Title: Association of urine mitochondrial DNA with clinical measures of COPD in the SPIROMICS cohort

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ABSTRACT

BACKGROUND. Mitochondrial dysfunction, a proposed mechanism of COPD pathogenesis, is associated with the leakage of mitochondrial DNA (mtDNA), which may be detected extracellularly in various bodily fluids. Despite evidence for the increased prevalence of chronic kidney disease in COPD subjects and for mitochondrial dysfunction in the kidneys of murine COPD models, whether urine mtDNA (u-mtDNA) associates with measures of disease severity in COPD is unknown.

METHODS. Cell-free u-mtDNA, defined as copy number of mitochondrially-encoded NADH dehydrogenase-1 (MTND1) gene, was measured by real-time quantitative PCR and normalized to urine creatinine in cell-free urine samples from participants in the Subpopulations and Intermediate Outcome Measures in COPD Study (SPIROMICS) cohort. Urine albumin/creatinine ratios (UACR) were measured in the same samples. Associations between u-mtDNA and UACR and clinical disease parameters, including FEV₁ % predicted, clinical measures of exercise tolerance, respiratory symptom burden, and chest CT measures of lung structure were examined.

RESULTS. U-mtDNA and UACR levels were measured in never smokers (n=64), smokers without airflow obstruction (n=109), participants with mild/moderate COPD (n=142), and participants with severe COPD (n=168). U-mtDNA was associated with increased respiratory symptom burden, especially among smokers without COPD. Significant sex differences in u-mtDNA levels were observed with females having higher u-mtDNA levels across all study subgroups. U-mtDNA associated with worse spirometry and CT emphysema in males only, and worse respiratory symptoms in females only. Similar associations were not found with UACR.

CONCLUSION. U-mtDNA levels may help to identify distinct clinical phenotypes and underlying pathobiological differences in males versus females with COPD.

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BRIEF SUMMARY

Urine mitochondrial DNA associates with distinct clinical phenotypes in COPD in a large multi-center cohort, with notable sex differences.
INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a heterogeneous lung disease resulting from an abnormal inflammatory and reparative response to toxic inhalants, in particular cigarette and biomass smoke. The marked heterogeneity of COPD manifests as distinct clinical phenotypes and trajectories, and likely starts very early in disease pathogenesis, in smokers who have yet to develop COPD by current spirometric criteria (1). Identifying disease mechanisms or endotypes to explain the heterogeneity of COPD more clearly or differentiate one patient from another is therefore of critical importance (2). The development of novel biomarkers and personalized therapies can occur only with a more nuanced understanding of the underlying biologic processes, especially at disease onset (3, 4).

One underlying biological mechanism that may provide insight into COPD pathogenesis and progression is mitochondrial dysfunction, which is consistently observed in cells derived from COPD subjects (5-13), as well as in experimental COPD models (11, 14). Such mitochondrial dysfunction is associated with the leakage of mitochondrial DNA (mtDNA) (15-19), a mitochondrial-derived danger-associated molecular pattern (mtDAMP) (20, 21). Intracellularly, mtDNA acts as an essential second messenger that activates pathogen recognition receptors (PRRs), in particular the NLRP3 inflammasome and the DNA sensor cGAS, eliciting a series of innate immune signaling cascades (21-24). When found extracellularly, cell-free mtDNA, which can be detected in a range of biological fluids including plasma, serum, urine, and bronchoalveolar lavage fluid (25-27), activates neutrophils (22), vascular endothelial cells (28) and alveolar macrophages (29), perpetuating inflammatory signals or cascades. MtDNA has been proposed as a biomarker and prognostic indicator for a range of diseases and syndromes (25, 26, 30-42). The source of this extracellular mtDNA is unknown but is assumed to arise from injured cells either in the process of cell death or repair (43). Extracellular mtDNA has not been examined for association with clinical phenotypes or outcomes in a large, well-characterized COPD cohort.
Based on the hypothesis that leakage of extracellular mtDNA is reflective of mitochondrial dysfunction in subjects with COPD, we sought to determine if extracellular mtDNA levels associate with unique COPD subgroups in the extensively characterized Subpopulations and Intermediate Outcome Measures in COPD Study (SPIROMICS). In this study, we chose to measure urine extracellular mtDNA (u-mtDNA) levels, as it may serve as an indicator of systemic mitochondrial damage. Additionally, recent studies from our group and others provide evidence that individuals with COPD have a higher incidence of chronic kidney disease (CKD) (44-55) and that mitochondrial dysfunction may be one of the mechanisms behind this finding (56). Specifically, we have recently shown that exposure to cigarette smoke induces mitochondrial swelling and loss of cristae in murine renal tubular epithelial cells (56), and that glomerulosclerosis and tubulointerstitial fibrosis occur more frequently in patients with COPD than in control subjects (44). U-mtDNA/creatinine ratios are higher in subjects with acute or chronic kidney injury, suggesting that u-mtDNA may offer potential as a unique biomarker for patients with kidney injury (26, 32-34, 36-38, 41, 42). For comparison, we also measured urine albumin, which has an established connection with CKD as well as lung function decline in COPD (44, 51-54). We herein show that u-mtDNA associates with important clinical outcomes in COPD, providing further evidence for mitochondrial dysfunction in COPD pathobiology.
RESULTS

SPIROMICS participant characteristics. Flow diagram for SPIROMICS and the characteristics of the participants for whom urine samples were analyzed are shown in Figure 1 and Table 1. Compared to participants in the overall SPIROMICS cohort, the participants in this study were less likely to be current smokers (p < 0.001); the severe COPD subgroup is also slightly over-represented (Supplementary Table 1). Urine albumin, creatinine, and cell-free u-mtDNA were measured in 483 participants, including 64 never smokers, 109 smokers without airflow obstruction, 142 participants with mild or moderate COPD, and 168 participants with severe COPD (Table 1). Compared to never smokers and smokers without airflow obstruction, participants with COPD were predominantly male and older, and in particular, participants with severe COPD had a lower BMI compared to the other subgroups. There was no difference between any of the subgroups in the rate of genitourinary conditions, which includes chronic kidney disease (p = 0.39, Table 1).

