SUPPLEMENTAL INFORMATION

Title: TILRR (Toll-like interleukin-1 receptor regulator), an important modulator of inflammatory responsive genes, is circulating in the blood

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Running Title: TILRR is detected in blood

[††] In memoriam

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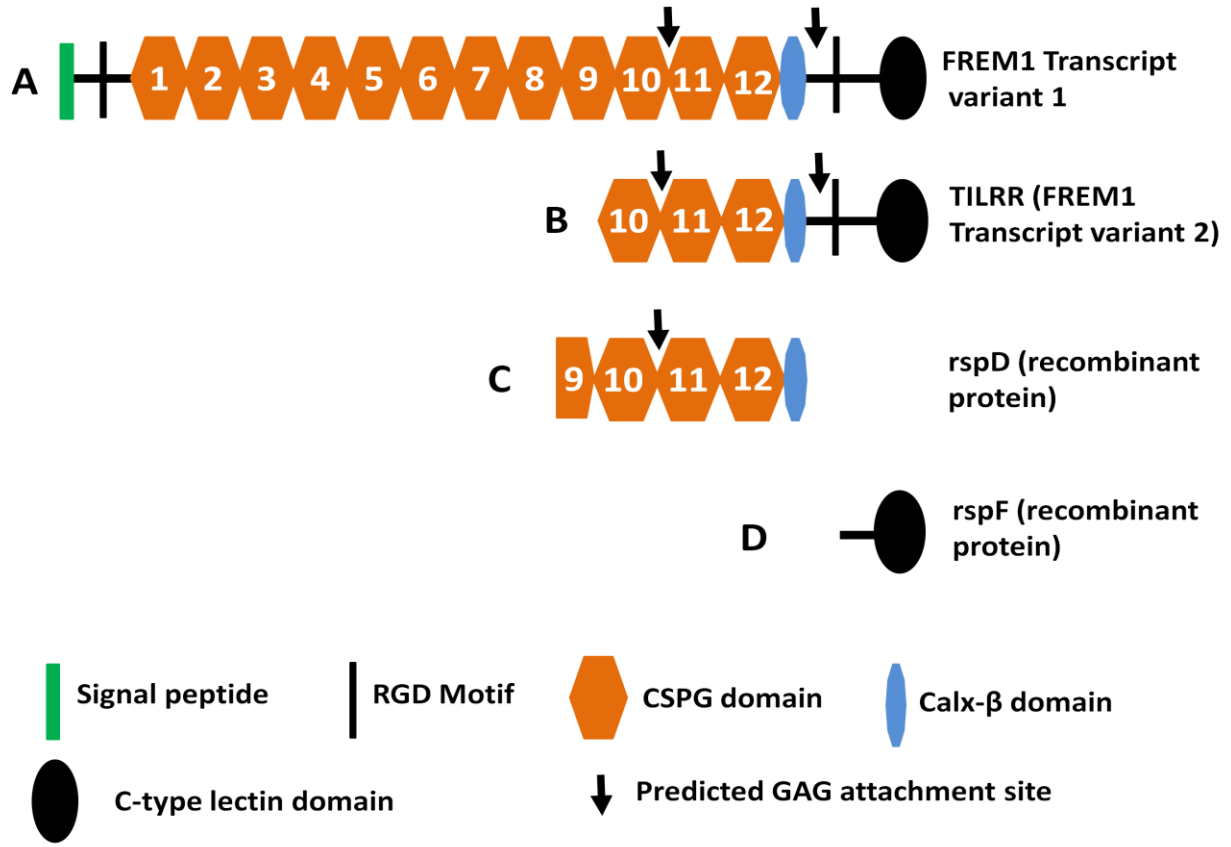


Figure S1: Diagram of FREM1 variants and recombinant proteins. **A)** Full-length FREM1 isoform 1; **B)** TILRR (FREM1 isoform 2); **C)** rFREM1 spD protein; and **D)** rFREM1 spF protein. FREM1, Fras-related extracellular matrix 1; TILRR, toll-like interleukin 1 receptor regulator; RGD, arginine-glycine-aspartic acid; CSPG, chondroitin sulfate proteoglycan; and GAG, glycosaminoglycan. This figure was adapted from Yuan et al. with permission.[1]

