

Mitochondrial metabolic dysfunction in airway epithelial cells promotes goblet cell metaplasia and more severe asthma

Xiuxia Zhou,^{1*} Krithika S. Rao,² Sruti Shiva,³ Catharina van Heusden,⁴ Qi Wei,¹ Nobuhiko Fukuda,¹ Mayoko Tsuji,¹ Angelina Kendi,² Laura R. Bradley,¹ Jinming Zhao,¹ John B. Trudeau,¹ Anuradha Ray,⁵ Richard C. Boucher,⁴ Sally E. Wenzel^{1*}

¹Department of Environmental and Occupational Health, School of Public Health, University of Pittsburgh, Pittsburgh, PA 15261 USA

²Vascular Medicine Institute, Division of Cardiology, Department of Medicine, School of Medicine, University of Pittsburgh, Pittsburgh, PA 15260 USA

³Vascular Medicine Institute, Division of Classical Hematology, Department of Medicine, School of Medicine, University of Pittsburgh, Pittsburgh, PA 15260 USA

⁴Marsico Lung Institute, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599 USA

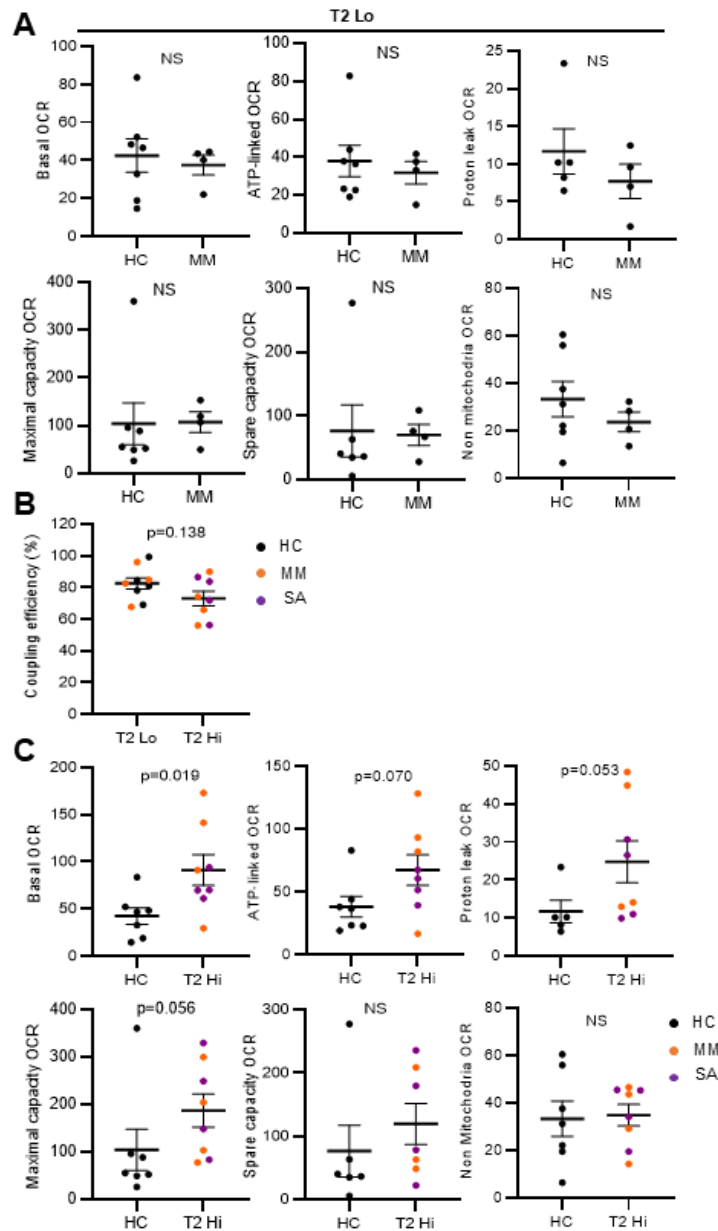
⁵Pulmonary, Allergy, Critical Care, and Sleep Medicine Division, Department of Medicine, School of Medicine, University of Pittsburgh, Pittsburgh, PA 15260 USA