Urine albumin/creatinine ratio as a COPD biomarker in the SPIROMICS cohort. We first examined the relationship between urine albumin/creatinine ratio (UACR), a marker of kidney injury, and disease severity in the SPIROMICS cohort. Consistent with previous reports utilizing different COPD cohorts (51, 53, 54, 57-59), UACR was significantly increased in subjects with mild/moderate (median = 10.4 mg/g, p = 0.010) and severe (median = 10.2 mg/g, p = 0.002) COPD, when compared to smokers without airflow obstruction (median = 7.0 mg/g) (Figure 2A). This association remained significant after adjusting for smoking status, age, sex, and BMI (\(\hat{\beta} = 0.38 [0.01,0.75], p = 0.042\) for mild/moderate COPD, \(\hat{\beta} = 0.53 [0.17,0.90], p = 0.004\) for severe COPD, Table 2). UACR was also significantly higher in currently smoking participants with COPD (median = 8.95 mg/g) compared to participants with COPD who are not currently smoking (median = 7.0 mg/g, p = 0.027, Figure 2B).

Albuminuria has been associated with lung function decline in large population-based longitudinal cohort studies (54). To validate these findings, we examined the relationship between
UACR levels and important clinical parameters in the SPIROMICS cohort. In SPIROMICS, UACR was inversely associated with post-bronchodilator FEV\textsubscript{1} % predicted in unadjusted analyses ($\hat{\beta} = -0.12 \ [ -0.20, -0.03 ]$, $p = 0.009$), but this association lost significance with adjustment ($\hat{\beta} = -0.08 \ [ -0.17, 0.01 ]$, $p = 0.069$) (Figure 2C, Supplementary Table 2). UACR was not significantly associated with six-minute walk distance (6MWD) or St. George’s Respiratory Questionnaire (SGRQ) score in unadjusted or adjusted analyses, but was higher in participants with more significant respiratory symptoms as defined by COPD Assessment Test (CAT) score $\geq 10$, an association that persisted with adjustment (unadjusted $\hat{\beta} = 0.37 \ [0.09, 0.65 ]$, $p = 0.009$; adjusted $\hat{\beta} = 0.31 \ [0.03, 0.59 ]$, $p = 0.033$ respectively; Figure 2D, Supplementary Figure 1A-B, Supplementary Table 2). UACR was not significantly associated with radiographic measures of emphysema in unadjusted or adjusted analyses, but was associated with radiographic small airway disease (PRM\textsuperscript{SAD}) in the unadjusted analyses ($\hat{\beta} = 0.01 \ [0.0008, 0.02 ]$, $p = 0.033$), although this was lost with adjustment (Supplementary Figure 1C-E, Supplementary Table 2).

U-mtDNA associates with respiratory symptoms and exercise tolerance in COPD, especially in smokers with early disease. Given the prior evidence for mitochondrial dysfunction in COPD, we next investigated the potential of u-mtDNA as a novel biomarker using the SPIROMICS cohort. U-mtDNA levels were similar among never smokers (median = $2.46 \times 10^9$ copies/g), smokers without airflow obstruction (median = $2.37 \times 10^9$ copies/g), and participants with COPD (median = $2.97 \times 10^9$ copies/g for mild/moderate COPD, $2.77 \times 10^9$ copies/g for severe COPD, respectively) in unadjusted analyses, although after adjustment participants with both mild/moderate and severe COPD had significantly higher u-mtDNA levels than smokers without airflow obstruction ($\hat{\beta} = 0.36 \ [0.00, 0.73 ]$, $p = 0.050$ for mild/moderate COPD, $\hat{\beta} = 0.40 \ [0.04, 0.77 ]$, $p = 0.032$ for severe COPD) (Figure 3A, Table 3). Current smoking status did not affect u-mtDNA levels, in contrast to UACR (Figure 3B). U-mtDNA was not significantly associated with FEV\textsubscript{1} % predicted, in the overall study cohort or within each
individual subgroup in unadjusted or adjusted analyses (Figure 3C, Supplementary Figure 2, Table 4). Notably, u-mtDNA and UACR were significantly associated in matched participants in the SPIROMICS cohort (Supplementary Figure 3).

We next examined the relationship between u-mtDNA and measures of respiratory symptom burden and exercise capacity. In contrast to UACR, u-mtDNA correlated with 6MWD ($\beta = -0.02 [-0.03, -0.01]$, $p = 0.002$) and SGRQ ($\beta = 0.01 [0.006, 0.01]$, $p = 0.045$), and was higher in participants with more severe symptoms by CAT ($\beta = -0.36 [-0.64, -0.08]$, $p = 0.013$); these associations remained significant after adjustment (Figure 4A-C and Table 4). In subgroup analysis, these correlations were most notable among smokers without airflow obstruction, for 6MWD ($\beta = -0.04 [-0.07, -0.01]$, $p = 0.004$), SGRQ ($\beta = 0.03 [0.01, 0.04]$, $p = 0.002$), and CAT $\geq 10$ ($\beta = 1.09 [0.48, 1.70]$, $p < 0.001$), although the relationships with 6MWD and SGRQ were no longer significant after adjustment, potentially due to a loss of power (Figure 4D-F and Table 4). Unadjusted u-mtDNA was not significantly associated with radiographic measures of small airway disease and emphysema; however, after adjustment for age, sex, BMI and smoking status, u-mtDNA was significantly associated with emphysema ($\beta = 0.01 [0.002, 0.03]$, $p = 0.028$ for PRM$^{EMPH}$, $\beta = 0.01 [0.002, 0.03]$, $p = 0.028$ for $\%$ low attenuation area/LAA $< -950$ HU, Supplementary Figure 4 and Supplementary Table 3).

