Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Pass HI, Levin SM, Harbut MR, et al. Fibulin-3 as a blood and effusion biomarker for pleural mesothelioma. N Engl J Med 2012;367:1417-27. DOI: 10.1056/NEJMoa1115050

Table of Contents: Supplemental Materials

Statement of Author Contributions

The study was designed by HIP; HIP, CG, DC, LG, GG, JM, GL, MST, MdeP, gathered the data; HIP, MT, and GL analyzed the data and vouch for the data and the analyses: all authors contributed to the writing of the paper; and HIP decided to publish the paper.

Supplemental Methods

Genomic Discovery: Total RNA was extracted from 37 matched MPM specimens and their corresponding peritoneum obtained at the time of extrapleural pneumonectomy. Using the GeneChip® Human Gene 1.0 ST Arrays, we found that EFEMP1 which codes for FBLN3 to be 37th out of 32,321 probe IDs with the highest fold change between MPM and peritoneum (7.36 fold change, $p=1.32 \times 10^{-9}$). These data were confirmed using GEO profiles

http://www.ncbi.nlm.nih.gov/geo/gds/profileGraph.cgi?&dataset=WSHPFJKFKIAATBbe68SWRL1LcTGRo58ohMk2pZrzCUbaHJeYL2S6l-&dataset=zzyyyyyxyycSymzzzzzzzyzyzzxyzzzzzyzzzzzztzzzyyzzyzyzzz$&gmin=12.800000&gmax=7806.900000&absc=49615&uid=10528370&gds=1220&idref=201842_s_at&annot=EFEMP1

and rtPCR amplification (manuscript in preparation).

***Immunohistochemistry Methods*:** In brief, sections were deparaffinized in xylene (3 changes), rehydrated through graded alcohols (3 changes 100% ethanol, 3 changes 95% ethanol) and rinsed in distilled water. Heat induced epitope retrieval was performed in 10mM citrate buffer pH 6.0 in a 1200-Watt microwave oven at 100% power for 20 minutes. Sections were allowed to cool for 30 minutes and then rinsed in distilled water. Slides were then washed in Tris-Buffered NaCl, Tween 20, pH7.6 (TBST Dako Carpentaria, CA USA). Fibulin-3 was diluted 1:50 and incubated overnight at 4˚C. TBST was substituted for negative control. Fibulin-3 was detected using rabbit anti-mouse Catalyzed Signal

Amplification (peroxidase/DAB) System (Dako Carpentaria, CA USA) following the manufacturer's instructions. Slide were counterstained with hematoxylin, and mounted with permanent media. Sections of squamous carcinoma of cervix and malignant glioma were used as positive controls for fibulin 3 staining[1,2]

Cut-Point Discovery: Effusion Fibulin Prognostication: Since the cutpoint for separation of survivals by effusion fibulin levels was determined empirically, it is possible that it may not be possible to generalize it to other populations. A secondary analysis to determine an optimal effusion prognostic cut-point was performed using X-Tile[3]. The statistical package separated the 54 cytoreduced effusion cohort into two cohorts with a cut-point of 870.2 ng/ml as seen in Figure S3. Table S3 demonstrates the cutpoint of 870.2 ng/ml as an independent variable for survival along with histology and stage.