* Corresponding authors

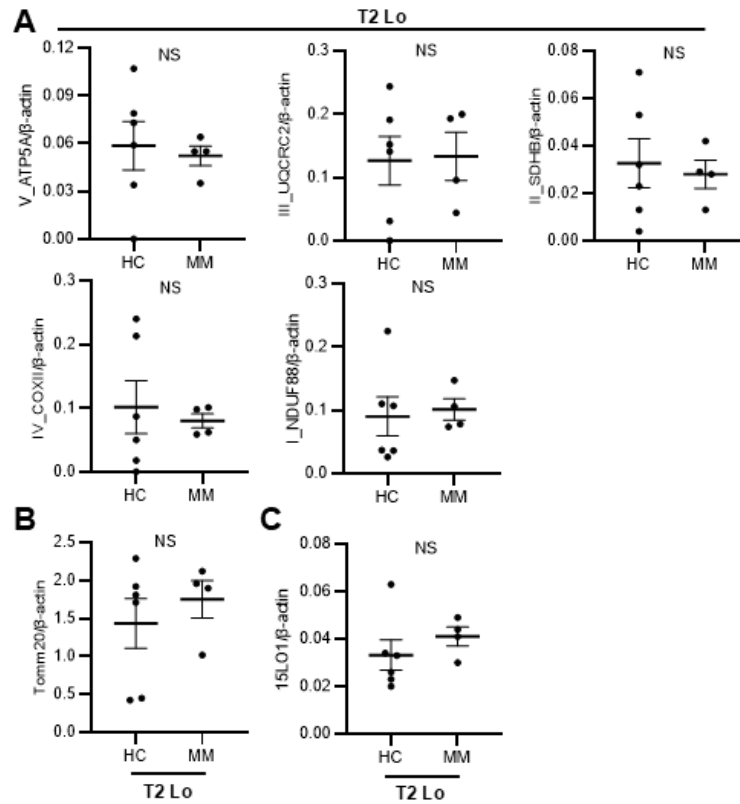
Sally E. Wenzel, MD
Department of Environmental and Occupational Health
School of Public Health
University of Pittsburgh
130 De Soto St, Pittsburgh, PA 15261
Phone: +1 (412) 624-9345
E-mail: swenzel@pitt.edu

Xiuxia Zhou, PhD
Department of Environmental and Occupational Health
School of Public Health
University of Pittsburgh
130 De Soto St, Pittsburgh, PA 15261
Phone: +1 (412) 624-3533
E-mail: xiz48@pitt.edu

Supplemental Figure 1

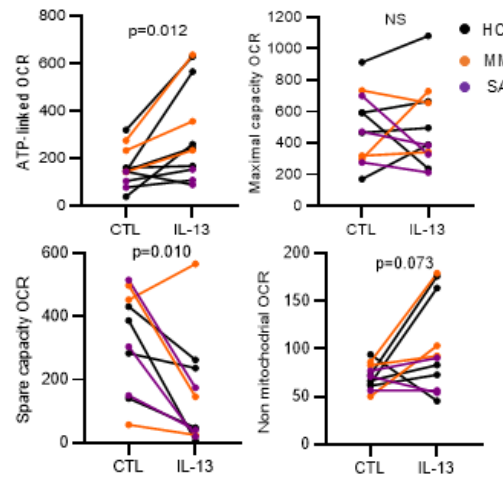


Supplemental Figure 1. Seahorse OCR parameters in freshly brushed human airway epithelial cells (AECs). (A) Seahorse parameters showed no significant differences between T2 Lo healthy controls (HC, n=7) and T2 Lo mild/moderate (MM, n=4). (B) Coupling efficiency (%) tended to be lower in T2 Hi group as compared with T2 Lo group even excluding the 3 subjects with coupling efficiency above 100%. (C) Seahorse parameters were elevated or showed a trend toward significantly higher levels in T2 Hi asthmatics compared with HC subjects. All comparisons used Student's t-tests. Individual data points are displayed as scatter dot plot. Normally distributed data are presented as mean $\pm$ SEM. NS, not significant.



Supplemental Figure 2. Comparison of electron transport chain (ETC) complexes and mitochondrial markers in freshly brushed human AECs. (A) Densitometric analysis of ETC complex proteins, normalized to β -actin, revealed no significant differences between healthy controls (HC) and mild/moderate (MM) asthmatics within the T2 Lo group. (B and C) Densitometric quantification of Tomm20 (B) and 15LO1 (C), normalized to β -actin, likewise showed no significant differences between HC and MM in the T2 Lo group. All comparisons used Student's t-tests. Individual data points are shown in scatter dot plot. Normally distributed data are presented as mean $\pm$ SEM, whereas non-normally distributed data are presented as median with Interquartile range (IQR). NS, not significant.

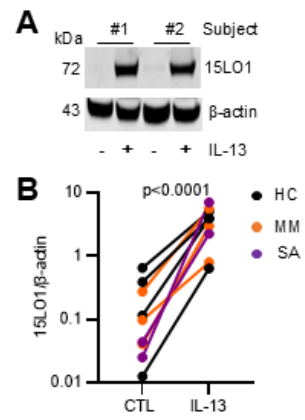
Supplemental Figure 3



Supplemental Figure 3. IL-13 stimulation alters mitochondrial respiration in ALI-cultured AECs.

