**ONLINE DATA SUPPLEMENT**

## Study Design

Subjects were recruited from pulmonary clinics and by advertising locally. Inclusion criteria included males and females, > 40 years, either nonsmokers or smokers of at least 10 pack-years in their lifetime. All subjects considered former smokers had to be abstinent of tobacco use for at least 12 months; whereas active smokers had to smoke at least one cigarette within 3 days of enrollment. Participation exclusion criteria included self-reported evidence of heart disease, creatinine elevation above normal reference value, use of antibiotics, systemic steroids or immunosuppressant agents within 12 weeks, presence of an exacerbation or acute upper respiratory infection within the previous 4 weeks and history of any lung condition other than COPD such as bronchiectasis, asthma, pulmonary malignancies or prior thoracic surgery. In addition, subjects had to be in stable clinical condition to undergo a bronchoscopy as per the physician investigators. All subjects underwent complete pulmonary function testing and filled in a detailed clinical and demographic questionnaire. We classified all smokers with airflow obstruction ($FEV_1/FVC \leq 0.70$) as having COPD.

## Antibodies

anti-rabbit α-klotho Ab, Abcam (Cambridge, MA, USA)

anti-rabbit FGFR4 antibody (sc-124), Santa Cruz Biotechnology, Dallas, TX, USA

anti-goat α-klotho antibody (sc-22220), Santa Cruz Biotechnology, Dallas, TX, USA

anti-rabbit phospho-PLCγ (8713S), Cell Signaling, Danvers, MA, USA

anti-rabbit PLCγ (2822S), Cell Signaling, Danvers, MA, USA

anti-β-actin antibody, Sigma-Aldrich, St. Louis, MO, USA

anti-rabbit phospho-ERK (9101S), Cell Signaling, Danvers, MA, USA

anti-rabbit ERK (4695S), Cell Signaling, Danvers, MA, USA

**Bronchoalveolar lavage and venipuncture**

All subjects underwent bronchoscopy via the nasal passage using a single flexible bronchoscope (Olympus, PA, USA) under conscious sedation in accordance with standard clinical practice. After the vocal cords and trachea were anesthetized with 1% lidocaine, the bronchoscope was directly inserted into the right middle lobe bronchus and wedged. Bronchoalveolar lavage (BAL) was collected with three instillations of 20 cc of saline and immediately aspirated using a collector trap on the suction port of the scope, centrifuged and the supernatant stored in 1 ml aliquots at -80$^{o}$C until analyzed. Transbronchial biopsies were obtained as well. The patients also underwent venipuncture and 5 ml of blood was collected, centrifuged at 1500 rpm for 5 minutes and the serum was stored in aliquots at -80$^{o}$C until analyzed.

**RNA Extraction and Quantitative RT-PCR**

Total RNA was extracted from ALI-cultured human airway epithelial cells using the RNeasy Protect mini kit (Qiagen, Valencia, CA, USA). Reverse transcription was done using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) with 1 μg of RNA according to the instructions of the manufacturer. Real-time quantitative PCR was performed using the following TaqMan probes: Hs00174097_m1 for IL-1β,

Hs00873651_m1 for MUC5AC, Hs00174103_m1 for IL-8, and Hs02758991_g1 for GAPDH.

**Western Blotting**

ALI-cultured human airway epithelial cells were lysed in radioimmune precipitation assay buffer containing protease inhibitors. Protein yield was measured by BCA assay (Pierce). Proteins were separated on a 4–20% precast Ready Gel (Bio-Rad) and blotted onto Immobilon-P membranes (Millipore, Billerica, MA). Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (pH 7.4) with 0.05% Tween 20 (TTBS) for 1 h. Primary antibodies were as follows: rabbit anti- phospho PLCγ and PLCγ antibodies (Cell Signaling), used as prescribed previously (1,2); rabbit total and phosphor-ERK1/2 (Cell Signaling); rabbit anti-FGFR4 and mouse anti-β-actin antibody. Secondary antibody was an anti-rabbit (Seracare, Milford, MA, USA) or anti-mouse (Seracare, Milford, MA, USA) horseradish peroxidase-linked antibody used at 1:5000 in TTBS for 1 h at room temperature. Positive signals were visualized by chemiluminescence on a ChemiDoc XRS system (Bio-Rad, Hercules, CA, USA).

