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on Lung Cancer**

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Elucidating the Expression & Therapeutic Targeting of the Cysteinyl Leukotriene Receptors in NSCLC

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Ireland

Science Without Boundaries: **Uniting the World Against Thoracic Cancer**

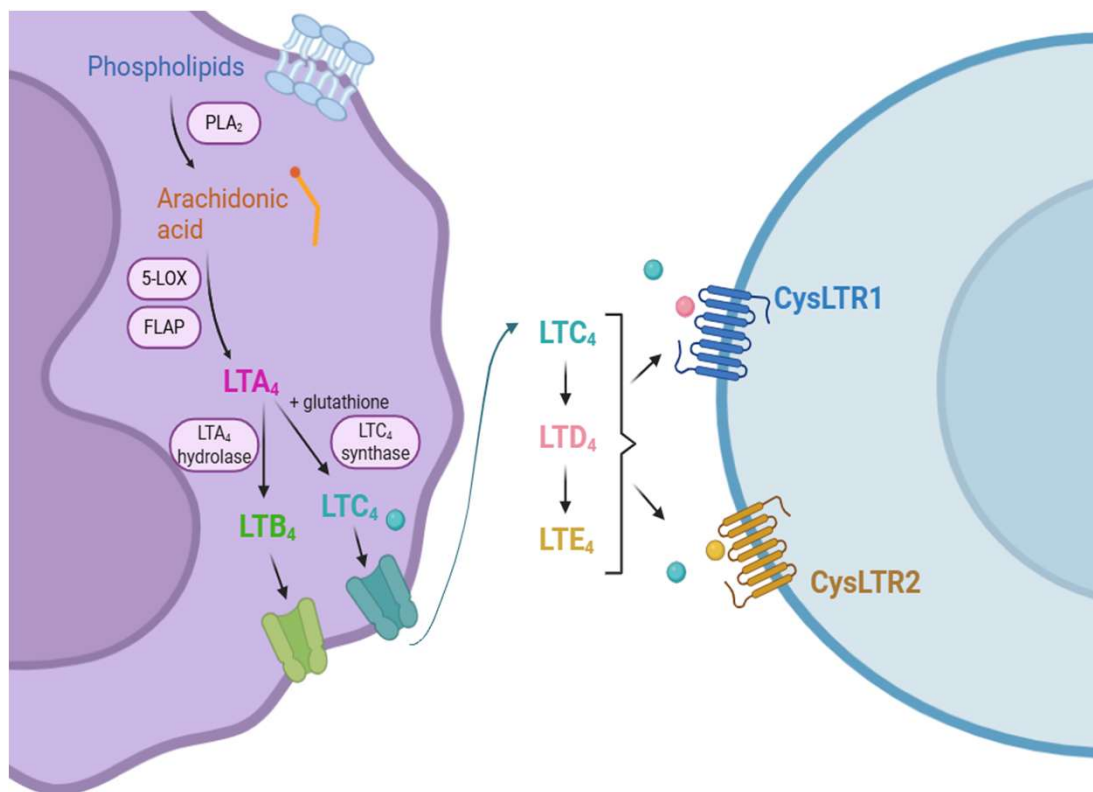
Disclosure

Artificial Intelligence (AI) Use Disclaimer

AI was not used in the development of material contained within this presentation.

Background

- Leukotrienes are lipid inflammatory mediators and are primarily synthesized by leukocytes including eosinophils, mast cells, neutrophils and macrophages (Cuzzo & Lappin, 2023; Tsai *et al.*, 2021).
- Cysteinyl leukotrienes exert their biological effects via binding to cysteinyl leukotriene receptor 1 (CysLTR1) and 2 (CysLTR2), and to a lesser extent CysLTR3 which has been less characterized.
- Different leukotrienes have varying affinities for cysteinyl leukotriene receptors, with CysLTR1 demonstrating higher affinity for LTD₄ while CysLTR2 displays similar affinity for both LTC₄ and LTD₄ while CysLTR3 preferentially binds to LTE₄.



Leukotriene synthesis pathway. Membrane phospholipids are metabolised by phospholipase A₂ (PLA₂) into arachidonic acid following stimulation by various factors. Arachidonic acid is subsequently metabolised by 5-lipoxygenase (5-LO) alongside 5-lipoxygenase-activating protein (FLAP) to generate the unstable epoxide leukotriene A (LTA₄). LTA₄ acts as a precursor for the generation of leukotriene B (LTB₄) via LTA₄ hydrolase and leukotriene C (LTC₄) mediated by LTA₄ synthase, which conjugates it with glutathione. LTC₄ is transported extracellularly and can be converted into leukotriene D (LTD₄) and leukotriene E (LTE₄). LTC₄, LTD₄ and LTE₄ are characterised as cysteinyl leukotrienes and exert their functions primarily by binding to cysteinyl receptors 1 and 2 (CysLTR1 and CysLTR2). Figure generated using <https://BioRender.com>

Cysteinyl Leukotriene signalling in cancer

- Leukotriene signalling has been associated with various cancers, including haematological malignancies and solid tumours such as colorectal, breast and lung.
- In breast and colorectal cancer, low CysLTR1 expression and high CysLTR2 expression have been associated with improved patient survival (Magnusson *et al.*, 2010; Liu *et al.*, 2011).
- In the clinical setting, the leukotriene pathway has been targeted in conditions such as asthma and allergic rhinitis via antagonism of CysLTR1 or inhibition of 5-lipoxygenase. These include the use of Zileuton, Montelukast, Zafirlukast and Pranlukast (Yokomizo *et al.*, 2018).
- Expression and targeting of the CysLTR's in lung cancer remains poorly defined.