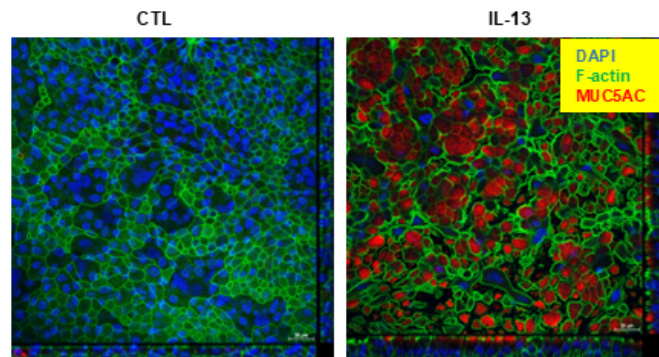
Quantification of OCR parameters reveals that IL-13 significantly increased ATP-linked OCR. Maximal capacity was not affected by IL-13; however, spare capacity OCR was reduced. Non mitochondrial OCR showed a trend toward an increase following IL-13 stimulation. Paired analyses were performed to assess the effects of IL-13 treatment. Data are presented as paired comparisons. NS, not significant.

Supplemental Figure 4



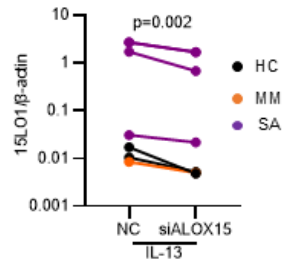
Supplemental Figure 4. IL-13 stimulation significantly increased 15LO1 protein in IL-13-treated vs CTL cells. (A) Representative Western blot of 15LO1 in the absence and presence of IL-13 treatment. (B) The densitometry of 15LO1 was presented as the ratio of 15LO1/β-actin. Paired analysis was performed between CTL and IL-13 (n=9) .

Supplemental Figure 5



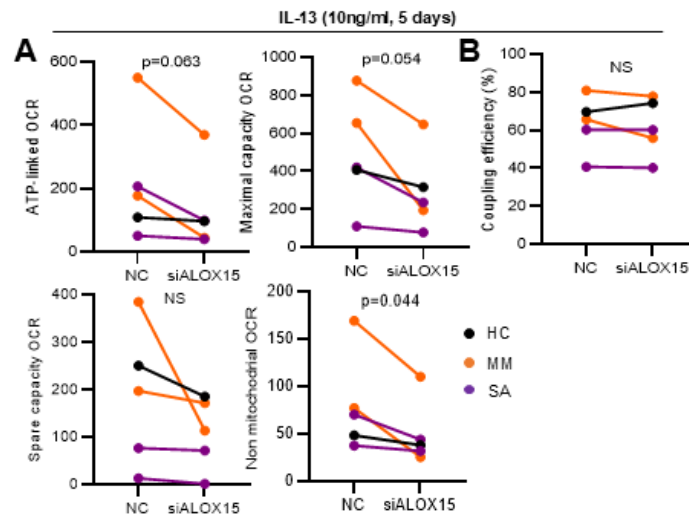
Supplemental Figure 5. IL-13 stimulation significantly increased MUC5AC expression, as demonstrated by immunofluorescence staining. Representative images show MUC5AC (red), F-actin (green), and nuclei stained with DAPI (blue). Enhanced red fluorescence intensity was observed in the IL-13 treated group compared with the CTL. Images were captured at 40X magnification.

