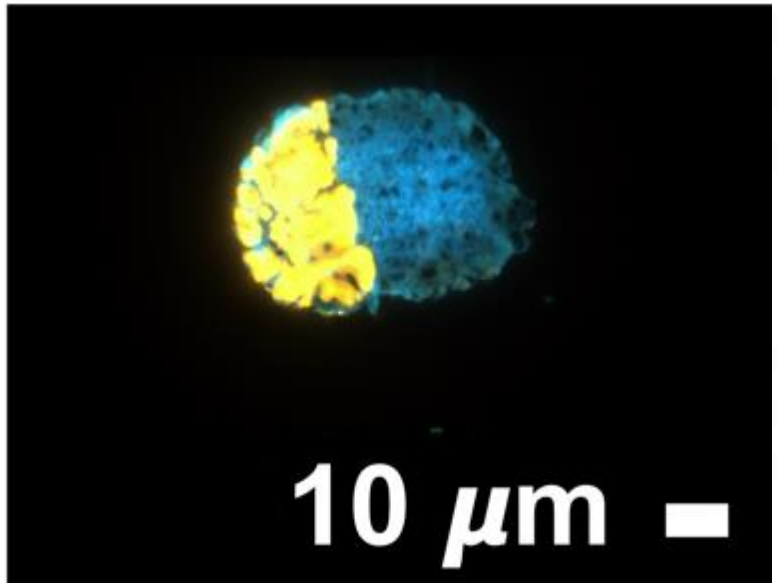




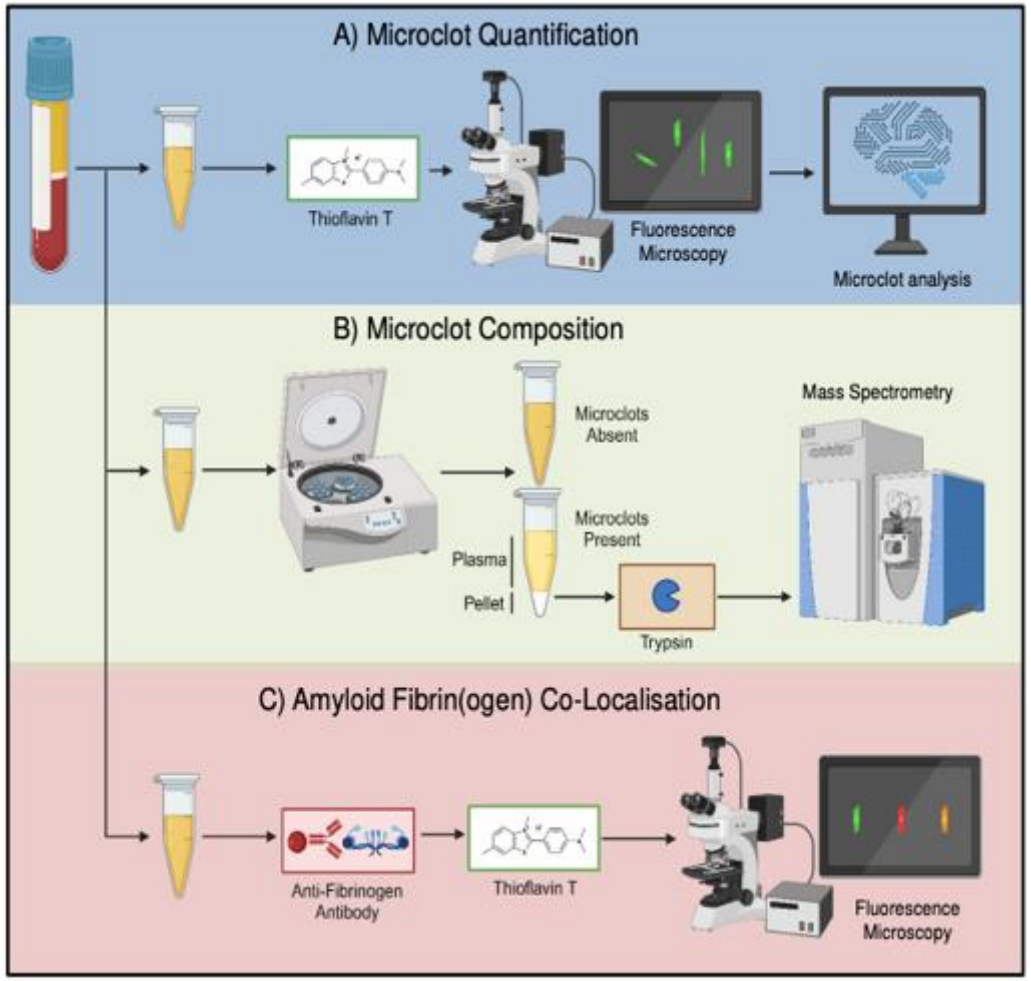


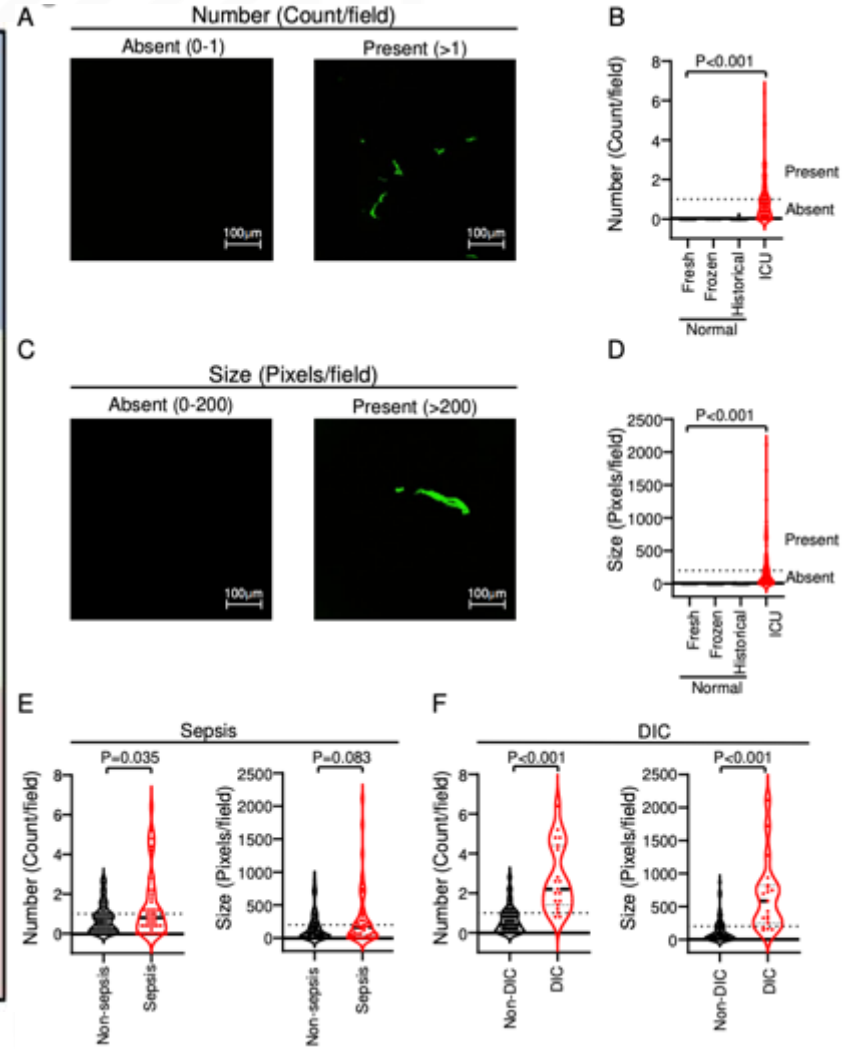


# Exciting research on microclots

## Microclots in sepsis

Schofield, J., Abrams, S.T., Jenkins, R., Lane, S., Wang, G., and Toh, C.H. (2024). Amyloid-Fibrinogen Aggregates ("Microclots") Predict Risks of Disseminated Intravascular Coagulation and Mortality. Blood Adv.