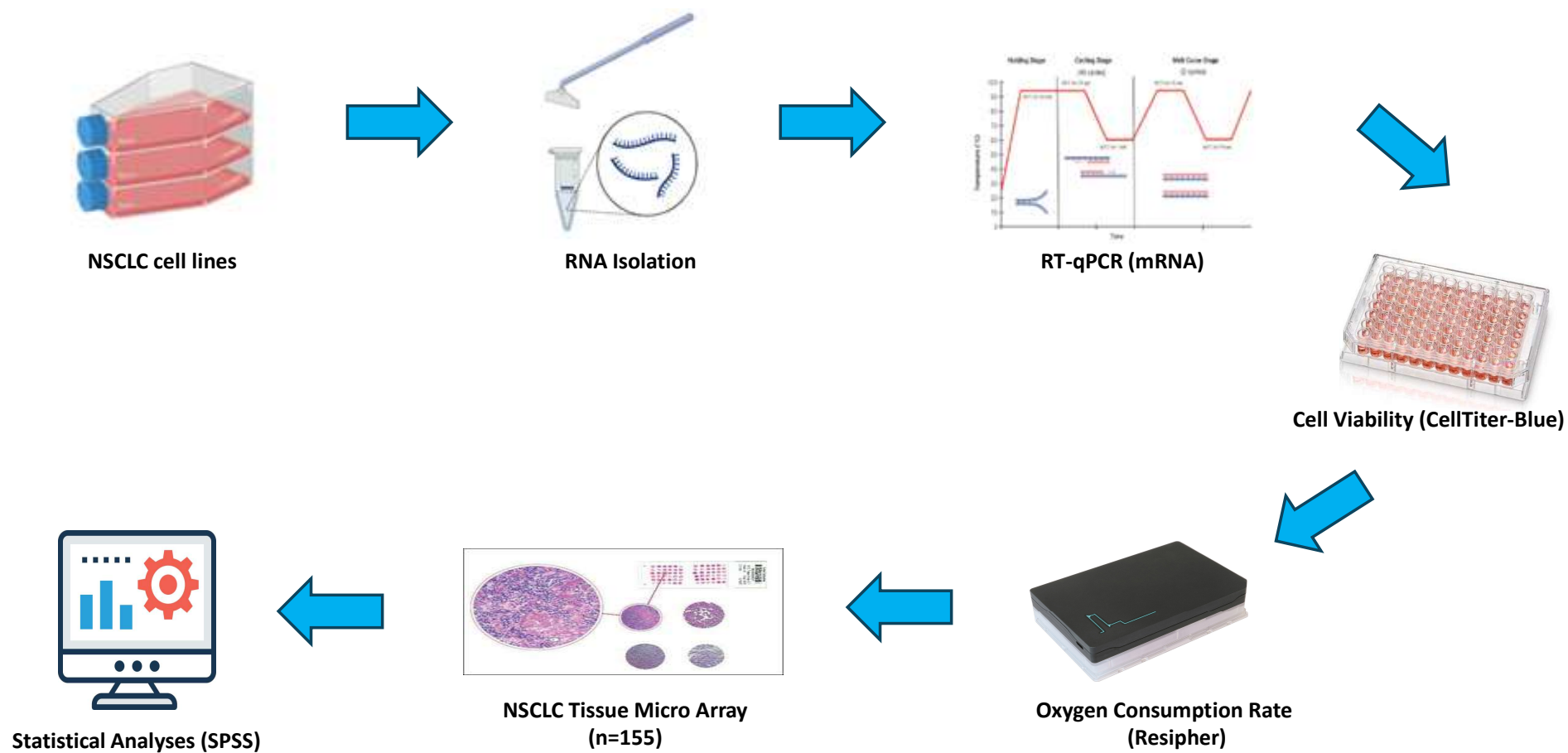
Hypothesis

Cysteinyl leukotriene receptors, CysLTR1 and CysLTR2, are expressed in NSCLC cell lines and tumour tissue, while the novel quininib analogue, 1,4-dihydroxy quininib (Q8) may be used as a therapeutic drug targeting these receptors.

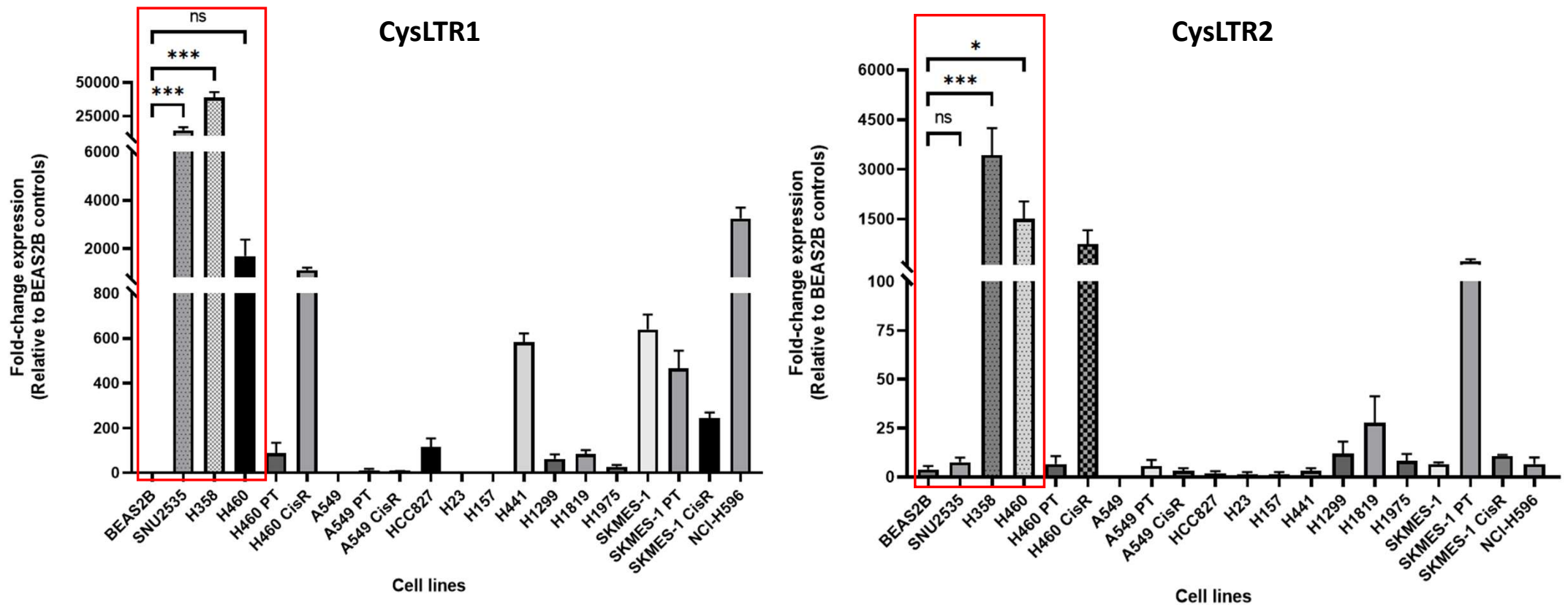
Study Aims

- To measure CysLTR gene expression, cell viability and oxygen consumption rate in a panel of wild-type and cisplatin resistant NSCLC cell lines (Barr *et al.*, 2013) at baseline and in response to treatment with 1,4-dihydroxy quininib (Q8).
- To examine CysLTR1 and CysLTR2 protein expression (IHC) and subcellular localisation in NSCLC cell lines and surgically resected treatment-naïve NSCLC tumours.
- To correlate cysteinyl leukotriene receptor expression with clinicopathological parameters and survival in NSCLC patients.

Methods

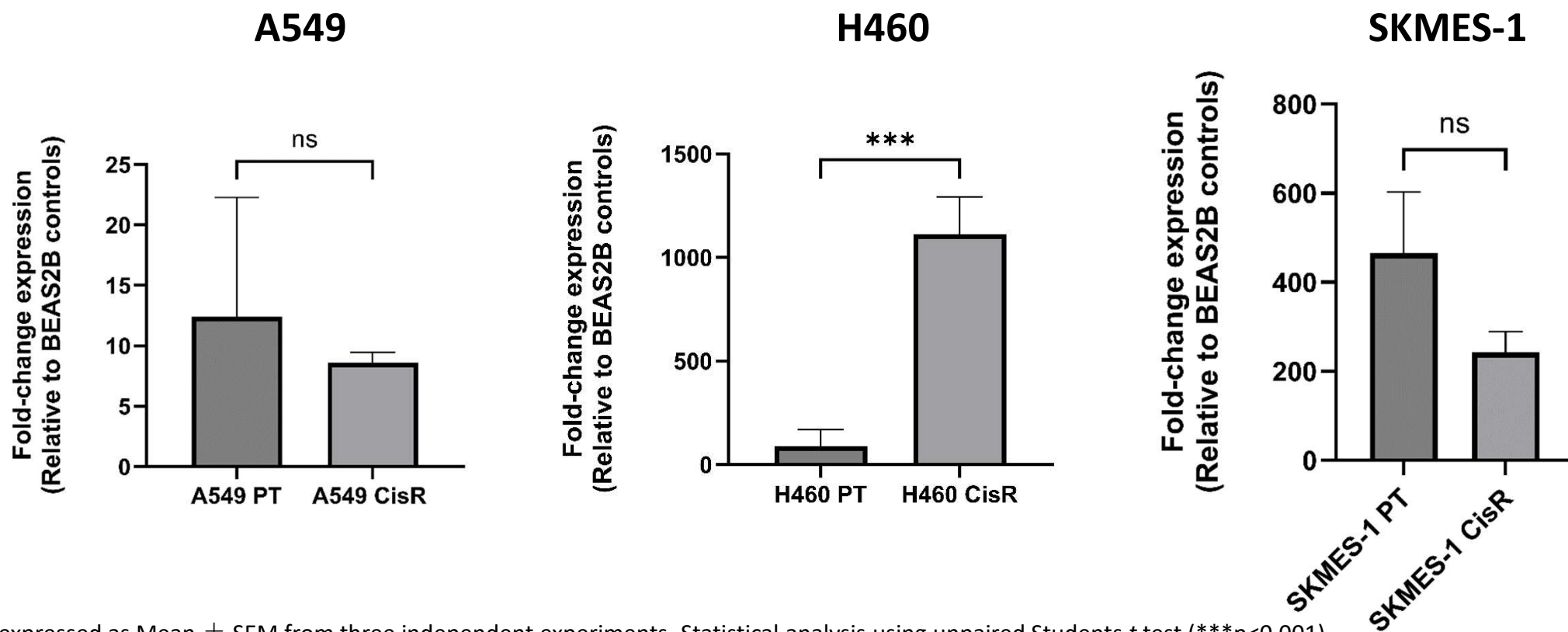


Differential expression of cysteinyl leukotriene receptors (mRNA) in a panel of NSCLC cell lines