U-mtDNA associates with different clinical outcomes in males and females. Despite the well-described sex differences in COPD presentation and progression (60-63), currently there are no validated biomarkers that parallel these clinical observations. In the SPIROMICS cohort, we found that u-mtDNA levels were significantly higher in female participants when compared to male participants (median = $3.94 \times 10^9$ copies/g vs. $2.13 \times 10^9$ copies/g, $p < 0.001$), and that this difference was consistent across all subgroups, including never smokers (median = $3.56 \times 10^9$ copies/g vs. $1.82 \times 10^9$ copies/g, $p = 0.003$), smokers without airflow obstruction ($4.13 \times 10^9$ copies/g vs. $1.59 \times 10^9$ copies/g,
p < 0.001), participants with mild or moderate COPD (3.92 x 10^9 copies/g vs. 2.38 x 10^9 copies/g, p <
0.001), and participants with severe COPD (4.13 x 10^9 copies/g vs. 2.29 x 10^9 copies/g, p < 0.001), in
unadjusted and adjusted analyses (Figure 5A-B and Table 5). In males only, u-mtDNA was
significantly associated with FEV1 % predicted ($\hat{\beta} = -0.13 [-0.24, -0.01], p = 0.027$), although this was
no longer significant after adjustment ($\hat{\beta} = -0.10 [-0.22, 0.02], p = 0.091$, Figure 5C and Table 6). In
females, while not associated with FEV1 % predicted, u-mtDNA was significantly associated with
worse 6MWD ($\hat{\beta} = -0.02 [-0.04, -0.004], p = 0.016$) and was higher in females with more significant
symptoms by SGRQ ($\hat{\beta} = 0.01 [0.0005, 0.02], p = 0.004$) and CAT ($\beta = 0.50 [0.11, 0.89], p = 0.012$
(Figure 5D-F). These associations largely remained significant in models that adjusted for age, BMI
and smoking status, and were not observed to be significant in males (Figure 5D-F, Table 6).
Conversely, there was a significant association between u-mtDNA and radiographic measures of
emphysema among males in models that adjusted for age, BMI and smoking status ($\hat{\beta} = 0.02$
[0.0007, 0.03], p = 0.041 for PRMEMPH, $\hat{\beta} = 0.02 [0.001, 0.03], p = 0.034$ for %LAA < -950 HU), which
was not observed in females (Figure 5G-I, Table 6). Notably, UACR was not significantly different
between males and females in the SPIROMICS cohort; while UACR was associated with more
significant respiratory symptoms by CAT in males ($\hat{\beta} = 0.41 [0.01, 0.81], p = 0.047$), there were no
other sex differences with UACR in relation to other clinical outcomes. (Supplementary Figure 5 and
Supplementary Table 4-5).
DISCUSSION

In this study, we found novel associations between u-mtDNA levels and clinical measures of disease severity, especially respiratory symptom burden, that were not observed in the more traditional urinary marker of kidney injury, the urine albumin/creatinine ratio. Strikingly, we not only observed stronger associations for u-mtDNA in “healthy” smokers without airflow obstruction, but also contrasting associations for u-mtDNA in males and females, with u-mtDNA associating more with respiratory symptoms in females and more with spirometric and emphysema severity in males. Our findings provide further evidence for a mechanistic role for mitochondrial dysfunction in COPD, and suggest that u-mtDNA may shed new insight on early COPD and the often described, but rarely explained connection between sex and COPD.

Given the ample evidence connecting kidney disease with COPD, with albuminuria in many studies associating with future spirometric progression, it is not surprising that there were significant correlations between urinary biomarkers and important clinical outcomes in this study (44, 51-54). Our results with UACR recapitulate these previous studies, although UACR did not associate strongly with other “early” intermediate outcomes or emphysema parameters in the SPIROMICS cohort. By comparison, u-mtDNA expanded upon the associations observed with UACR, with higher u-mtDNA associating with worse 6MWD, SGRQ, and CAT scores in both unadjusted and adjusted analyses. These associations were especially prominent in smokers without airflow obstruction, which may suggest that a pathologic process has already started in these “healthy” subjects, and supports previous results that show that increased symptom burden in this population is not benign (64).

Although UACR and u-mtDNA were associated with different clinical parameters in this study, they were associated with each other, perhaps suggesting that they are different outputs of a common pathologic process in COPD.

