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Plasma Mitochondrial DNA is Associated with Extrapulmonary Sarcoidosis

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Contributions

CR performed experiments, performed statistical analysis, and analyzed data; CB performed experiments and analyzed data, TA performed experiments, BH performed statistical analysis, DWK performed experiments, MY performed experiments, EPM analyzed data, AW performed experiments, BR performed experiments, HP assisted with statistical analysis, JW performed experiments, MM analyzed data, CSDC analyzed data, NK analyzed data, MG recruited subjects, procured biospecimens, and analyzed data, and ELH conceived experimental design and analyzed data. All authors participated in manuscript preparation and provided final approval of the submitted work.

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Take Home Message: Extrapulmonary Sarcoidosis is a devastating disease phenotype that disproportionately affects African-Americans (AAs). Enrichments in plasma mitochondrial DNA are seen in Sarcoidosis and are associated with extrapulmonary disease and AA descent.

ABSTRACT

Sarcoidosis is an unpredictable granulomatous disease in which African-Americans (AAs) disproportionately experience aggressive phenotypes. Mitochondrial DNA (mtDNA) released by cells in response to various stressors contributes to tissue remodeling and inflammation. While extracellular mtDNA has emerged as a biomarker in multiple diseases, its relevance to sarcoidosis remains unknown. We aimed to define an association between extracellular mtDNA and clinical features of sarcoidosis.

Extracellular mtDNA concentrations were measured using qPCR for the human MT-ATP6 gene on bronchoalveolar (BAL) and plasma samples from healthy control and sarcoidosis subjects from The Yale Lung Repository; associations between MT-ATP6 concentrations and Scadding Stage, extrapulmonary disease, and demographics were sought. Results were validated in the Genomic Research in Alpha-1 Antitrypsin Deficiency and Sarcoidosis cohort.

Relative to Controls, MT-ATP6 concentrations in sarcoidosis subjects were robustly elevated in the BAL and plasma, particularly in the plasma of subjects with extrapulmonary disease. Relative to Caucasians, AAs displayed excessive MT-ATP6 concentrations in the BAL and plasma, where the latter compartment correlated with significantly high odds of extrapulmonary disease.

Enrichments in extracellular mtDNA in sarcoidosis are associated with extrapulmonary disease and AA descent. Further study into the mechanistic basis of these clinical findings may lead to novel pathophysiologic and therapeutic insights.

Word Count: 200

Key Words: (1) Mitochondrial DNA, (2) Sarcoidosis, (3) Biomarkers

INTRODUCTION

Sarcoidosis is a granulomatous disease of unknown etiology with an unpredictable clinical course where some patients experience self-limited or stable disease and others develop progressive, debilitating impairment with multiorgan involvement [1]. For unknown reasons, African-American (AA) patients experience significant morbidity and mortality from increased rates of fibrotic lung disease and extrapulmonary manifestations [2]. Identification of mechanistic biomarkers predicting the development of fibrotic and/or extrapulmonary disease represents an unmet need as presently, there are no accepted biomarkers identifying patients at-risk for these aggressive disease phenotypes [3, 4].

Although it is widely accepted that granuloma formation involves an adaptive immune response, [1], the pathobiologic contribution of innate immunity remains less defined [5]. While studies show that alterations in macrophage proliferation [6] and activation [7] mediate granuloma formation, and differential expression of innate immune receptors [8], particularly Toll Like Receptor 9 (TLR9) [9], demonstrate diagnostic properties in sarcoidosis [10], the mechanism(s) through which innate immune processes are involved remain unknown. Innate immunity is activated by receptor mediated recognition of agonists such as pathogen associated molecular patterns (PAMPS), which arise from infectious agents, and danger associated molecular patterns (DAMPS) that are generated by injured cells [11]. Most sources agree that sarcoidosis results from the host response to infectious or environmental exposures [12, 13], but the innate immune response to endogenous ligands is unknown. One potential innate immune ligand is the unmethylated, CpG-rich mitochondrial DNA (mtDNA) that functions as an endogenous TLR9 agonist [14-16]. Released either non-specifically by necrotic cells [17] with the nuclear DNA binding

protein High Mobility Group Box 1 (HMGB1) [18, 19] or via regulated processes by stressed but viable cells [14, 20], extracellular mtDNA mediates both antimicrobial and pro-inflammatory responses [21]. Experimental exposure to mtDNA or synthetic analogs activates macrophages [22] and TLR9 [10], but little is known regarding the association between mtDNA and granulomatous processes. Thus, elucidation of its relevance in sarcoidosis - particularly regarding severe disease phenotypes - may provide mechanistic insight.

Disparate rates of fibrotic and extrapulmonary disease between Caucasian and AA sarcoidosis patients remain poorly understood [1, 23]. Epidemiologic studies indicate that socioeconomic status and environmental factors do not fully account for these observations [24], and genome-wide association studies (GWAS) have demonstrated an increased incidence of fibroprolierative disorders among AAs, including a subgroup of sarcoidosis [24, 25]. However, correlating genetic variants with specific, clinically relevant disease phenotypes requires further study [4]. Thus, identification of biomarkers reflective of the sarcoidosis disease state might enhance our understanding of the biological basis behind the worsened clinical outcomes observed among AAs.

While the diagnostic and prognostic significance of extracellular mtDNA has been demonstrated in various diseases [14, 26, 27], a relationship with sarcoidosis is unknown. In this study, we used bronchoalveolar (BAL) and plasma samples from subjects obtained from two independent sarcoidosis cohorts to define an association between extracellular mtDNA and severe clinical phenotypes in this enigmatic disease.

MATERIALS AND METHODS

Human Subjects

Discovery cohort: BAL and plasma specimens and corresponding clinical data were obtained from The Yale Lung Repository (TYLR) housed within the ILD Center of Excellence at Yale School of Medicine. *Validation cohort:* BAL and plasma specimens and corresponding clinical data were obtained from the Genomic Information Center (GIC) of the Genomic Research in Alpha-1 Antitrypsin Deficiency and Sarcoidosis (GRADS) study. The study rationale and procedures have been previously described [28].

Control group: Biospecimens from healthy subjects without known inflammatory or lung disease were obtained from TYLR [26]. All human studies were performed with informed consent on protocols approved by the Institutional Review Board at each participating institution and by the GRADS GIC. Sarcoidosis diagnosis was based on current consensus guidelines [28, 29]. Clinical data included disease duration, pulmonary function testing results for percent predicted forced vital capacity (FVC%), forced expiratory volume after 1 second (FEV1%), FEV1/FVC, diffusion capacity of the lung for carbon monoxide (D_{LCO}%), Scadding Stage, the presence of extrapulmonary disease, active or recent use of systemic therapy, and the patient-centered outcome of fatigue, as determined by the Fatigue Assessment Scale (FAS) [30].

Mitochondrial DNA Quantification

Isolation and quantification of mtDNA from biospecimens were performed [14, 26] as outlined in the online supplement.

Toll like Receptor 9 Detection

Commercially available human TLR9-expressing HEK 293 cells were cultured and assayed for TLR9 activation [14] as outlined in the online supplement.

High Mobility Group Box 1 Quantification

Quantification of plasma HMGB1 concentrations were performed using a commercially available enzyme-linked immunosorbent assay (ELISA) [31] as outlined in the online supplement.

Statistical Analysis

Data distribution was assessed using D'Agostino-Pearson omnibus test. Categorical data was analyzed with Fischer's exact test. Parametric comparisons were made using Student's t-test, and non-parametric data were compared using Mann-Whitney. Multivariate analysis was completed with multiple linear regression. Receiver operator curve (ROC) analysis was performed to determine a threshold value MT-ATP6 value for extrapulmonary disease. Logistic regression models were developed to determine odds ratios. These evaluations were performed using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA), MedCalc (MedCalc Software, Belgium), or SAS 9.4 (SAS Institute Inc., Cary, NC).

RESULTS

Patient population

We analyzed BAL and plasma specimens from control and sarcoidosis subjects from Yale, and then validated our findings with GRADS subjects [28]. Demographic and clinical characteristics are shown in Table 1. For the derivation cohort, control and sarcoidosis subjects were recruited from the Greater New Haven area, where controls were demographically matched by age, gender, and race. For the validation cohort, we leveraged resources from the GRADS study. Notably, although Yale was a GRADS site, for our purposes, we excluded the Yale GRADS subjects since they had been evaluated previously. Interestingly, Yale sarcoidosis and GRADS subjects had significantly higher rates of smoking than controls. However, there was no association found between smoking and mtDNA concentrations (Figure S1A-E).

Extracellular mtDNA is elevated in the BAL and plasma of sarcoidosis subjects in the discovery cohort

Elevated extracellular mtDNA concentrations present in the BAL and plasma of various diseases lend clinically relevant insight [32]. To determine if similar findings are seen in sarcoidosis, we began by evaluating BAL and plasma samples from the relatively small Yale sarcoidosis cohort and demographically matched controls using a well validated method that measures copy numbers of the mtDNA specific gene, MT-ATP6 [14, 26]. We found significant increases in both the BAL (3.805 vs 4.716 log copies/µl, p=0.008, Figure 1A) and plasma (3.174 vs 5.032 log copies/µl, p<0.0001, Figure 1B) of sarcoidosis subjects, independent of age, gender, AA race, smoking status, and treatment status. Importantly, in this cohort, mtDNA concentrations were not associated with platelet counts, a common confounder of mtDNA assays [33] (Spearman r=-0.017, p=0.793, Figure S2A), nor associated with leukocyte counts (Spearman r=0.047, p=0.818, Figure S2B). Interestingly, while there was no correlation between MT-ATP6 copy numbers in the BAL and plasma of matched samples (Spearman r=0.344, p=0.149, Figure 1C), median MT-ATP6 concentrations were an order of magnitude lower in the BAL than their respective plasma sample (4.716 vs 5.060 log copies/µl, p=0.029, Figure 1D). These data show that local and circulating concentrations of mtDNA are enriched in sarcoidosis, suggesting a connection to the disease state.

Extracellular mtDNA is elevated in the BAL and plasma of sarcoidosis subjects in the validation cohort

Sarcoidosis is a heterogeneous disease that displays differences in clinical phenotypes that somewhat varies by geographic region [34]. Thus, it was necessary to validate our findings in a larger, more heterogeneous cohort, and we employed

the NIH sponsored GRADS study, which enrolled and characterized subjects with various forms of sarcoidosis across nine U.S. institutions from 2013 to 2015 [28]. When we repeated the above studies in the GRADS samples, we found similar increases in the BAL (3.805 vs 4.178 log copies/μl, p=0.022, Figure 2A) and plasma (3.174 vs 5.343 log copies/μl, p<0.0001, Figure 2B) that, like the Yale subjects, were independent of age, gender, AA race, smoking status, and treatment status. As with the derivation cohort, there was also no correlation between MT-ATP6 copy numbers in the BAL and plasma (Spearman r=0.010, p=0.888, Figure 2C), and median MT-ATP6 concentrations were similarly lower in the BAL than plasma (4.178 vs 5.331 log copies/μl, p<0.0001, Figure 2D). To understand the functional relevance of mtDNA in the circulation, we then stimulated the above TLR9-expressing HEK 293 cells with control and GRADS plasma. Relative to plasma from healthy controls, plasma obtained from GRADS subjects robustly resulted in TLR9 activation (0.193 vs 0.409 absorbance at 640 nm, p<0.0001, Figure 3A) that significantly correlated with plasma MT-ATP6 concentrations (Spearman r=0.410, p<0.0001, Figure 3B), suggesting the presence of an TLR9 ligand, *specifically mtDNA*, in the plasma of sarcoidosis subjects. Moreover, among GRADS subjects, plasma mtDNA copy numbers did not correlate with their respective plasma concentration of the DNA binding protein HMGB1 (Spearman r=0.138, p=0.016, Figure 3C), indicating an active process by which mtDNA is released into the circulation. These data confirm that the TLR9 agonist mtDNA is elevated in the lungs and blood of sarcoidosis subjects.

Extracellular mtDNA is not increased in Pulmonary Sarcoidosis

Having detected substantial increases in local and circulating mtDNA in two sarcoidosis cohorts, we next sought an association with clinical phenotypes.