Data expressed as Mean $\pm$ SEM from three independent experiments. Statistical analysis using one-way ANOVA with Bonferroni post-hoc test (* $p < 0.05$, *** $p < 0.001$)

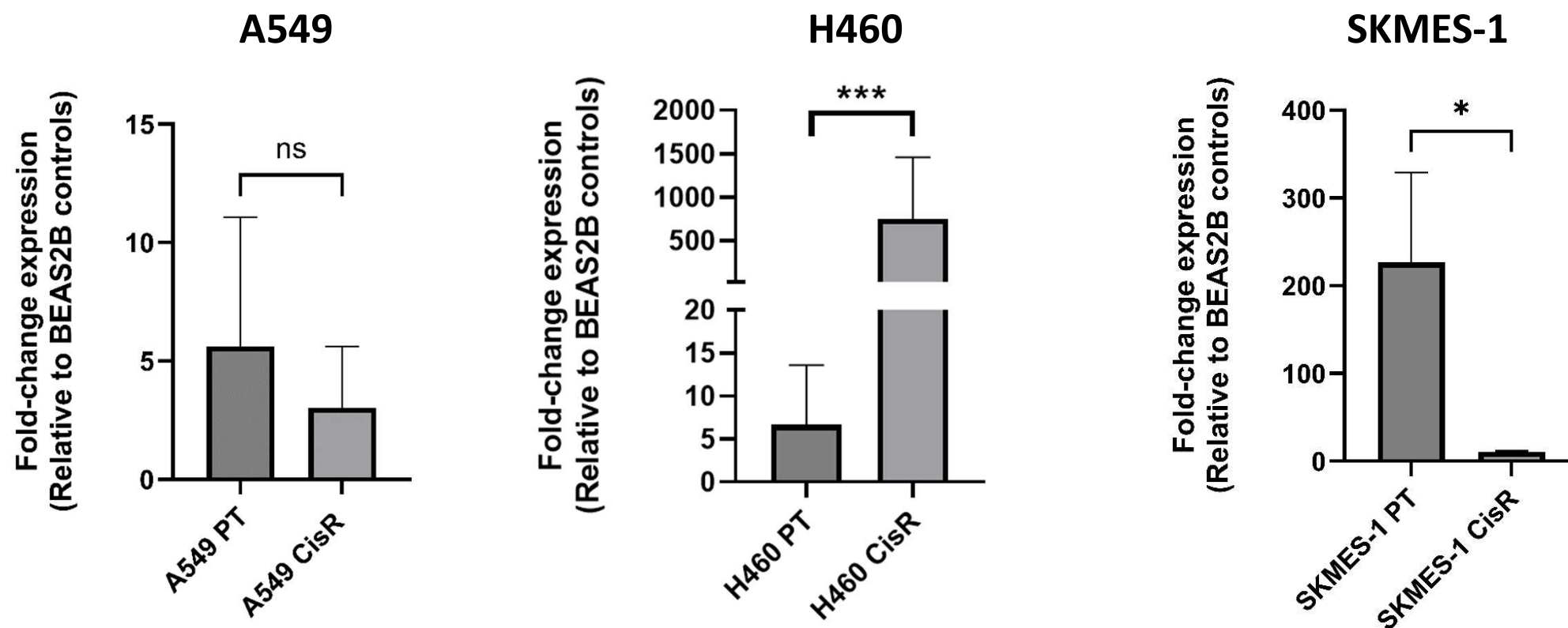
CysLTR1 gene expression in cisplatin sensitive & resistant NSCLC cells



Data expressed as Mean $\pm$ SEM from three independent experiments. Statistical analysis using unpaired Students *t* test (***p*<0.001)

PT=parental, CisR=cisplatin resistant

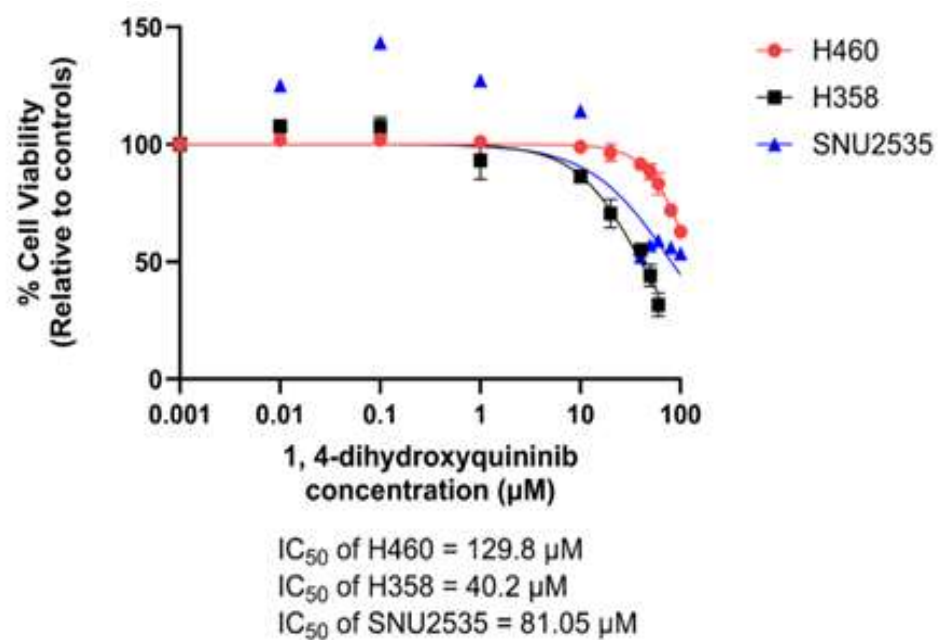
CysLTR₂ gene expression in cisplatin sensitive & resistant NSCLC cells



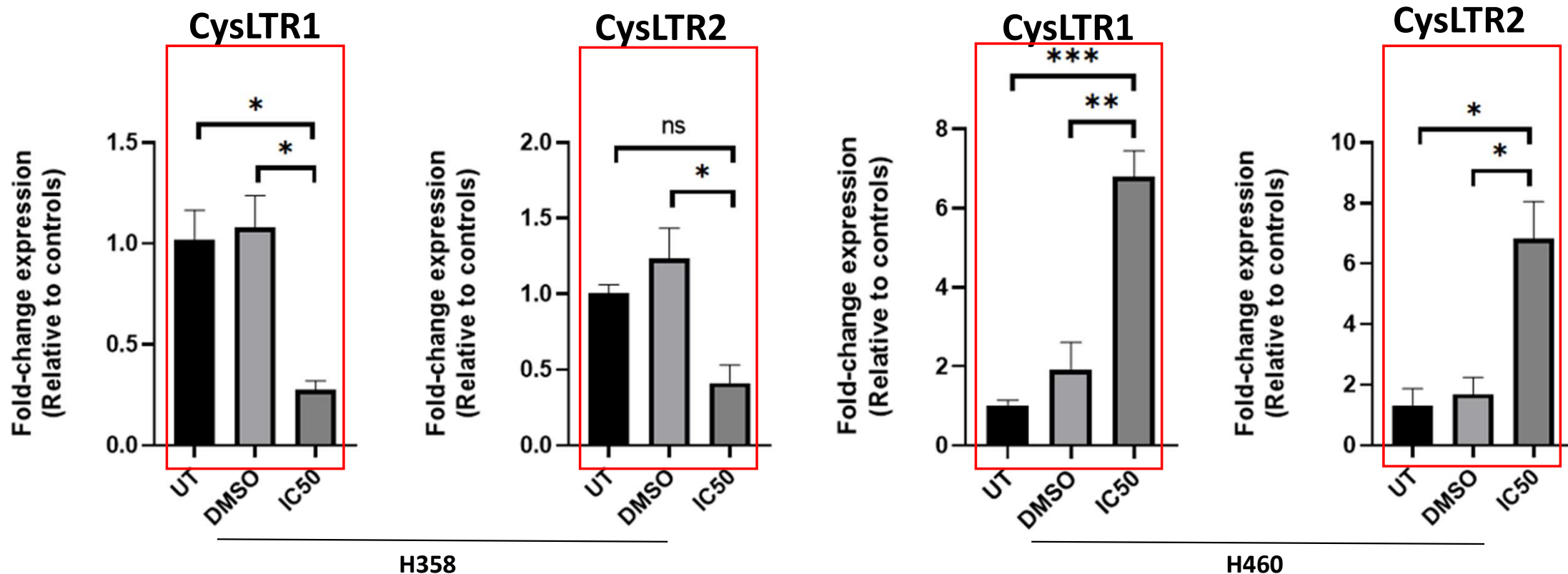
Data expressed as Mean $\pm$ SEM from three independent experiments. Statistical analysis using unpaired Students *t* test (**p*<0.05, ****p*<0.001)

