

Title:

Immunoglobulin G Complexes from Post-infectious ME/CFS, including post-COVID ME/CFS Disrupt Cellular Energetics and Alter Inflammatory Marker Secretion

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Supplementary Methods

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Drp1 anti-human antibody	Santa Cruz Biotechnology	Cat#sc-101270; RRID:AB_2093545
Mfn1 anti-human antibody	Santa Cruz Biotechnology	Cat#sc-166644; RRID:AB_2142616
Mfn2 anti-human antibody	Santa Cruz Biotechnology	Cat#sc-515647; RRID:AB_2811176
Mitofilin anti-human antibody	Abcam	Cat#ab245764
PLD6 anti-human antibody	Abcam	Cat#ab237612
p53 anti-human antibody	Santa Cruz Biotechnology	Cat#sc-126; RRID:AB_628082
FAM73A (Miga1) anti-human antibody	Invitrogen	Cat#PA553611; RRID:AB_2641452
TOMM20 anti-human antibody	Santa Cruz Biotechnology	Cat#sc-17764; RRID:AB_628381
TIM23 anti-human antibody	Santa Cruz Biotechnology	Cat#sc-514463; RRID:AB_2923126
LC3 β anti-human antibody	Santa Cruz Biotechnology	Cat#sc-376404; RRID:AB_11150489
LC3 β anti-human antibody	Proteintech	Cat#18725-1-AP; RRID:AB_2137745
PINK1 anti-human antibody	Novus Biologicals	Cat#NB100-644; RRID:AB_10001270
Actin anti-human antibody	Santa Cruz Biotechnology	Cat#sc-8432; RRID:AB_626630
Vinculin anti-human antibody	Santa Cruz Biotechnology	Cat#sc-73614; RRID:AB_1131294
HRP Rabbit anti-human IgG (whole molecule)	Sigma-Aldrich	Cat#A8792; RRID:AB_258414
HRP Goat anti-human IgG (Fc specific)	Sigma-Aldrich	Cat#A0170; RRID:AB_257868
HRP Goat anti-mouse IgG antibody	Sigma-Aldrich	Cat#12-348; RRID:AB_390191
HRP Goat anti-rabbit IgG antibody	Sigma-Aldrich	Cat#12-349; RRID:AB_390192
HRP anti-mouse antibody	Bio-Techne	Cat#DM-002; RRID:AB_3095298
HRP anti-rabbit antibody	Bio-Techne	Cat#DM-001; RRID:AB_3095297
Anti-Human IgG (H+L) CF594	Sigma-Aldrich	Cat#SAB4600097
Fluorescent anti-mouse antibody	Bio-Techne	Cat#DM-009
Fluorescent anti-rabbit antibody	Bio-Techne	Cat#DM-007

Azide Free Fc Receptor Blocker	Innovex Biosciences	Cat#NB335
Biological samples		
Healthy human blood	Riga Stradiņš University Ambulanz	Peripheral blood
Healthy control, ME/CFS, PCS-CFS, MS serum samples	Outpatient clinic of the Charité Universitätsmedizin, Berlin	Frozen serum samples
Systemic sclerosis serum samples	Clinic for Rheumatology and Clinical Immunology, University Hospital Schleswig-Holstein, Lübeck, Germany	Frozen serum samples
U2-OS human osteosarcoma cells	ATCC	Cat.HTB-96; RRID:CVCL_0042
HUVEC-TERT2 human endothelial cells	Evercyte, Austria	Cat.CHT-006-0008; RRID:CVCL_9Q53
HFF-TERT (Human foreskin fibroblast cells)	Cambridge Institute for Medical Research, University of Cambridge, UK	N/A
Critical commercial assays		
Simple Western 12-230 kDa gels	Bio-Techne	Cat#SM-W004
Simple Western 2-40 kDa gels	Bio-Techne	Cat#SM-W004
Protein G Sepharose 4 Fast Flow beads	Cytiva	Cat#17061801
Poly-Prep Chromatography Columns	BIO-RAD	Cat#731-1550
Slide-A-Lyzer MINI Dialysis Devices	Thermo Fisher Scientific	Cat#88404
Slide-A-Lyzer MINI Dialysis Devices	Thermo Fisher Scientific	Cat#88401
Easy-Titer Human IgG (H+L) Assay Kit	Thermo Fisher Scientific	Cat#23310
Pierce Fab Micro Preparation Kit	Thermo Fisher Scientific	Cat#44685
XFe96/XF pro cell culture microplates	Agilent	Cat#103793-100
Pierce BCA Protein Assay Kit	Thermo Fisher Scientific	Cat#23225
IL-6 ELISA kit	Thermo Fisher Scientific	Cat# 88-7066-88
IL-1 β ELISA kit	Thermo Fisher Scientific	Cat# 88-7346-88
TNF- α ELISA kit	Thermo Fisher Scientific	Cat# 88-7261-88
Milliplex MAP Human Cytokine, Chemokine, and Growth Factor Panel A Magnetic Bead Panel kit	Millipore	Cat.HCYTA-60K
Human Ficolin-2 ELISA Kit	Invitrogen	Cat#EH192RB
Human Transferrin ELISA Kit	Invitrogen	Cat#EHTF
Human Prekallikrein 1B ELISA Kit	Abcam	Cat#ab202405