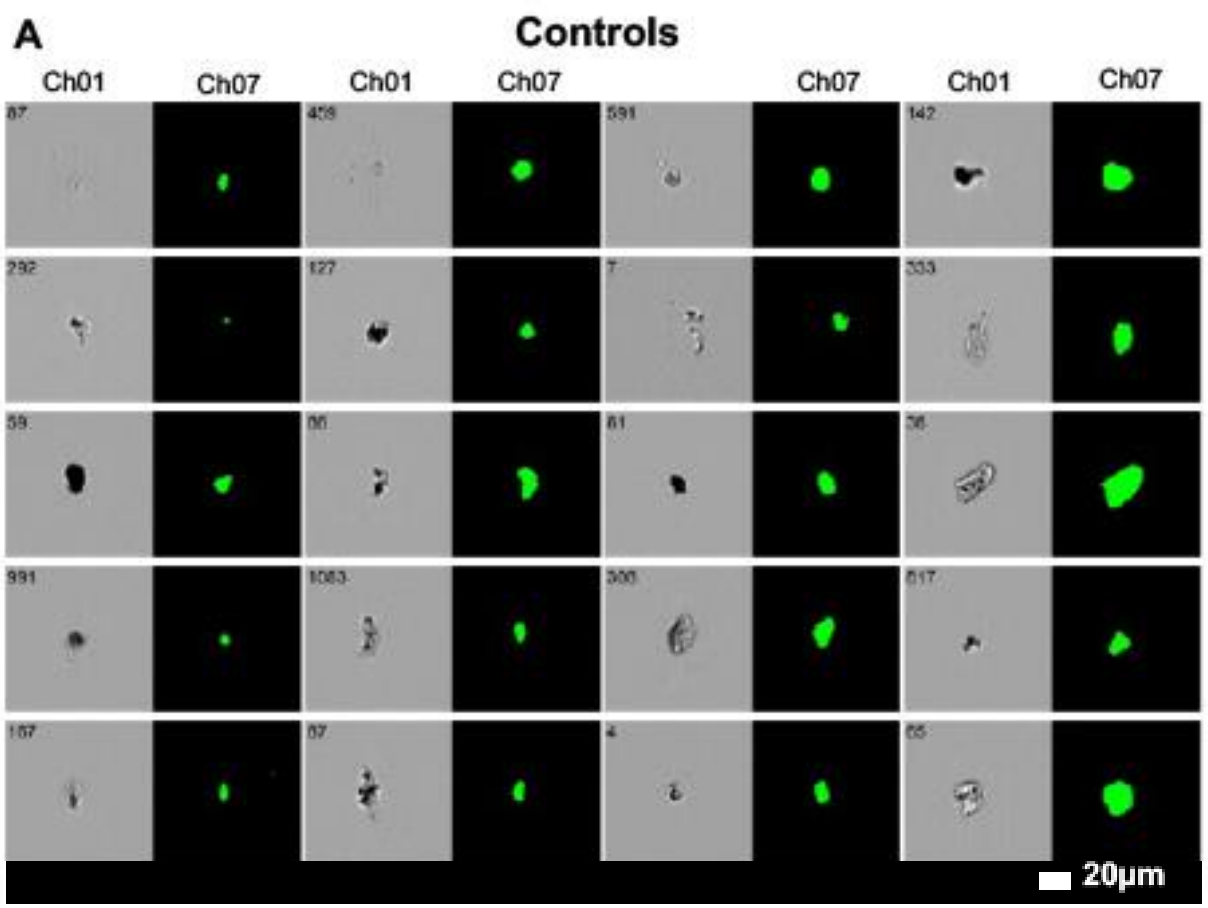
# Microclots, their content and diagnosis for hypercoagulation in Long COVID and ME/CFS: a measurable biomarker in clinical trials