PT=parental, CisR=cisplatin resistant

1,4-dihydroxy quininiib (Q8) significantly reduces cell viability of NSCLC cells at 24 h

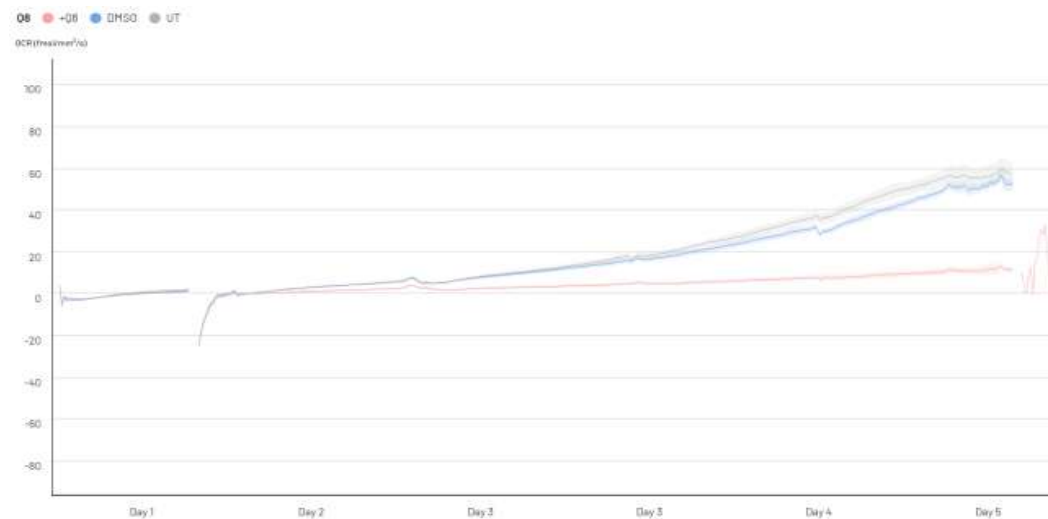
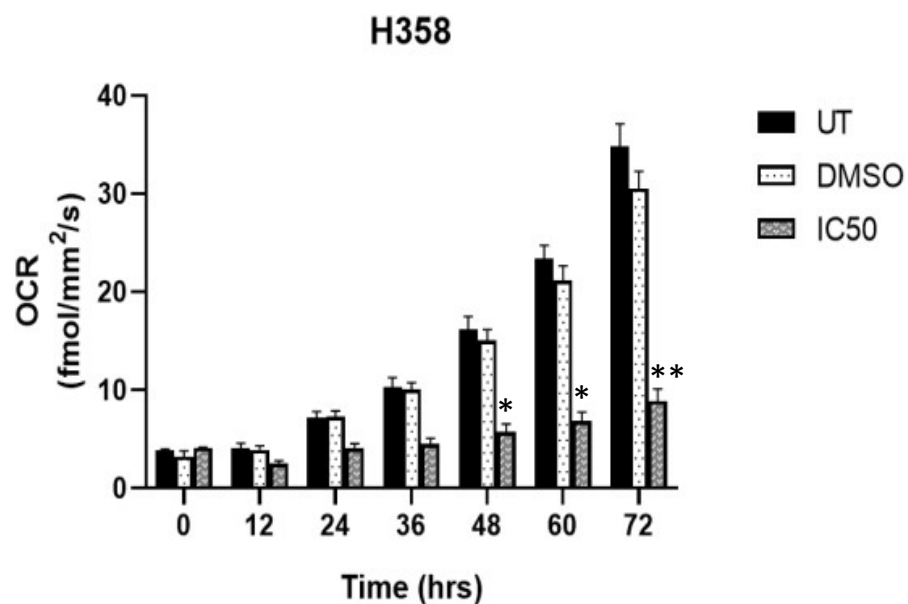


CysLTR1 and CysLTR2 gene expression is significantly altered in NSCLC cells in response to treatment with 1,4-dihydroxy quininib (IC₅₀)



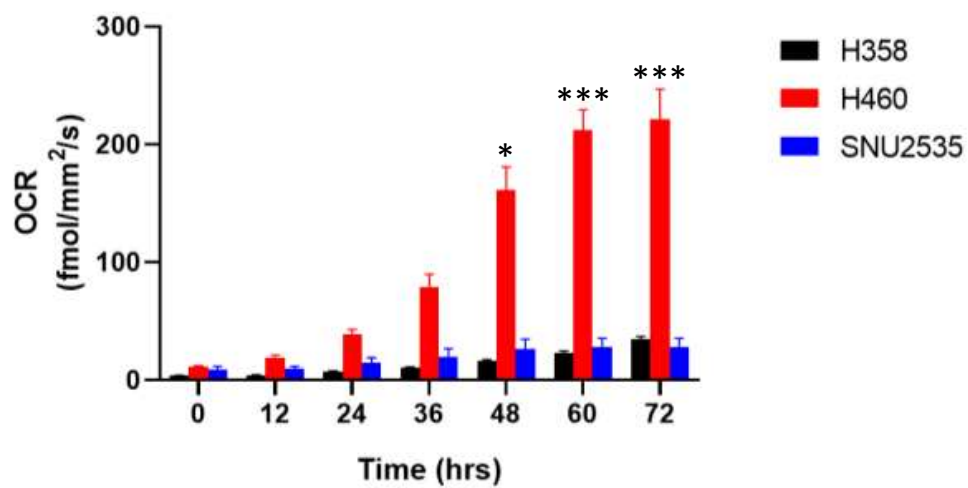
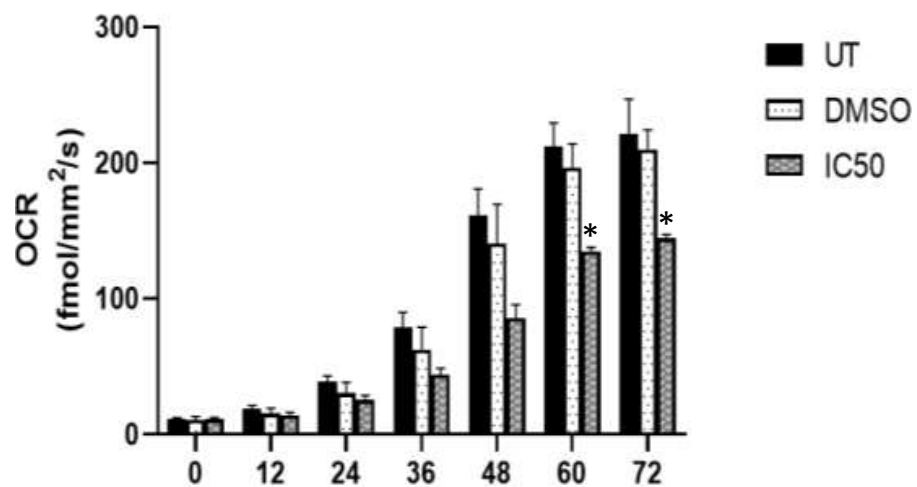
Data expressed as Mean $\pm$ SEM from three independent experiments. Statistical analysis using one-way ANOVA with Bonferroni post-hoc test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

1,4-dihydroxy quininiib (IC_{50}) decreases the oxygen consumption rate of NSCLC cells