Management strategies differentiate patients with and without lung involvement based on Scadding Stage, which despite its limitations, remains the clinical standard [35]. To determine whether mtDNA in the BAL or plasma are elevated in subjects with known lung involvement, we stratified the Yale cohort into those lacking detectable lung involvement (Stage 0/I) vs those with clear lung involvement (Stages II, III, and IV). Somewhat surprisingly, this approach failed to discern significant differences in the BAL (4.385 vs 4.812 log copies/µl, p=0.100, Figure S3A) or plasma (5.038 vs 5.208 log copies/µl, p=0.537, Figure S3B), a finding that was also seen in the GRADS BAL (4.134 vs 4.169 log copies/µl, p=0.775, Figure S3C) and plasma (5.348 vs 5.327 log copies/µl, p=0.119, Figure S3D). When Stage I subjects were omitted from the analysis, there was no significant differences between subjects with Stage 0 disease versus those with Stage II, III, or IV disease in the BAL (5.127 vs 5.321 log copies/µl, p=0.588) or plasma (5.208 vs 5.327 log copies/µl, p=0.865) of the GRADS cohort, although this approach did trend towards statistical significance. Moreover, neither BAL nor plasma MT-ATP6 concentrations showed any correlation with commonly used measures of lung function in both cohorts, including FEV1% (Figures S4A-D), FVC% (Figures S5A-D), and D_{LCO}% (Figures S6A-D). These data show that detection of extracellular mtDNA is increased in the BAL and plasma of Sarcoidosis subjects in a manner that is independent of pulmonary involvement, indicating a potential relationship with other disease features.

Plasma mtDNA is elevated in extrapulmonary disease

A clinically significant indicator of disease severity is the presence of extrapulmonary involvement, which portends significant morbidity and mortality [36, 37]. The diagnosis of organ involvement followed modified ACCESS criteria per the GRADS protocol [28], and the extrapulmonary organ systems involved are shown for Yale

(Table S1) and GRADS (Table S2), where dermatologic, cardiac, and joint involvement were the three most commonly reported extrapulmonary manifestations in both cohorts. To determine whether mtDNA was associated with this complication, BAL and plasma specimens from the Yale subjects without and with extrapulmonary disease were analyzed. As shown in Figure 4A-B, MT-ATP6 concentrations in both cohorts were similar in the BAL regardless of the absence or presence of extrapulmonary disease (Yale: 4.730 vs 4.692, log copies/μl, p=0.773; GRADS: 4.064 vs 4.160, log copies/μl, p=0.279). However, when comparing the plasma of Yale subjects with lung-restricted disease to subjects with extrapulmonary involvement, MT-ATP6 concentrations were substantially elevated (4.522 vs 5.066 log copies/μl, p=0.001, Figure 4C). These findings were recapitulated in the GRADS cohort (5.152 vs 5.377 log copies/μl, p=0.019, Figure 4D). Not surprisingly, in a functional investigation of this mitochondrial-related DAMP, relative to the plasma from GRADS subjects lacking extrapulmonary involvement, plasma from those with extrapulmonary disease exhibited greater TLR9 activation (0.375 vs 0.426 absorbance at 640 nm, p=0.001, Figure 5A). Furthermore, these observations did not appear to be related to necrosis as plasma MT-ATP6 copy numbers did not correlate with plasma HMGB1 concentrations among subjects with extrapulmonary disease (Spearman r=0.130, p=0.058, Figure 5B). Interestingly, no organ-specific associations with plasma MT-ATP6 concentrations were found in either cohort when evaluating those without and with dermatologic (Figures S7A-B), cardiac (Figures S7C-D), or joint disease (Figures S7E-F). These findings demonstrate an association between plasma mtDNA concentrations and extrapulmonary disease.

Elevated plasma mtDNA is associated with high odds of extrapulmonary disease

We then evaluated the clinical relevance of these results since, as patients with extrapulmonary sarcoidosis are often asymptomatic [38], an easily measured blood biomarker identifying patients at-risk for extrapulmonary disease will be of great clinical utility. ROC analysis on the Yale cohort revealed that a plasma MT-ATP6 copy number of 4.71 log copies/µl can reliably stratify subjects for low odds (<4.71 log copies/µl) or high odds (≥4.71 log copies/µl) of extrapulmonary disease (area under the curve (AUC): 0.836, p=0.001, Figure S8). Subjects with plasma MT-ATP6 concentrations ≥4.71 log copies/µl had substantially increased odds of extrapulmonary disease at the time of evaluation (odds ratio (OR) 8.500, 95% confidence interval (CI) 1.964-36.790, p=0.004, Figure 5C), independent of age, gender, AA race, smoking, treatment, and Stage IV disease. The predictive ability of this threshold value was validated in the GRADS cohort, where following adjustment for relevant covariates, subjects with plasma MT-ATP6 concentrations ≥4.71 log copies/µl were also more likely to have extrapulmonary involvement (OR 2.021, 95% CI 1.494-2.735, p<0.0001, Figure 5C). In keeping with these data, a significant association was found between plasma mtDNA and the patient centered outcome of fatigue in both cohorts. Here, relative to participants reporting normal fatigue scores, subjects who reported elevated fatigue scores displayed significantly higher levels of plasma MT-ATP6 concentrations (Yale: 4.791 vs 5.516 log copies/µl, p=0.004, Figure 5D; GRADS: 5.341 vs 5.534 log copies/µl, p=0.005, Figure 5E). These findings show that the elevated plasma mtDNA is marker of both extrapulmonary disease and fatigue, perhaps reflecting the ongoing systemic inflammation that contributes to this condition.

Extracellular mtDNA is increased in the BAL and plasma of AA Sarcoidosis subjects

The data presented above indicate that extracellular mtDNA is a marker of extrapulmonary disease in two sarcoidosis cohorts. This led to question whether elevated extracellular mtDNA might also be seen in AA subjects, who relative to their Caucasian counterparts, are at higher risk for severe disease phenotypes [2]. To this end, BAL and plasma mtDNA concentrations were compared between participants of Caucasian and AA descent in both cohorts. Subject characteristics between Caucasians and AAs are shown for Yale (Table S3) and GRADS (Table S4). In the Yale cohort, compared to Caucasians with sarcoidosis, BAL MT-ATP6 concentrations were not increased in AAs (4.385 vs 4.874 log copies/μl, p=0.221, Figure 6A). However, MT-ATP6 concentrations were substantially elevated in the plasma of AAs in the Yale (4.792 vs 5.564 log copies/μl, p=0.004, Figure 6B) and GRADS (4.793 vs 5.010 log copies/μl, p=0.006, Figure 6C) cohorts. These findings in the plasma were independent of age, gender, smoking, treatment, Scadding Stage, and extrapulmonary disease. Additionally, relative to the plasma from Caucasian GRADS subjects, AA GRADS plasma demonstrated greater TLR9 activation (0.394 vs 0.448 absorbance at 640 nm, p<0.0001, Figure 7A), and plasma MT-ATP6 concentrations were independent of plasma HMGB1 concentrations (Spearman r=0.035, p=0.799, Figure 7B). These data demonstrate that the TLR9 agonist mtDNA is elevated in the circulation of AA sarcoidosis subjects in two independent cohorts.

Extracellular mtDNA provides race-specific associations with extrapulmonary disease

After finding that plasma mtDNA is elevated in AA sarcoidosis subjects, we then explored whether this provided race-specific associations with extrapulmonary disease, a frequent complication observed among AAs [2]. Only the GRADS cohort

met a sample size suitable for this analysis, and here, profound racial differences emerged. Relative to Caucasian race, AA race was independently associated with extrapulmonary disease (OR 2.497, 95% CI: 1.271-4.905, p=0.008). This was further reflected in the plasma; when compared to Caucasians with extrapulmonary disease, AAs with extrapulmonary disease had higher MT-ATP6 concentrations (5.348 vs 5.482 log copies/μl, p=0.021, Figure 7C). A logistic regression model was then developed to determine the odds of extrapulmonary disease based on the previously derived threshold MT-ATP6 copy number of 4.71 log copies/μl and AA race, and ROC analysis of this multivariate model revealed an AUC of 0.611. AAs whose plasma MT-ATP6 concentrations exceeded ≥ 4.71 log copies/μl had the highest odds of having extrapulmonary disease (OR 4.700, 95% CI: 2.375-9.300, p<0.0001, Figure 7D). In fact, Caucasians with plasma mtDNA concentrations exceeding 4.71 log copies/μl were less likely to have extrapulmonary disease than their AA counterparts (OR 2.293, 95% CI: 1.084-4.849, p=0.030, Figure 7D). These findings show that elevated plasma mtDNA concentrations convey important information regarding the odds of AA sarcoidosis subjects being afflicted with extrapulmonary disease.

DISCUSSION

In novel analysis of two independent sarcoidosis cohorts, we found a significant association between excessive extracellular mtDNA concentrations, extrapulmonary involvement, and racial differences in disease. Specifically, enriched mtDNA copy numbers were found in the BAL and plasma of subjects with sarcoidosis, where increases in the latter compartment was robustly associated with extrapulmonary disease. In addition, increased mtDNA concentrations were also seen in AA subjects, an at-risk population for poor disease outcomes.

Since initially reported as a mediator of inflammatory joint disease, the pathogenic significance of extracellular mtDNA has been increasingly recognized [32]. The extracellular release of mtDNA occurs either non-specifically, typically in response to cellular stress or necrosis, or actively through extracellular vesicles (EVs) [32]. Because we failed to detect an association between levels of MT-ATP6 and the commonly used necroptosis marker HGMB1, we believe this extracellular mtDNA accumulation results from a mechanism other than or in addition to necroptosis. Interestingly, trafficking of mtDNA via EVs mediates cell-to-cell signal transduction as part of the inflammatory response [39], likely via activation of TLR9 [14] and STING [40] pathways. Because we found that sarcoidosis plasma showed robust TLR9 activating capacity, we believe that the most likely functional ramification of our work relates to activation of this pathway. However, alternate explanations may exist. As some postulate an infectious etiology for sarcoidosis [34], it is possible that extracellular mtDNA is acting in an antimicrobial facility by activating the NLRP3 inflammasome [41], forming extracellular traps [42], or provoking mitochondrial antiviral signaling [43]. As a mediator of inflammatory, infectious, and fibrosing processes, further mechanistic and functional study of mtDNA in sarcoidosis can yield novel insights into its enigmatic etiology.

Our work also presents new observations regarding the association between mtDNA and sarcoidosis phenotypes. The finding that circulating mtDNA was elevated in subjects with extrapulmonary disease, but not pulmonary granuloma formation, may reflect previously unrecognized differences in the mechanism between the granulomatous response to inhaled vs systemically delivered antigen. It should be noted that plasma mtDNA concentrations were an order of magnitude higher than that of the BAL, which may reflect true biology, technical artifact in BAL

sampling and processing (such as dilutions involved in obtaining BAL fluid), or a yet to be identified experimental or clinical factor(s). Additional work is required to determine how mtDNA, pulmonary sarcoidosis, and extrapulmonary involvement are linked.

Perhaps our most exciting finding is the observed racial differences in mtDNA, especially with aggressive clinical phenotypes. GWAS have identified susceptible genetic variants for sarcoidosis among AAs [44], and racial differences in innate immunity have been demonstrated, particualrly in TLR9 polymorphisms related to breast cancer [45] and infectious granulomas [46], and in TLR9 expression in systemic lupus erythematosus [47]. An association between TLR9 and sarcoidosis has been reported [48], although racial differences have not been explored. However, an association with race and TLR4 variants has been shown [49], and because crosstalk exists between TLR4 and TLR9 [50], it is possible that an endognous TLR9 agonist results in excessive inflammation that then feeds into TLR4, leading to significant injury and extrapulmonary involvement. This hypothesis will require further study with overexpression and knockdown strategies.

While novel and provocative, our study has several limitations. We have not identified the tissue(s) from which mtDNA originates, which will include identifying the cell of origin, mechanism(s) of release, association with mitochondrial function, and regulation of extracellular trafficking. Despite these relatively minor limitations, our work provides strong evidence supporting circulating mtDNA as a biomarker of organ involvement and racially disparate clinical presentations of sarcoidosis. Further investigation may lead to new therapeutic avenues for this poorly understood and difficult to treat disease.

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TABLES

Table 1. Baseline characteristics of control and Sarcoidosis subjects.