Human Azurocidin/CAP37 ELISA Kit	Invitrogen	Cat#EH39RB
Human von Willebrand Factor (VWF) ELISA Kit	Invitrogen	Cat#EHVWF
Deposited data		
Immune complex mass spectrometry proteomics data		ProteomeXchange Consortium PRIDE dataset identifier PXD065439
Software and algorithms		
Fiji	https://imagej.net/software/fiji/	RRID:SCR_002285
MaxQuant version 1.6.2.2	https://www.maxquant.org/	RRID:SCR_014485
R package	https://www.R-project.org	RRID:SCR_001905
Scikit-learn (v0.23.2) Python package	https://pypi.org/project/scikit-learn/0.23.2/	RRID:SCR_008394
fgsea (version 1.32.2)	https://doi.org/10.1101/060012	RRID:SCR_020938
clusterProfiler (version 4.14.4)	https://doi.org/10.1089/omi.2011.0118	RRID:SCR_016884
DAVID (v2023q4) web tools	https://davidbioinformatics.nih.gov/	RRID:SCR_001881
LuxScan 3.0	https://www.capitalbiotechnology.com	N/A
Other		
McCoy's 5A cell culture media	Gibco	Cat16600082
DMEM cell culture media	Gibco	Cat11965092
RPMI 1640 cell culture media	ThermoFisher Scientific	Cat#22400-089
Fetal bovine serum (FBS)	Sigma-Aldrich	CatS0615
Penicillin-streptomycin	Gibco	Cat15140122
EBM basal medium	Lonza	Cat#CC-3121
EGM SingleQuot Kit	Lonza	Cat# CC-4133
Seahorse XF RPMI medium	Agilent	Cat#103576-100
D-glucose	Sigma Aldrich	Cat#G8769
D-Galactose	Sigma Aldrich	Cat#G5388
L-glutamine	Gibco	Cat#A2916801
Sodium pyruvate	Gibco	Cat#11360070
ProLong Glass Antifade Mountant with NucBlue Stain	Invitrogen	Cat#P36981
Histopaque-1077	Sigma-Aldrich	Cat#10771

Statistical analysis and modelling to study the correlation between disease severity and mitochondrial alterations

STEP 1: Group Analysis, including all the study groups

We fitted a multiple linear regression model to evaluate the relationship between mitochondrial morphology and disease severity as measured by the Bell disability scale. The response variable was the Bell score (lower scores indicate greater severity), and the predictors included mean mitochondrial surface area, age, gender, and disease group (healthy controls [HC] as the reference category). As Bell scores are not determined in routine clinical practice for healthy individuals, we have rounded the Bell scores to 100 for those healthy controls for whom values were unavailable.