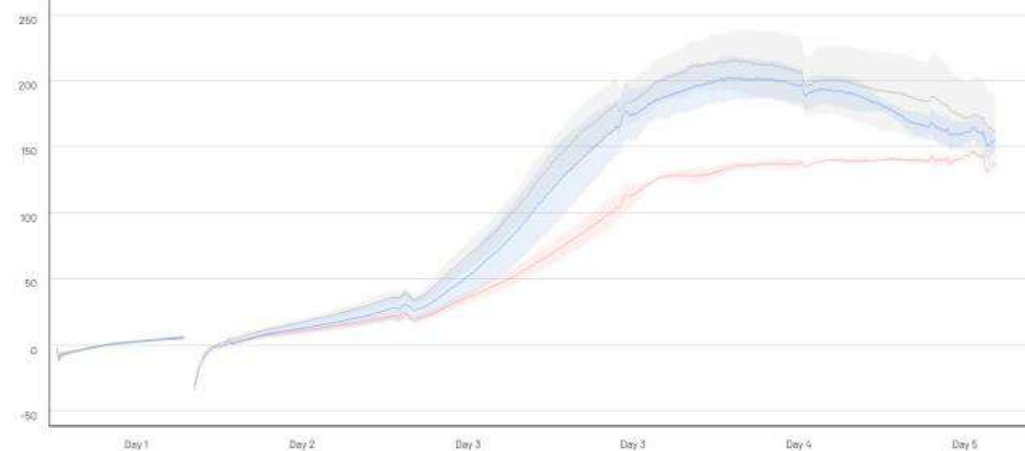


Data expressed as Mean $\pm$ SEM from three independent experiments. Statistical analysis using one-way ANOVA with Bonferroni post-hoc test (* $p < 0.05$, ** $p < 0.01$)

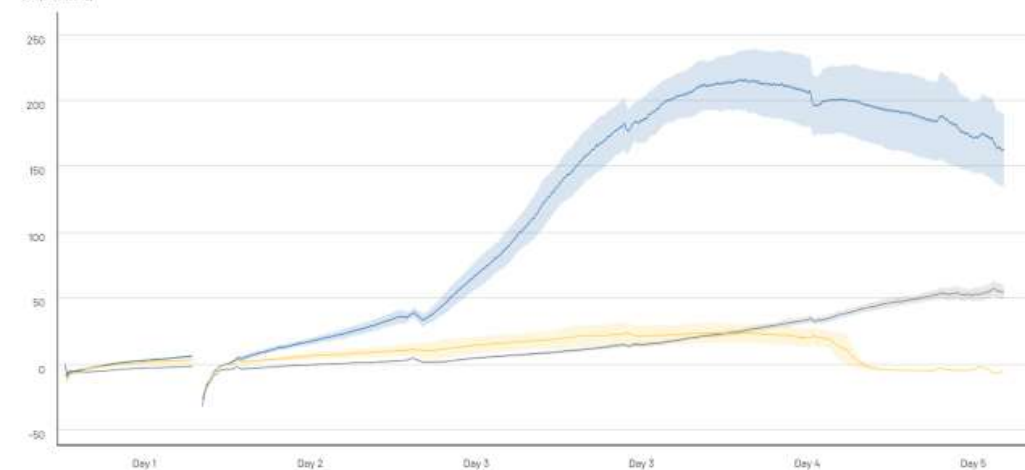
H460



08 ● -08 ● DMSO ● UT

OCR (fmol/mm²/s)

Cell Type ● H460 ● H358 ● SNU2535

OCR (fmol/mm²/s)

Clinicopathological parameters	Number of Patients (n=155)	Percentage (%)
Age, Years		
<65	68	43.87
≥65	87	56.13
Median (Range)	66.19 (29.47-86.19)	
Sex		
Male	101	65.16
Female	54	34.84
Smoking Status		
Current smokers	71	45.81
Ex-smokers	63	40.65
Never-smokers	21	13.55
Histology		
Adenocarcinoma	66	42.58
Squamous cell carcinoma	89	57.42
Current Status		
Alive	7	4.52
Dead	148	95.48
Stage		
I	76	50.97
II	37	23.87
III	41	26.45
IV	1	0.65

Grade		
1	11	7.09
2	85	54.84
3	59	38.06

Patient Characteristics Table (Tissue Micro Array, n=155)

A.

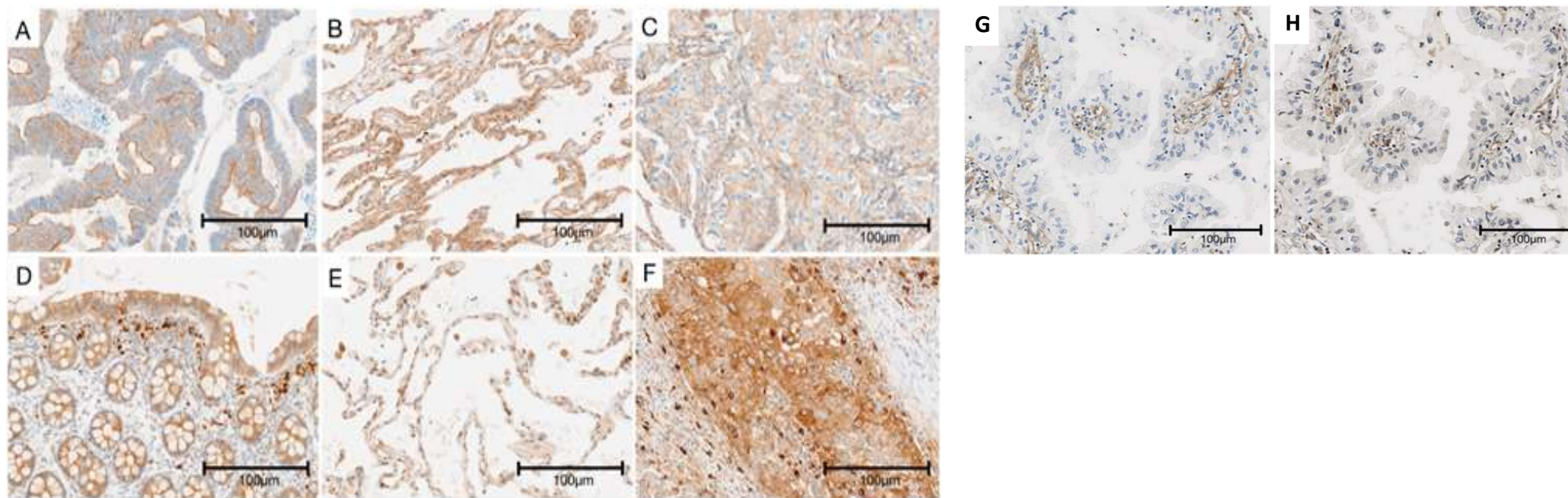
Antibody Dilution (μL:μL)	Protocol	Ultra CC1 (Antigen Retrieval at 100°C, min)	Peroxidase inhibitor (min)	Antibody exposure (min)	OptiView HQ Universal Linker (min)	OptiView HRP Multimer (min)	OptiView DAB IHC Detection Kit	Bluing & Haematoxylin (min/min)
1:100	A	16	10	8	8	8	8	8/8
	B	32	10	16	8	8	8	8/8
	C	64	10	32	8	8	8	8/8
1:200	A	16	10	8	8	8	8	8/8
	B	32	10	16	8	8	8	8/8
	C	64	10	32	8	8	8	8/8
1:250	A	16	10	8	8	8	8	8/8
	B	32	10	16	8	8	8	8/8
	C	64	10	32	8	8	8	8/8

B.