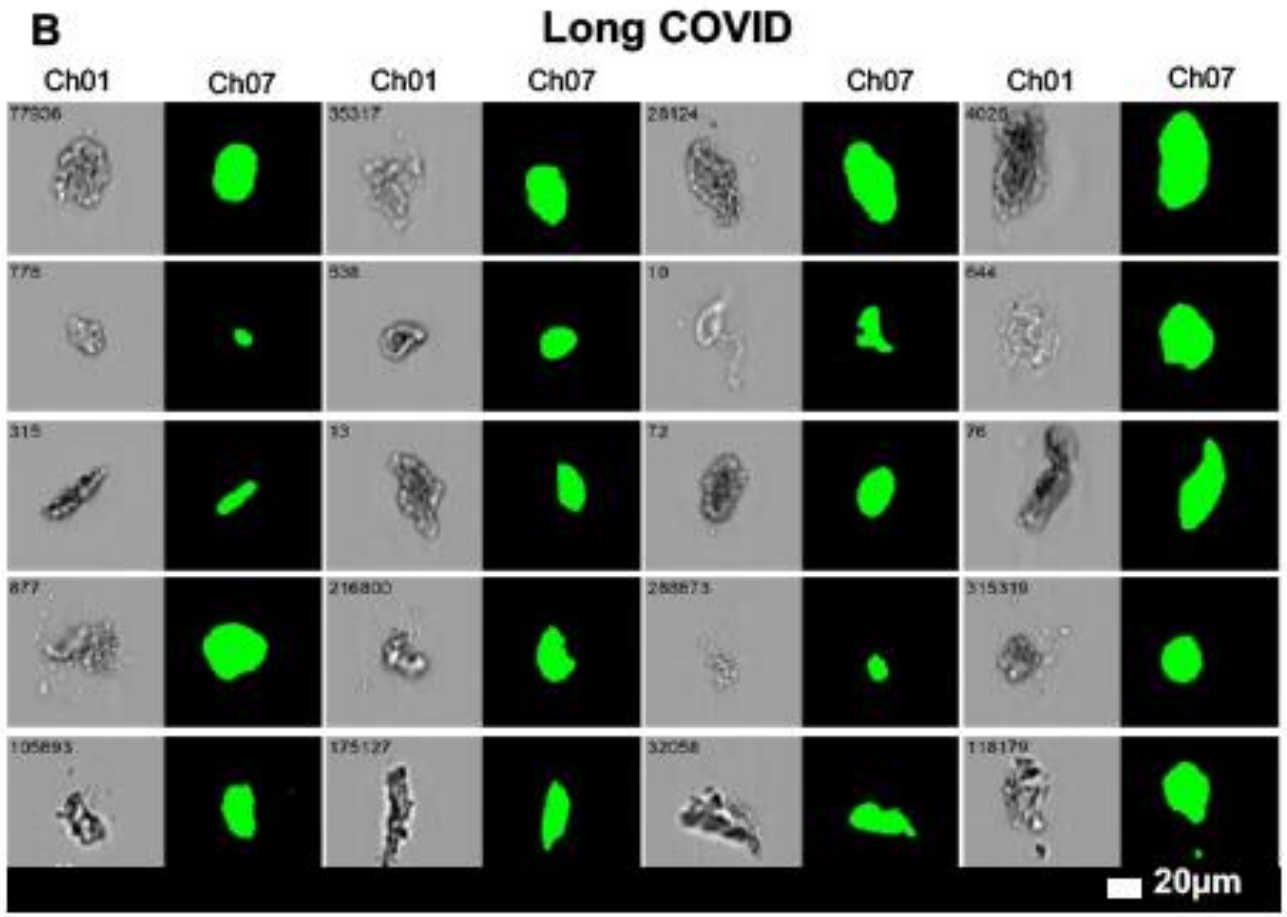
## Microclot Testing a new diagnostic frontier

Our patented diagnostic technology detects abnormal microclots in the blood, indicating poor vascular health, inflammation, and an increased risk for thrombotic endotheliallitis. This pioneering test has been used to detect and aid in the treatment of microclots in thousands with Long Covid, a debilitating condition impacting millions worldwide and deeply affecting global health, society, and the economy.