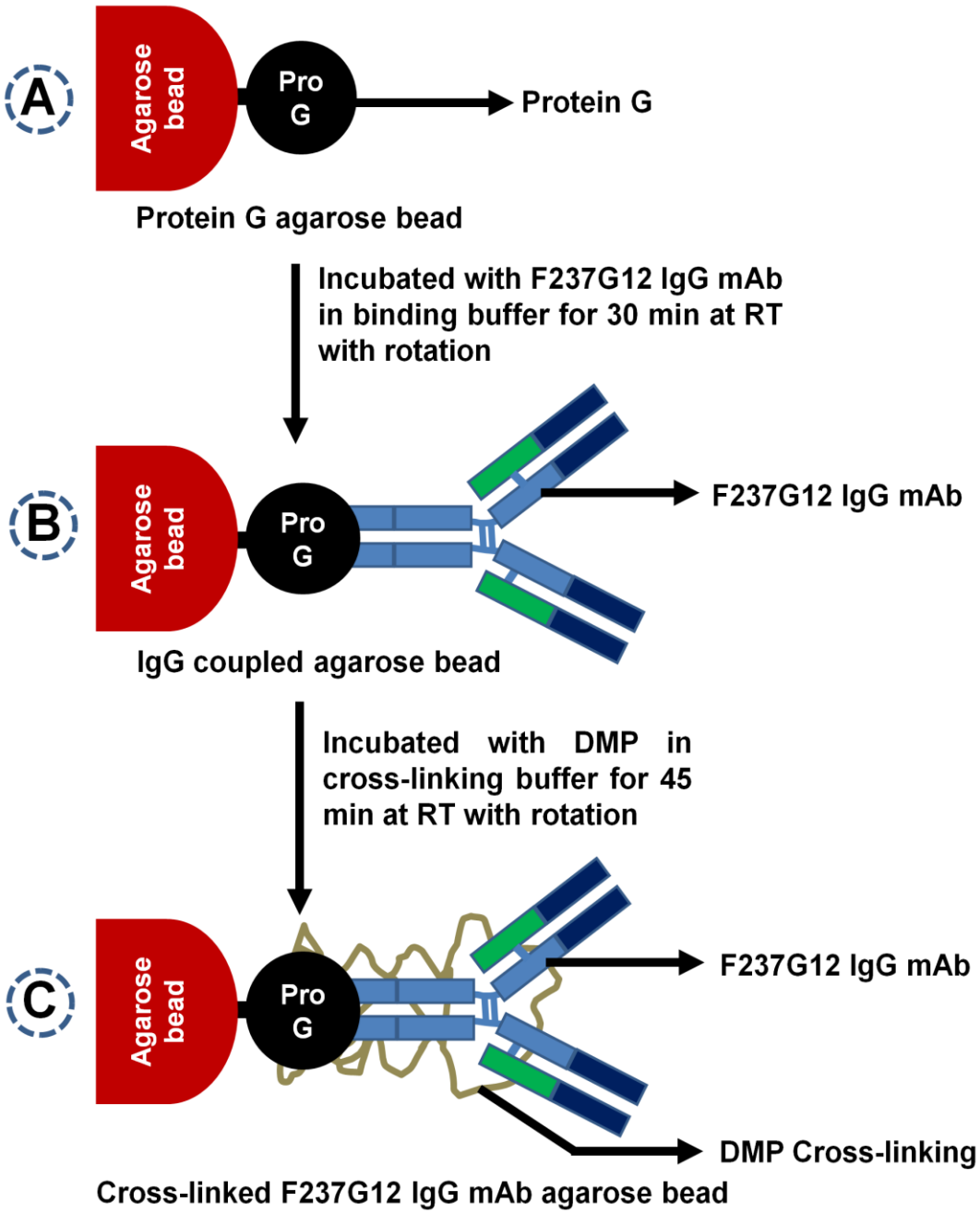


Figure S2: Graphical representation of anti-FREM1 F237G12 IgG mAb cross-linking procedure with Protein G agarose beads. A) Protein G coupled agarose bead, **B)** Protein G bound with Fc region of anti-FREM1 F237G12 mAb, **C)** Anti-FREM1 F237G12 mAb cross-linked with protein G agarose bead by DMP. FREM1, Fras-related extracellular matrix 1; mAb, monoclonal antibody; IgG, immunoglobulin G; DMP, Dimethyl pimelimidate dihydrochloride; and RT, room temperature.