Supplemental Figure 6



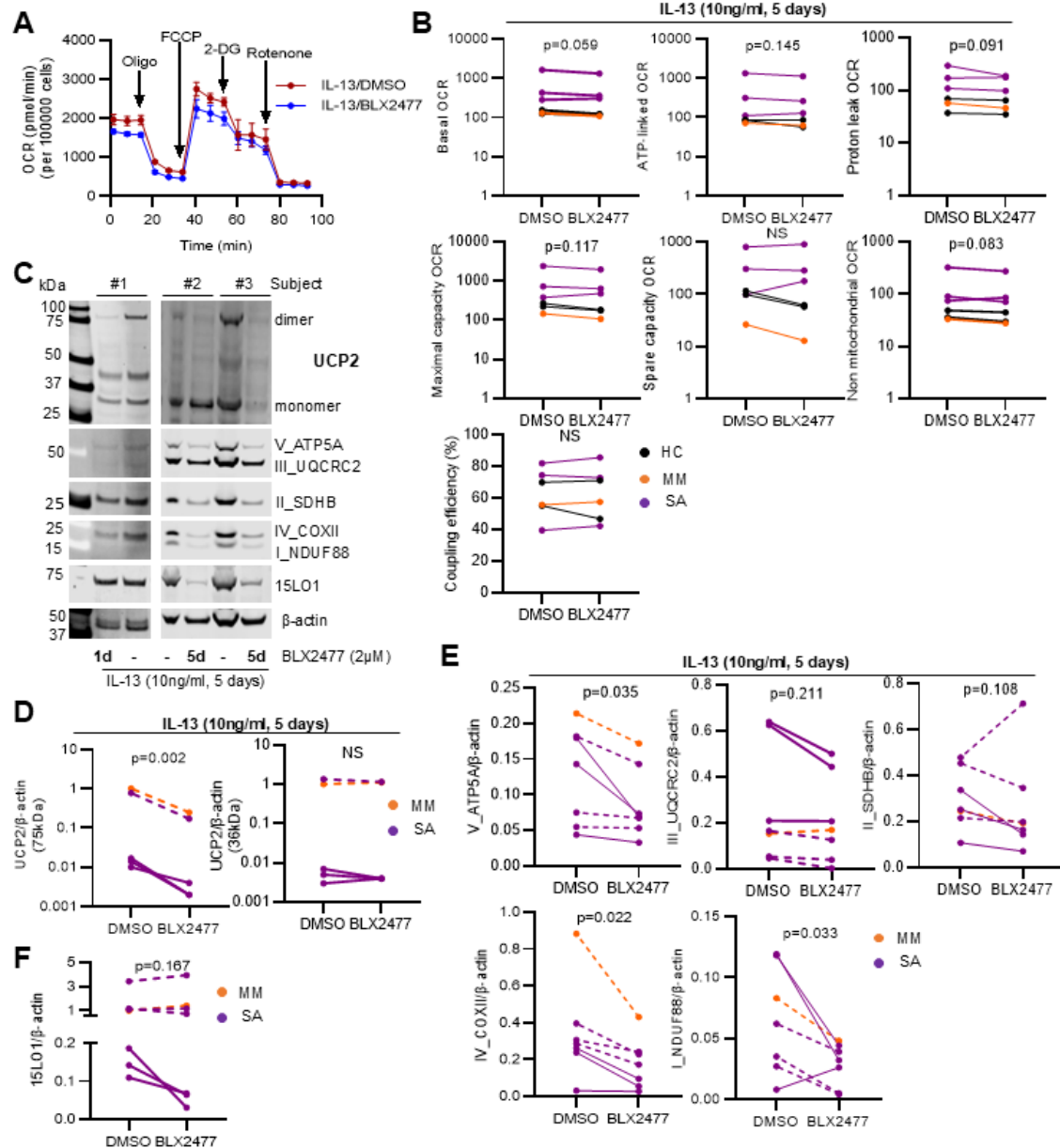
Supplemental Figure 6. Knockdown (KD) of ALOX15 on 15LO1 protein level under IL-13 stimulation in ALI cultured AECs. Densitometric analysis of 15LO1 (indexed to β -actin) showed that 15LO1 protein was silenced using siALOX15 in the presence of IL-13 (see Figure 7C). The data were normally distributed after log transformation. A paired t test was used to compare siALOX15 with negative control (NC) on log transformed data. Y-axis showed the ratio of 15LO1 densitometry to β -actin. The raw data were presented in the figures.

Supplemental Figure 7



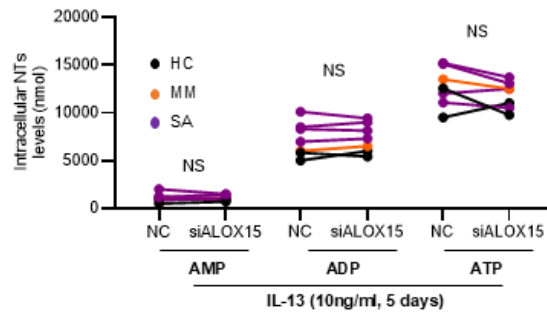
Supplemental Figure 7. Knockdown (KD) of ALOX15 on mitochondrial respiration under IL-13 stimulation in ALI cultured AECs. 15LO1 was silenced using siALOX15 in the presence of IL-13. A paired t test was used to compare siALOX15 with negative control (NC). **(A)** ALOX15 KD tended to decrease ATP-linked OCR, maximal capacity OCR but not Spare capacity OCR, whereas decreased non mitochondrial OCR. **(B)** ALOX15 KD had no effect on coupling efficiency. Paired t-tests on normally distributed data or log transformed data assessed the effects of siALOX15. The raw data were presented in the figures. The p values were labeled in each graph. NS, not significant.