License Microclot Technology

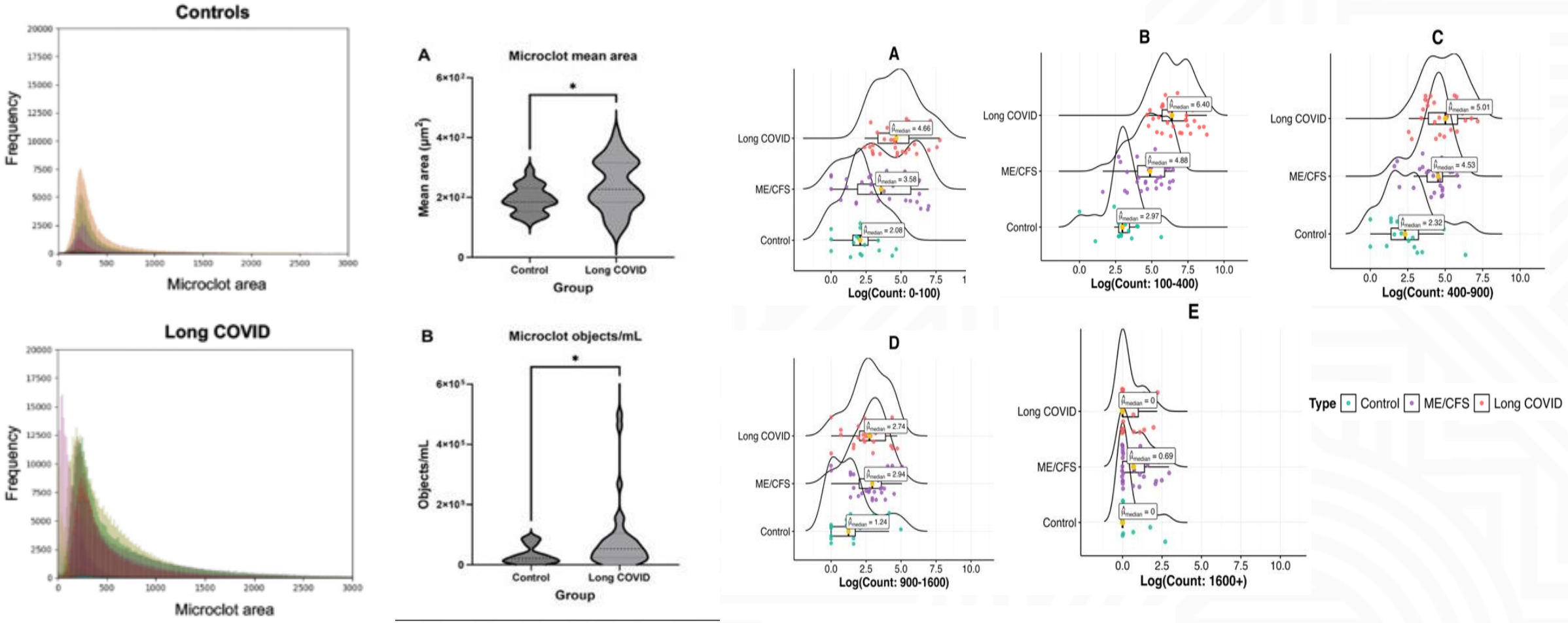
# A place for flow cytometry? (Balvi Foundation, funded together with KERNLS and Polybio Research Foundation)