The source of u-mtDNA in our study is unknown; despite u-mtDNA being examined previously in a variety of clinical scenarios including both acute and chronic kidney injury (26, 32, 33, 36-42), it is not clear if mtDNA detected in urine arises from the kidney intrinsically, or whether it is filtered and
secreted (or at least not reabsorbed) from circulating extracellular mtDNA. In at least one study, exogenous mtDNA given intravascularly to rodents did not increase circulating mtDNA but increased u-mtDNA (26). U-mtDNA may also originate from renal cells, including glomerular or tubular epithelial cells (56), endothelial cells (44), immune cells (6, 12), as well as lung-derived cell sources (5-11, 16) or muscle (65); further mechanistic studies are needed to identify the source of extracellular mtDNA in COPD. Whether u-mtDNA in COPD is a marker of disease or is pathogenic in itself remains to be determined; some studies have shown that giving mice exogenous mtDNA may increase markers of inflammation but does not induce bona fide kidney injury (26, 66).

The contrasting associations between u-mtDNA and clinical outcomes in male and female subjects are particularly interesting and thought provoking. It is unclear why u-mtDNA would differ by sex, an effect that is independent of smoking and is observed amongst never-smokers. Mitochondrial DNA is maternally inherited, and it is possible that our results are supportive of potentially different heritable genetic risk factors for COPD between men and women (67, 68). In this study, u-mtDNA correlated with increased respiratory symptom burden in females only, which may be consistent with studies showing that women report greater dyspnea (60, 61) and have an increased rate of exacerbation (63) than men. In general, women are more susceptible to smoke exposure, have more severe disease despite lower cumulative tobacco consumption, and experience earlier onset of COPD, faster decline in FEV₁, and more frequent exacerbations (69-73). In addition, females develop a different type of COPD to males with less severe emphysematous destruction, but thicker small airway walls (70). Mitochondria-related heritable factors as well as metabolite differences have been differentially observed between males and females (67, 74). Our findings herein add to the body of literature that exists that highlight sex differences in not only the clinical presentation but also the underlying pathobiology of COPD (75-77). Future studies using animal models of COPD may be insightful in elucidating the mechanisms behind these observations (78).

Our study is the first to examine u-mtDNA in COPD, and benefited from having a very large, well-characterized, prospective cohort that is rich in clinical information. The SPIROMICS cohort is
additionally enriched for participants with early COPD and milder disease, which allows for the exploration of “early” mechanisms in this population. We have extensive experience in studies involving extracellular mtDNA, having examined the utility of this biomarker in other diseases, and have over time refined our protocols to allow for accurate quantitation (25, 79). We normalized raw u-mtDNA copy numbers with urine creatinine from the same subjects, and further adjusted for BMI, a representation of muscle mass which is known to affect creatinine levels (80, 81).

Our study has several limitations. The smoking status was determined by self-report. Levels of urine cotinine, a biomarker of cigarette smoke exposure, have been assessed in the SPIROMICS population but were not analyzed in this study to verify individual smoking status. The surveys given at each study visit screen broadly for genitourinary conditions, including diseases of the bladder and prostate, and therefore do not capture accurately the proportion of patients with true renal disease. We chose to normalize our data to urine creatinine in the spot (untimed) urine samples, in a similar fashion as UACR to assess albuminuria, as 24-hour urine was not collected in the SPIROMICS cohort. The conventional 24-hour urine collection can also be a cumbersome procedure that is subject to errors from improper or incomplete collection. We analyzed UACR and u-mtDNA in models adjusted for age, sex (where appropriate), BMI, and smoking, but not study site. This decision stemmed from the fact that despite opportunistically sampling the available inventory, many sites were heavily underrepresented in our measured samples, an imbalance which would likely reduce our statistical power significantly with adjustment. Instead of removing these data points or grouping them together, we decided to analyze the entire dataset without adjusting for clinical site. Aside from BMI and urine creatinine, we have no other biological rationale to believe that study subject location would affect u-mtDNA, although we recognize the potential for unmeasured factors that may be unaccounted for in our analyses. We performed many statistical tests in this study and recognize the potential for type I errors given our level of significance; while these analyses are largely exploratory, the clustering of significant associations in related clinical parameters (such as 6MWD, SGRQ, and CAT) suggests to us that there is a true biological phenomenon underlying our findings.
In conclusion, extracellular urine mtDNA associates with an increased respiratory burden phenotype in COPD, especially in smokers and women. Further research is needed to uncover the source and meaning of u-mtDNA, as well as the role of mitochondrial dysfunction in the biological processes underlying COPD pathogenesis and progression, especially with regards to sex differences in disease presentation.
METHODS

Study design and sample collection. SPIROMICS (ClinicalTrials.gov NCT01969344) is an on-going longitudinal, prospective, multicenter observational study (82) that recruited never smokers (≤1 pack-year of tobacco-smoking history), current or former smokers (ever smokers, ≥ 20 pack-years) without airflow obstruction, and ever-smokers with airflow obstruction (64). Clean-catch urine was collected and stored at -80 °C prior to thawing and processing. For this study, urine samples were opportunistically sampled from available inventory. Clinical data were collected at the baseline and follow-up study visits, including demographics, comorbidities, questionnaires, cigarette smoke exposure and 6-minute walk distance (6MWD) (82). Symptom burden was quantified with the COPD Assessment Test (CAT) and health-related quality of life (HRQL) with the St. George’s Respiratory Questionnaire (SGRQ) total score. The extent of emphysema and airway-wall abnormality was characterized with HRCT scans of the lung, using Imbio (parametric response mapping, PRM) and VIDA Diagnostics software; percent emphysema was defined as low attenuation area (LAA) less than -950 Hounsfield Units at total lung capacity (%LAA < -950 HU) (83).