**16 HBE Cell Culture**

16HBE cells, a SV40-immortalized human bronchial epithelial cell line, generously provided by the UAB Cystic Fibrosis Center, were utilized for this study. Cells were grown in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% heat-inactivated fetal bovine serum (Atlas Biologicals; Fort Collins, CO) and without antibiotics, hereafter referred to as complete medium (CM).

**NFAT Luciferase Reporter Assay**

To access NFAT transcriptional activity following FGF23 treatment, 16HBE cells were seeded at a density of 1.5 x$10^4$cells/well in 96-well plates. After 24 hours, cells were transfected with the constitutively-active *Renilla*-luciferase construct, as a transfection control, along with 100 ng/well of a Fire Fly Luciferase reporter construct, either a NFAT reporter or a null promoter construct serving as a negative control as provided with the NFAT Cignal Reporter Assay Kit (Qiagen; Hilden, Germany). Transfection was performed under serum-free conditions in OptiMEM using Lipofectamine 2000 transfection reagent (Thermo Scientific, Grand Island, NY, USA). After an overnight incubation, the medium was replaced with CM. When required, cells were pre-incubated for 30 min with 10 μM of the FGFR4 inhibitor BLU9931 (Selleck Chemicals, Houston, TX, USA). Immediately thereafter, varying dilutions of recombinant human FGF23 in the presence or absence of BLU9931 were added to the wells. Following an additional 24 hr incubation, a Luciferase assay was performed using the Dual-Luciferase Reporter Assay System (Promega; Madison, WI, USA) as directed by the manufacturer. Briefly, the cells were washed with phosphate buffered saline, pH 7.4 (PBS), then lysed in 80 μL of passive lysis buffer at room temperature with shaking for 15 min. 20 μL lysate was loaded into each well of an opaque 96-well plate and relative light units were measured utilizing a SpectraMax i3x plate reader equipped with dual injectors (Molecular Devices; Sunnyvale, CA, USA).

**NFAT knockdown Experiments**

The effect of NFAT knockdown on IL-1β induction following FGF23 and cigarette smoke extract (CSE) treatment was tested on 16HBE cells. 6 x $10^4$ cells were seeded in 24-well plates. The next day, medium was replaced with 0.5mL OptiMEM (Thermo Scientific, Grand Island, NY, USA) and the cells were transfected with 5 nmol of either AllStar negative control, a mix of two NFATC2 siRNAs, or a mix of two NFATC3 siRNAs using 3 μL/well of HiPerFect transfection reagent (Qiagen; Hilden, Germany). Following a 6 hour incubation, medium was replaced following an additional 48 hr incubation to allow for NFAT knockdown, cells were treated with FGF-23 (40 ng/mL), 5% CSE or vehicle (DMSO or CM) control. After 24 hours, wells were washed with 1.0 mL cold phosphate buffered saline (pH 7.4) and RNA was extracted using the GeneJET RNA purification kit (Thermo Scientific, Grand Island, NY, USA). cDNA was generated using the Maxima H Minus First Strand cDNA Synthesis Kit and transcript levels for NFAT and IL-1β were quantified using Taqman assays (Thermo Scientific, Grand Island, NY, USA).

**Morphometric Analysis and BALF in mice**

Morphometric analyses were conducted in a standard fashion as previously reported assessing mean linear intercepts after fixation of the murine lung tissue (3). BALF was obtained following established protocols of the lab (4). Briefly, a tracheal cannula was inserted and the BAL procedure was performed under direct visualization of lung distension (maximum 2 ml), as previously described (5,6). Cells were pelleted by centrifugation (1,100 × *g* for 5 min at 4° C), resuspended in PBS and stained with modified Wright-Giemsa for differential cell counts.