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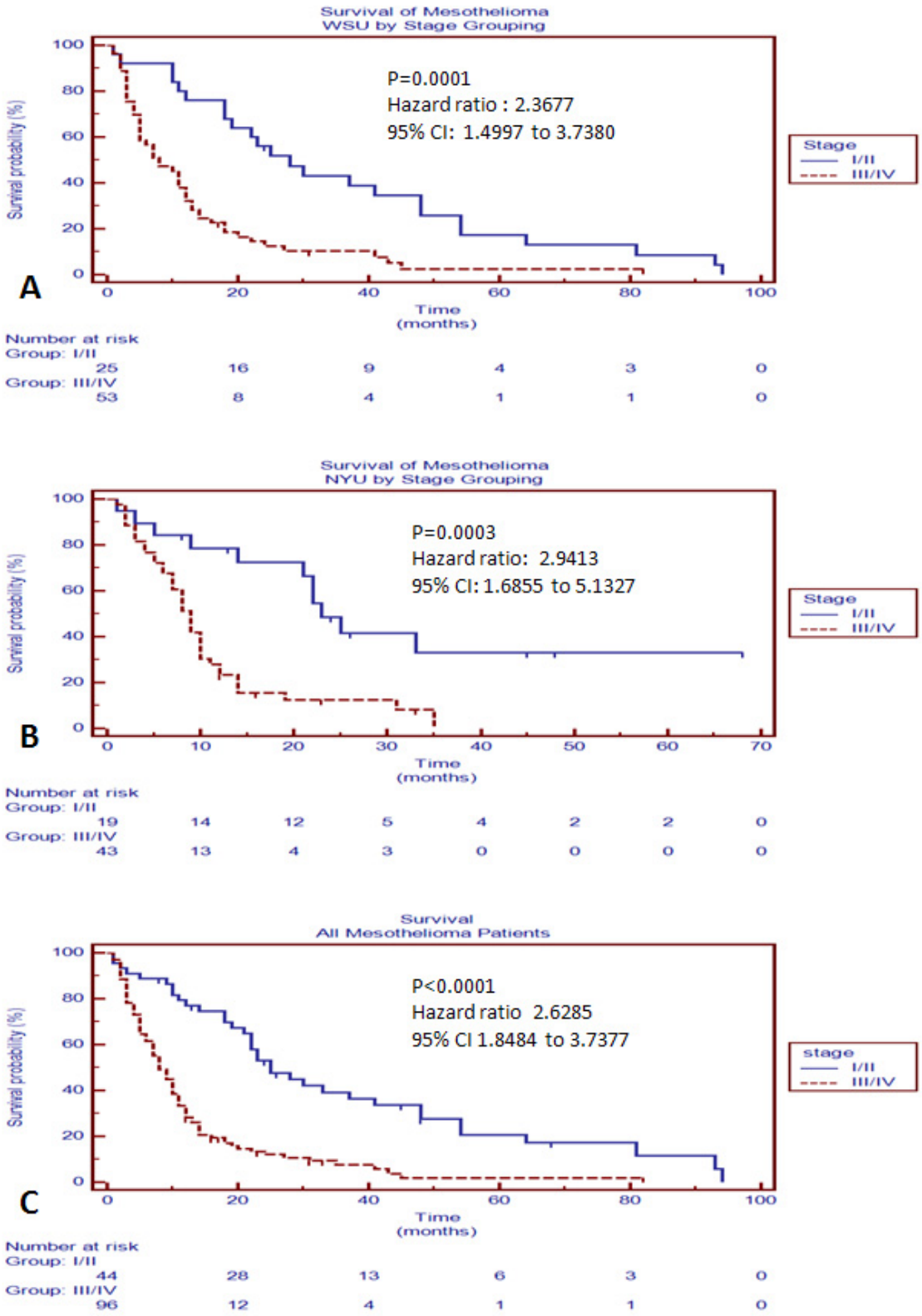

Figure S1: Survival of mesothelioma cohorts by stage grouping to demonstrate similarity of cohorts and consistency with other cohorts in the literature. A) WSU; B) NYU C) WSU and NYU combined