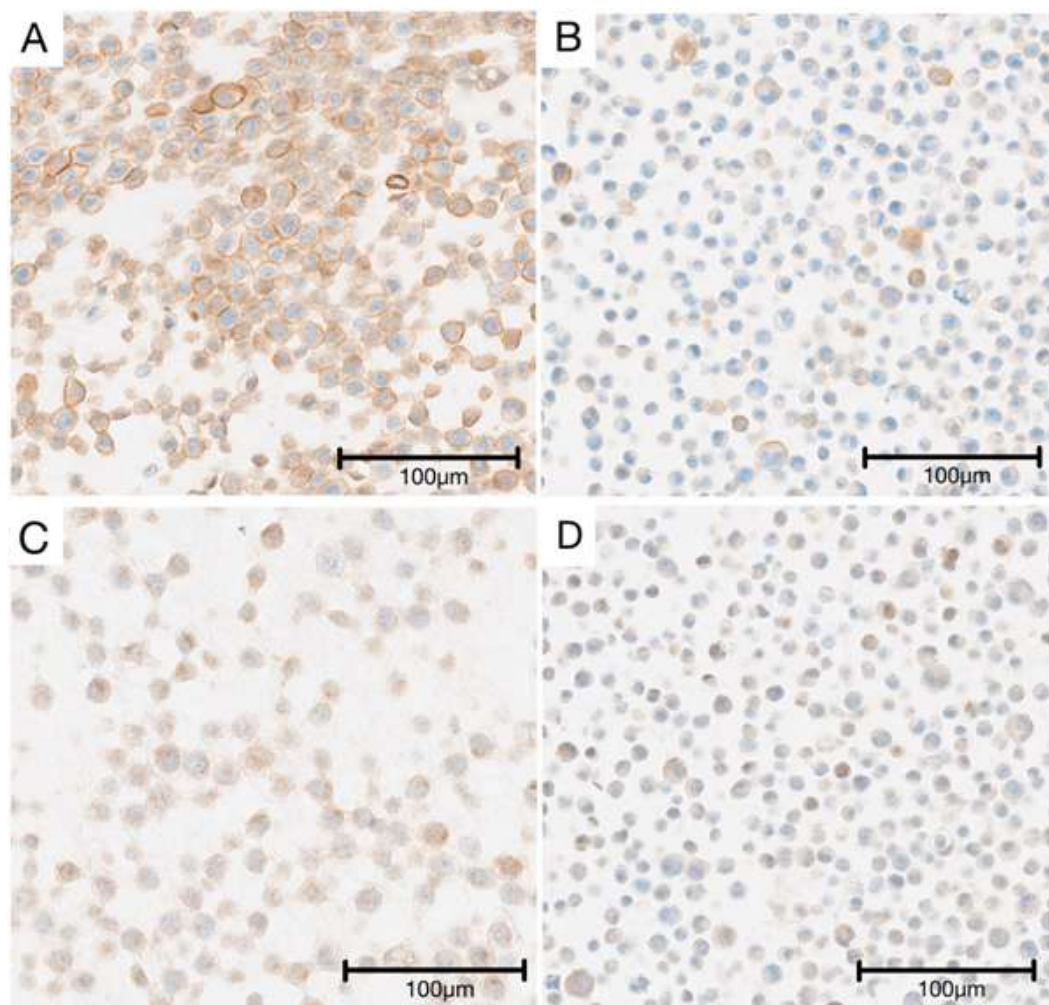
Antibody Dilution (μL:μL)	Protocol	Ultra CC1 (Antigen Retrieval at 100°C, min)	Peroxidase inhibitor (min)	Antibody exposure (min)	OptiView HQ Universal Linker (min)	OptiView HRP Multimer (min)	OptiView DAB IHC Detection Kit	Bluing & Haematoxylin (min/min)
1:120	A	16	10	8	8	8	8	8/8
	B	32	10	16	8	8	8	8/8
	C	64	10	32	8	8	8	8/8
1:200	A	16	10	8	8	8	8	8/8
	B	32	10	16	8	8	8	8/8
	C	64	10	32	8	8	8	8/8
1:250	A	16	10	8	8	8	8	8/8
	B	32	10	16	8	8	8	8/8
	C	64	10	32	8	8	8	8/8

**CysLTR1 (A) and CysLTR2 (B)
antibody optimizations using
the Ventana BenchMark
ULTRA system (Roche
Diagnostics)**

Representative IHC images for CysLTR1 and CysLTR2 staining in matched normal and tumour lung tissues following antibody optimization

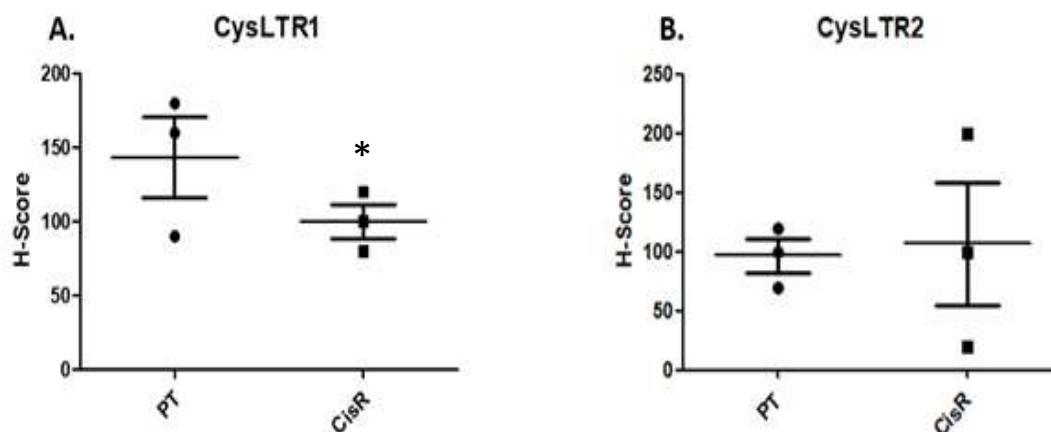


(A) Colon tissue (positive control), CysLTR1 (1:120), **(B, C)** Matched normal and tumour lung tissue, CysLTR1 (1:120), **(D)** Colon tissue (positive control), CysLTR2 (1:200), **(E, F)** Matched normal and tumour lung tissue, CysLTR2 (1:200). Adenocarcinoma cells staining negative for **(G)** CysLTR1 and **(H)** CysLTR2. Magnification 20×