	Control Subjects	Yale Sarcoidosis	GRADS Sarcoidosis	p-value
N	50	27	304	
Age (years)				
Mean ± SD	53.48 ± 19.92	50.56 ± 13.38	54.78 ± 9.82	0.138
Gender n (%)				
Female	27 (54.00)	10 (37.04)	141 (46.38)	0.353
Race n (%)				
Caucasian	44 (88.00)	20 (74.07)	238 (78.29)	0.231
African-American	6 (12.00)	7 (25.93)	66 (21.71)	
Smoking Status n (%)				
Ever/Current	4 (8.00)	12 (44.44)	99 (32.56)	**0.001**
Never	46 (92.00)	15 (55.55)	205 (67.43)	
Institution				
National Jewish Health			75 (24.67)	
University of California-San Francisco			55 (18.09)	
Johns Hopkins University			44 (14.47)	
University of Pennsylvania			43 (14.14)	
Vanderbilt University			34 (11.18)	
University of Pittsburgh			26 (8.55)	
Arizona Health Science Center			24 (7.87)	
Medical University of South Carolina			3 (0.01)	
Disease Duration (mean ± SD)		5.93 ± 8.08	11.92 ± 18.03	**0.001**
Extrapulmonary Disease n (%)		20 (74.07)	214 (70.39)	0.827
Scadding Stage				
Stage 0 n (%)		6 (22.22)	34 (11.18)	0.116
Stage I n (%)		4 (14.81)	67 (22.04)	0.471
Stage II n (%)		12 (44.44)	90 (29.61)	0.129
Stage III n (%)		0 (0.00)	41 (13.49)	**0.034**
Stage IV n (%)		5 (18.52)	72 (23.68)	0.641
FVC (mean percent predicted ± SD)		89.67 ± 16.48	87.18 ± 16.27	0.476
FEV1 (mean percent predicted ± SD)		87.37 ± 20.77	83.88 ± 20.64	0.485
FEV1/FVC (mean ± SD)		0.78 ± 0.04	0.75 ± 0.15	0.379
D_{LCO} (mean percent predicted ± SD)		75.30 ± 16.67	81.35 ± 23.49	0.181
Immunosuppressant Therapy n (%)		13 (48.15)	267 (87.83)	**<0.0001**

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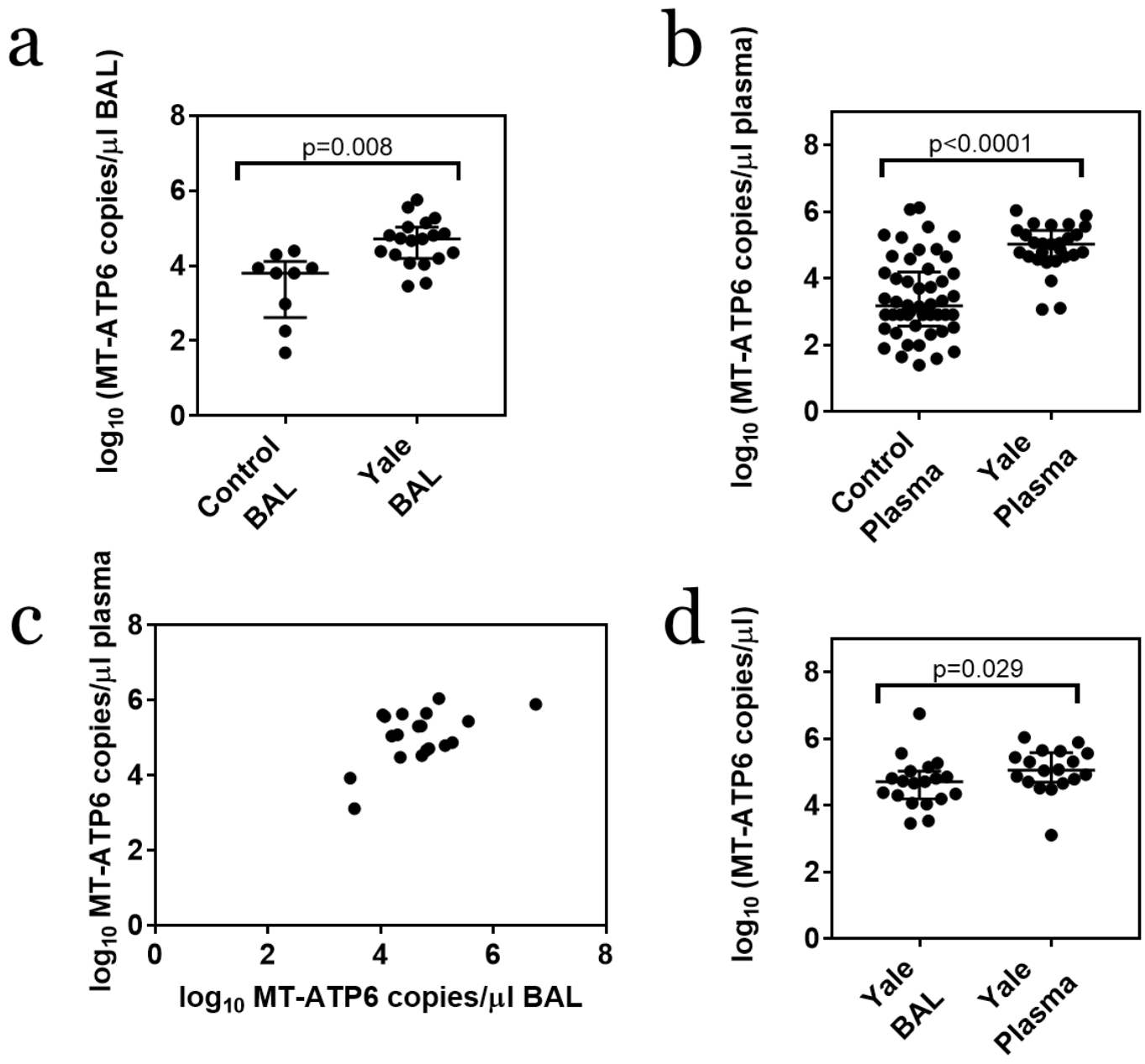

Figure 1. Extracellular mtDNA is elevated in the BAL and plasma of subjects in the Yale Sarcoidosis cohort. Relative to normal controls (BAL: n=9, plasma: n= 50), subjects with Sarcoidosis in the Yale cohort displayed significantly elevated concentrations of MT-ATP6 in the **(a)** BAL (n=19) and **(b)** plasma (n=27). **(c)** While there was no correlation in MT-ATP6 concentrations between matched BAL and plasma samples (n=19, Spearman r=0.344, p=0.149), **(d)** median MT-ATP6 concentrations were an order of magnitude lower in the BAL than their respective plasma sample. Data are presented graphically as log base 10 of the raw values of MT-ATP6 copies per μl of BAL or plasma with median value and interquartile range.

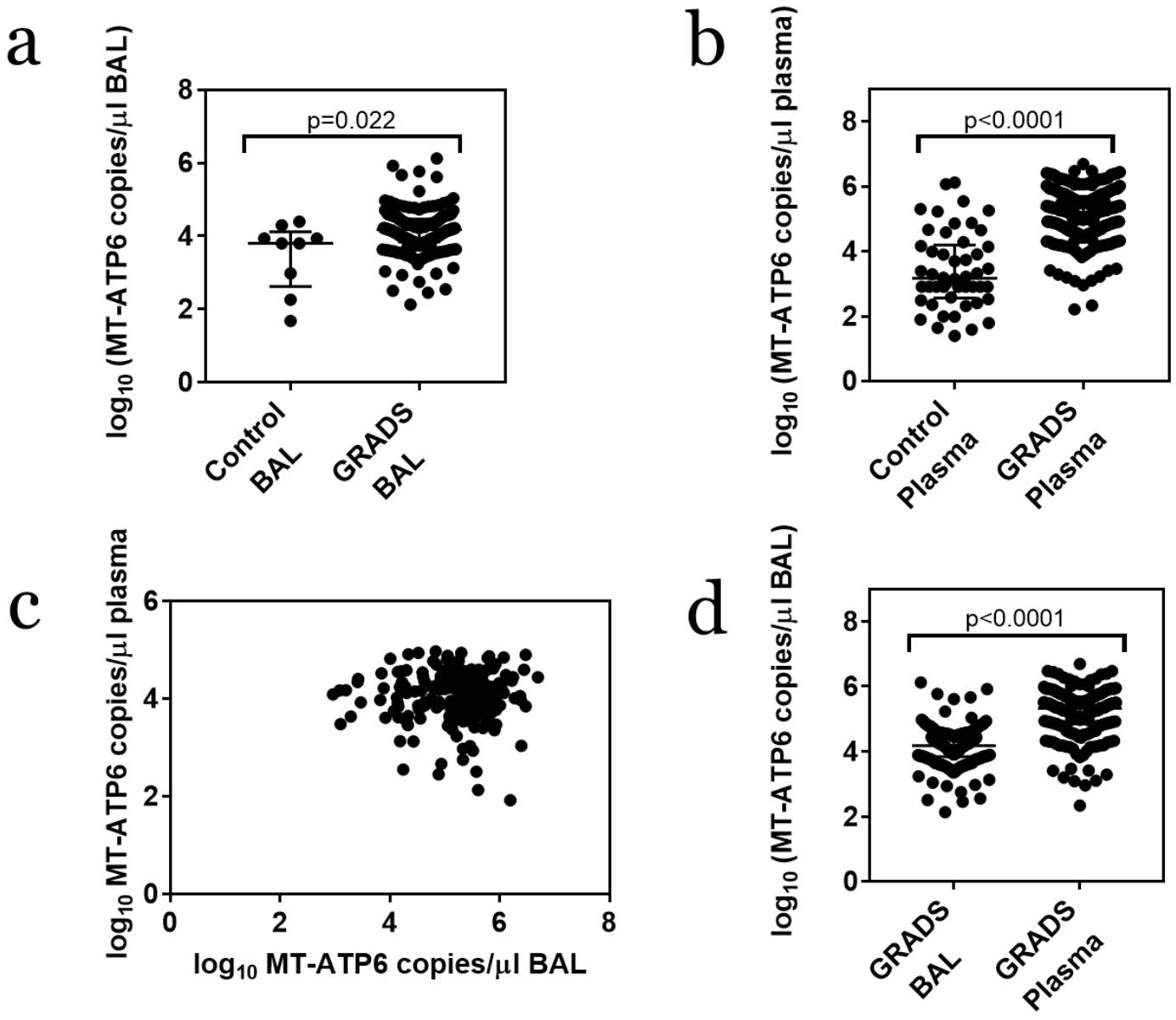

Figure 2. Elevations in extracellular mtDNA in the BAL and plasma of subjects with Sarcoidosis is validated in the GRADS cohort. Relative to normal controls (BAL: n=9, plasma: n= 50), elevated concentrations of MT-ATP6 in the **(a)** BAL (n=205) and **(b)** plasma (n=304) were recapitulated in the GRADS cohort. As similarly seen with the Yale cohort, **(c)** there was no correlation in MT-ATP6 concentrations between matched BAL and plasma samples (n=205, Spearman r=0.010, p=0.888), and **(d)** median MT-ATP6 concentrations were an order of magnitude lower in the BAL than their respective plasma sample in the GRADS cohort. Data are presented graphically as log base 10 of the raw values of MT-ATP6 copies per µl of BAL or plasma with median value and interquartile range.