Model Specification:

```
model <- lm(BellScore ~ MeanSurfaceArea + Age + Gender + DiseaseGroup, data = df2)
```

```
> model4 <- lm(BellScore ~ MeanSurfaceArea + Age + Gender + DiseaseGroup , data = df2)
> summary(model4)
```

Call:

```
lm(formula = BellScore ~ MeanSurfaceArea + Age + Gender + DiseaseGroup,
    data = df2)
```

Residuals:

Min	1Q	Median	3Q	Max
-33.302	-5.095	0.516	3.390	32.723

Coefficients:

	Estimate	Std. Error	t value	Pr(> t)
(Intercept)	86.93426	9.87912	8.800	4.85e-14 ***
MeanSurfaceArea	4.97632	3.60216	1.381	0.170
Age	0.01166	0.10985	0.106	0.916
GenderM	-2.02036	2.41645	-0.836	0.405
DiseaseGroupME/CFS	-66.58689	2.73469	-24.349	< 2e-16 ***
DiseaseGroupMS	-43.29126	3.88099	-11.155	< 2e-16 ***
DiseaseGroupPCS/CFS	-67.47335	3.62103	-18.634	< 2e-16 ***

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 11.22 on 98 degrees of freedom

Multiple R-squared: 0.8964, Adjusted R-squared: 0.8901

F-statistic: 141.3 on 6 and 98 DF, p-value: < 2.2e-16

Our analysis suggests that there is no strong relationship between average mitochondrial surface area and Bell score after adjusting for other factors (MeanSurfaceArea +4.97 (p = 0.17), **not statistically significant**). Similarly, Age +0.01 (p = 0.916) showed **no significant** association with Bell score (disease severity). We found no evidence of correlation between sex differences and disease severity in our model (Gender -2.02 (p = 0.405), **Not significant**).

As expected, all the three disease groups showed strong alterations in disease severity. DiseaseGroupME/CFS -66.58 (p < 2e-16) showed a **strong and significant** reduction in Bell score relative to HD.DiseaseGroupMS -43.29 (p = 2e-16) **also** showed a **significant reduction** relative to HD.DiseaseGroupPCS_CFS -67.47 (p < 2e-16) showed the **most severe reduction** among all the three disease groups.

Overall summary:

a) Disease Group is Highly Predictive

- All disease groups (ME/CFS, MS, PCS-CFS) have strong, significant negative coefficients. Bell score is 44–67 points lower in these groups vs HC, even after adjusting for surface area, age, and gender.

b) Age and Gender Are Not Predictive

- Age: virtually no effect (slope ~ 0.01), $p = 0.91$; GenderM: effect -2.0 , $p = 0.41$, i.e., no significant sex difference.
- Mean mitochondrial surface area is positively associated with Bell score (suggesting greater surface area may correspond to less severe disease), but this effect is not statistically significant ($p = 0.17$) in the presence of stronger covariates, such as disease group.

Based on these analyses, we concluded that even if average mitochondrial surface area had predictive value, changes in mitochondrial surface area are not significantly associated with disease severity.

STEP 2: ME/CFS disease group Analysis**HD vs ME/CFS**

```
df_sub <- df2 %>% filter(DiseaseGroup %in% c("HD", "ME/CFS"))
> summary(model_hc_mecfs)
```

```
> summary(model_hc_mecfs)
```

Call:

```
lm(formula = BellScore ~ MeanSurfaceArea + Age + Gender + DiseaseGroup,
    data = df_hc_mecfs)
```

Residuals:

Min	1Q	Median	3Q	Max
-20.8767	-2.0323	0.5087	2.5745	30.1148

Coefficients:

	Estimate	Std. Error	t value	Pr(> t)
(Intercept)	91.13974	8.33124	10.940	<2e-16 ***
MeanSurfaceArea	4.42440	3.00891	1.470	0.146
Age	-0.08135	0.09400	-0.865	0.390
GenderM	1.09577	2.08303	0.526	0.600
DiseaseGroupME/CFS	-66.91610	2.13035	-31.411	<2e-16 ***

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 8.61 on 75 degrees of freedom

Multiple R-squared: 0.9436, Adjusted R-squared: 0.9405

F-statistic: 313.5 on 4 and 75 DF, p-value: < 2.2e-16

Results:

- MeanSurfaceArea: Estimate = +4.42, p = 0.146 (**not significant**)
- GenderM: Estimate = +1.10, p = 0.60 (**not significant**)
- DiseaseGroup ME/CFS: (**Highly significant (-66.9, p < 2e-16)**)

Based on these results, we concluded that ME/CFS disease severity is not associated with mitochondrial surface area or gender.