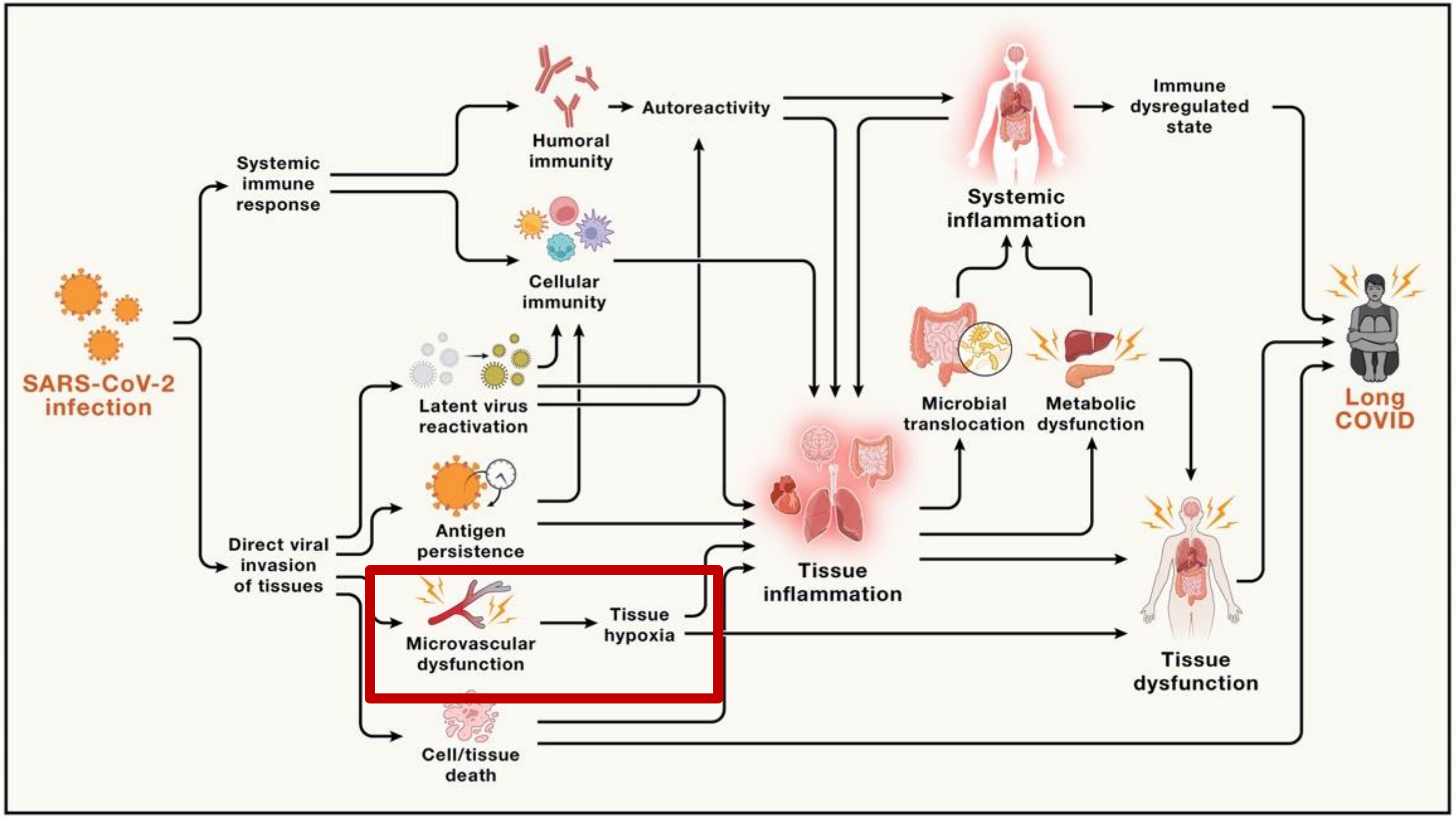




Turner, S., Laubsher, HG.J., Khan, M.A., Kell, D.B., and Pretorius, E. (2023). Accelerating Discovery: A Novel Flow Cytometric Method for Detecting Fibrin(ogen) Amyloid Microclots Using Long COVID as a Model. Heliyon *9* e19605.