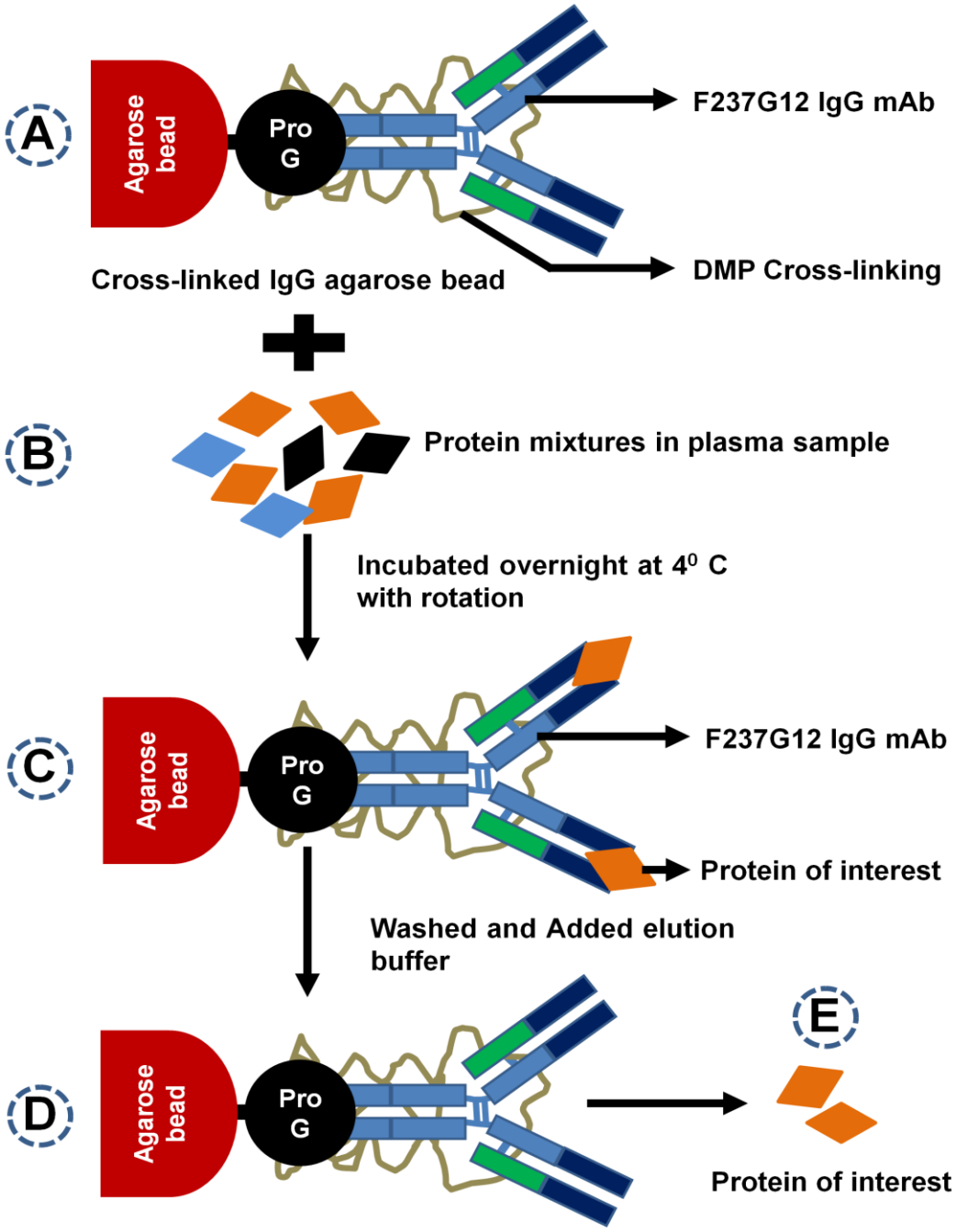
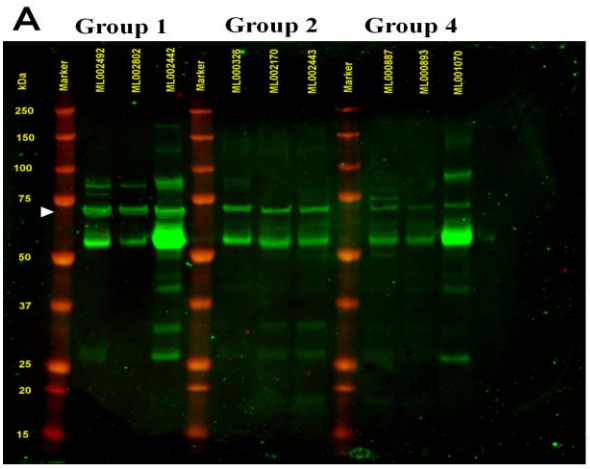
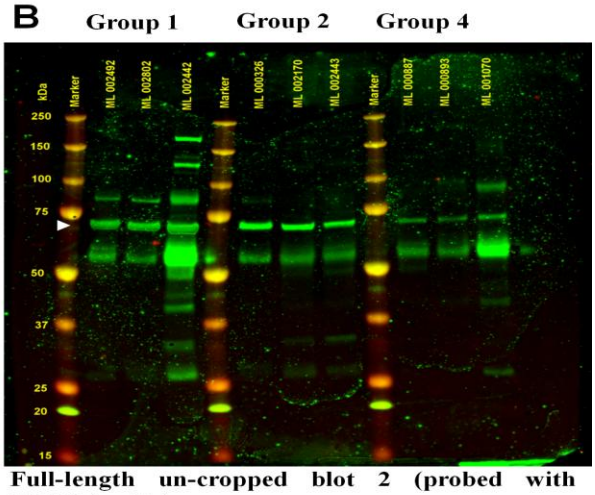