ME/CFS only

```
df_mecfs <- df2 %>% filter(DiseaseGroup == "ME/CFS")
```

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Call:

```
lm(formula = BellScore ~ MeanSurfaceArea + Age + Gender, data = df_mecfs)
```

Residuals:

	Min	1Q	Median	3Q	Max
	-21.1843	-8.6902	-0.8667	7.2933	31.4118

Coefficients:

	Estimate	Std. Error	t value	Pr(> t)
(Intercept)	25.3761	12.8645	1.973	0.0561 .
MeanSurfaceArea	5.9333	4.7362	1.253	0.2182
Age	-0.1889	0.2087	-0.905	0.3711
GenderM	1.0015	3.7780	0.265	0.7924

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 11.21 on 37 degrees of freedom

Multiple R-squared: 0.06234, Adjusted R-squared: -0.01368

F-statistic: 0.82 on 3 and 37 DF, p-value: 0.4911

Results:

- MeanSurfaceArea: Estimate = +5.93, p = 0.218 (**not significant**).
- GenderM: Estimate = +1.00, p = 0.79 (**not significant**).
- Also, $R^2 = 0.06$, which is a **very weak model fit**.

Based on these results, we concluded that even within ME/CFS disease group, disease severity is not significantly associated with mitochondrial surface area and gender.

STEP 3:

Interaction model:

Does the effect of average mitochondrial surface area (MeanSurfaceArea) depend on disease group?

```
> summary(model_interact)
```

Call:

```
lm(formula = BellScore ~ MeanSurfaceArea * DiseaseGroup + Age +
    Gender, data = df2)
```

Residuals:

```
      Min       1Q   Median       3Q      Max
-31.174  -3.849   0.646   2.872  31.720
```

Coefficients:

	Estimate	Std. Error	t value	Pr(> t)	
(Intercept)	98.3621	17.4939	5.623	1.88e-07	***
MeanSurfaceArea	0.9296	6.9370	0.134	0.893684	
DiseaseGroupME/CFS	-78.7322	19.4621	-4.045	0.000106	***
DiseaseGroupMS	-139.7897	43.0619	-3.246	0.001616	**
DiseaseGroupPCS/CFS	-51.9106	28.5035	-1.821	0.071724	.
Age	-0.0343	0.1096	-0.313	0.754933	
GenderM	-1.4781	2.3988	-0.616	0.539254	
MeanSurfaceArea:DiseaseGroupME/CFS	5.1799	8.3642	0.619	0.537203	
MeanSurfaceArea:DiseaseGroupMS	40.7208	18.0917	2.251	0.026704	*
MeanSurfaceArea:DiseaseGroupPCS/CFS	-7.7618	12.7689	-0.608	0.544722	

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 11.03 on 95 degrees of freedom

Multiple R-squared: 0.9029, Adjusted R-squared: 0.8937

F-statistic: 98.19 on 9 and 95 DF, p-value: < 2.2e-16

Results:

- **MeanSurfaceArea:DiseaseGroupMS: Estimate = +40.72, p = 0.027 is significant.**
- All other interactions (including ME/CFS × SurfaceArea): Not significant
So, average mitochondrial surface area **does significantly predict Bell score in MS**, but not in ME/CFS or PCS-CFS.

Conclusions:

- So the original conclusion — that **disease severity (Bell score) is not significantly associated with average mitochondrial surface area** — holds even when:
 - PCS-CFS and MS groups are excluded.
 - The model is restricted to just HD vs ME/CFS.
 - The model is limited to ME/CFS only.
- This confirms that the non-significance is not an artifact of mixing disease groups.

- Gender is **consistently non-significant across all models**, including full cohort, HC vs ME/CFS, and ME/CFS-only — suggesting no evidence for gender differences in disease severity.
- The only group where average mitochondrial surface area is significantly associated with Bell score is **MS**, where a larger surface area correlates with a higher (better) functional status.
- This suggests that the role of mitochondrial morphology in predicting disease severity is disease-specific (in MS); in MS, mitochondrial fragmentation may directly relate to functional status, whereas in ME/CFS and PCS-CFS, disease severity may be driven by other factors not captured by mitochondrial surface area alone.

In summary, disease severity as measured by Bell score is not significantly associated with average mitochondrial surface area and gender in ME/CFS and PCS-CFS. However, for MS, disease severity is significantly predictive of average mitochondrial surface area.