Urine processing and quantification of u-mtDNA and albumin/creatinine ratio (UACR). Samples were thawed on ice and vortexed briefly to mix. 50 µL of urine was diluted with 170 µL of PBS, and then centrifuged at 700 g at 4 °C for 5 minutes to remove cells and cellular debris. DNA was isolated using 180 µL of the supernatant with the QIAamp DSP DNA Blood Mini Kit (#61104; Qiagen), according to the manufacturer’s microcentrifuge protocol. 200 µL of the provided elution buffer was used to collect the DNA. Prior to quantification, the DNA solution was diluted 1:5 with nuclease-free deionized, distilled H₂O. MtDNA levels were measured by SYBR Green dye-based qPCR assay using a PRISM 7500 sequence detection system (Applied Biosystems) as previously described (25). Briefly, the following primer sequences were used: human NADH dehydrogenase 1 gene (forward 5’-ATACCCATGGCCAACCTC-3’, reverse 5’-GGGCCTTTGCGTAGTTGTAT-3’). For absolute
quantitation of mtDNA, a standard curve was generated from DNA plasmid constructs in serial dilutions (ORIGENE #SC101172 and GenScript NM_173708). 5 µL of sample was added to each well in triplicate along with a no-template control. The thermal profile was carried out as follows: 2 minutes at 50 °C, 10 minutes at 95 °C, 40 cycles for 15 seconds at 95 °C, and 1 minute at 60 °C. Urine albumin/creatinine ratio (UACR) was determined by immunoassay with Siemens DCA Microalbumin/Creatinine Reagent Kit (6011A) using a separate sample aliquot, according to the manufacturer’s instructions. The concentration of mtDNA in urine was calculated in copies per µL as previously described (25), followed by normalization to urine creatinine; so that final values were reported in copies of mtDNA per g of creatinine.

Statistical analysis. Clinical characteristics of SPIROMICS participants for whom urine creatinine and u-mtDNA were measured were compared to those of all SPIROMICS participants summarized using medians and interquartile intervals, or counts and percentages as appropriate. Both u-mtDNA and UACR were analyzed on the log2 scale to account for right-skewed distributions. Post-hoc pairwise differences between subgroups were evaluated using Tukey’s range test on the transformed data, and are presented in the figures and tables. For comparison, untransformed medians were provided in the text, and the nonparametric Kruskal-Wallis test was implemented and presented as well. Associations between u-mtDNA, UACR and baseline characteristics were performed using unadjusted linear models. Associations with post-bronchodilator FEV₁ % predicted, 6MWD, SGRQ, CAT, and CT measures of lung structure were further evaluated with linear models adjusted for age, sex, BMI, and current smoking status at baseline, overall and within COPD subgroups. Given the previous literature showing differences in mtDNA between males and females, an a-priori subgroup analysis for sex was performed. The effect size from the linear models is reported as a beta coefficient with a corresponding 95% confidence interval, representing the change in the outcome variable for a unit change in the explanatory value, holding all other variables in the model constant.
Analysis was conducted in R (84) and figures were produced using the package ggplot2 (85). All statistical tests were 2-sided and were performed using a significance level of $p < 0.05$.

**Study approval.** The study protocols for SPIROMICS were approved by the IRBs at all participating sites. All participants provided written informed consent prior to inclusion in the respective study.

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REFERENCES


Included in SPIROMICS Core v4.0 Data Set (n=2974)

Urine samples obtained/analyzed
Allocated into groups (n=483)

- Never Smokers (n=64)
- Smokers without Airflow Obstruction (n=109)
- Mild/Moderate COPD (n=142)
- Severe COPD (n=168)

Urine sample not obtained/analyzed (n=2491)
FIGURE LEGENDS

Figure 1. Flow diagrams for the participants in this SPIROMICS sub-study. COPD, chronic obstructive pulmonary disease.
Figure 2
Figure 2. Urine albumin/creatinine ratios (UACR) are higher in subjects with COPD and correlate with worse spirometry and respiratory symptom severity by the CAT score. (A) UACR (log2 mg/g) was measured in SPIROMICS participants and compared between never smokers (n=64), smokers without airflow obstruction (n=109), participants with mild/moderate COPD (n=139), and severe COPD (n=168). (B) UACR in participants with COPD who are current non-smokers (n=219) and current (n=82) smokers. (C) UACR and association with post-bronchodilator FEV1% predicted in all participants, including never smokers (n=63; red), smokers without airflow obstruction (n=109; green), participants with mild/moderate COPD (n=139; blue), and severe COPD (n=168; purple). (D) UACR compared in participants with mild symptoms (“not significant”, n=156) and those with more severe respiratory symptoms (“impactful”, n=294) using COPD Assessment Test (CAT) scores of ≥ 10 as a cut-off for impactful symptoms. Data (A, B, D) are presented as median with box indicating upper and lower quartiles, whiskers indicating extrema, and with p-values calculated by Tukey’s range test (A) or unadjusted linear regression models (B, D). Linear associations (C) were implemented with unadjusted linear regression models.
Figure 3

A) Log$_2$ urine mtDNA/creatinine ratio (copies mtDNA/g creatinine) for different smoking status and COPD severity groups. 
B) Log$_2$ urine mtDNA/creatinine ratio for non-smokers and smokers, with no significant difference (p = 0.59). 
C) Log$_2$ urine mtDNA/creatinine ratio vs. FEV$_1$ % predicted, showing a trend of higher ratios in smokers without airflow obstruction (β = -0.036, p = 0.43).
Figure 3. Urine mtDNA (u-mtDNA) levels do not associate with smoking status or spirometric severity in the SPIROMICS cohort. (A) U-mtDNA (log 2 copies mtDNA/g creatinine) was measured in SPIROMICS participants and compared between never smokers (n=64), smokers without airflow obstruction (n=109), participants with mild/moderate COPD (n=139), and severe COPD (n=168). (B) U-mtDNA in participants with COPD who are current non-smokers (n=219) and current (n=82) smokers. (C) U-mtDNA and association with post-bronchodilator FEV₁ % predicted in all participants, including never smokers (n=63; red), smokers without airflow obstruction (n=109; green), participants with mild/moderate COPD (n=142; blue), and severe COPD (n=168; purple). Data (A-B) are presented as median with box indicating upper and lower quartiles, whiskers indicating extrema, and with p-values calculated by Tukey’s range test (A) or unadjusted linear regression models (B). Linear associations (C) were implemented with unadjusted linear regression models.
Figure 4