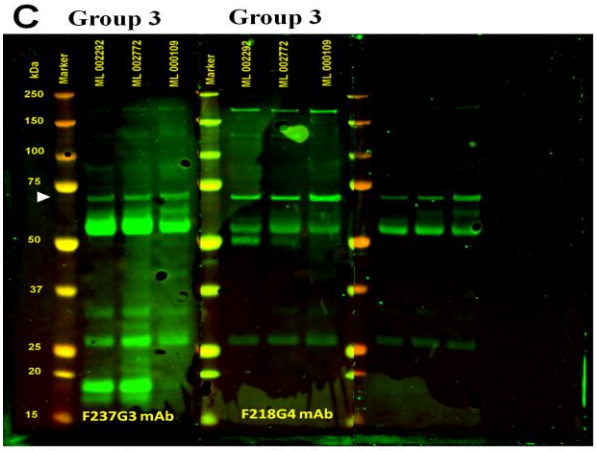
Figure S3: Affinity purification procedure of TILRR protein. A) Anti-FREM1 F237G12 mAb cross-linked protein G agarose bead, **B)** Mixture of proteins in plasma samples, **C)** Protein of interest (TILRR) bound with Fab region of anti-FREM1 mAb, **D)** Neutralized cross-linked anti-FREM1 mAb following affinity purification of target protein, **E)** Target protein (TILRR). FREM1, Fras-related extracellular matrix 1; TILRR, toll-like interleukin 1 receptor regulator; mAb, monoclonal antibody; IgG, immunoglobulin G; and DMP, Dimethyl pimelimidate dihydrochloride.



Full-length un-cropped blot 1 (probed with F237G3 mAb)

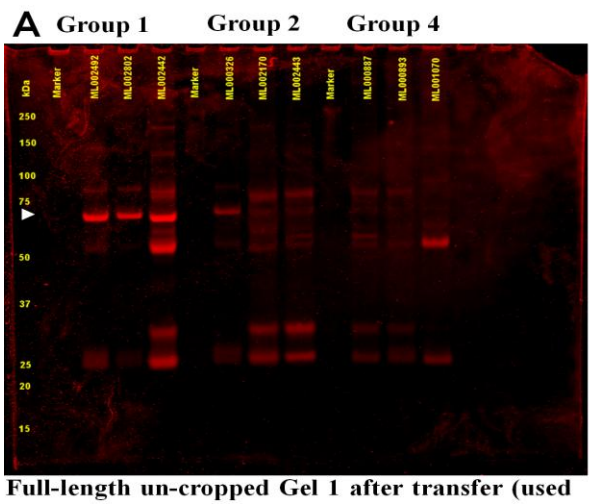


Full-length un-cropped blot 2 (probed with F218G4 mAb)

