

## ***Appendix 1: Detailed Methodology for Functional Assays of Fibrin and Fibrinogen.***

### *Fibrin clot turbidity*

We determined fibrin clot turbidity, which corresponds to fibrin network fibril dispersal and branching as measured by microscopy,<sup>38</sup> in fibrin clots that were formed by combining fibrinogen, thrombin, and calcium chloride, as described.<sup>27</sup> In microplate wells 100µl fibrin clots were made with fibrinogen (1 mg/mL), plasminogen (20 µg/mL) and thrombin (0.5 units/mL) in the presence of calcium chloride (10 mM) and allowed to clot for 2 hours in a humidity chamber. Turbidity was measured in a plate reader at 405 nm 25°C.

### *Fibrin clot susceptibility to lysis*

We measured clot lysis rates through turbidity decreases, measured every 10 minutes for four hours after clots were exposed to a mixture of plasminogen (20 µg/mL) and TPA (1 µg/mL). Lysis rate was defined as the relative rate of decrease in turbidity per minute during the predominant time period of lysis, in which the rate was linear.<sup>27</sup> Fibrin residua after partial lysis was reflected by the turbidity after 2 hours of lysis divided by the maximum turbidity.<sup>27</sup>

### *Fibrin clot permeability*

We measured fibrin clot permeability, which reflects the organization with which fibrin polymers are formed, as previously described.<sup>29</sup> Fibrin clots were made by mixing fibrinogen (1 mg/mL, 300 µl) with thrombin (0.5 units/mL) in the presence of calcium chloride (10 mM). The clots were formed in solid phase extraction columns and allowed to clot for 2 hours in a humidity chamber.

Permeation buffer (50 mM Tris, 150 mM NaCl, 1 mg/mL BSA, pH 7.0) was added to the top of the column and kept at a constant pressure head for one hour while flow through the clots was recorded. The surface of the clot allowing flow through the network was represented by its Darcy constant,  $K_s$ :

$$K_s = Q \cdot L \cdot n / t \cdot A \cdot \Delta P ,$$

where  $Q$  was the volume collected (in mL),  $L$  was the clot length (1.176 cm),  $n$  was the viscosity ( $0.001 \text{ kg} \cdot \text{m}^{-1} \cdot \text{s}^{-1}$ ),  $t$  was the collection time (3600 sec),  $A$  was the clot cross sectional area (0.255 cm) and  $\Delta P$  was the pressure differential across the clot (6.5 cm H<sub>2</sub>O). More organized fibrin networks with large pores will have higher flow (and hence larger  $K_s$ ) compared to disorganized fibrin networks with small pores.

#### *Fibrin crosslinking*

We measured the degree that fibrin crosslinks its  $\alpha$ -chains as a reflection of the potential to form stabilized clots during thrombosis. Fibrinogen samples (1mg/mL final) were clotted for two hours in a humidity chamber with thrombin (0.5 units/mL) in the presence of calcium chloride (10 mM). Fibrin clots formed under these conditions reach a maximum degree of crosslinking within two hours, which typically involves about one third of the  $\alpha$ -chains. The clots were then reduced and denatured using a 2% lithium dodecyl sulfate and 50 mM dithiothreitol solution for 60 min. The respective fibrinogen sample and reduced/denatured clot (2  $\mu$ g) were run on SDS PAGE under reducing conditions and stained using colloidal blue stain. Gels were scanned and densitometry was performed using ImageJ software (Rasband, W.S., NIH, Bethesda, Maryland).

The amount of crosslinking was determined as one minus the density representing the (uncrosslinked)  $\alpha$ -chains in the clotted sample relative to the amount present in the fibrinogen sample.

#### *Fibrin $\beta_{15-42}$ accessibility*

We measured accessibility within the fibrin clots of the fibrin  $\beta$ -chain amino termini (residues 15-42 of the fibrinogen B $\beta$ -chain), which are implicated in a variety of physiological events that affect thrombus remodeling into intravascular scars.<sup>39-41</sup> Antibodies specific for the  $\beta_{15-42}$  peptide sequence chain (anti- $\beta_{15-42}$ ) were I<sup>125</sup>-labeled in Pierce pre-coated iodination tubes (Thermo Scientific®). The percent incorporation of I<sup>125</sup> to anti- $\beta_{15-42}$  was determined by separation of the unbound I<sup>125</sup> through a 30 KDa cut off Centrifee filter (Millipore).

Immediately after each clot permeability assay, I<sup>125</sup>-labeled anti- $\beta_{15-42}$  (100  $\mu$ L, 66.7  $\mu$ g/mL) was added to the top of the clot in the permeability column and allowed to percolate through the matrix. When the flow stopped, clots were washed 3 times by passing 800  $\mu$ L of buffer (50 mM Tris pH7.5, 150 mM NaCl, 0.1% BSA) through the columns. The amount of anti- $\beta_{15-42}$  retained within each fibrin clot was determined in a Captus 3000 gamma counter (Southern Scientific, West Sussex, UK).

#### *Fibrinogen mass variants*

We measured precise fibrinogen masses with liquid chromatography, followed by mass spectrometry (LC/MS). Fibrinogen (4mg/mL, 50  $\mu$ L) was precipitated with a 50% ethanol/citrate buffer (17  $\mu$ L) and separated by centrifugation. Samples were reduced and denatured in tris/urea/dithiothreitol

buffer (200  $\mu$ l) for 4 hours at 37°C and then acidified with 10% trifluoroacetic acid (20  $\mu$ l) to prevent disulfide bonds from reforming. Samples were frozen at -70°C and then analyzed in batch via LC/MS utilizing a pre-defined and validated algorithm.<sup>27</sup> First, post-translational modifications were identified using a Thermo Fisher LTQ Orbitrap Velos Mass Spectrometer equipped with electron transfer dissociation capability. The patient sample measurements of fibrinogen were acquired on an Agilent 6520 LC- Q-TOF system equipped with an Agilent Chip Cube for nanoflow chromatography on microfluidic devices (Agilent Technologies, Santa Clara, CA). Mass spectra were recorded in profile mode using Masshunter Acquisition v. B 04.00 from 300 to 3000 m/z at 1.5s per scan. Between each pair of samples, a HPLC protein standard and a blank were run to minimize the risk of carryover. Data were analyzed using Mass Hunter Qualitative Analysis Software with Bioconfirm v. B.03.00 (Agilent Technologies). Chromatographic peaks for the three chains of fibrinogen were integrated manually and the corresponding spectra were extracted. The profile spectra were background subtracted and “deconvoluted” using the Maximum Entropy deconvolution routine considering data only between 600 and 1600 m/z.

We analyzed the LC/MS spectra representing the fibrinogen A $\alpha$ -chains (65900-66500 Da), B $\beta$ -chains (54000-54700 Da), and  $\gamma$ -chains (48200-48900 Da) and identified mass peaks corresponding to post-translational modification variants. The proportion of each variant was represented by the proportional height of the corresponding mass peaks. Based on our previous observations with predominant mass variants in CTEPH-associated dysfibrinogenemias, we

prospectively measured the following subtypes: A  $\alpha$  -chain aphosphorylation, monophosphorylation and diphosphorylation; B  $\beta$  -chain monosialylation and disialylation; B  $\beta$  -chain hydroxylation; B $\beta$ -chains from which the C-terminus glutamine had been proteolyzed (des-gln461 B $\beta$ -chains);  $\gamma$  -chain monosialylation and disialylation;  $\gamma$  ' ; and any other mass peaks that represented 10% or more of the associated fibrinogen chains.