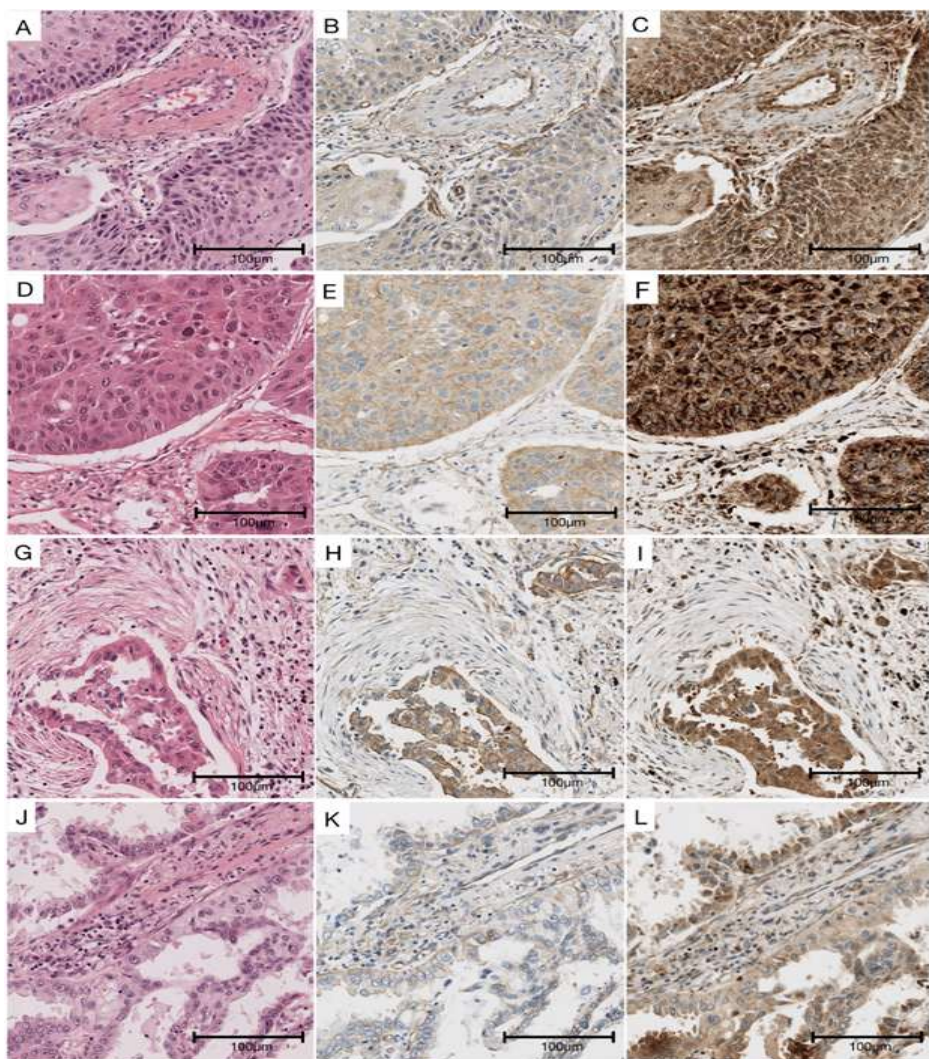


Representative immunohistochemistry images of CysLTR1 and CysLTR2 protein expression in H460 parental (PT) and cisplatin-resistant (CisR) NSCLC cell lines. CysLTR1: **(A)** Strong membranous staining in H460 PT cells stained with anti-CysLTR1 antibody (H-score: 180), **(B)** Weak membranous and cytoplasmic staining of H460 CisR cells (H-score: 80). CysLTR2: **(C)** Weak membranous and cytoplasmic staining of H460 PT cells (H-score: 70), **(D)** Weak cytoplasmic staining of H460 CisR cells (H-score: 20). Magnification 20×

CysLTR1 and CysLTR2 protein expression in parental (PT) and cisplatin-resistant (CisR) NSCLC cell lines

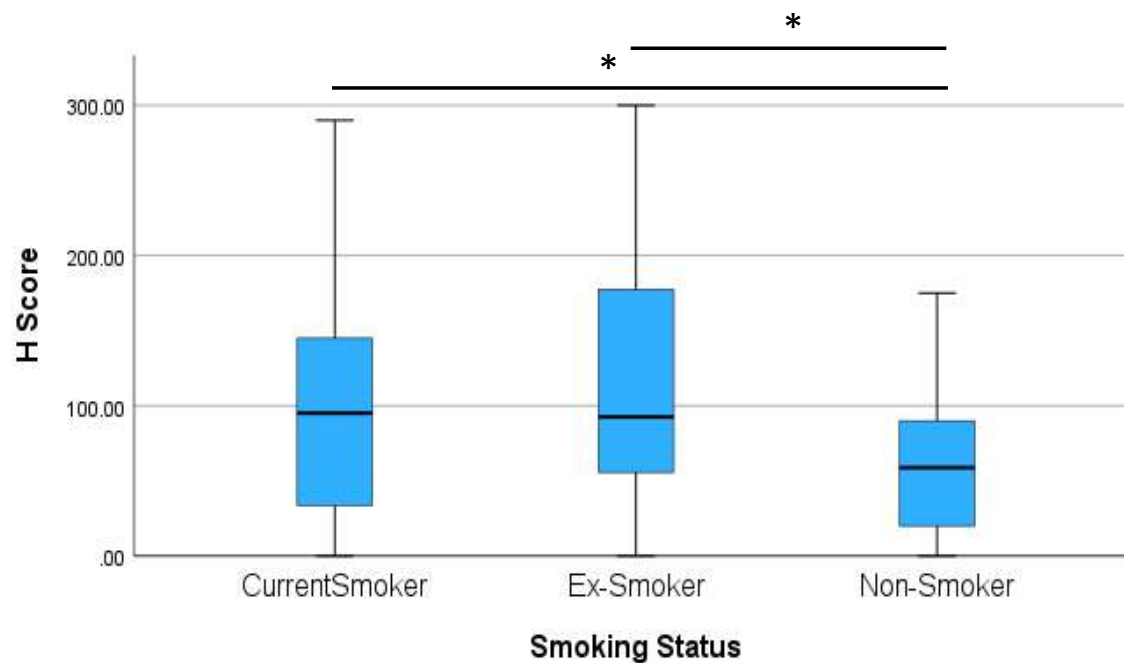


Cell blocks were prepared for H460, A549 and SKMES-1 PT and matched CisR cell sublines and stained for **(A)** CysLTR1 and **(B)** CysLTR2 using optimized antibody dilutions and experimental conditions. H-scores for CysLTR1 and CysLTR2 expression were averaged for each cell line. Statistical analysis was carried for three independent immunocytochemistry analyses using a Student's *t*-test (* $p < 0.05$).



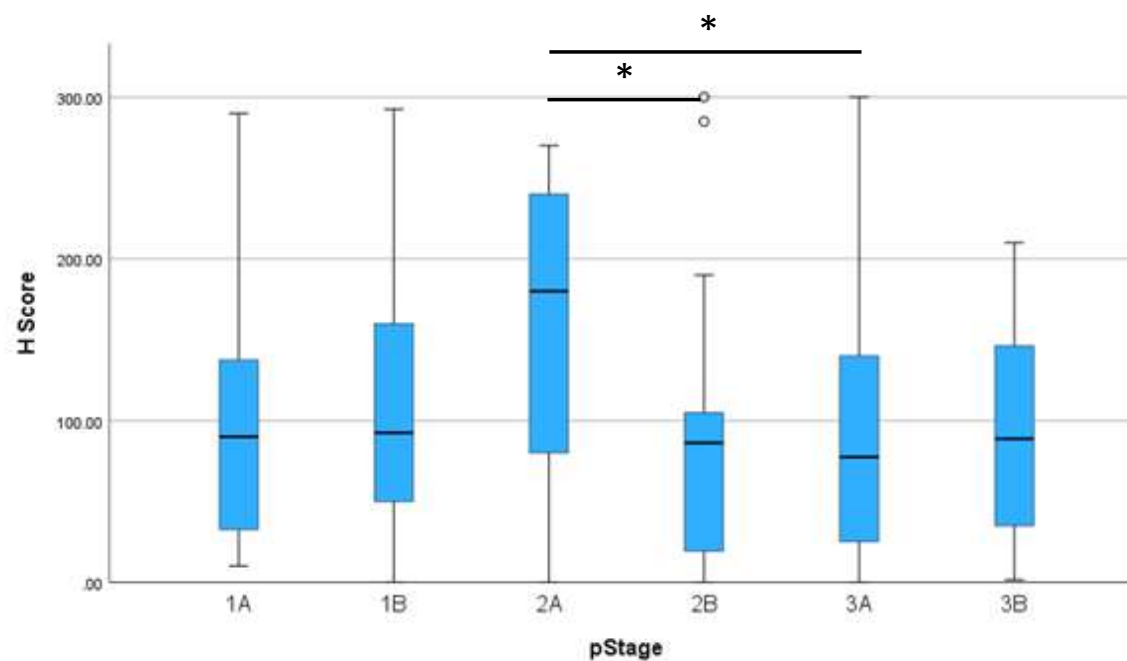
H&E and IHC tissue images representing CysLTR1 and CysLTR2 protein expression in squamous cell carcinoma (SqCC) and adenocarcinoma (ADC) tissue cores from the NSCLC TMA. (A) H&E staining of SqCC lung tissue, (B) Weak CysLTR1 expression in SqCC tumour cells (H-Score: 40), (C) Moderate CysLTR2 protein expression in SqCC tumour cells (H-Score: 160), (D) H&E staining of ADC lung tissue, (E) Moderate CysLTR1 protein expression in ADC tumour cells (H-Score: 200), (F) Strong CysLTR2 protein expression in ADC tumour cells with granular staining pattern (H-Score: 300), (G) H&E staining of ADC lung tissue, (H) Strong CysLTR1 expression in ADC tumour cells (H-Score: 270), (I) Moderate CysLTR2 expression in ADC tumour cells (H-Score: 180), (J) H&E staining of ADC lung tissue, (K) Moderate CysLTR1 expression in ADC tumour cells (H-Score: 160), (L) Weak CysLTR2 expression in ADC tumour cells (H-Score: 60). Magnification at 20×