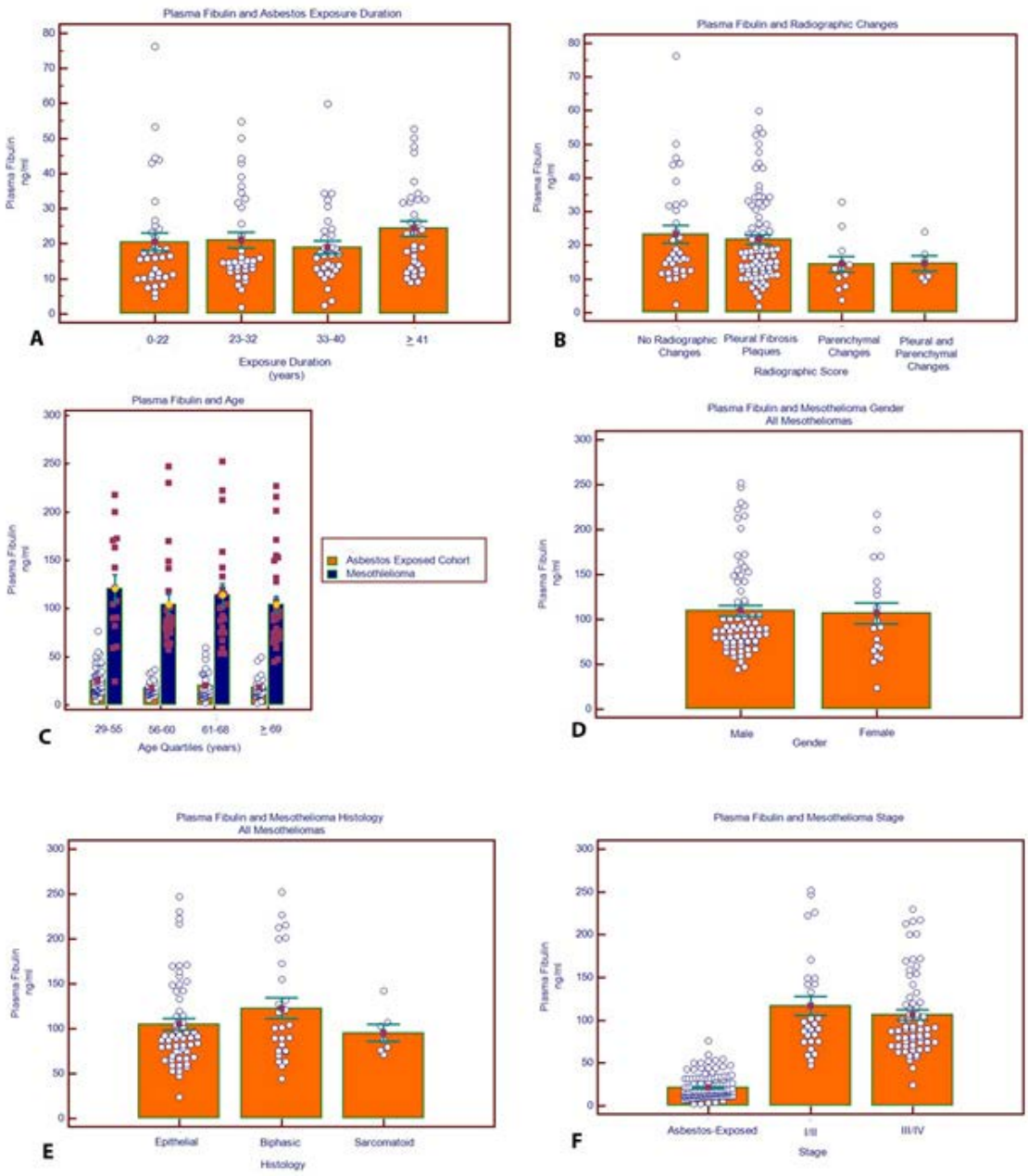

Figure S2: Plasma FBLN3 levels as a function of cohort demographics A) There was no correlation between level of FBLN3 and duration of asbestos exposure (in years, by quartiles); B) Plasma FBLN3 levels did not vary according to the severity of radiographic changes that were compatible with asbestos exposure; C) Plasma FBLN3 levels of mesothelioma patients or asbestos exposed non- malignant individuals were not influenced by age; D) No changes were seen in plasma FBLN3 levels of males compared to females; E) No difference was seen in plasma FBLN3 levels regarding the histologic type of mesothelioma studied; F) Mesothelioma patients had significantly higher levels of plasma **FBLN3 than** asbestos exposed individuals, and there was no difference between early stage (I/II) and late stage (III/IV) mesothelioma plasma FBLN3 levels.