**Air-liquid interface (ALI) cell culture and cigarette smoke exposure**

Human bronchial epithelial cells (HBEC) from age-matched nonsmoker and COPD lungs were isolated and de-differentiated through expansion as described previously (7,8). Fully differentiated HBEC were exposed to cigarette smoke (Kentucky 3R4F cigarettes) using a Vitrocell VC-10 smoking robot (Vitrocell Systems GMBH, Waldkirch, Germany) (8). Murine tracheal epithelial cells (MTEC) were obtained from wild type mice as well as klotho hypomorphic mice (SV129 background) (9). MTEC from wild type and kl+/-, kl-/- mice (10)were cultured and differentiated according to an adapted protocol of You et al. (7,11). Cells were fully differentiated after approximately 2-3 weeks and then used for experiments.

**Acute cigarette smoke exposure in mice**

Klotho overexpressing mice and their wild type littermates (10) were exposed to cigarette smoke (3R4F, University of Kentucky) twice daily (1 hour smoke – 1h break – 1h smoke) for a total of 5 days with increasing amounts of cigarettes (Day 1: 4 cigarettes/day, Day 2: 6 cigarettes/day, Day3: 8 cigarettes/day, Day 4: 10 cigarettes/day and Day 5: 12 cigarettes/day).


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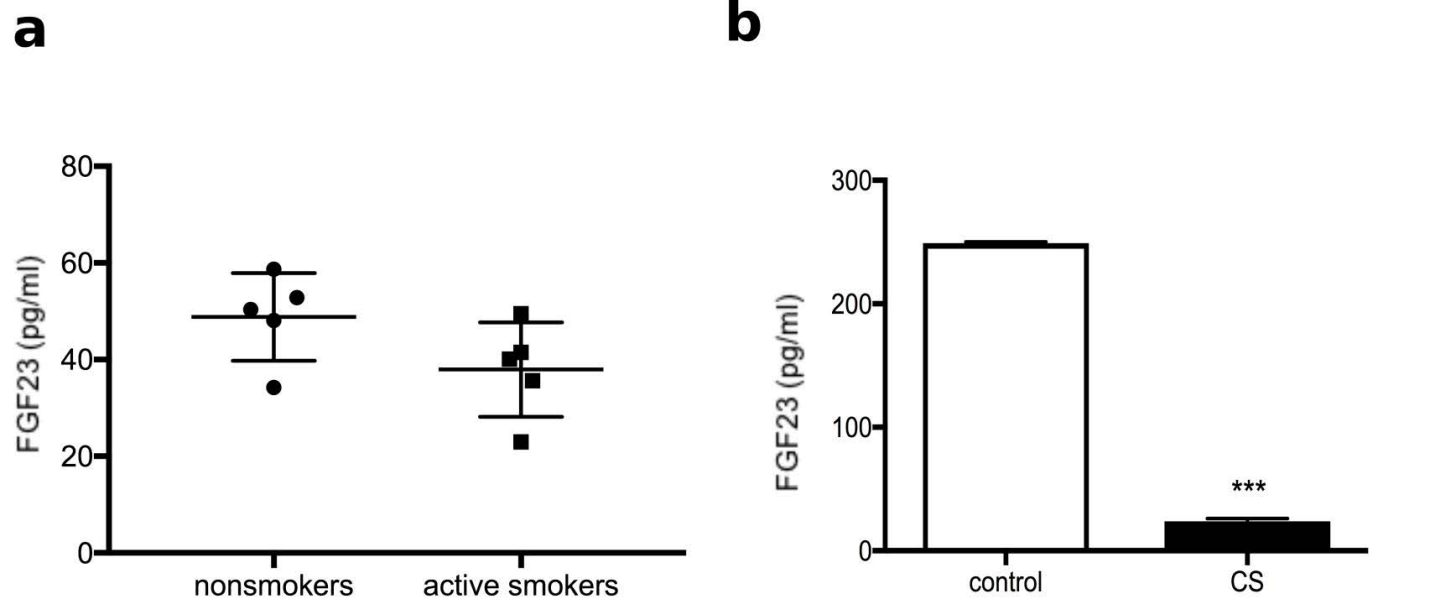
## Supplemental Table 1. Clinical Characteristics