Supplemental Figure 8

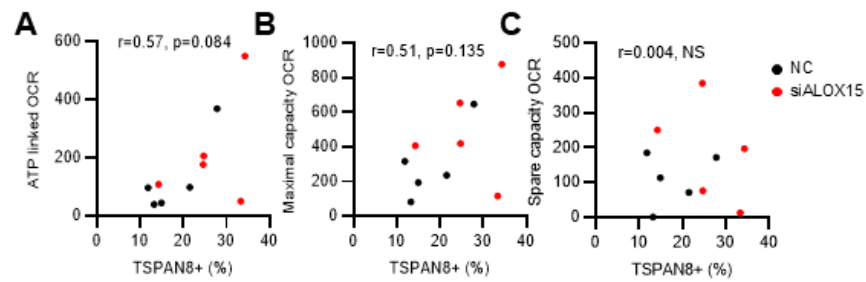


Supplemental Figure 8. 15LO1 inhibition (BLX2477, 2μM) reduces mitochondrial respiration, UCP2, and ETC Complex protein expression in IL-13 treated ALI cultured AECs. (A) Representative Seahorse traces from IL13-treated cells with or without BLX2477. (B) BLX2477 treatment (1 day) decreased or tended to decrease the level of OCR parameters under IL-13 conditions. (C) Representative Western blots showing UCP2, ETC Complexes and 15LO1 after 1 or 5 days of BLX2477 treatment. (D) Densitometric analysis of UCP2. (E) Densitometric analysis of ETC Complexes, showing decreased or trending lower expression with BLX2477 treatment. (F) Densitometric analysis of 15LO1. Statistical significance was determined using paired t-tests on normally distributed or log-transformed data. Raw data are shown and p values are indicated in each graph. NS, not significant. ---- BLX2477 1 day; — BLX2477 5 days

Supplemental Figure 9

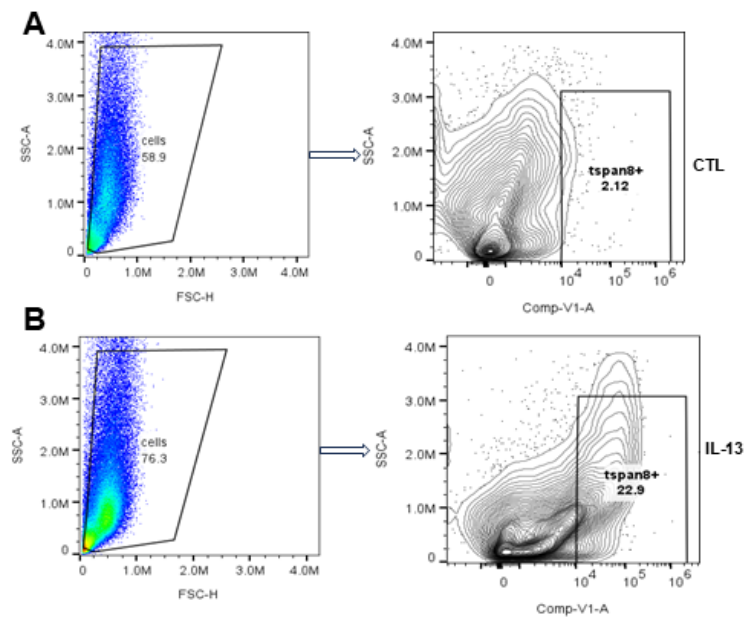


Supplemental Figure 9. Knockdown (KD) of ALOX15 by siALOX15 does not affect AMP, ADP and ATP levels under IL-13 stimulation in ALI cultured AECs (n=7). Paired t-tests were applied to compare the AMP, ADP and ATP levels between siALOX15 and negative control (NC). NS, not significant.



Supplemental Figure 10. Correlations between the percentage of TSPAN8+ cells and Seahorse OCR parameters following ALOX15 KD under IL-13 stimulation. (A and B) The percentage of TSPAN8+ cells showed a positive trend with ATP-linked OCR (A) and maximal capacity OCR (B). **(C)** No correlation was observed between the percentage of TSPAN8+ cells and spare capacity OCR. Pearson correlation analyses were performed using normally distributed or log transformed data, as appropriate. Individual data points are shown in each graph, and the corresponding p values are indicated. NS, not significant.

Supplemental Figure 11



Supplemental Figure 11. Flow cytometric analysis gating strategy. Cells were first gated on the main population using a polygon gate based on forward/side scatter characteristics (left panels), excluding debris and outliers. The gated population was then further refined using a secondary polygon gate in a downstream parameter plot to isolate the target TSPAN8+ subset and remove residual background events (right panels). (A) CTL; (B) IL-13.

Supplemental Table 1. Demographic characteristics of participants included in *In vitro* experiments

	T2 Low HC	T2 Low MM	T2 High MM & SA	T2 High Biologic, SA
HC/MM/SA	12/0/0	0/6/0	0/6/8	0/0/8
Age (yrs)	41.4 ± 4.4	43.0 ± 6.3	46.2 ± 4.1	56.1 ± 5.4
Sex (M/F)	3/9	1/5	4/10	3/5
Race (W/AA/O)	9/1/2	3/3/0	13/1/0	7/1/0
BMI	30.1 ± 1.9	31.3 ± 3.0	30.1 ± 1.8	27.3 ± 2.4
FEV1% Pred.	98.3 ± 6.2	77.5 ± 8.7	78.7 ± 5.7	71.7 ± 7.6
FeNO (ppb)	17.0 (13.5-19.3)	14.5 (10.0-20.0)	37.5 (28.5-54.5)	20.5 (10.0-33.8)
Blood eosinophils (/μl)	100 (55-200)	86 (39-147)	383 (145-493)	83 (14-470)
OCS (Y/N)	NA	1/5	5/9	6/2
ICS Y/N)	NA	3/3	8/6	5/3

- Sex (M/F): Male/Female
- Race (W/AA/O): White/African American/Others
- Age, BMI and FEV1% Pred. are normally distributed and presented as Mean ± SEM; whereas FeNO and blood eosinophils are not normally distributed and presented as Median with interquartile range (25-75 IQR)
- T2 High Biologic includes T2 Hi with biologic treatments (anti IL-4R, anti IL-5). After biologic treatment, both FeNO and blood eosinophils reduced.