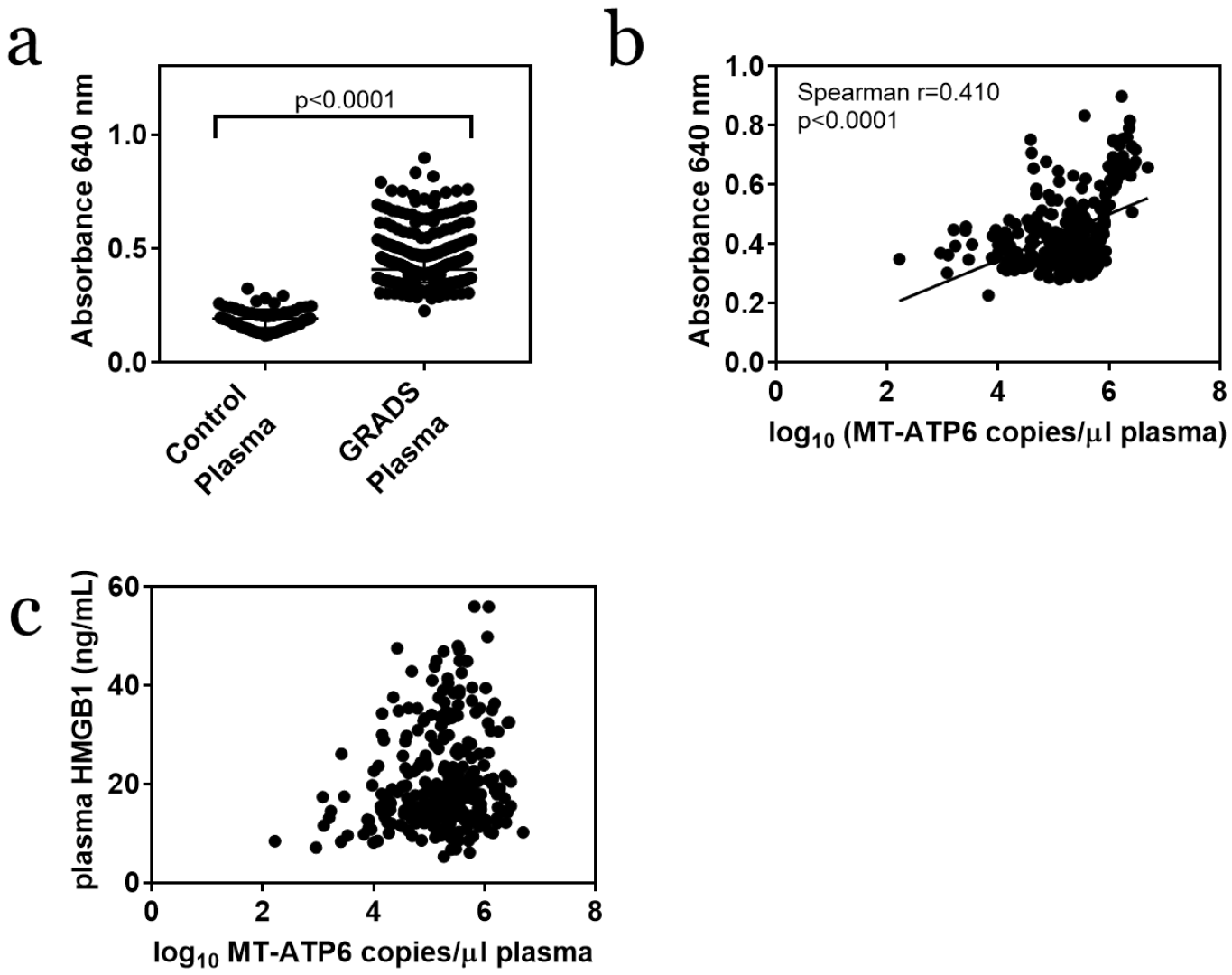

Figure 3. GRADS plasma results in TLR9 activation that correlates with plasma mtDNA concentrations. (a) Relative to plasma from normal controls (n=50), plasma from GRADS subjects (n=304) resulted in substantial TLR9 activation that **(b)** significantly correlated with plasma MT-ATP6 concentrations (log base 10 of the raw values of MT-ATP6 copies per µl of plasma). TLR9 activation is presented graphically as median absorbance at 640 nm with interquartile range. **(c)** Plasma MT-ATP6 copy numbers did not correlate with their respective plasma concentration of the DNA binding protein HMGB1 (ng/mL, Spearman r=0.138, p=0.016).

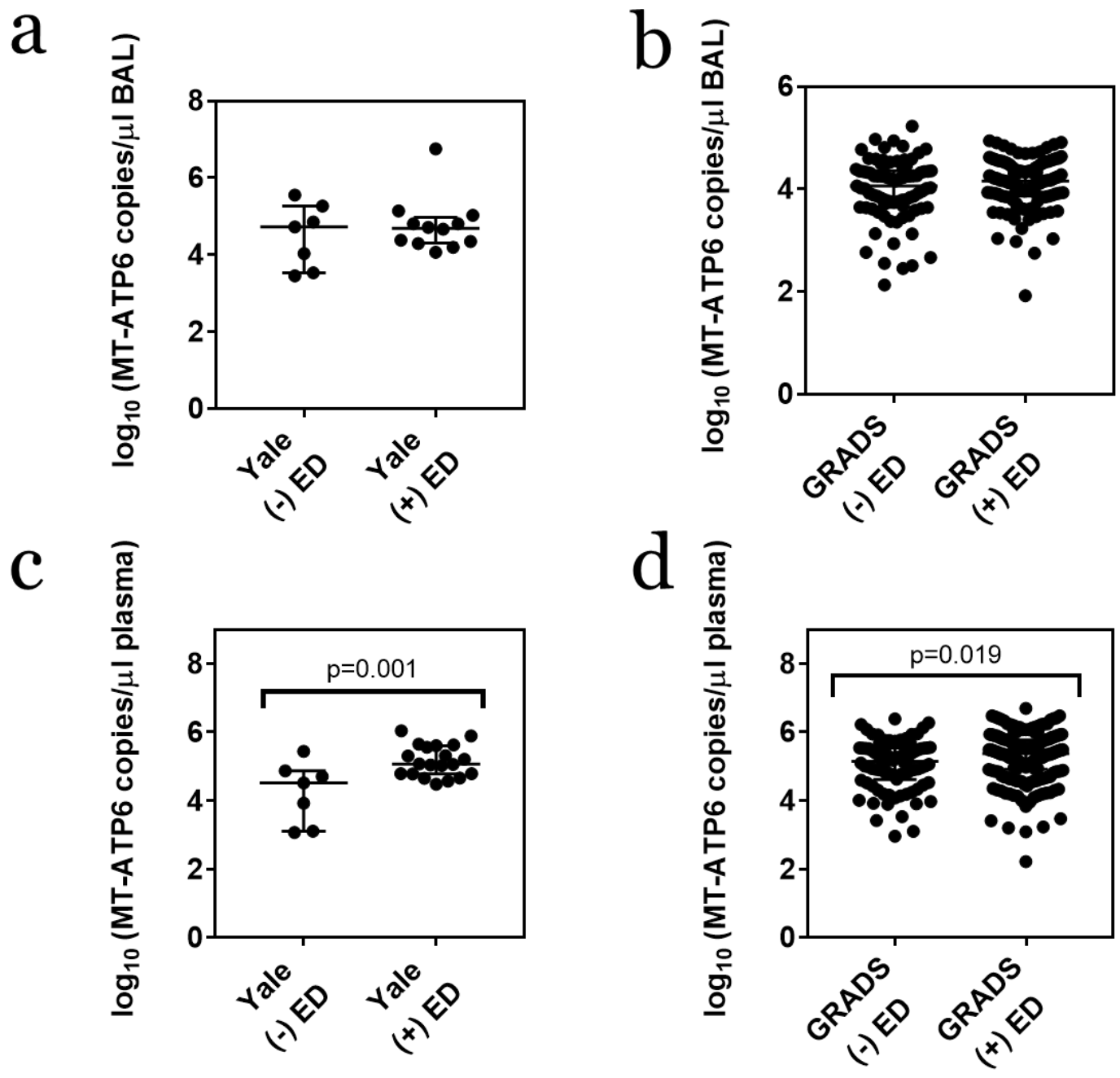

Figure 4. Extracellular mtDNA is elevated in the plasma of Sarcoidosis subjects with extrapulmonary disease in both cohorts. Median MT-ATP6 concentrations were similar in the BAL between subjects without extrapulmonary disease (ED) and those with ED in both the **(a)** Yale (n=7 vs 12, p=0.773) and **(b)** GRADS (n=83 vs 121, p=0.279) cohorts. **(c)** However, in the Yale cohort, relative to subjects with disease limited to the lung (n=7), median plasma concentrations of MT-ATP6 were significantly elevated in subjects with extrapulmonary disease (ED, n=20). **(d)** Similar results were seen in the GRADS cohort (n=90 vs 214). Data are presented graphically as log base 10 of the raw values of MT-ATP6 copies per µl of plasma with median value and interquartile range.

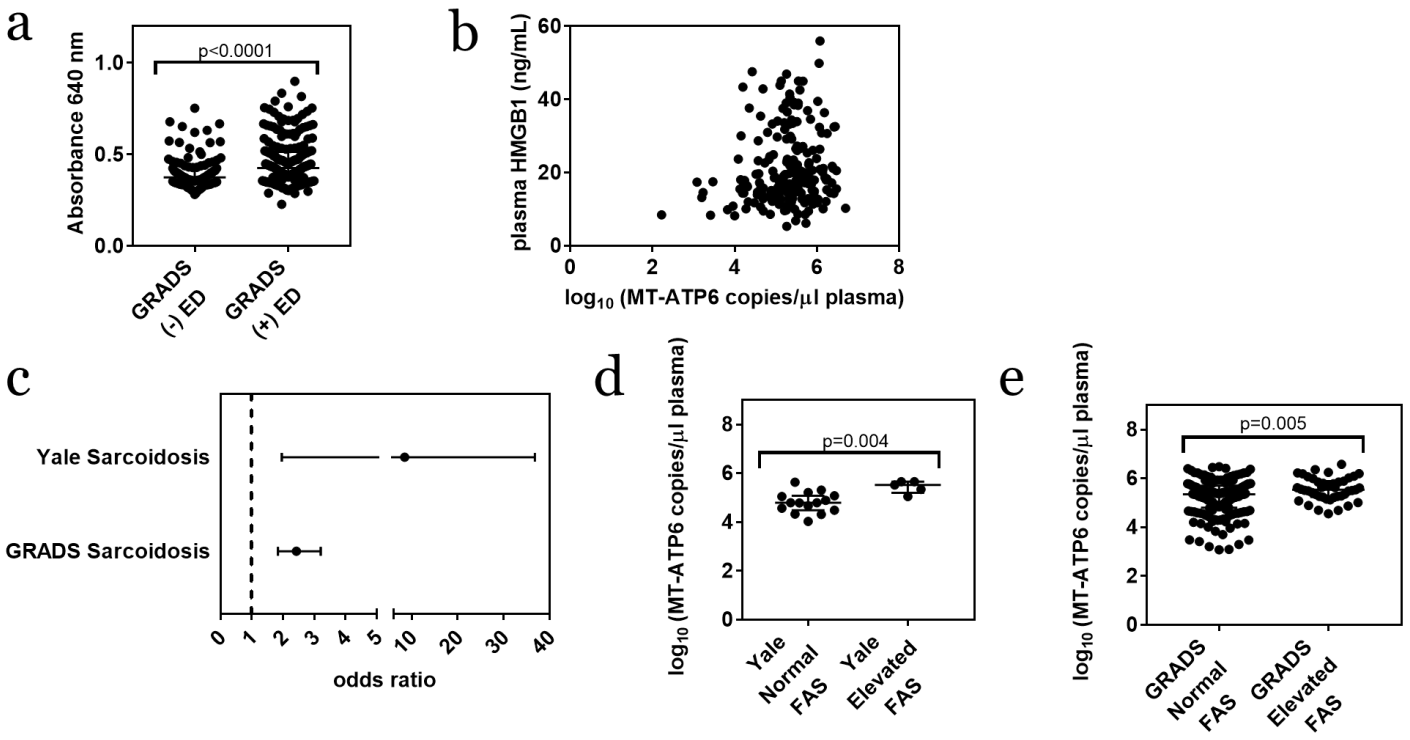

Figure 5. Elevated plasma mtDNA is associated with high odds of extrapulmonary disease and excessive fatigue. (a) Relative to plasma obtained from GRADS subjects lacking extrapulmonary involvement (n=90), plasma from those with extrapulmonary disease (n=214) exhibited greater TLR9 activation. Data presented graphically as median absorbance at 640 nm with interquartile range. **(b)** These observations did not appear to be related to necrosis as plasma MT-ATP6 concentrations (log base 10 of the raw values of MT-ATP6 copies per µl of plasma) did not correlate with plasma HMGB1 concentrations (ng/mL) among these subjects with extrapulmonary disease (Spearman r=0.130, p=0.058). In evaluating the clinical relevance of these findings, receiver operator curve (ROC) analysis of the Yale cohort revealed that a plasma MT-ATP6 copy number of 4.71 log copies/µl reliably stratifies subjects for low or high odds of extrapulmonary disease; **(c)** subjects whose plasma MT-ATP6 concentrations exceeded this threshold value had significantly increased odds of extrapulmonary disease in the Yale (odds ratio (OR) 8.500, 95% confidence interval (CI) 1.964-36.790, p=0.004) and GRADS (OR 2.429, 95% CI 1.839-3.208, p<0.0001) cohorts. Among subjects with extrapulmonary disease, relative to participants reporting normal fatigue scores, as measured by the Fatigue Assessment Scale (FAS), subjects who reported elevated fatigue scores displayed significantly higher levels of plasma MT-ATP6 in the **(d)** Yale (n=15 vs 5) and **(e)** GRADS (n=171 vs 43) cohorts. Data are presented graphically as log base 10 of the raw values of MT-ATP6 copies per µl of plasma with median value and interquartile range.