| | Non-COPD (n = 10) | Mild-to-moderate COPD (n = 9) | Severe COPD (n = 9) | P value |
|---|---|---|---|---|
| Age, years | 53 ± 9 | 57 ± 10 | 63 ± 2 | (p=0.29, p=0.02) |
| Male sex | 3 (33%) | 8 (80%) | 8 (80%) | (p=0.008, p<0.008) |
| FEV1% | 100 ± 17 | 64 ± 15 | 44 ± 17 | (p=0.0002, p<0.0001) |
| Current smoker | 5 (56%) | 4 (40%) | 2 (20%) | (p>0.9999, p=0.45) |
| FGF23 (pg/ml) | 42 ± 10 | 59 ± 12 | 51 ± 18 | (p=0.004, p=0.42) |

Data expressed as mean ± standard deviation or n (%). FEV1% = post-bronchodilator forced-expiratory volume in 1-second percent predicted. P values show comparison of control vs mild-moderate COPD and control vs severe COPD using 1-way ANOVA and either Tukey post test or Kruskal Wallis (for gender and smoking status).


a
80
60
40
20
0
FGF23 (pg/ml)
nonsmokers
active smokers
b
300
200
100
0
FGF23 (pg/ml)
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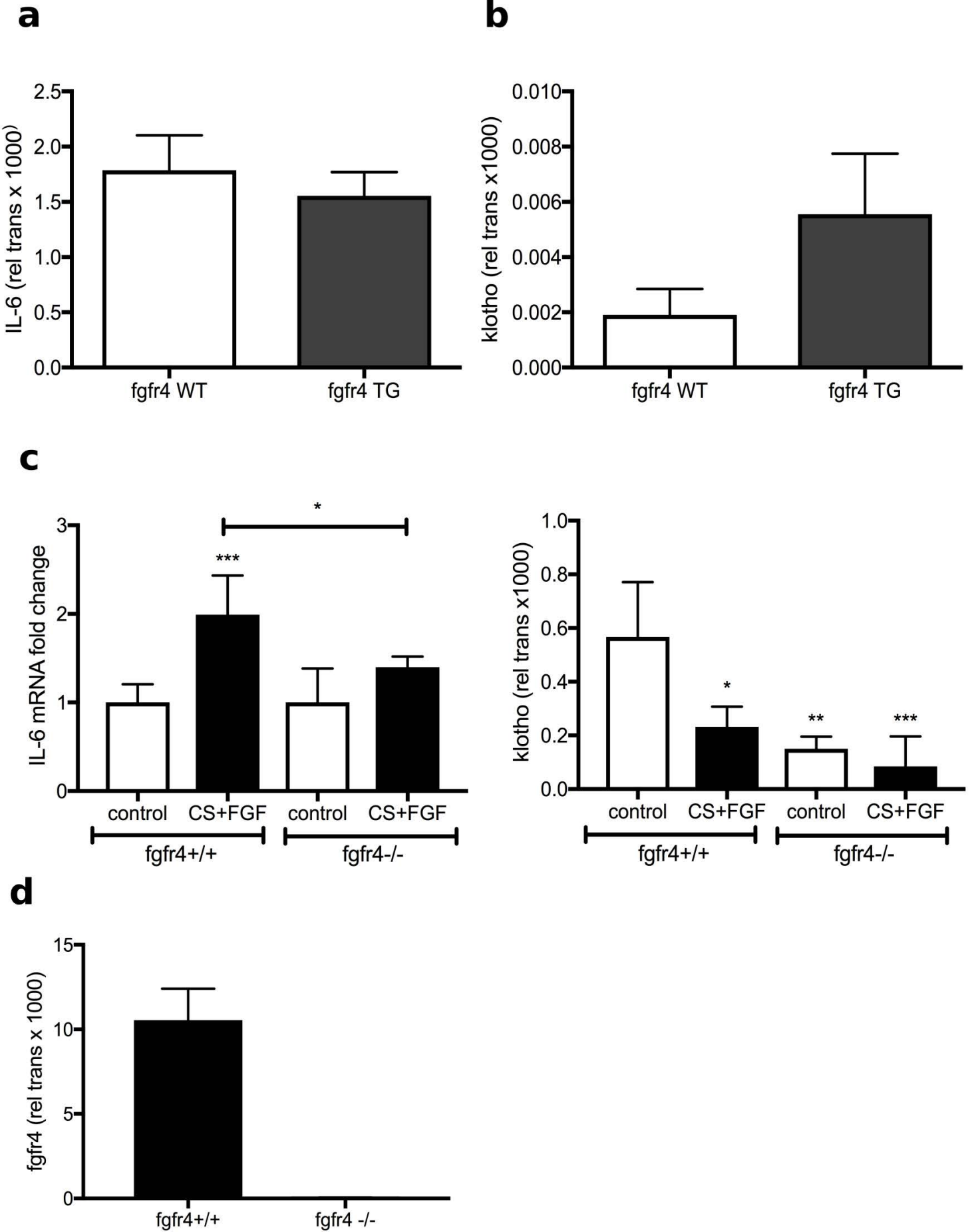
control
CS