Supplemental Table 2. Resources and functions of OCR and ECAR inhibitors

Inhibitors	Abbre	Resources	Concentration	Functions
Oligomycin	Oligo	Cat # 75351 (Sigma)	2 μ M	quantify respiration coupled to ATP production and proton leak
carbonyl cyanide-4 -(trifluoromethoxy) -phenylhydrazone	FCCP	Cat # 0453 (Tocris)	1.5 μ M	assess maximal cellular respiratory
2-deoxy-D-glucose	2-DG	Cat# D8375 (Sigma)	100 mM	measure the ECAR capacity
Rotenone		Cat# 13995 (Cayman)	15 μ M	assess non mitochondria respiration

Supplemental Table 3. Calculations of Seahorse OCR parameters

Parameters	Calculation formula
Basal OCR	Last reading of baseline OCR– lowest reading after Rotenone injection
ATP-linked OCR	Last reading of baseline OCR – lowest reading after Oligo injection
Proton leak OCR	Lowest reading after Oligo injection – lowest reading after Rotenone injection
Maximal capacity OCR	The maximum reading after FCCP injection – lowest reading after Rotenone injection
Spare capacity OCR	The maximum reading after FCCP injection - last reading of baseline
Non mitochondria OCR	Lowest reading after Rotenone injection
Coupling efficiency (%)	$\frac{\text{ATP-linked OCR} \times 100\%}{\text{Basal OCR}}$

Supplemental Table 4. Function of ETC complexes and associated with total OXPHOS antibody cocktail

Subunit in OXPHOS antibody	ETC Complex	ETC names	Key Function
NDUF88	Complex I	NADH Dehydrogenase	Transfers electrons from NADH to CoQ and pumps protons into the intermembrane space
SDHB	Complex II	Succinate Dehydrogenase	Transfers electrons from FADH ₂ to CoQ; does not pump protons
UQCRC2	Complex III	Cytochrome bc ₁ Complex / Cytochrome c reductase	Transfers electrons from reduced CoQ to cytochrome c; pumps protons
COXII	Complex IV	Cytochrome c Oxidase	Transfers electrons from cytochrome c to oxygen, reducing it to water; pumps protons
ATP5A	Complex V	ATP Synthase /F0F1-ATP synthase	Utilizes the proton gradient generated by complex I, III and IV to synthesize ATP from ADP

Supplemental Table 5. Ex vivo correlations among OCR parameters, ETC Complexes, Tomm20 and 15LO1 in T2 Lo and T2 Hi participants (without BIO treatment)

Variables	Basal OCR	ATP-linked OCR	Proton leak	Max OCR	Spare capacity OCR	I	II	III	IV	V	Tomm20	15LO1
Basal OCR	1.00	0.94	0.59	0.62	0.49	0.14	0.55	0.42	0.01	0.55	0.14	0.72
ATP-linked OCR	0.94	1.00	0.39	0.62	0.53	0.08	0.60	0.44	0.06	0.60	0.23	0.60
Proton leak	0.59	0.39	1.00	0.22	0.06	0.40	0.29	0.25	0.01	0.27	-0.11	0.62
Max OCR	0.62	0.62	0.22	1.00	0.88	0.44	0.59	0.55	0.36	0.61	0.65	0.44
Spare capacity OCR	0.49	0.53	0.06	0.88	1.00	0.13	0.63	0.56	0.20	0.73	0.46	0.28
I	0.14	0.08	0.40	0.44	0.13	1.00	0.50	0.57	0.48	0.35	0.51	0.27
II	0.55	0.60	0.29	0.59	0.63	0.50	1.00	0.89	0.35	0.94	0.34	0.27
III	0.42	0.44	0.25	0.55	0.56	0.57	0.89	1.00	0.21	0.88	0.33	0.24
IV	0.01	0.06	0.01	0.36	0.20	0.48	0.35	0.21	1.00	0.15	0.41	0.10
V	0.55	0.60	0.27	0.61	0.73	0.35	0.94	0.88	0.15	1.00	0.30	0.22
Tomm20	0.14	0.23	-0.11	0.65	0.46	0.51	0.34	0.33	0.41	0.30	1.00	0.22
15LO1	0.72	0.60	0.62	0.44	0.28	0.37	0.27	0.24	0.10	0.22	0.22	1.00

- The correlation coefficients between each variable are presented in the table. p values less than 0.05 are shown in red font, whereas p values greater than 0.05 but less than 0.10 are shown in green font.
- The data were normalized after log transformed, and Pearson correlations were performed.