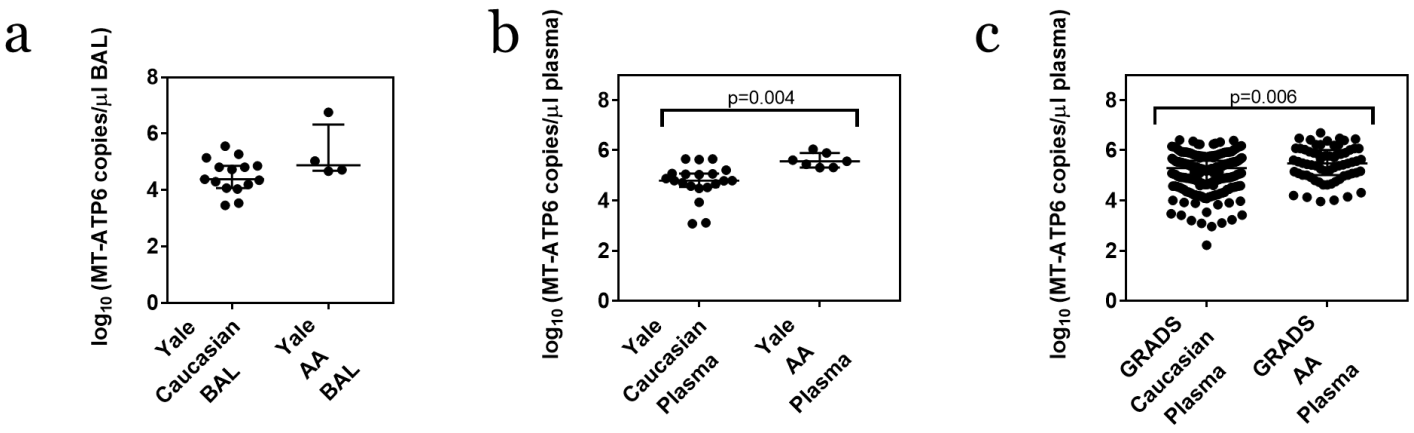

Figure 6. Extracellular mtDNA is elevated in the plasma of African-American (AA) Sarcoidosis subjects. (a) Median MT-ATP6 concentrations were similar in the BAL between Caucasian (n=15) and AA (n=4) Sarcoidosis subjects in the Yale cohort. **(b)** However, in the plasma, relative to Caucasian subjects (n=20), AA subjects (n=7) had robustly increased MT-ATP6 concentrations; these findings were subsequently validated in the much larger and more diverse **(c)** GRADS cohort (n=238 vs 66). Data are presented graphically as log base 10 of the raw values of MT-ATP6 copies per μl of BAL or plasma with median value and interquartile range.

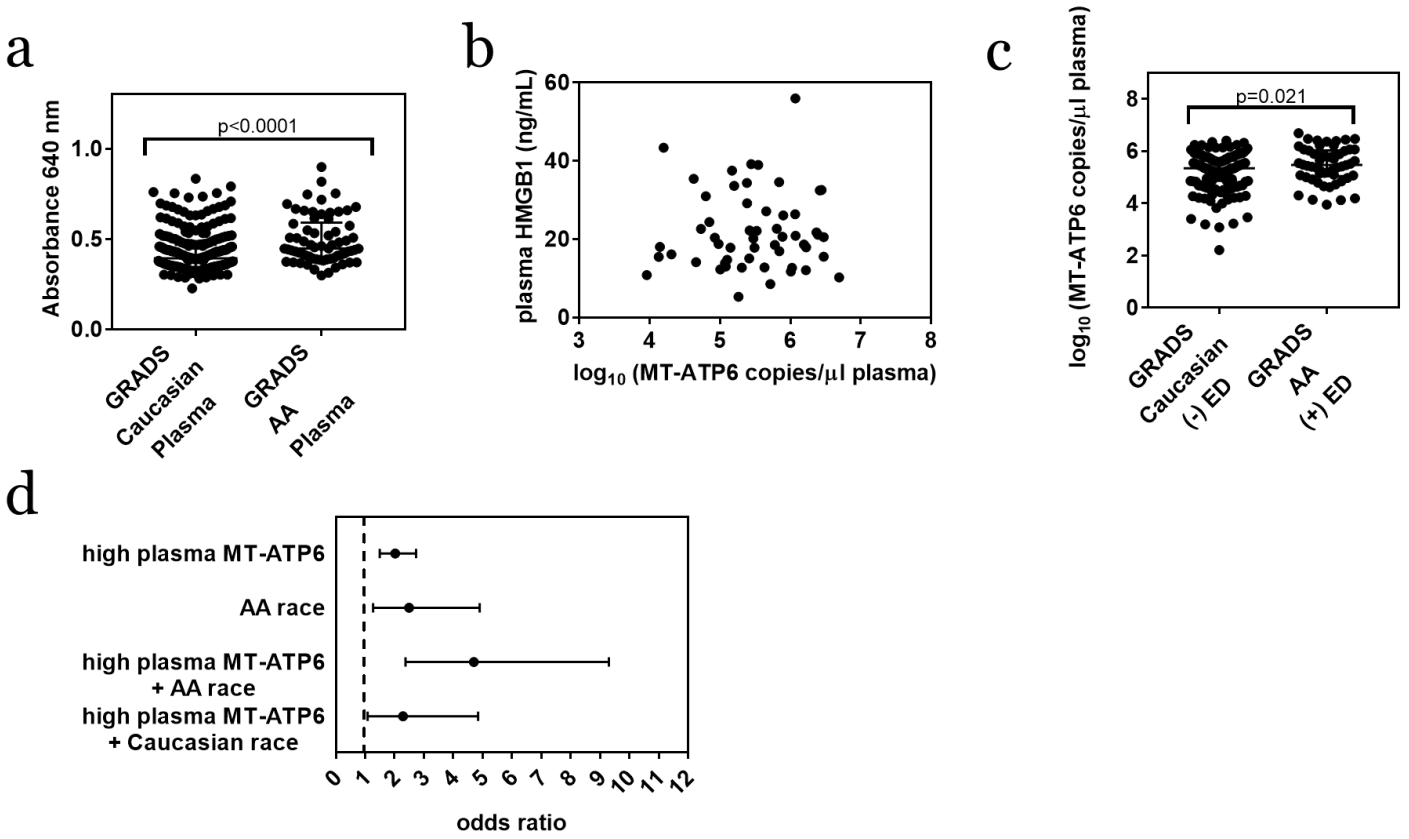

Figure 7. Plasma mtDNA provides race-specific associations with extrapulmonary disease. (a) Relative to plasma obtained from Caucasian GRADS subjects with extrapulmonary disease (n=160), plasma from AA GRADS subjects (n=54) resulted in greater TLR9 activation. Data presented graphically as median absorbance at 640 nm with interquartile range. **(b)** Plasma MT-ATP6 concentrations (log base 10 of the raw values of MT-ATP6 copies per µl of plasma) were independent of plasma HMGB1 concentrations (ng/mL, Spearman r=0.035, p=0.799). **(c)** Relative to Caucasian GRADS subjects with extrapulmonary disease, AA GRADS subjects with extrapulmonary disease had significantly elevated median concentrations of MT-ATP6. Data are presented graphically as log base 10 of the raw values of MT-ATP6 copies per µl of plasma with median value and interquartile range. **(d)** Forrest plot depicting odds of extrapulmonary disease based on high plasma MT-ATP6 concentrations (≥ 4.71 log copies/µl, OR 2.021, 95% CI: 1.494-2.735, p<0.0001), AA race (OR 2.497, 95% CI: 1.271-4.905, p=0.008), and high plasma MT-ATP6 with AA race (OR 4.700, 95% CI: 2.375-9.300, p<0.0001), which imparted the highest odds of extrapulmonary disease. Caucasian subjects with high plasma MT-ATP6 had lower odds of extrapulmonary disease than their AA counterparts (OR 2.293, 95% CI: 1.084-4.849, p=0.030).

ONLINE SUPPLEMENT

METHODS

Yale BAL Isolation

Bronchoalveolar lavage (BAL) specimens were obtained from excess clinical samples obtained from patients at Yale without parenchymal lung disease in whom infection had been specifically ruled out, and from patients with Sarcoidosis. Upon arrival in the laboratory, BAL samples were subject to two centrifugations at 1000 RPM of 30 minutes to ensure separation of the cellular pellet. The subsequent cell-free supernatant of these BAL samples was aliquoted and stored at -80C until time of analysis.

Yale Plasma Isolation

Blood was collected on BD Vacutainer™ Glass Mononuclear Cell Preparation (CPT) Tubes (ThermoFisher Scientific, Waltham, MA) containing sodium citrate from healthy volunteers and patients with Sarcoidosis. Within an hour of collection, CPT tubes were centrifuged at 1500 revolutions per minute (RPM) at room temperature for 8 minutes. The plasma layer (top layer) was then carefully aliquoted and stored at -80C until time of analysis.

GRADS BAL Collection

BAL obtained from GRADS participants was centrifuged at 300 g for 7 minutes; the subsequent cell-free supernatant of these BAL samples was aliquoted and stored at -80C until time of analysis.

GRADS Plasma Isolation

Blood was collected on BD Vacutainer™ Glass Mononuclear Cell Preparation (CPT) Tubes (ThermoFisher Scientific, Waltham, MA) containing sodium citrate from GRADS participants. Within an hour of collection, CPT tubes were centrifuged at

2750 RPM at room temperature for 25 minutes. The plasma layer (top layer) was then carefully aliquoted and stored at -80C until time of analysis.

Toll like Receptor 9 (TLR9) Detection

Human TLR9-expressing HEK 293 cells obtained from Invivogen (Invivogen, San Diego, CA) were cultured with DMEM/2%FBS/1%Pen-Strep and passaged twice, at which point selective antibiotics Blasticidin and Zeocin were added. Cells were plated onto 96-well plates (approximately 80,000 cells per well) with the HEK-Blue™ Detection medium and was stimulated with either healthy control or GRADS plasma for 6 hours. TLR9 activation was measured by reading the optical density at 640 nm using the Gen5™ Microplate Data Collection and Analysis Software (BioTek Instruments, Inc, Winooski, VT)

High Mobility Group Box 1 (HMGB1) Quantification

Quantification of plasma HMGB1 concentrations were performed using an enzyme-linked immunosorbent assay (ELISA) from Aviva Systems Biology (Aviva Systems Biology, Corp, San Diego, CA) per their protocol. In brief, healthy control or GRADS plasma samples were diluted in a 1:10 ratio and plated onto the pre-coated HMGB1 microplate. The optical density was read at 450 nm using the Gen5™ Microplate Data Collection and Analysis Software (BioTek Instruments), and concentrations of HMGB1 were determined based on a standard curve developed from serial dilutions of the HMGB1 assay standards.

DNA Isolation and Quantification

DNA was extracted from BAL or plasma using the QiaAMP DNA Mini-Kit (Qiagen, Germantown, MD) per their protocol; in brief, 200 µl of BAL or plasma was used to elute 100µl of DNA. The presence of mtDNA in each sample of DNA was assayed by real-time qPCR for the human MT-ATP6 gene, using commercially available primers

for MT-ATP6 (ThermoFisher Scientific) and probes (SsoAdvanced Universal Probes Supermix, Bio-Rad Laboratories, Hercules, CA). The LightCycler 480 (Roche, Indianapolis, IN) system was employed with the following cycling conditions: incubation at 95° for 10 minutes followed by 50 amplification cycles of 95° for 10 seconds, 60° for 30 seconds, and 72° for 1 second, and finally cooling at 40° for 30 seconds. The number of MT-ATP6 copies per microliter (copies/µl) was determined based on a standard curve developed from serial dilutions of a commercially available DNA plasmid (OriGene, Rockville, MD) with complementary DNA sequences for human MT-ATP6.