A) $eta = -0.02$, $p = 0.0015$

B) $eta = 0.01$, $p = 0.045$

C) $p = 0.013$

D) $p = 0.80$

E) $p = 0.81$

F) $p = 0.001$
Figure 4. U-mtDNA levels associate with exercise capacity and symptom severity in the SPIROMICS cohort, especially among smokers without COPD. (A-C) Association between u-mtDNA (log 2 copies mtDNA/g creatinine) in all participants (never smokers, n=61-64; smokers without airflow obstruction/“smokers”, n=104-109; mild/moderate COPD, n=135-137; severe COPD, n=149-162) and respiratory symptoms as measured by (A) six-minute walk distance (6MWD, m=meters), (B) St. George’s Respiratory Questionnaire (SGRQ), and (C) COPD Assessment Test (CAT) (“not significant” n=156; “impactful” n=297). (D-F) Association between u-mtDNA and respiratory symptoms as measured by (D) 6MWD, (E) SGRQ, and (F) CAT within never smokers (n=56-64), smokers without airflow obstruction/“smokers” (n=101-109), mild/moderate COPD (n=134-137), severe COPD (n=149-162). Linear associations (A, B, D, E) were implemented with unadjusted linear regression models. Data (C, F) are presented as median with box indicating upper and lower quartiles, whiskers indicating extrema, and with p-values calculated by unadjusted linear regression models.
Figure 5
Figure 5. U-mtDNA levels are higher in women and higher urine mtDNA levels associate with worse exercise capacity and more severe respiratory symptoms in women but not men. (A) U-mtDNA (log 2 copies mtDNA/g creatinine) levels in males (n=258) compared to females (n=225) in all participants and (B) within each subgroup (never smokers male n=20, female n=44; smokers without airflow obstruction/"smokers" male n=48, female n=61; mild/moderate COPD male n=83, female n=59; severe COPD male n=107, female n=61). (C-I) U-mtDNA and association with post-bronchodilator FEV₁ % predicted (C), 6MWD (D), SGRQ (E), CAT ("not significant" male n=82, female n=74; “impactful” male n=162, female n=135) (F), % low attenuation area/LAA < -950 HU (HU=Hounsfield units) (G), emphysema by parametric response mapping (PRM$^{EMPH}$) (H), and functional small airway disease by parametric response mapping (PRM$^{FSAD}$) (I) within males and females (never smokers male n=18-20, female n=36-44; smokers without airflow obstruction male n=43-48, female n=53-61; mild/moderate COPD male n=76-83, female n=50-59; severe COPD male n=94-107, female n=51-61). Data (A, B, F) are presented as median with box indicating upper and lower quartiles, whiskers indicating extrema, and with p-values calculated by unadjusted linear regression models. Linear associations (C-E, G-I) were implemented with unadjusted linear regression models.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Never smokers (n=64)</th>
<th>Smokers without airflow obstruction (n=109)</th>
<th>Mild/moderate COPD (n=142)</th>
<th>Severe COPD (n=168)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, median [IQR]</td>
<td>57.0 [50.8-66.2]</td>
<td>60.0 [51.0-67.0]</td>
<td>66.0 [60.2-71.0]</td>
<td>65.0 [59.0-71.0]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Male N (%)</td>
<td>20 (31.2)</td>
<td>48 (44.0)</td>
<td>83 (58.5)</td>
<td>107 (63.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Race N (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>American Indian</td>
<td>0 (0.00)</td>
<td>0 (0.00)</td>
<td>0 (0.00)</td>
<td>1 (0.60)</td>
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<tr>
<td>Asian</td>
<td>1 (1.56)</td>
<td>1 (0.93)</td>
<td>4 (2.82)</td>
<td>2 (1.20)</td>
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<tr>
<td>Black</td>
<td>16 (25.0)</td>
<td>33 (30.6)</td>
<td>13 (9.15)</td>
<td>24 (14.5)</td>
<td></td>
</tr>
<tr>
<td>Mixed</td>
<td>2 (3.12)</td>
<td>5 (4.63)</td>
<td>1 (0.70)</td>
<td>3 (1.81)</td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>45 (70.3)</td>
<td>69 (63.9)</td>
<td>124 (87.3)</td>
<td>136 (81.9)</td>
<td></td>
</tr>
<tr>
<td>Current non-smoker N (%)</td>
<td>64 (100)</td>
<td>54 (50.9)</td>
<td>91 (66.4)</td>
<td>128 (78.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FEV₁ % predicted, median [IQR]</td>
<td>101 [94.7-108]</td>
<td>95.8 [86.8-105]</td>
<td>70.5 [61.4-77.8]</td>
<td>33.2 [26.6-43.4]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Genitourinary condition N (%)</td>
<td>20 (32.3%)</td>
<td>33 (30.6%)</td>
<td>56 (40.6%)</td>
<td>58 (35.6%)</td>
<td>0.392</td>
</tr>
</tbody>
</table>