CysLTR₁ is significantly higher in current and former smokers



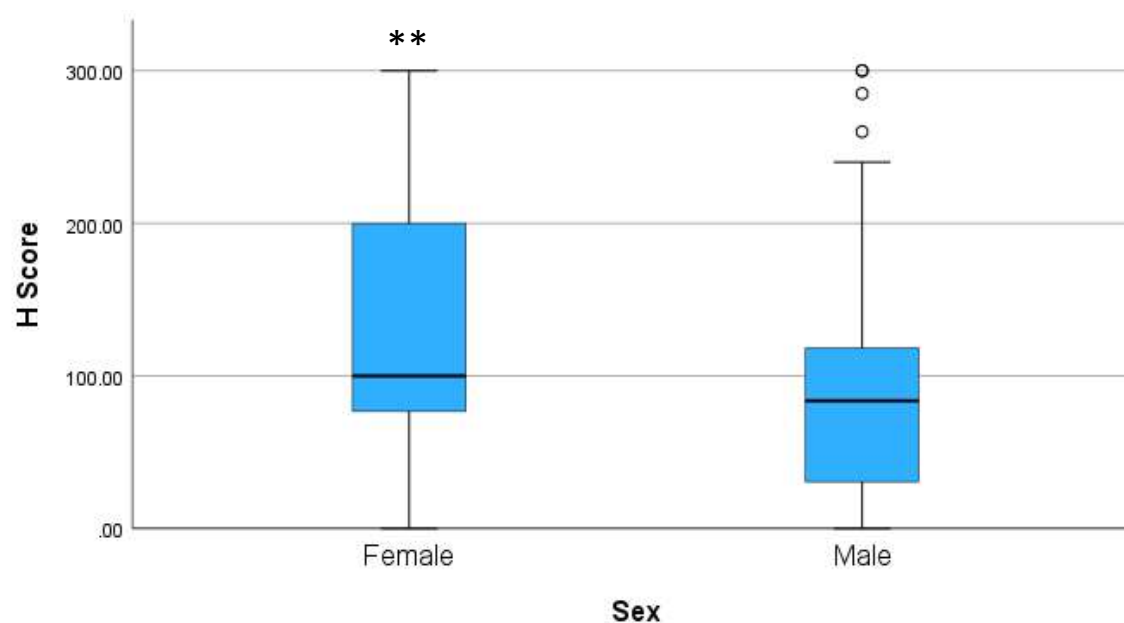
H-scores for CysLTR1 in TMA NSCLC tissues (n=155) based on smoking status were compared between current smokers vs never smokers ($p=0.046$) and ex-smokers vs never smokers ($p=0.015$), * $p<0.05$

CysLTR1 is significantly higher in patients with stage IIA and IIIA disease



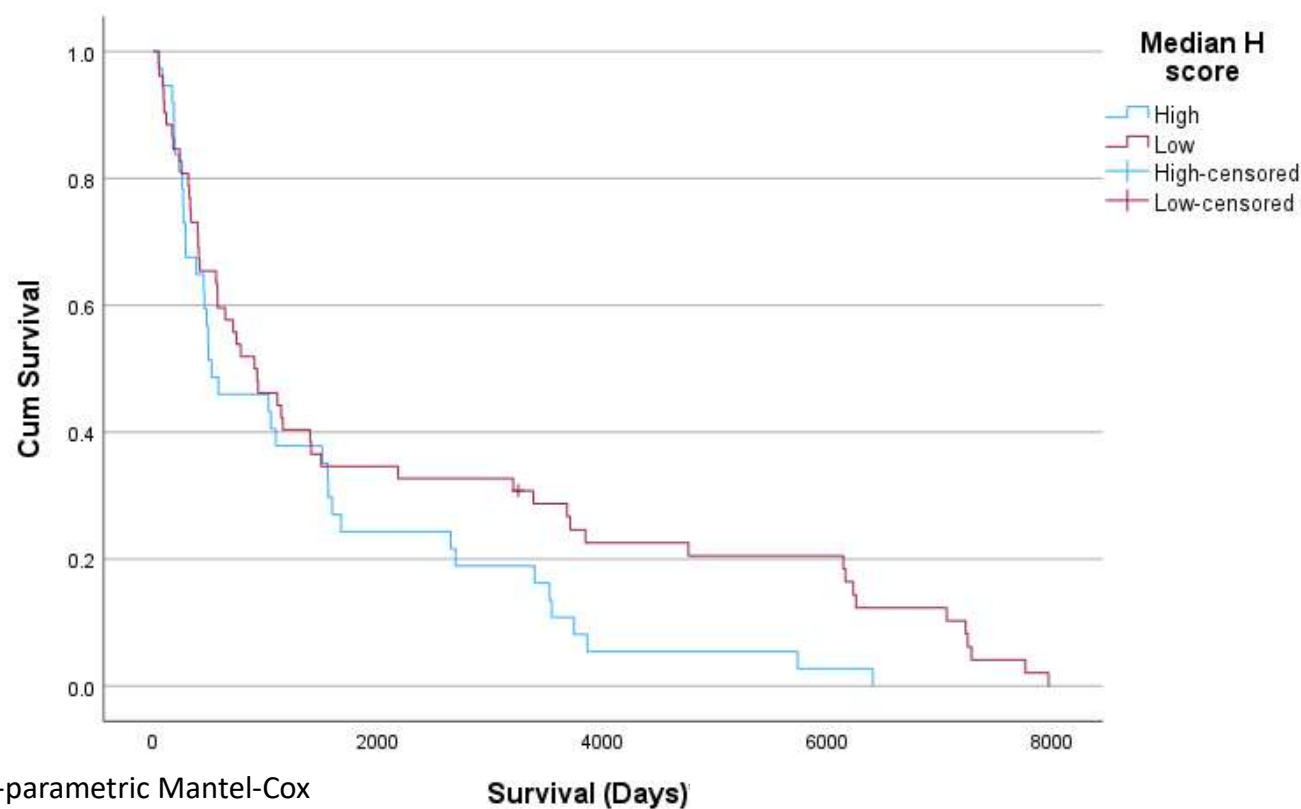
CysLTR1 H-scores for NSCLC patients (n=155) were compared across different tumour stages using the Kruskal-Wallis test, where stage 2A vs 2B ($p=0.034$) and 2A vs 3A ($p=0.046$), * $p<0.05$

CysLTR2 is significantly higher in females vs males



Staining intensity for CysLTR2 based on H-scores (TMA) were compared between female and male NSCLC patients (n=155) using the Mann-Whitney U test, female vs male (p=0.001), **p<0.05

NSCLC patients with squamous cell histology and high CysLTR1 expression have worse survival outcomes



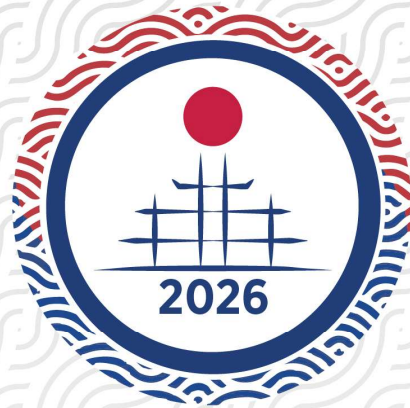
Kaplan-Meier survival curve, non-parametric Mantel-Cox
log-rank test ($p=0.065$)

Conclusions

- CysLTR's show promise as potential biomarkers in NSCLC, supported by our *in vitro* findings demonstrating that the novel quininib analogue, 1,4-dihydroxyquininib (Q8), significantly inhibits the growth of cisplatin-resistant NSCLC cells.
- This study supports our hypothesis that cysteinyl leukotriene receptors, CysLTR1 and CysLTR2 are expressed in NSCLC tumour tissues and are significantly associated with, at least in part, smoking status, tumour stage, gender and overall survival in patients with NSCLC.
- While further research in this area is warranted, our data provide new information on the role of the CysLTR's in NSCLC and lay the foundation for future studies in assessing Q8 on lung tumour growth and in reversing drug resistance, a significant clinical challenge in the treatment of lung cancer.

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Thank You.

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