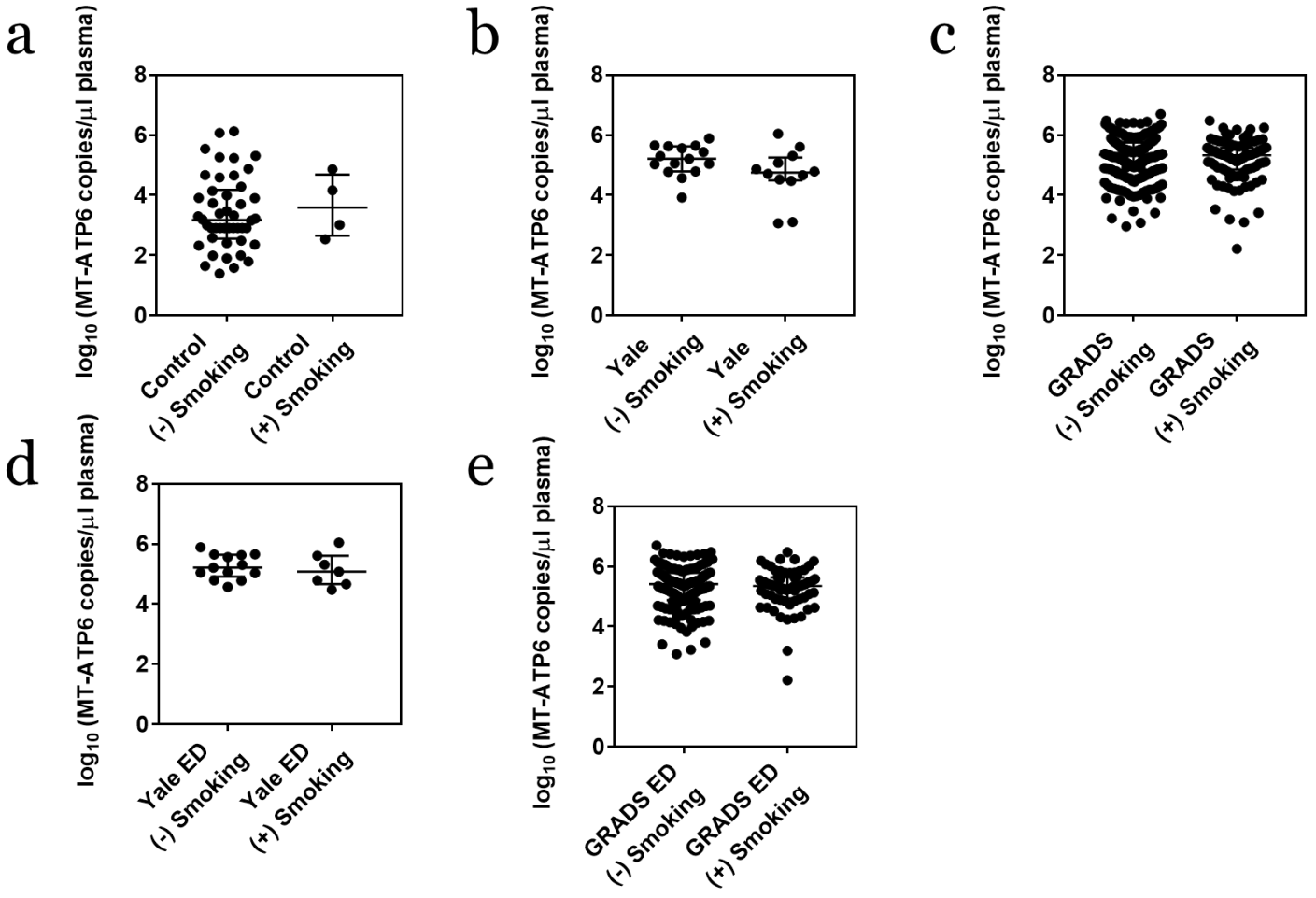

Figure S1. There was no association between smoking and plasma mtDNA concentrations among healthy controls or Sarcoidosis subjects. (a) Among healthy controls, there was no difference in plasma MT-ATP6 concentrations between non-smokers (n=46) and smokers (n=4, 3.174 vs 3.590 log copies/µl, p=0.616). **(b)** Among Yale Sarcoidosis subjects, there was no difference in plasma MT-ATP6 concentrations between non-smokers (n=15) and smokers (n=12, 5.208 vs 4.751 log copies/µl, p=0.103). **(c)** Among GRADS subjects, there was no difference in plasma MT-ATP6 concentrations between non-smokers (n=205) and smokers (n=99, 5.343 vs 5.331 log copies/µl, p=0.471). **(d)** Among Yale Sarcoidosis subjects with extrapulmonary disease (ED), there was no difference in plasma MT-ATP6 concentrations between non-smokers (n=13) and smokers (n=7, 5.208 vs 5.076 log copies/µl, p=0.699). **(e)** Among GRADS subjects with ED, there was no difference in plasma MT-ATP6 concentrations between non-smokers (n=146) and smokers (n=68, 5.419 vs 5.350 log copies/µl, p=0.577). Data are presented graphically as log base 10 of the raw values of MT-ATP6 copies per µl of plasma with median value and interquartile range.

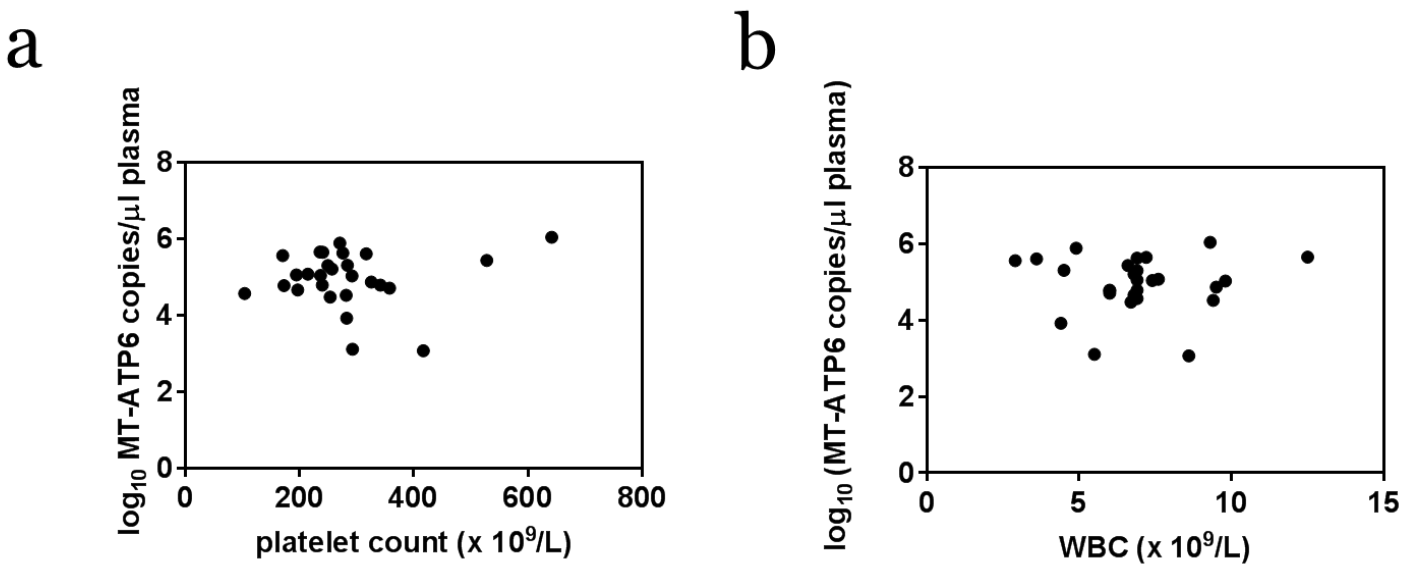

Figure S2. Plasma mtDNA is not associated with platelet or white blood cell counts in the Yale cohort. There was no correlation between plasma MT-ATP6 concentrations and **(a)** platelet counts (x 10^9/L, n=27, Spearman r=-0.017, p=0.933) or **(b)** white blood cell counts (x 10^9/L, n=27, Spearman r=0.047, p=0.818) in the Yale cohort.

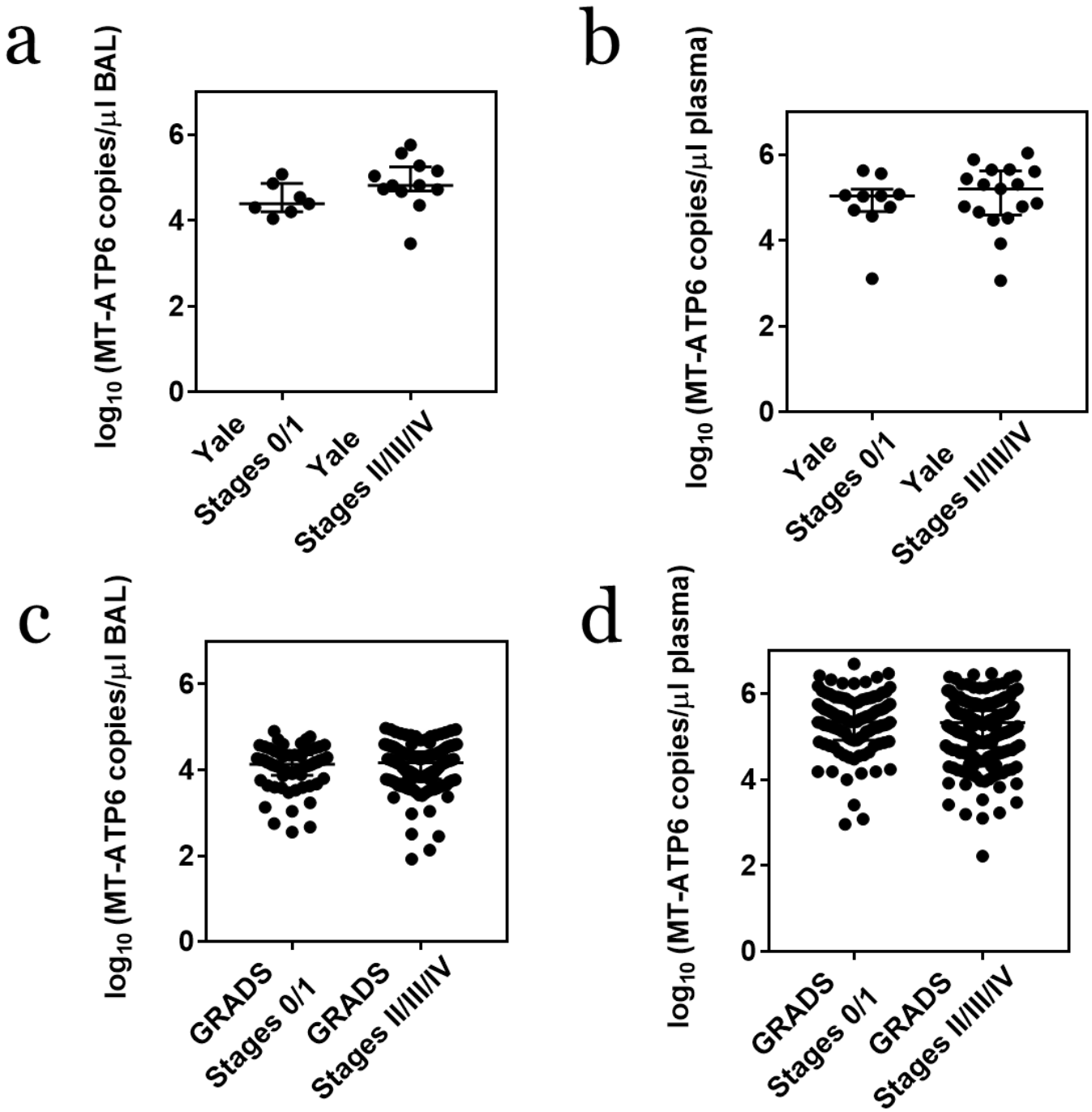

Figure S3. The presence of pulmonary Sarcoidosis does not affect BAL or plasma mtDNA in both cohorts. When subjects of the Yale cohort with Stage 0/I disease were compared to those with Stage II/III/IV disease, there were no significant differences detected in the **(a)** BAL (n=7 vs 12, p=0.100) or **(b)** plasma (n=10 vs 17, p=0.537). Similar findings were recapitulated in the GRADS **(c)** BAL (n=69 vs 135, p=0.775) and **(d)** plasma (n=101 vs 203, p=0.119). Data are presented graphically as log base 10 of the raw values of MT-ATP6 copies per µl of BAL or plasma with median value and interquartile range.