*Kruskal-Wallis test, Chi-square test, or Fisher’s exact test comparing participants within each subgroup, as appropriate
Abbreviations: IQR=interquartile range; 25% and 75% percentiles, FEV₁=forced expiratory volume in 1 second
<table>
<thead>
<tr>
<th>Subgroups compared</th>
<th>Unadjusted</th>
<th>Adjusted for age, sex, BMI, &amp; smoking status</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\hat{\beta}$ (CI)</td>
<td>$P$-value $^*$</td>
</tr>
<tr>
<td>Never smokers – Smokers without airflow obstruction</td>
<td>-0.02 (-0.46, 0.42)</td>
<td>0.935</td>
</tr>
<tr>
<td>Smokers without airflow obstruction – Mild/moderate COPD</td>
<td>0.47 (0.11, 0.82)</td>
<td>0.010</td>
</tr>
<tr>
<td>Smokers without airflow obstruction – Severe COPD</td>
<td>0.55 (0.21, 0.90)</td>
<td>0.002</td>
</tr>
<tr>
<td>Mild/moderate COPD – Severe COPD</td>
<td>0.09 (-0.23, 0.41)</td>
<td>0.600</td>
</tr>
</tbody>
</table>

$^*$Pairwise contrasts of strata using Tukey’s range test (unadjusted and adjusted)

Abbreviations: BMI=body mass index, $\hat{\beta}$=estimated change log₂ UACR with a one unit increase in clinical characteristic, CI=confidence interval
Table 3. Urine mtDNA between subgroups

<table>
<thead>
<tr>
<th>Subgroups compared</th>
<th>Unadjusted</th>
<th>Adjusted for age, sex, BMI, &amp; smoking status</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\hat{\beta}$ (CI)</td>
<td>$P$-value*</td>
</tr>
<tr>
<td>Never smokers – Smokers without airflow obstruction</td>
<td>-0.05 (-0.51,0.41)</td>
<td>0.832</td>
</tr>
<tr>
<td>Smokers without airflow obstruction – Mild/moderate COPD</td>
<td>0.24 (-0.13,0.54)</td>
<td>0.207</td>
</tr>
<tr>
<td>Smokers without airflow obstruction – Severe COPD</td>
<td>0.16 (-0.19,0.52)</td>
<td>0.368</td>
</tr>
<tr>
<td>Mild/moderate COPD – Severe COPD</td>
<td>-0.07 (-0.40,0.26)</td>
<td>0.661</td>
</tr>
</tbody>
</table>

*Pairwise contrasts of strata using Tukey’s range test (unadjusted and adjusted)

Abbreviations: BMI=body mass index, $\hat{\beta}$=estimated change log2 urine mtDNA with a one unit increase in clinical characteristic, CI=95% confidence interval
Table 4. Urine mtDNA and clinical characteristics within subgroups

<table>
<thead>
<tr>
<th>Clinical characteristic</th>
<th>Unadjusted</th>
<th>Adjusted for age, sex, BMI, &amp; smoking status*</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>( \hat{\beta} ) (CI)</td>
<td>( P )-value*</td>
</tr>
<tr>
<td><strong>FEV(_1) % predicted</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>-0.04 (-0.12,0.05)</td>
<td>0.428</td>
</tr>
<tr>
<td>Never smokers</td>
<td>0.31 (-0.19,0.82)</td>
<td>0.230</td>
</tr>
<tr>
<td>Smokers without airflow obstruction</td>
<td>-0.28 (-0.72,0.16)</td>
<td>0.214</td>
</tr>
<tr>
<td>Mild/moderate COPD</td>
<td>0.02 (-0.30,0.33)</td>
<td>0.915</td>
</tr>
<tr>
<td>Severe COPD</td>
<td>0.08 (-0.34,0.50)</td>
<td>0.712</td>
</tr>
<tr>
<td><strong>6MWD (meters)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>-0.02 (-0.03,-0.01)</td>
<td>0.002</td>
</tr>
<tr>
<td>Never smokers</td>
<td>-0.03 (-0.07,-0.001)</td>
<td>0.048</td>
</tr>
<tr>
<td>Smokers without airflow obstruction</td>
<td>-0.04 (-0.07,-0.01)</td>
<td>0.004</td>
</tr>
<tr>
<td>Mild/moderate COPD</td>
<td>-0.01 (-0.04,0.01)</td>
<td>0.229</td>
</tr>
<tr>
<td>Severe COPD</td>
<td>-0.01 (-0.03,0.01)</td>
<td>0.295</td>
</tr>
<tr>
<td><strong>SGRQ</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>0.01 (0.006,0.01)</td>
<td>0.045</td>
</tr>
<tr>
<td>Never smokers</td>
<td>0.01 (-0.02,0.03)</td>
<td>0.650</td>
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<tr>
<td>Smokers without airflow obstruction</td>
<td>0.03 (0.01,0.04)</td>
<td>0.002</td>
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<tr>
<td>Mild/moderate COPD</td>
<td>-0.002 (-0.01,0.01)</td>
<td>0.767</td>
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<tr>
<td>Severe COPD</td>
<td>0.003 (-0.01,0.02)</td>
<td>0.688</td>
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<tr>
<td><strong>CAT (≥10)</strong></td>
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<tr>
<td>All</td>
<td>0.36 (0.08,0.64)</td>
<td>0.013</td>
</tr>
<tr>
<td>Never smokers</td>
<td>-0.09 (-0.84,0.66)</td>
<td>0.812</td>
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<tr>
<td>Smokers without airflow obstruction</td>
<td>1.09 (0.48,1.70)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mild/moderate COPD</td>
<td>0.06 (-0.43,0.55)</td>
<td>0.804</td>
</tr>
<tr>
<td>Severe COPD</td>
<td>0.16 (-0.65,0.98)</td>
<td>0.699</td>
</tr>
</tbody>
</table>