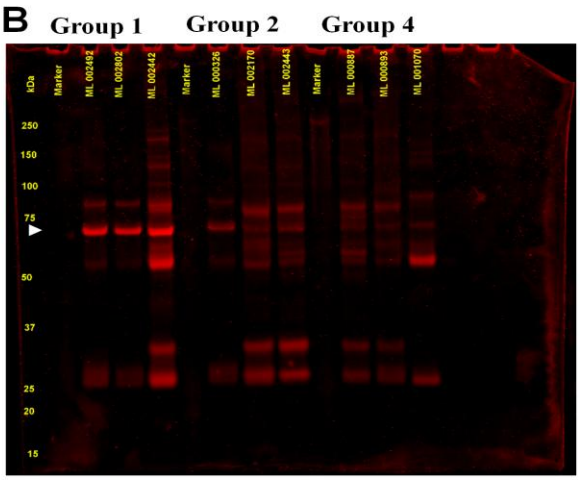


Full-length un-cropped blot 3 (Lanes 1-8 probed with F237G3 and F218G4 mAbs as indicated in the blot). This membrane was cut into three pieces and lanes 1-8 was incubated with two antibodies separately. Lanes 9-12 used for other purposes.

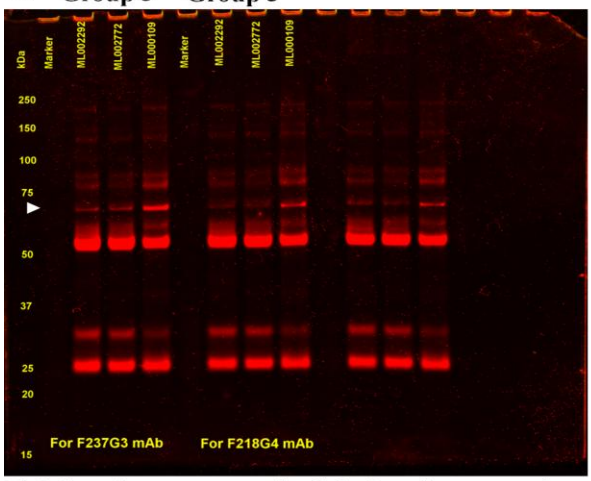
Figure S4: Full-length un-cropped blots of Western blot analysis. TILRR protein was confirmed by Western blot analysis in plasma of patients' group-1, -2, -3, and 4 (patient -1[ML002492], -2[ML002802], -3[ML002442], -4[ML000326], -5[ML002170], -6[ML002443], -7[ML2292], -8[ML2772], -9[ML000109], -10[ML000887], -11[ML000893], and -12[ML001070]) using affinity-purified protein (n=12; each group shows 3 patients data). The images show the bands in the blot for TILRR protein detected by two different anti-FREM1 IgG mAbs, such as F237G3 mAb **(A)**, F218G4 mAb **(B)**, whereas blot 3 **(C)** was cut in three slices and then probed with F237G3 mAb and F218G4 mAb separately as indicated. Western blot images were acquired by Odyssey CLx imaging system (LI-COR, USA) with auto channels (both 700 and 800), 42 µm resolution, high image quality, and 0mm focal offset. A white-colored arrowhead indicates the 70 kDa-sized TILRR protein band. Other observed bands (<70kDa or >70 kDa) in the blot could be the additional variants of FREM1 (yet uncharacterized). Patients' ID# and groups are mentioned on the top of the blots. FREM1, Fras-related extracellular matrix 1; TILRR, toll-like interleukin 1 receptor regulator; ID#, identification number; and kDa, kiloDalton.



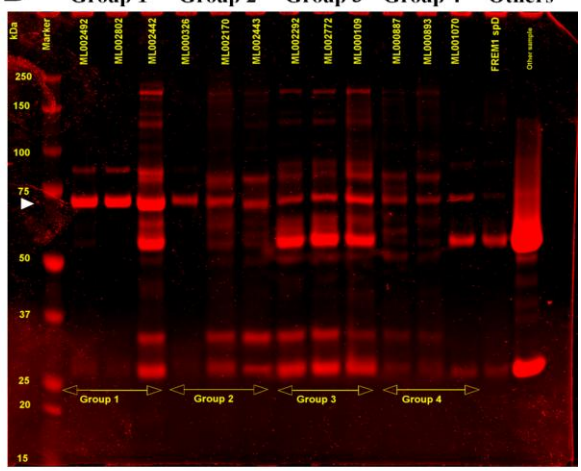
Full-length un-cropped Gel 1 after transfer (used for F237G3 mAb)