Supplemental Table 6. *Ex vivo* correlations between 15LO1 expression and mitochondrial/metabolic parameters in T2-Lo and T2-Hi groups

15LO1/β-actin	n	r values	p values
Basal OCR	16	0.72	0.002
ATP-linked OCR	16	0.60	0.014
Proton leak OCR	16	0.62	0.010
Maximal capacity OCR	16	0.44	0.091
Spare capacity OCR	14	0.28	0.333

Supplemental Table 7. *Ex vivo* correlations among clinical parameters and Seahorse parameters in T2 Lo and T2 Hi participants (without biologic treatment)

	FEV1 (% Pred.)		No&short cilia (%)		Coupling efficiency (%)	
	r value	p value	r value	p value	r value	p value
FEV1(% Pred)	1.00				0.53	0.018
No & short cilia (%)	-0.56	0.013	1.00		-0.53	0.018
Basal OCR	-0.34	0.154	0.27	0.256	-0.23	0.352
ATP-linked OCR	-0.17	0.498	0.09	0.713	0.11	0.652
Coupling efficiency (%)	0.53	0.018	-0.53	0.018	1.00	
Proton leak OCR	-0.49	0.046	0.44	0.077	-0.64	0.006
Maximal OCR	-0.30	0.218	0.32	0.179	0.04	0.988
Spare capacity OCR	-0.26	0.312	-0.02	0.950	0.14	0.590

- The correlation coefficients between clinical parameters and Seahorse parameters are presented in the table.
- p values less than 0.05 are shown in red font, whereas p values greater than 0.05 but less than 0.10 are shown in green font.
- The Pearson correlations are performed using normally distributed or log transformed data.

Supplemental Table 8. In vitro correlations among OCR parameters, ETC Complexes, Tomm20 and 15LO1 in CTL and IL-13 conditions

Variables	Basal OCR	ATP-linked OCR	Coupling efficiency %	Proton leak OCR	Max OCR	Spare capacity OCR	I	II	III	IV	V	Tomm 20	15LO1
Basal OCR	1.00	0.94	-0.23	0.82	0.60	0.14	-0.29	-0.46	-0.60	0.11	-0.47	0.28	0.66
ATP-linked OCR	0.94	1.00	0.13	0.60	0.68	0.28	-0.14	-0.33	-0.55	0.31	-0.34	0.44	0.80
Coupling efficiency %	-0.23	0.13	1.00	-0.61	0.12	0.34	0.52	0.55	0.42	0.45	0.54	0.70	0.10
Proton leak OCR	0.82	0.60	-0.61	1.00	0.37	-0.17	-0.40	-0.60	-0.56	-0.08	-0.57	-0.02	0.22
Max OCR	0.60	0.68	0.12	0.37	1.00	0.60	-0.07	-0.12	-0.41	0.24	-0.29	0.11	-0.43
Spare capacity OCR	0.14	0.28	0.34	-0.17	0.60	1.00	0.07	0.35	-0.06	0.08	0.11	-0.18	-0.68
I	-0.29	-0.14	0.52	-0.40	-0.07	0.07	1.00	0.51	0.78	0.78	0.83	0.69	0.25
II	-0.46	-0.33	0.55	-0.60	-0.12	0.35	0.51	1.00	0.47	0.41	0.82	0.44	0.39
III	-0.60	-0.55	0.42	-0.56	-0.41	-0.06	0.78	0.47	1.00	0.30	0.77	0.80	0.57
IV	0.11	0.31	0.45	-0.08	0.24	0.08	0.78	0.41	0.30	1.00	0.63	0.56	-0.07
V	-0.47	-0.34	0.54	-0.57	-0.29	0.11	0.83	0.82	0.77	0.63	1.00	0.67	0.39
Tomm20	0.28	0.44	0.70	-0.02	0.11	-0.18	0.69	0.44	0.80	0.56	0.67	1.00	0.73
15LO1	0.66	0.80	0.10	0.22	-0.43	-0.68	0.25	0.39	0.57	-0.07	0.39	0.73	1.00

- ETC complexes I-V, Tomm20 and 15LO1: Western blot densitometry data indexed to β -actin.
- Pearson correlation analysis was performed using combined CTL and IL-13 data after log transformation.
- The correlation coefficients between each variable are shown in the table. Red font indicates p values < 0.05; and green font indicates p values > 0.05 but < 0.10.

Supplemental Table 9. In vitro correlations among TSPAN8+ (%) and OCR parameters, ETC complexes, Tomm20, 15LO1 under CTL and IL-13 conditions

TSPAN8 + (%)	n	r value	p value
Basal OCR	16	0.65	0.009
ATP linked OCR	16	0.64	0.007
Proton leak OCR	16	0.60	0.015
Maximal OCR	16	0.28	0.288
Spare capacity OCR	6	-0.84	0.037
I_NDUF88	4	-0.04	0.960
II_SDHB	4	-0.90	0.096
III_UQCRC2	4	0.16	0.836
IV_COXII	4	-0.18	0.823
V_ATP5A	4	-0.77	0.229
Tomm20	6	0.15	0.780
15LO1	2	NA	NA

- TSPAN8+ (%): the percentage of TSPAN8+ cells by Flow cytometric analysis.
- Pearson correlation analyses were performed using log transformed non-normally distributed data or originally normally distributed data.
- Correlation analysis was performed using combined CTL and IL-13 data.
- Red font indicates p values < 0.05; and green font indicates p values > 0.05 but < 0.10.