# A place for (imaging) flow cytometry?



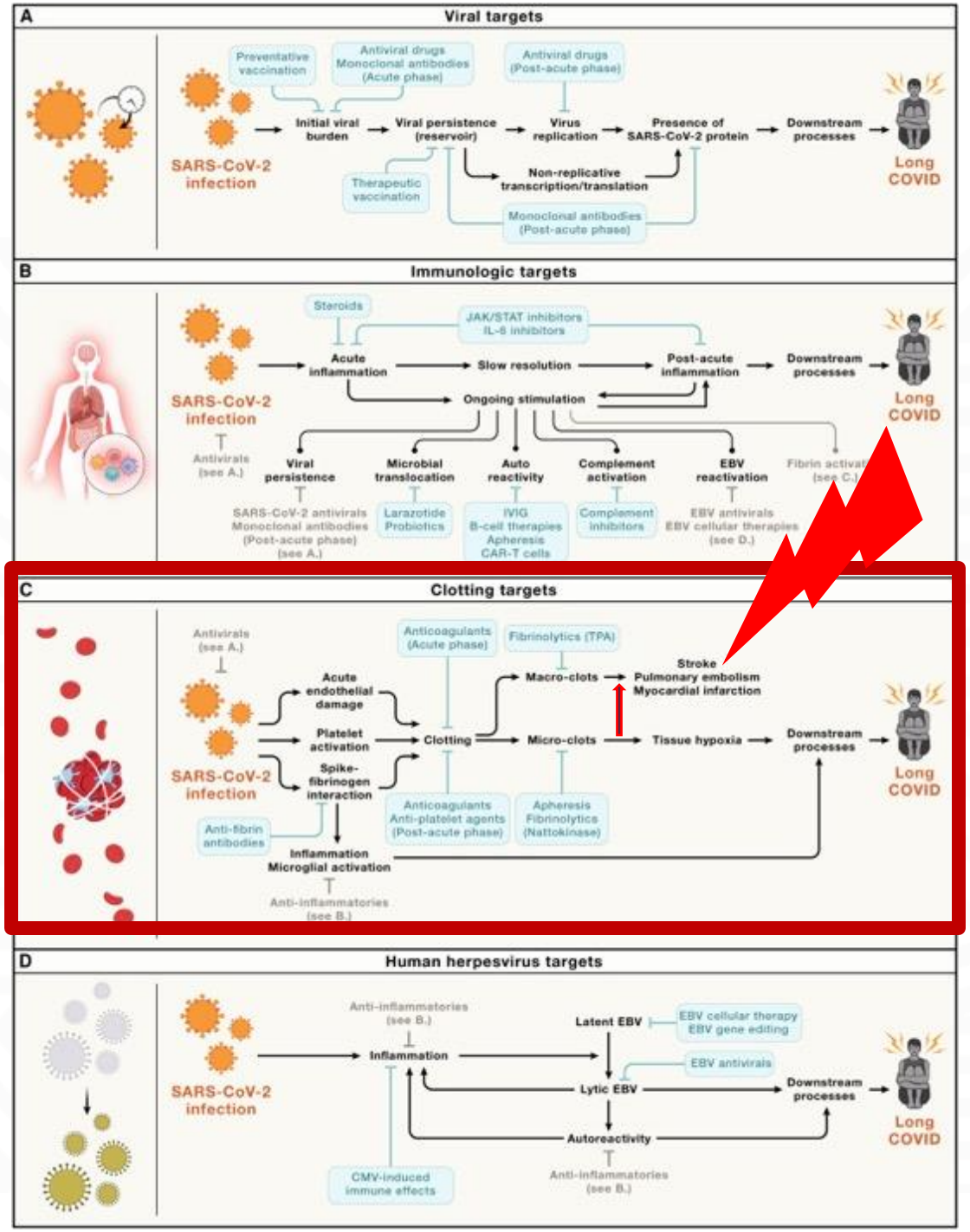

Turner, S., Laubsher, HG.J., Khan, M.A., Kell, D.B., and Pretorius, E. (2023). Accelerating Discovery: A Novel Flow Cytometric Method for Detecting Fibrin(ogen) Amyloid Microclots Using Long COVID as a Model. Heliyon *9* e19605.