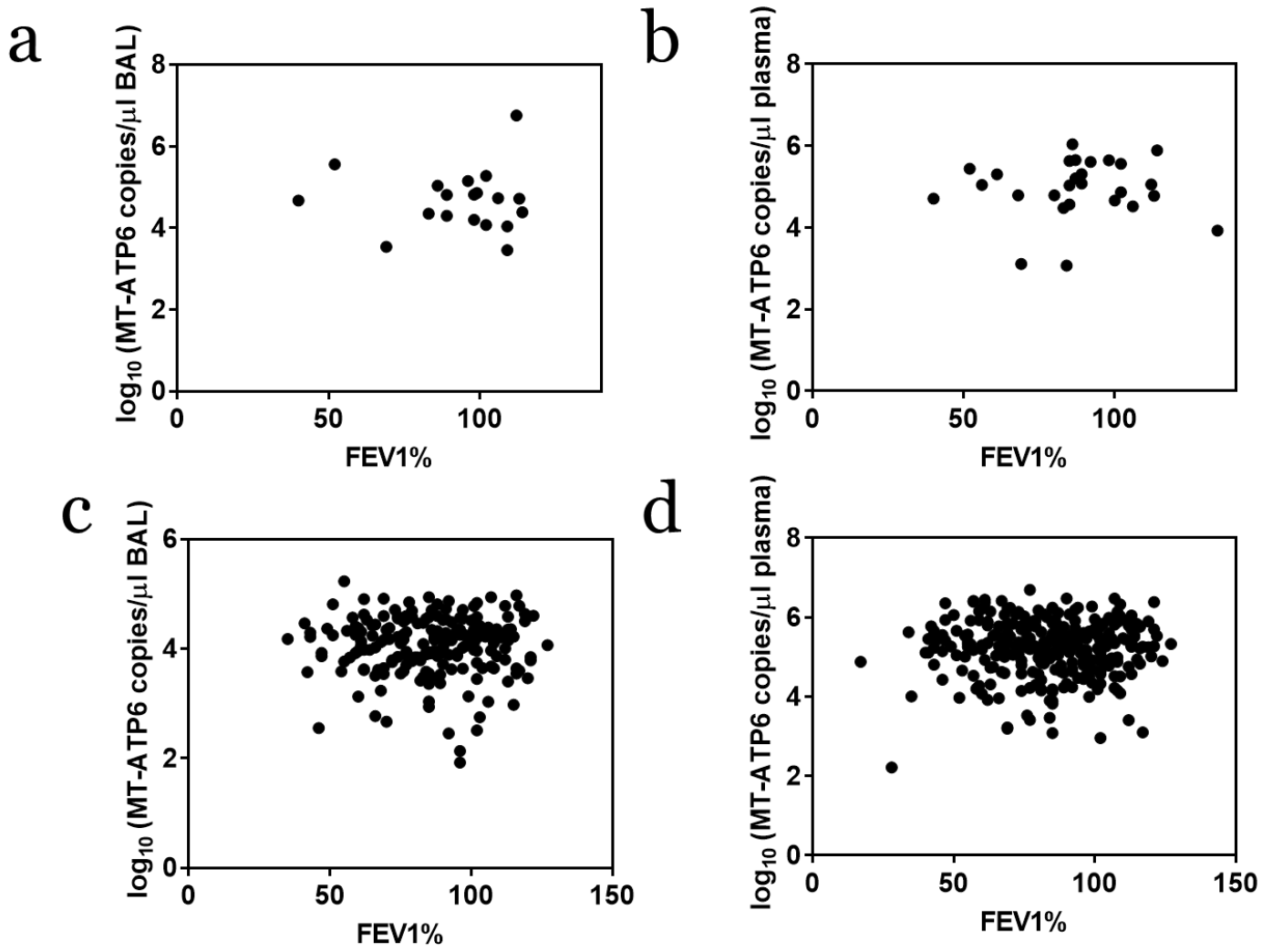

Figure S4. Neither BAL nor plasma mtDNA correlates with FEV1% in both cohorts. MT-ATP6 concentrations did not correlate with percent predicted forced expiratory volume after 1 second (FEV1%) in the **(a)** BAL (n=19, Spearman r=-0.453, p=0.051) and **(b)** plasma (n=27, Spearman r=-0.127, p=0.528) of the Yale cohort, or in the **(c)** BAL (n=204, Spearman r=0.013, p=0.857) and **(d)** plasma (n=304, Spearman r=-0.017, p=0.763) of the GRADS cohort. Data are presented graphically as log base 10 of the raw values of MT-ATP6 copies per µl of BAL or plasma.

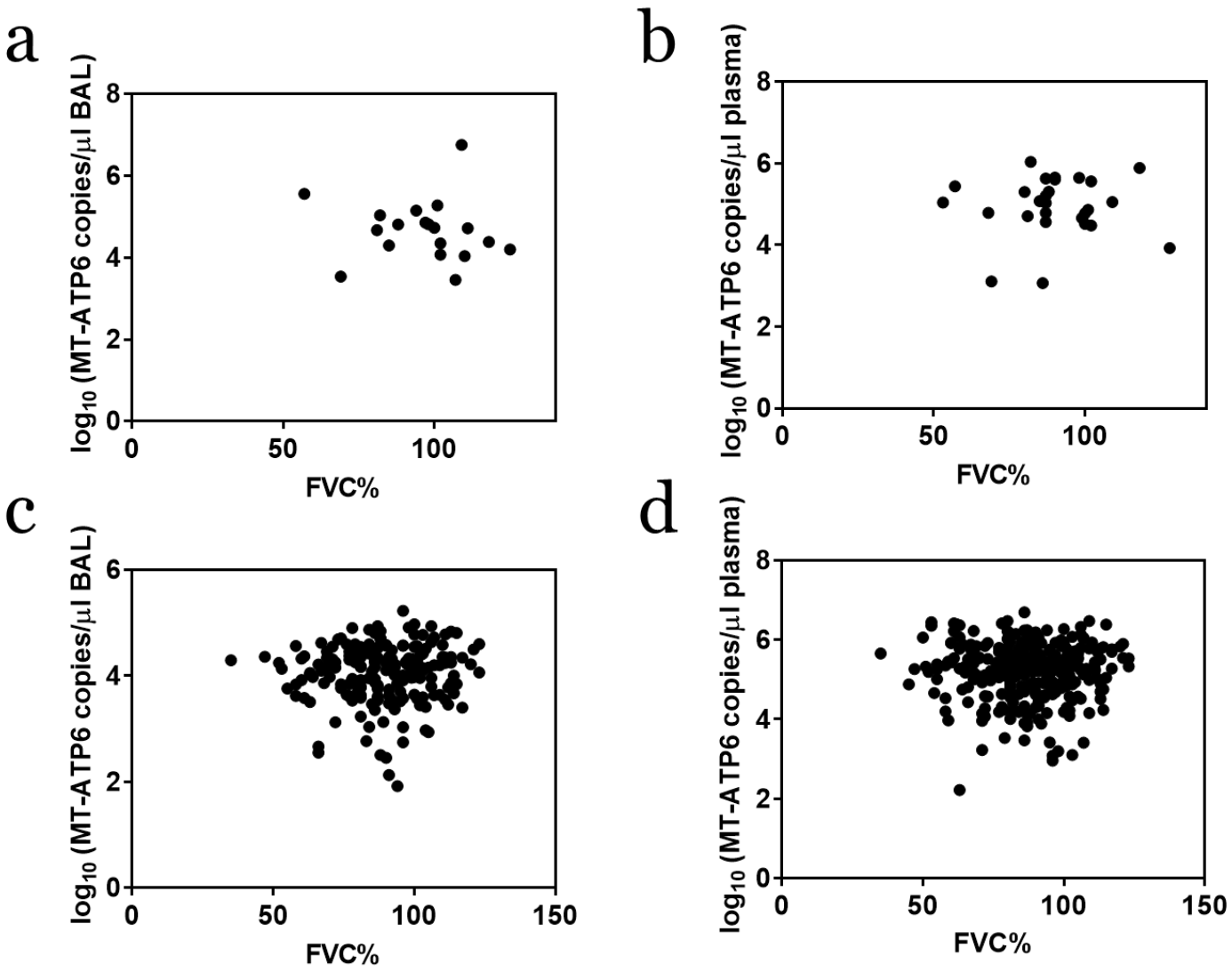

Figure S5. Neither BAL nor plasma mtDNA correlates with FVC% in both cohorts. MT-ATP6 concentrations did not correlate with percent predicted forced vital capacity (FVC%) in the **(a)** BAL (n=19, Spearman r=-0.276, p=0.252) and **(b)** plasma (n=27, Spearman r=-0.000, p>0.999) of the Yale cohort, or in the **(c)** BAL (n=204, Spearman r=0.037, p=0.602) and **(d)** plasma (n=304, Spearman r=-0.012, p=0.840) of the GRADS cohort. Data are presented graphically as log base 10 of the raw values of MT-ATP6 copies per µl of BAL or plasma.

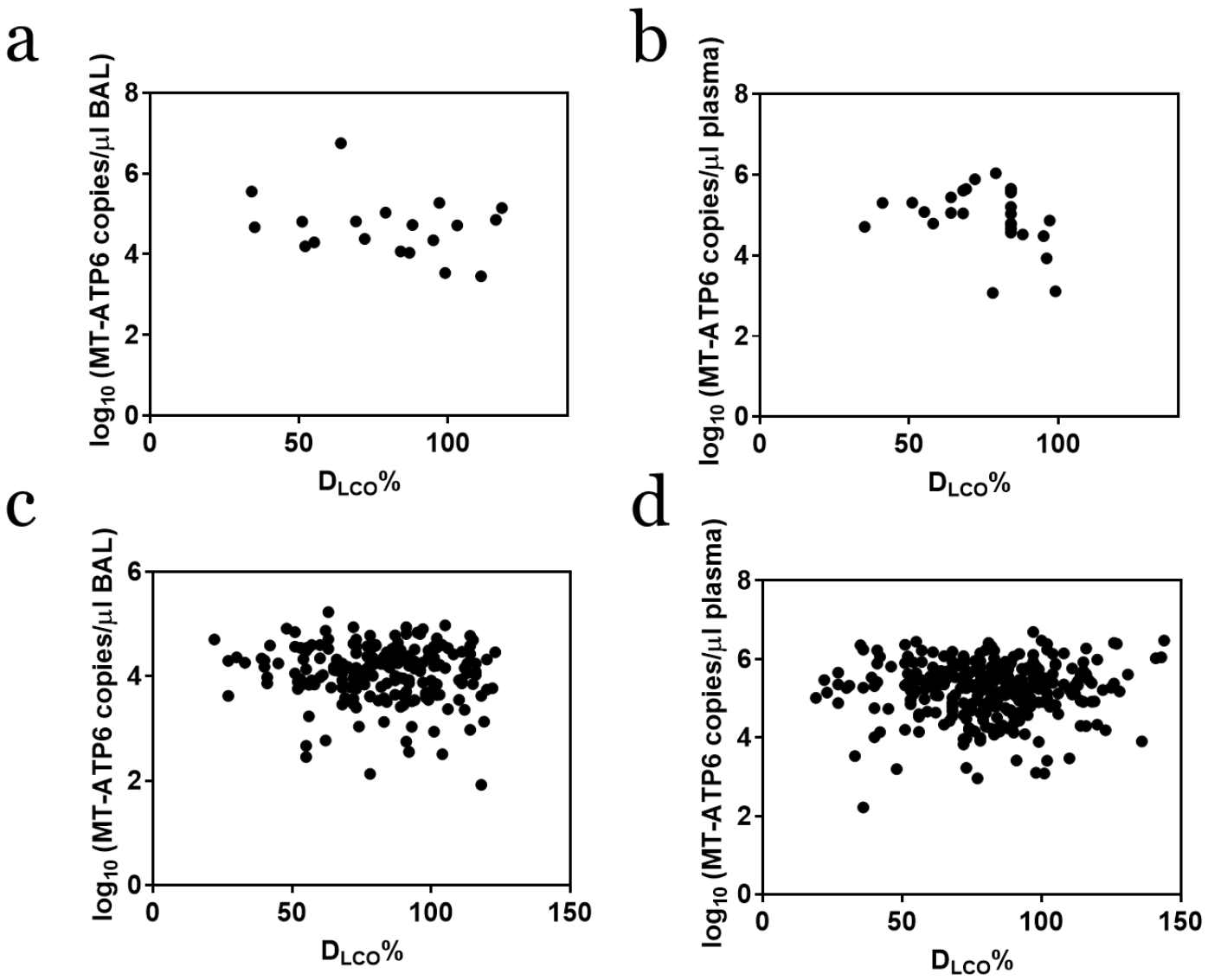

Figure S6. Neither BAL nor plasma mtDNA correlates with D_{LCO}% in both cohorts. MT-ATP6 concentrations did not correlate with diffusion capacity of the lung for carbon monoxide (D_{LCO}%) in the **(a)** BAL (n=19, Spearman r=-0.135, p=0.581) and **(b)** plasma (n=27, Spearman r=-0.390, p=0.054) of the Yale cohort, or in the **(c)** BAL (n=204, Spearman r=-0.087, p=0.217) and **(d)** plasma (n=304, Spearman r=0.002, p=0.979) of the GRADS cohort. Data are presented graphically as log base 10 of the raw values of MT-ATP6 copies per µl of BAL or plasma.