Supplemental Table 10. *In vitro* correlations among OCR parameters, ETC complexes, Tomm20 and 15LO1 in NC and ALOX15 KD under IL-13 treatment

Variables	Basal OCR	ATP linked OCR	Proton leak OCR	Max OCR	Spare capacity OCR	I	II	III	IV	V	Tomm20	15LO1
Basal OCR	1.00	0.98	0.88	0.91	0.39	0.03	-0.06	-0.18	-0.07	-0.31	0.41	-0.35
ATP linked OCR	0.98	1.00	0.78	0.94	0.50	-0.11	-0.21	-0.32	-0.22	-0.45	0.27	-0.42
Proton leak OCR	0.88	0.78	1.00	0.67	0.09	0.41	0.34	0.24	0.34	0.12	0.75	-0.06
Max OCR	0.91	0.94	0.67	1.00	0.70	-0.35	-0.44	-0.54	-0.44	-0.65	-0.03	-0.65
Spare capacity OCR	0.39	0.50	0.09	0.70	1.00	-0.94	-0.93	-0.89	-0.92	-0.86	-0.87	-0.73
I	0.03	-0.11	0.41	-0.35	-0.94	1.00	0.83	0.78	0.96	0.77	0.74	-0.26
II	-0.06	-0.21	0.34	-0.44	-0.93	0.83	1.00	0.96	0.68	0.95	0.72	0.16
III	-0.18	-0.32	0.24	-0.54	-0.89	0.78	0.96	1.00	0.62	0.99	0.80	0.23
IV	-0.07	-0.22	0.34	-0.44	-0.92	0.96	0.68	0.62	1.00	0.63	0.63	-0.37
V	-0.31	-0.45	0.12	-0.65	-0.86	0.77	0.95	0.99	0.63	1.00	0.75	0.33
Tomm20	0.41	0.27	0.75	-0.03	-0.87	0.74	0.72	0.80	0.63	0.75	1.00	0.19
15LO1	-0.35	-0.42	-0.06	-0.65	-0.73	-0.26	0.16	0.23	-0.37	0.33	0.19	1.00

- ETC complexes I-V, Tomm20 and 15LO1: Western blot densitometry data indexed to β -actin.
- Pearson correlation analysis was performed using combined NC and ALOX15 KD under IL-13 treatment after log transformation.
- The correlation coefficients between each variable are shown in the table. Red font indicates p values < 0.05; and green font indicates p values > 0.05 but < 0.10.

Supplemental Table 11. *In vitro* correlations among TSPAN8+ and OCR parameters, ETC complexes, Tomm20 and 15LO1 in NC and siALOX15 KD conditions under IL-13 treatment

TSPAN8+ (%)	n	r value	p value
Basal OCR	10	0.69	0.029
ATP linked OCR	10	0.57	0.084
Proton leak OCR	10	0.77	0.010
Maximal OCR	10	0.51	0.135
Spare capacity OCR	10	0.00	0.992
I_NDUF88	6	0.24	0.654
II_SDHB	6	0.13	0.804
III_UQCRC2	6	0.01	0.986
IV_COXII	6	0.12	0.824
V_ATP5A	6	-0.14	0.791
Tomm20	4	0.40	0.597
15LO1	4	-0.36	0.643

- TSPAN8+ (%): the percentage of TSPAN8+ cells by Flow cytometric analysis.
 - Pearson correlation analyses were performed using log transformed non-normally distributed data or originally normally distributed data.
 - Correlation analysis was performed using combined NC and siALOX15.
 - Red font indicates p values < 0.05; and green font indicates p values > 0.05 but < 0.10.
-

Supplemental Table 12. Flow antibody details

Antigen	Gene	Fluorophore	Isotype	Source	Cat#	test (μ l)	Target cell
CD271	NGFR	PeCy7	mlgG1	BioLegend	345110	1	Basal
Cd66c	CEACAM6	BUV395	mlgG1	BD	742689	1	Secretory
TSPAN8	TSPAN8	AF405	ratlgG2b	R&D systems	FAB4734V	2	Goblet
TUBA1A	TUBA1A	AF647	mlgG2b	Santa Cruz	sc-23950	1	Ciliated

Definition:

NGFR: Nerve growth factor receptor

CEACAM6: Carcinoembryonic Antigen Related Cell Adhesion Molecule 6

TSPAN8: Tetraspanin 8

TUBA1A: Acetylated α -tubulin