Full-length un-cropped Gel 2 after transfer (used for F218G4 mAb)

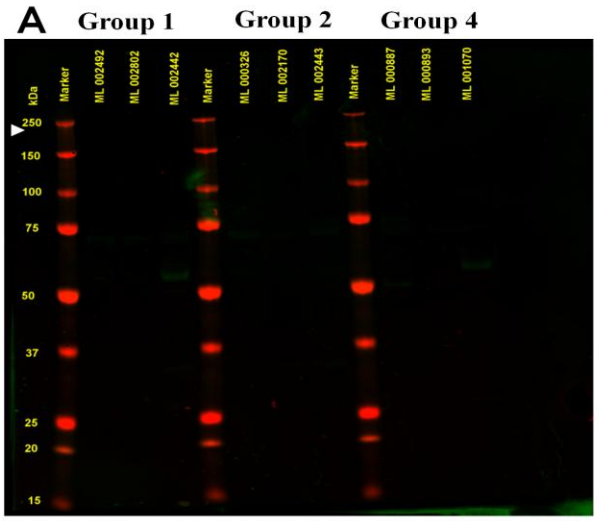


Full-length un-cropped Gel 3 after transfer (Lanes 1-8 used for F237G3 and F218G4 mAbs as indicated in the gel). Lanes 9-12 used for other purposes.

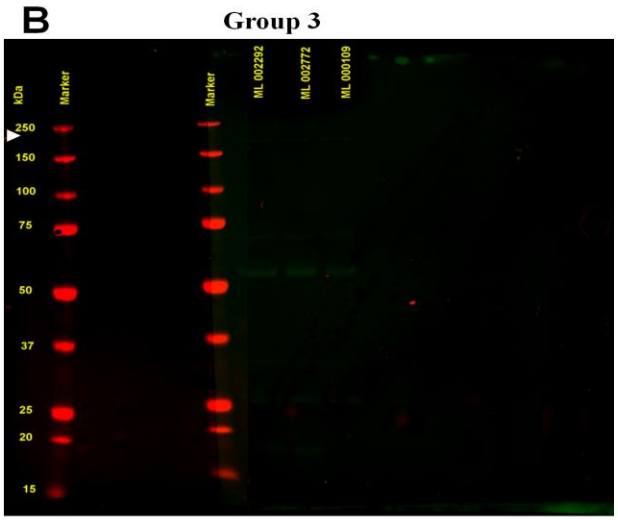


Full-length un-cropped gel before iBlot transfer to the nitrocellulose membrane 3 after transfer (Lanes 14-15 used for other purposes)

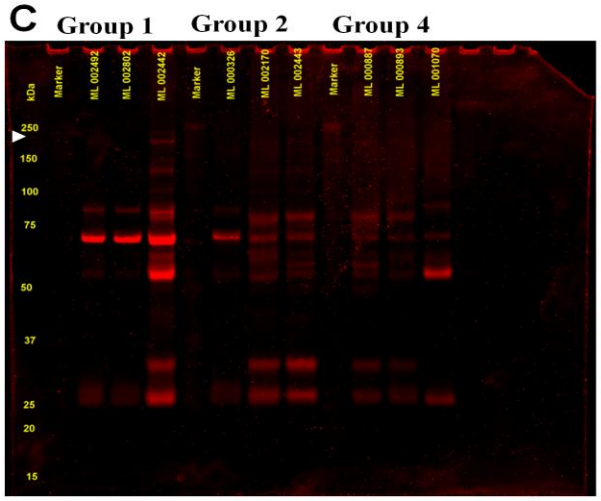
Figure S5: Full-length un-cropped gels of Coomassie blue staining. Non-transferred proteins in the gel after and before iBlot transfer in plasma of patients' group-1, -2, -3, and 4 (patient -1[ML002492], -2[ML002802], -3[ML002442], -4[ML000326], -5[ML002170], -6[ML002443], -7[ML2292], -8[ML2772], -9[ML000109], -10[ML000887], -11[ML000893], and -12[ML001070]) (n=12; each group shows 3 patients data). Gels were used to transfer proteins onto Nitrocellulose membrane that was probed by F237G3 mAb **(A)**, and F218G4 mAb **(B)**, except Gel3 **(C)**, which was used for both F237G3 and F218G4 mAbs. **D)** After the electrophoresis, the gel was directly stained with Coomassie blue staining without transferring to Nitrocellulose membrane. The images show the bands in the gel for the different proteins including a 70 kDa TILRR protein (White-colored arrowhead). Coomassie blue staining gel images were acquired by Odyssey CLx imaging system (LI-COR, USA) with auto channel (700 channel), 42 µm resolution, high image quality, and 0.5mm focal offset. Patients' ID# and groups are mentioned on the top of the blots. ID#, identification number; and kDa, kiloDalton.