a
IL-6 (rel trans x 1000)
2.5
2.0
1.5
1.0
0.5
0.0
fgfr4 WT
fgfr4 TG
b
klotho (rel trans x1000)
0.010
0.008
0.006
0.004
0.002
0.000
fgfr4 WT
fgfr4 TG
c
IL-6 mRNA fold change
3
2
1
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control
CS+FGF
control
CS+FGF
fgfr4+/+
fgfr4-/-
klotho (rel trans x1000)
1.0
0.8
0.6
0.4
0.2
0.0
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control
CS+FGF
control
CS+FGF
fgfr4+/+
fgfr4-/-
d
fgfr4 (rel trans x 1000)
15
10
5
0
fgfr4+/+
fgfr4 -/-

## SUPPLEMENTARY FIGURE LEGENDS

**Table 1**

Patient characteristics of our study population, divided into patients without (FEV1/FVC > 0.7) and with mild – moderate or severe COPD (FEV1/FVC < 0.7), including age, gender, FEV1 % predicted post bronchodilator, smoking status and FGF23 plasma levels (shown as mean ± S.E.M.).

**Supplementary Figure 1.** (a) Dot plot showing plasma FGF23 levels in the control cohort, subdivided in 5 non-smokers and 5 active smokers showing no significant difference when unpaired Student's t-test was used for comparison ($p = 0.1047$). (b) Bar graphs indicating serum FGF23 levels in control mice compared to mice exposed to cigarette smoke for a total of 6 months (3 mice per group). (shown as mean ± S.E.M. with ***$p<0.005$).

**Supplementary Figure 2.** (a) mRNA levels of IL-6 and (b) klotho in fgfr4 WT and TG MTECs. (c) IL6 and klotho mRNA fold change 24h after stimulation with cigarette smoke (2 cigarettes) ± FGF23 (25 ng/ml) in MTECs from $fgfr4^{+/+}$ and $fgfr4^{-/-}$ MTECs. (d) fgfr4 transcript levels in $fgfr4^{+/+}$ and $fgfr4^{-/-}$ MTECs. All bar graphs are mean ± S.E.M. *$P<0.05$, **$P<0.01$ and ***$P<0.005$, compared to control (ctrl) group.