*Univariable or multivariable linear regression with log2 urine mtDNA as among all participants and within each group.
*Never smokers are adjusted only for age, sex, & BMI

Abbreviations: BMI=body mass index, \( \hat{\beta} \)=estimated change log2 urine mtDNA with a one unit increase in clinical characteristic, CI=95% confidence interval, FEV\(_1\)=forced expiratory volume in 1 second, 6MWD=six-minute walk distance, SGRQ=St. George’s Respiratory Questionnaire, CAT=COPD Assessment Test
<table>
<thead>
<tr>
<th></th>
<th>Unadjusted</th>
<th></th>
<th>Adjusted for age, BMI, &amp; smoking status*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\hat{\beta}$ (CI)</td>
<td>$P$-value*</td>
<td>$\hat{\beta}$ (CI)</td>
</tr>
<tr>
<td>All</td>
<td>0.97 (0.72,1.22)</td>
<td>&lt;0.001</td>
<td>1.01 (0.75,1.26)</td>
</tr>
<tr>
<td>Never smokers</td>
<td>0.99 (0.37,1.61)</td>
<td>0.003</td>
<td>1.12 (0.49,1.75)</td>
</tr>
<tr>
<td>Smokers without airflow obstruction</td>
<td>1.40 (0.83,1.97)</td>
<td>&lt;0.001</td>
<td>1.43 (0.86,2.01)</td>
</tr>
<tr>
<td>Mild/moderate COPD</td>
<td>0.75 (0.33,1.17)</td>
<td>&lt;0.001</td>
<td>0.75 (0.32,1.19)</td>
</tr>
<tr>
<td>Severe COPD</td>
<td>1.11 (0.64,1.58)</td>
<td>&lt;0.001</td>
<td>1.18 (0.69,1.67)</td>
</tr>
</tbody>
</table>

*Univariable or multivariable linear regression with log2 mtDNA as dependent variable.
*Never smokers are adjusted only for age & BMI
Abbreviations: BMI=body mass index, $\hat{\beta}$=estimated difference in log2 urine mtDNA in females compared to males, CI=95% confidence interval
<table>
<thead>
<tr>
<th></th>
<th>Unadjusted</th>
<th>Adjusted for age, BMI, &amp; smoking status</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \hat{\beta} ) (CI)</td>
<td>( P)-value*</td>
</tr>
<tr>
<td><strong>FEV(_1) % predicted</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>-0.13 (-0.24,-0.01)</td>
<td>0.027</td>
</tr>
<tr>
<td>Females</td>
<td>-0.05 (-0.18,0.08)</td>
<td>0.463</td>
</tr>
<tr>
<td><strong>6MWD (meters)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>-0.01 (-0.02,0.004)</td>
<td>0.181</td>
</tr>
<tr>
<td>Females</td>
<td>-0.02 (-0.04,-0.004)</td>
<td>0.016</td>
</tr>
<tr>
<td><strong>SGRQ</strong></td>
<td></td>
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<tr>
<td>Males</td>
<td>0.001 (-0.01,0.01)</td>
<td>0.799</td>
</tr>
<tr>
<td>Females</td>
<td>0.01 (0.004,0.02)</td>
<td>0.004</td>
</tr>
<tr>
<td><strong>CAT (≥10)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>0.26 (-0.10,0.62)</td>
<td>0.161</td>
</tr>
<tr>
<td>Females</td>
<td>0.50 (0.11,0.89)</td>
<td>0.012</td>
</tr>
<tr>
<td><strong>%LAA (&lt;-950 HU)</strong></td>
<td></td>
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</tr>
<tr>
<td>Males</td>
<td>0.01 (-0.0001,0.03)</td>
<td>0.053</td>
</tr>
<tr>
<td>Females</td>
<td>0.004 (-0.01,0.02)</td>
<td>0.613</td>
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<tr>
<td><strong>PRM(_{EMPH})</strong></td>
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</tr>
<tr>
<td>Males</td>
<td>0.01 (-0.0003,0.03)</td>
<td>0.057</td>
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<tr>
<td>Females</td>
<td>0.01 (-0.01,0.02)</td>
<td>0.526</td>
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<tr>
<td><strong>PRM(_{FSAD})</strong></td>
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<tr>
<td>Males</td>
<td>0.01 (-0.0004,0.02)</td>
<td>0.060</td>
</tr>
<tr>
<td>Females</td>
<td>0.002 (-0.01,0.02)</td>
<td>0.753</td>
</tr>
</tbody>
</table>

*Univariable or multivariable linear regression with log2 urine mtDNA as among all participants and within each group.

Abbreviations: BMI=body mass index, \( \hat{\beta} \)=estimated change log2 urine mtDNA with a one unit increase in clinical characteristic, CI=95% confidence interval, FEV\(_1\)=forced expiratory volume in 1 second, 6MWD=six-minute walk distance, SGRQ=St. George’s Respiratory Questionnaire, CAT=COPD Assessment Test, LAA=low attenuation area, HU=Hounsfield units, PRM\(_{EMPH}\)=emphysema by parametric response mapping, PRM\(_{FSAD}\)=functional small airway disease by parametric response mapping