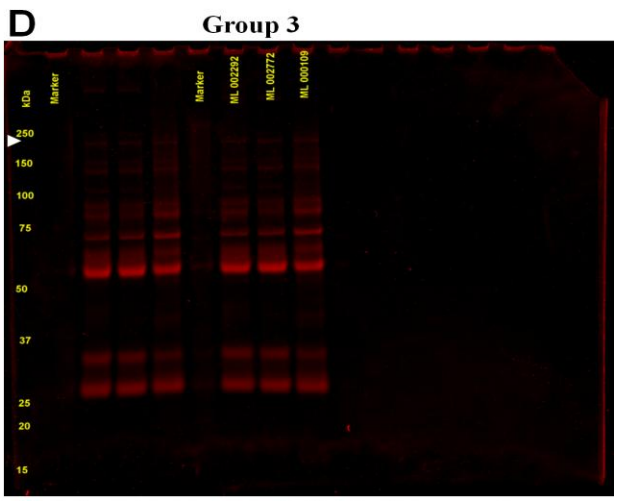
Full-length un-cropped blot 1 (probed with F237G1 mAb)



Full-length un-cropped blot 2 (probed with F237G1 mAb). Lanes 1-4 used for other purposes.



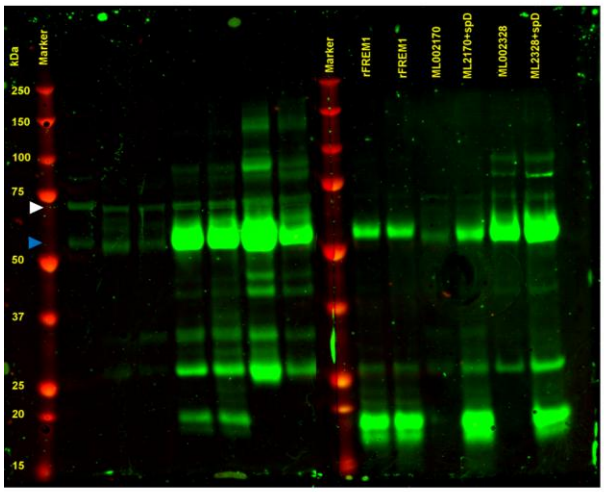
Full-length un-cropped Gel 1 after transfer (used for F237G1 mAb)



Full-length un-cropped Gel 2 after transfer (used for F237G1 mAb). Lanes 1-4 used for other purposes.

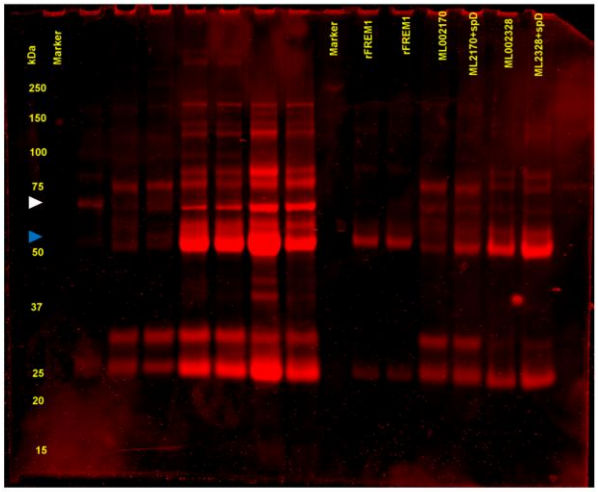
Figure S6: Western blot verification of full-length FREM1 protein after affinity purification. The blot was separately probed with primary antibody (anti-FREM1 mAb F237G1 targeting CSPG9 domain of FREM1) to identify the full-length FREM1 protein from affinity-purified protein of patients' plasma (patient -1[ML002492], -2[ML002802], -3[ML002442], -4[ML000326], -5[ML002170], -6[ML002443], -7[ML2292], -8[ML2772], -9[ML000109], -10[ML000887], -11[ML000893], and -12[ML001070]). **A-B)** Figure A shows the blot for group-1, -2, and -4 and Figure B for group-3. No band of ~235 kDa was detected (indicated by a white-colored arrowhead). **C-D)** Corresponding gels were used to transfer proteins onto the nitrocellulose membrane. Western blot and Coomassie blue staining images were acquired by Odyssey CLx imaging system (LI-COR, USA) with auto channels (both 700 and 800), 42 µm resolution, high image quality, and 0 mm focal offset for blots and 0.5 mm focal offset for gels. Patients' ID# and groups are mentioned on the top of the blots. FREM1, Fras-related extracellular matrix 1; ID#, identification number; and kDa, kiloDalton.