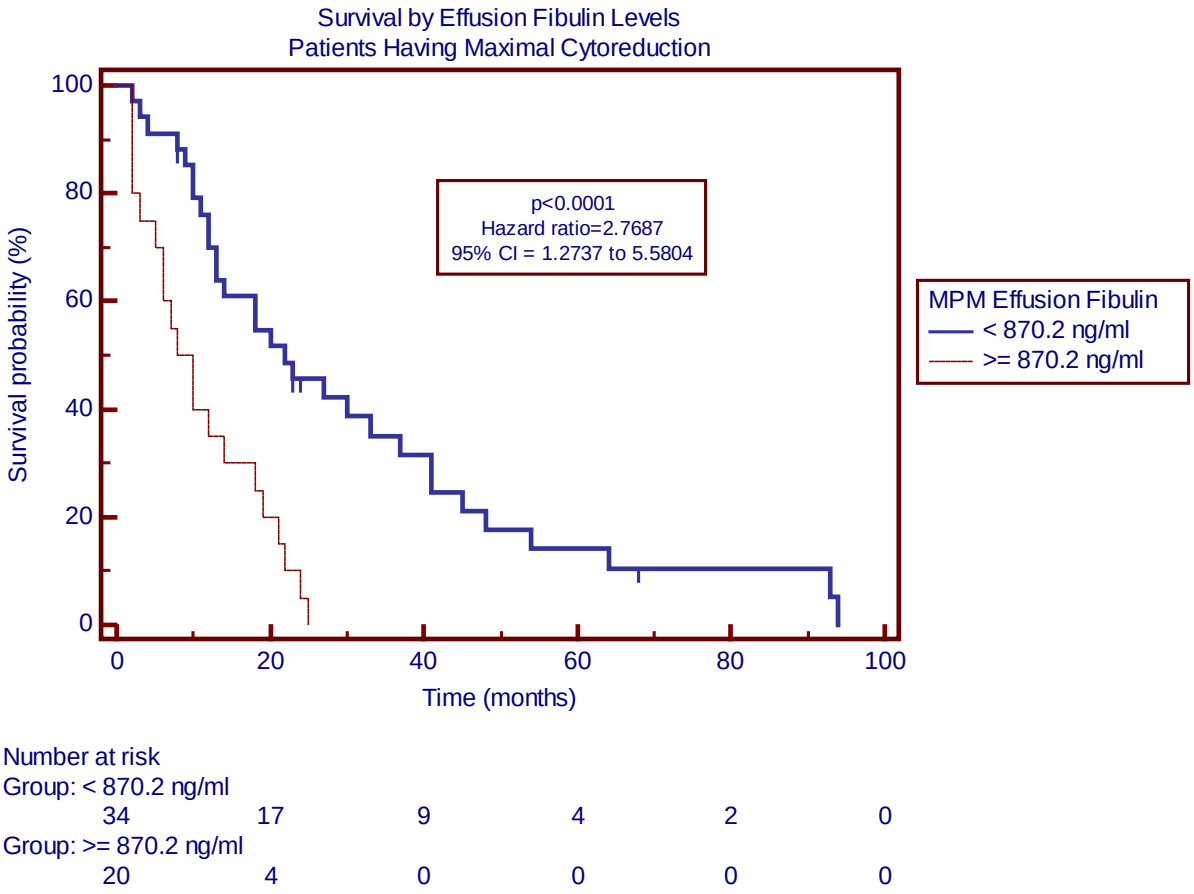

Figure S3: Alternate cutpoint determined by X-Tile statistical analysis for MPM effusion prognostication. Two distinct groups are seen with median survivals of 22 months and 8 months.