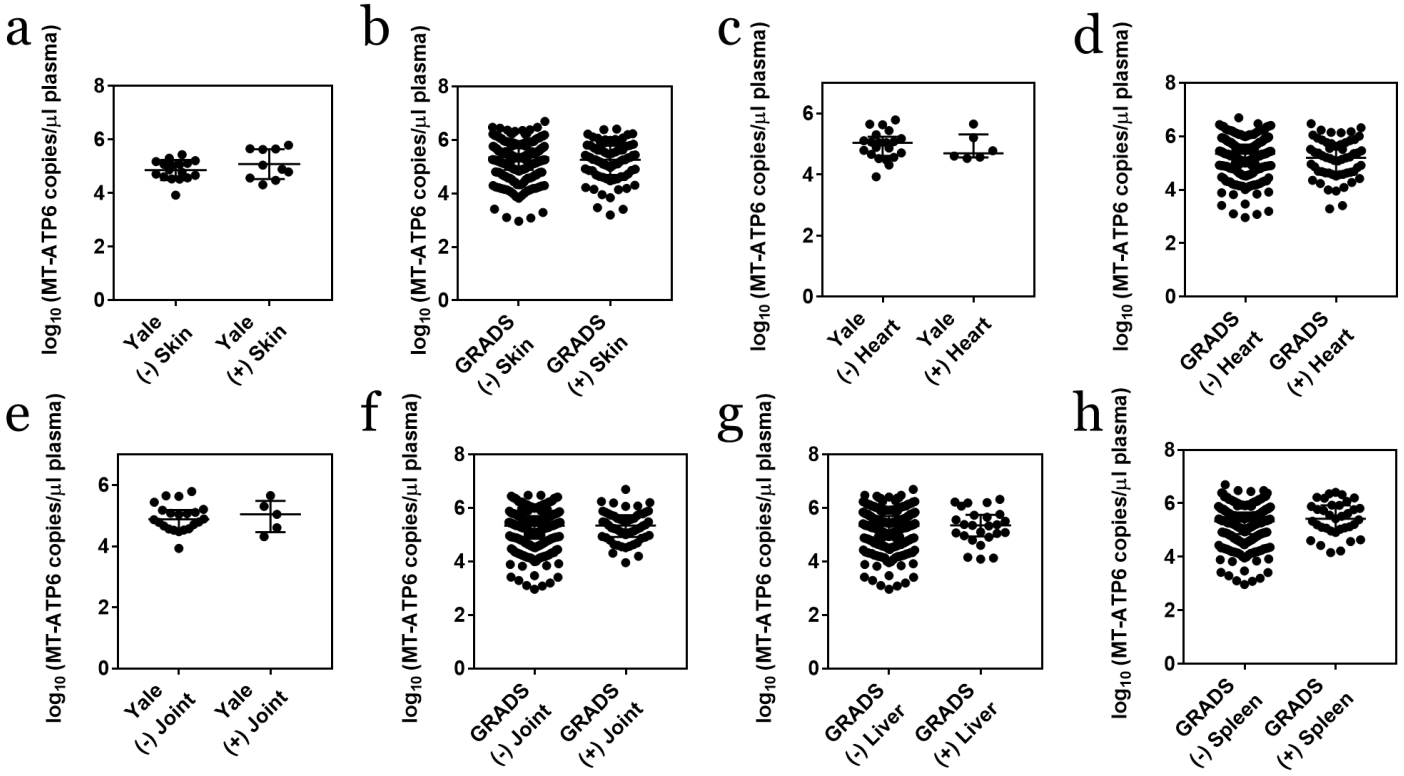

Figure S7. No organ-specific associations with plasma mtDNA concentrations were found in both cohorts. (a) Plasma MT-ATP6 concentrations were similar between subjects without dermatologic (n=17) and with dermatologic disease (n=10, 4.869 vs 4.967 $\log_{10}$ copies/µl, p=0.474) in the Yale Sarcoidosis cohort and in the **(b)** GRADS cohort (n=228 vs 75, 5.348 vs 5.263 $\log_{10}$ copies/µl, p=0.642). **(c)** Median plasma MT-ATP6 concentrations were similar between subjects without cardiac (n=21) and with cardiac disease (n=6, 5.041 vs 4.693 $\log_{10}$ copies/µl, p=0.755) in the Yale Sarcoidosis cohort and in the **(d)** GRADS cohort (n=246 vs 57, 5.348 vs 5.196 $\log_{10}$ copies/µl, p=0.232). **(e)** Median plasma MT-ATP6 concentrations were similar between subjects without joint (n=22) and with joint disease (n=5, 4.879 vs 5.044 $\log_{10}$ copies/µl, p=0.833) in the Yale Sarcoidosis cohort and in the **(f)** GRADS cohort (n=247 vs 56, 5.334 vs 5.346 $\log_{10}$ copies/µl, p=0.591). **(g)** Among GRADS subjects, median plasma MT-ATP6 concentrations were similar between subjects without hepatic (n=278) and with hepatic (n=25, 5.333 vs 5.351 $\log_{10}$ copies/µl, p=0.791). **(h)** Among GRADS subjects, median plasma MT-ATP6 concentrations were similar between subjects without splenic (n=268) and with splenic (n=35, 5.332 vs 5.422 $\log_{10}$ copies/µl, p=0.144).

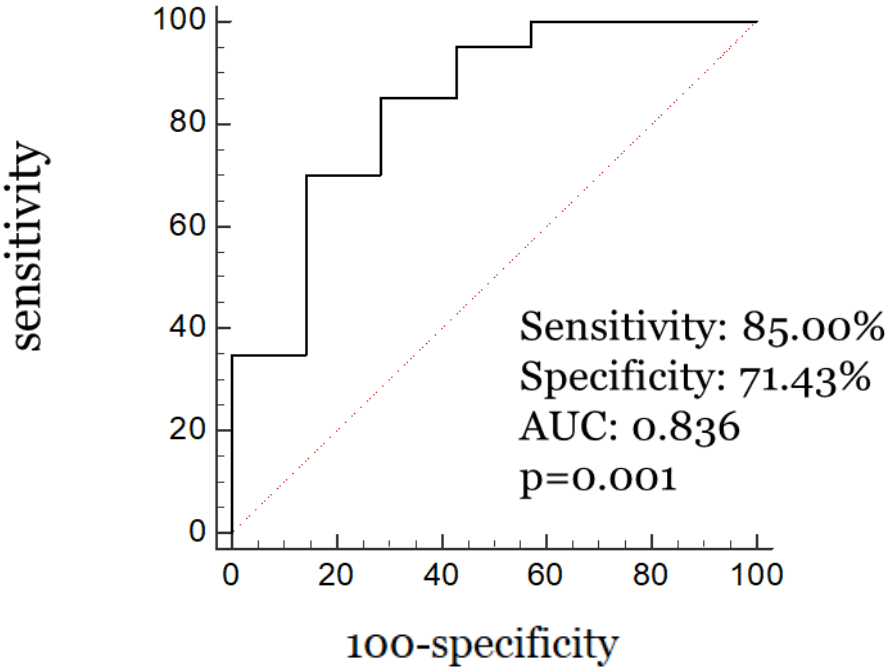

Figure S8. Receiver operator curve (ROC) analysis of the Yale Sarcoidosis cohort. ROC analysis of the Yale cohort revealed that a plasma MT-ATP6 copy number of 4.71 $\log_{10}$ copies/µl can reliably stratify subjects for low odds (<4.71 $\log_{10}$ copies/µl) or high odds (≥4.71 $\log_{10}$ copies/µl) for extrapulmonary disease.

TABLES

Table S1. Involved extrapulmonary organ systems in the Yale Sarcoidosis cohort.

Organ System	n (%)
Dermatologic	10 (37.04)
Cardiac	6 (22.22)
Joint	5 (18.52)
Ocular	4 (14.81)
Hepatic	3 (11.11)
Splenic	3 (11.11)
Bone	3 (11.11)
Neurologic	2 (7.41)
Otolaryngoscopic	1 (3.70)
Renal	0 (0.00)

Table S2. Involved extrapulmonary organ systems in the GRADS cohort.

Organ System	n (%)
Dermatologic	77 (35.98)
Cardiac	57 (26.64)
Joint	56 (26.17)
Ocular	47 (21.96)
Splenic	35 (16.36)
Hepatic	27 (12.62)
Otolaryngoscopic	26 (12.15)
Neurologic	17 (7.94)
Bone	11 (5.14)
Renal	9 (4.21)

Table S3. Comparison of clinical characteristics between Yale Caucasian and African-American Sarcoidosis subjects.

	Yale Caucasian	Yale AA	p-value
N	20	7	
Age (years)			
Mean ± SD	49.97 ± 14.19	52.40 ± 10.13	0.687
Gender n (%)			
Female	8 (40.00)	2 (28.57)	0.678
Smoking n (%)			
Ever/Current	9 (45.00)	3 (42.86)	>0.999
Never	11 (55.00)	4 (57.14)	>0.999
Disease Duration (mean ± SD)	5.25 ± 7.88	7.88 ± 8.97	0.468
Extrapulmonary Disease n (%)	14 (70.00)	6 (85.71)	0.633
Scadding Stage			
Stage 0 n (%)	5 (25.00)	1 (14.29)	>0.999
Stage I n (%)	4 (20.00)	0 (0.00)	0.557
Stage II n (%)	9 (45.00)	3 (42.86)	>0.999
Stage III n (%)	0 (0.00)	0 (0.00)	>0.999
Stage IV n (%)	2 (10.00)	3 (42.86)	0.293
FVC (mean percent predicted ± SD)	90.20 ± 16.02	88.14 ± 18.98	0.881
FEV1 (mean percent predicted ± SD)	88.15 ± 20.91	85.14 ± 21.84	0.882
FEV1/FVC (mean ± SD)	0.78 ± 0.05	0.79 ± 0.03	0.773
D_{LCO} (mean percent predicted ± SD)	78.70 ± 16.14	65.57 ± 15.20	**0.042**
Immunosuppressant Therapy n (%)	12 (60.00)	1 (14.29)	0.077

Table S4. Comparison of clinical characteristics between GRADS Caucasian and African-American Sarcoidosis subjects.

	GRADS Caucasian	GRADS AA	p-value
N	238	66	
Age (years)			
Mean ± SD	54.13 ± 10.13	53.69 ± 8.71	0.747
Gender n (%)			
Female	120 (50.42)	21 (31.82)	**0.008**
Smoking n (%)			
Ever/Current	78 (32.77)	21 (31.82)	>0.999
Never	160 (67.23)	45 (68.18)	>0.999
Institution			
National Jewish	68 (28.57)	7 (10.61)	**0.002**
UCSF	52 (21.85)	3 (4.55)	**0.001**
Johns Hopkins	27 (11.34)	17 (25.76)	**0.001**
Penn	31 (13.03)	12 (18.18)	0.319
Vanderbilt	21 (8.82)	13 (19.69)	**0.025**
Pittsburgh	20 (8.40)	6 (9.09)	0.807
Arizona	17 (7.14)	7 (10.61)	0.437
MUSC	2 (0.84)	1 (1.52)	0.522
Disease Duration (mean ± SD)	11.04 ± 18.60	15.08 ± 15.49	0.107
Extrapulmonary Disease n (%)	160 (67.23)	54 (81.82)	**0.023**
Scadding Stage			
Stage 0 n (%)	31 (13.03)	3 (4.55)	0.075
Stage I n (%)	56 (23.53)	11 (16.67)	0.314
Stage II n (%)	70 (29.41)	20 (30.30)	0.880
Stage III n (%)	32 (13.45)	9 (13.64)	>0.999
Stage IV n (%)	49 (20.59)	23 (34.85)	**0.022**
FVC (mean percent predicted ± SD)	89.51 ± 15.52	78.77 ± 16.27	**<0.0001**
FEV1 (mean percent predicted ± SD)	86.45 ± 20.74	74.59 ± 17.51	**<0.0001**
FEV1/FVC (mean ± SD)	0.76 ± 0.16	0.74 ± 0.13	0.313
D_{LCO} (mean percent predicted ± SD)	85.03 ± 21.68	68.14 ± 25.11	**<0.0001**
Immunosuppressant Therapy n (%)	208 (87.03)	59 (89.39)	0.682