A



Full-length un-cropped blot (Lanes 9-15 probed with F237G3 mAb). Lanes 1-8 used for other purposes. This membrane was cut into two pieces and lanes 9-15 were incubated with F237G3 mAb.

B



Full-length un-cropped Gel after transfer (Lanes 9-15 used for F237G3 mAb). Lanes 1-8 used for other purposes.

Figure S7: Full-length un-cropped blot and gel. A) The blot was cut into two pieces and the second piece was incubated with anti-FREM1 F237G3 mAb. A clear band of ~57 kDa (blue-colored arrowhead) rFREM1 spD was observed in all samples. A 70 kDa band (white-colored arrowhead) of TILRR protein is also observed in plasma alone and plasma with spiked rFREM1 spD protein. **B)** Corresponding gel following iBlot transfer onto Nitrocellulose membrane stained with Coomassie blue. The white arrowhead indicates 70kDa and the blue arrowhead represents ~57 kDa. Western blot and Coomassie blue staining images were acquired by Odyssey CLx imaging system (LI-COR, USA) with auto channels (both 700 and 800), 42 µm resolution, high image quality, and 0 mm focal offset for blot and 0.5 mm focal offset for gel. Patients' ID# and groups are mentioned on the top of the blots. FREM1, Fras-related extracellular matrix 1; ID#, identification number; and kDa, kiloDalton.

Table S1: Demographic characteristics of the study subjects

Characteristics (at sample collection)	All subjects (n=640) Median (IQR)	Plasma group 1 (n=186) Median (IQR)	Plasma group 2 (n=303) Median (IQR)	Plasma group 3 (n=19) Median (IQR)	Plasma group 4 (n=132) Median (IQR)	p-value
Age, in years	35 (30-40) (n=624[a])	33 (28.5-37) (n=179)	35 (30-40) (n=297)	35 (30-38.75) (n=18)	39 (33-45) (n=130)	0.0142[b] <0.0001[c,d] 0.0147[e]
	% (n)	**% (n)**	**% (n)**	**% (n)**	**% (n)**	
Sexually transmitted infection (STIs)	27.00 (172/637[f])	29.03 (54/186)	24.67 (74/300)	42.11 (8/19)	28.03 (37/132)	NS
Genital ulcer	6.12 (39/637[g])	5.91 (11/186)	3.67 (11/300)	15.79 (3/19)	10.61 (14/132)	0.0124[h] 0.0044[i]
Oral contraceptive used	22.45 (143/637[g])	20.43 (38/186)	21.00 (63/300)	31.58 (6/19)	27.27 (36/132)	NS

[a]Samples with known age at the time of collection. Age was unknown for 16-samples.
[b]Student t-test was conducted between group 1 and group 2.
[c]Student t-test was conducted between group 1 and group 4.
[d]Student t-test was conducted between group 2 and group 4.
[e]Student t-test was conducted between group 3 and group 4.
[f]Samples with a history of known STIs (Gonorrhea, Syphilis, Chlamydial infection, and bacterial vaginosis). 3-samples do not have a history of STIs.
[g]Samples with a history of vaginal discharge, genital ulcer, and use of oral contraceptives. History was unknown for 3-samples.
[h]Chi-Square test was conducted between group 2 and group 3.
[i]Chi-Square test was conducted between group 2 and group 4.
IQR, interquartile range; n, sample#; NS, not statistically significant

REFERENCES

1. Yuan XY, Liu LR, Krawchenko A, et al. Development of monoclonal antibodies to interrogate functional domains and isoforms of FREM1 protein. *Monoclon Antib Immunodiagn Immunother.* Apr 2014;33(2):129-140.