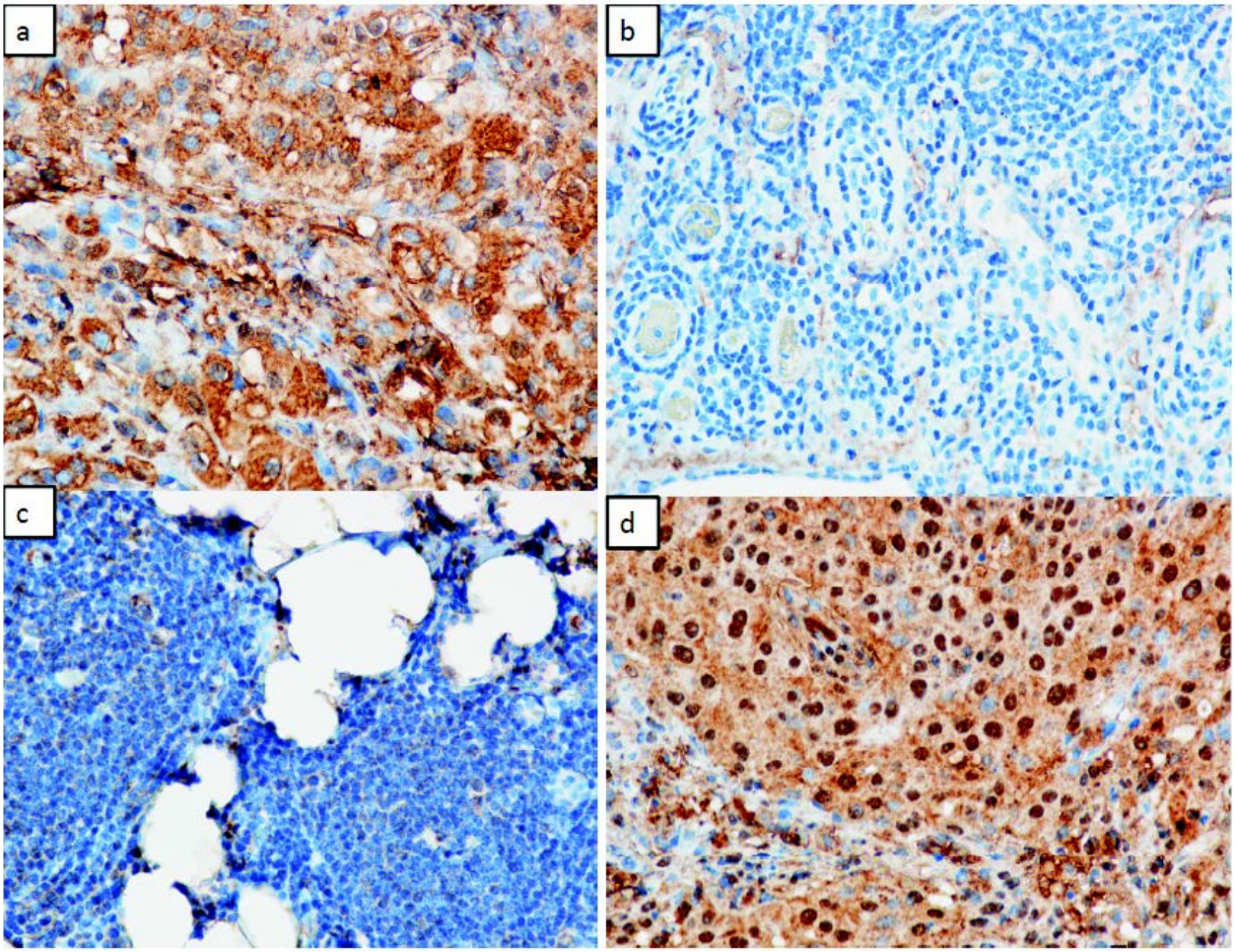

Figure S4: a) Epithelioid mesothelioma with diffuse strong cytoplasmic expression of fibulin-3 (400x), b) Mesothelial and submesothelial tissue including dense lymphoid tissue with minimal weak expression of fibulin-3 in vessel walls (400 x) c) Additional sample of cellular pleural tissue containing lymphoid tissue with only weak expression of fibulin-3 in macrophage type cells (400 x) d) Another case of epithelioid mesothelioma with diffuse strong cytoplasmic and nuclear expression of fibulin-3 (400x)

Table S1: WSU and NYU Cohort Comparisons of Effusion Fibulin				
			MPM vs non-MPM Effusions	
WSU	**AUC**		**0.95**	
	Cut-off of Maximum Sensitivity and Specificity		**378**	
	Sensitivity at 100% Specificity	**Cut-off**	**7.6**	**1007**
	Specificity at 100% Sensitivity	**Cut-off**	**24.5**	**97.1**
NYU	**AUC**		**0.91**	
	Cut-off of Maximum Sensitivity and Specificity		**346.0**	
	Sensitivity at 100% Specificity	**Cut-off**	**47.6**	**494.1**
	Specificity at 100% Sensitivity	**Cut-off**	**62.9**	**131.2**

Table S2: Multivariable Analysis of Prognostic Factors for Survival					
Covariate	**b**	**SE**	**P**	**Exp(b)**	**95% CI of Exp(b)**
cut733.4	0.8811	0.3897	0.0238	2.4136	1.1289 to 5.1605
gender	-0.08121	0.3858	0.8333	0.9220	0.4345 to 1.9563
histology	0.9364	0.3343	0.0051	2.5507	1.3291 to 4.8952
stage	1.4182	0.3793	0.0002	4.1297	1.9712 to 8.6521

Table S3: Multivariable Analysis of Prognostic Factors for Survival, Alternate Cut-point					
Covariate	**b**	**SE**	**P**	**Exp(b)**	**95% CI of Exp(b)**
cut870.2	0.9565	0.3483	0.0060	2.6025	1.3196 to 5.1326
gender	-0.09718	0.3835	0.7999	0.9074	0.4296 to 1.9167
histology	1.0043	0.3341	0.0026	2.7299	1.4231 to 5.2368
stage	1.5844	0.3731	<0.0001	4.8762	2.3557 to 10.0935

Reference List

1. Hu, B., et al. 2009. Fibulin-3 is uniquely upregulated in malignant gliomas and promotes tumor cell motility and invasion. Mol. Cancer Res. 7: 1756-1770.

2. En-lin, S., et al. 2010. The expression of EFEMP1 in cervical carcinoma and its relationship with prognosis. Gynecol. Oncol. 117: 417-422.

3. Camp RL, Dolled-Filhart M, Rimm DL. X-tile: a new bio-informatics tool for biomarker assessment and outcome-based cut-point optimization. Clin Cancer Res 2004;10(21):7252-7259