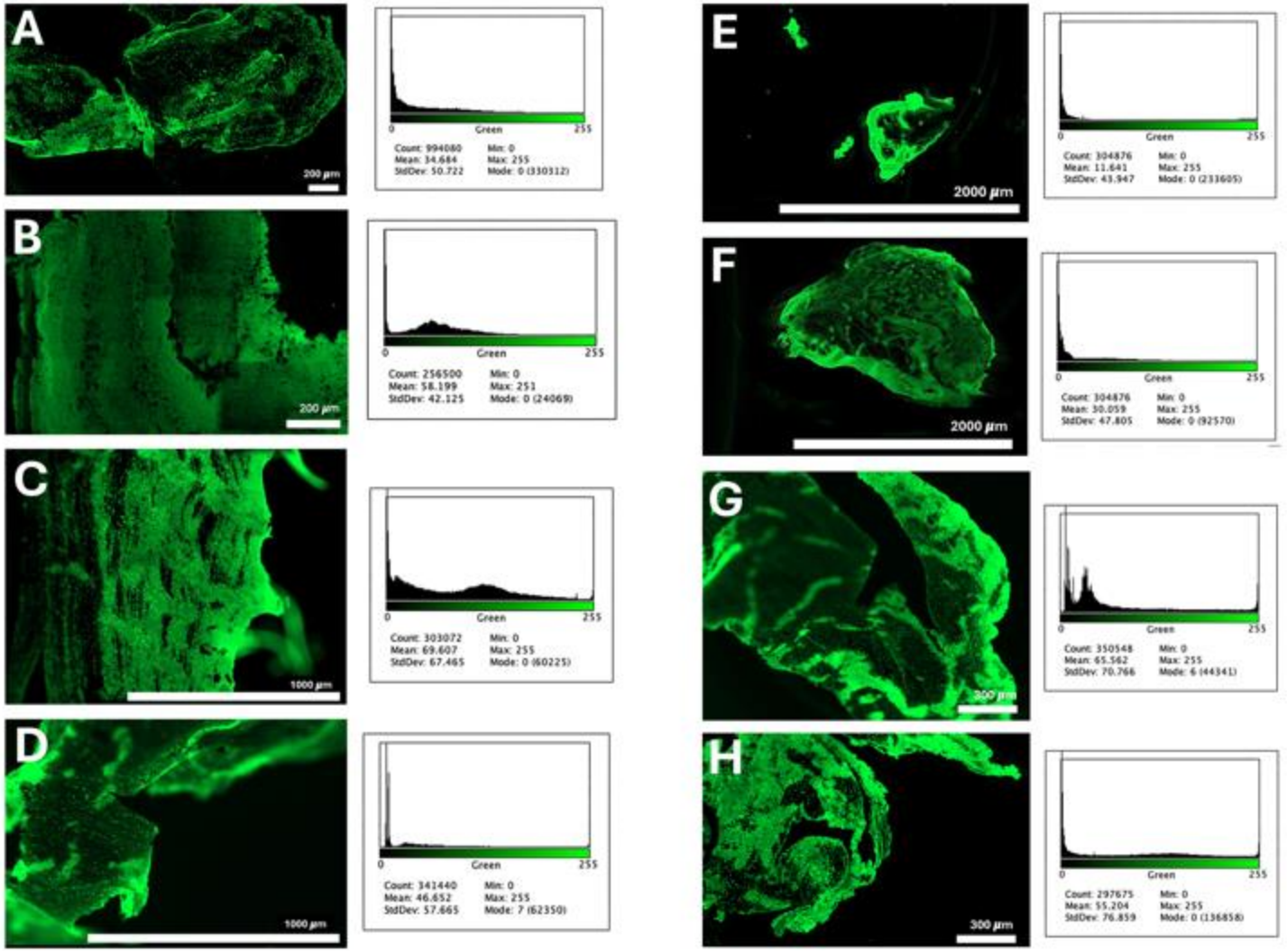

M. J. Peluso and S. G. Deeks 2024 Mechanisms of long COVID and the path toward therapeutics Cell https://doi.org/10.1016/j.cell.2024.07.054

# Mechanistic Targets of Long COVID

- Viral Targets
- Immunologic Targets
- Clotting Targets
- Human Herpesvirus Targets



# Extracted stroke clot analysis



# Microclots: A measurable biomarker as indicator of endothelial damage

- It is **not only (simply) the size/numbers of microclots** present in healthy vs Long COVID (or any other disease with circulating inflammatory molecules), but their:
  - *content (inflammatory molecules of interest for a trial)*
  - *activity*
  - *biochemical characteristics*
- We cannot ignore the role of hyperactivated platelets
- Ultimately these 2 pathologies are driving thrombotic endothelialitis


Kell, D.B., and Pretorius, E. (2022). The potential role of ischaemia-reperfusion injury in chronic, relapsing diseases such as rheumatoid arthritis, Long COVID, and ME/CFS: evidence, mechanisms, and therapeutic implications. Biochem J *479*, 1653-1708.

Turner, S., Khan, M.A., Putrino, D., Woodcock, A., Kell, D.B., and Pretorius, E. (2023). Long COVID: pathophysiological factors and abnormalities of coagulation. Trends Endocrinol Metab *34*, 321-344.

Pretorius, E., and Kell, D.B. (2023). A Perspective on How Fibrinaloid Microclots and Platelet Pathology May be Applied in Clinical Investigations. Semin Thromb Hemost.

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# Thank you
# Enkosi
# Dankie