HUMAN CELL-BASED miRNA INVESTIGATION OF OCCUPATIONAL ALLERGIC ASTHMA PATHWAYS

F.C. Passoni[1], F. Liviero[2], G. Melzi[1], *E. Corsini*[1], M. Marinovich[1], and *V. Galbiati*[1]

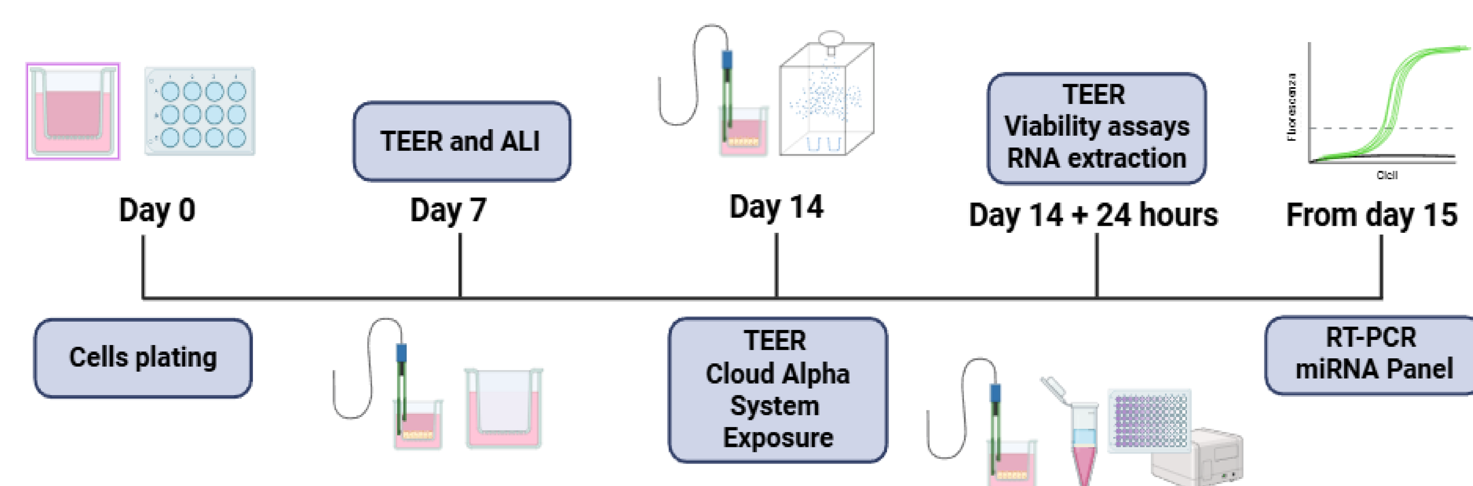
[1]*Universita Degli Studi Di Milano, Milan, Italy;* [2]*Universita' Di Padova, Padova, Italy*

For more information: francesca.passoni@unimi.it - valentina.galbiati@unimi.it

BACKGROUND & AIM

Asthma is a heterogeneous and chronic disease of the lower airways that affects around 300 millions people worldwide. The most common and well-studied form of asthma is the allergic type affecting both children and adults. Recent evidences reported alterations in miRNA expression in a variety of lung diseases, including allergic asthma. Several miRNAs have been associated with asthma and airways inflammation, but target identification remain not yet determined for the majority of the studies. The specific aim of this study, entirely based on human cells, is to identify miRNA patterns and consequently which specific targets are involved in the occupational allergic asthma disease

MATERIALS & METHODS



Cell culture: human bronchial epithelium Calu-3 cell line (AddexBio Technologies) was used for *in vitro* exposure. Calu-3 were cultured with MEM + GlutaMAX (Immunological Sciences), supplemented with 10% FBS, 1% NEAA solution (Capricorn) and 1% penicillin/streptomycin in an incubator with 5% CO2 at 37° C. Upon reaching about 80% confluence, 0.5 mL cells suspension (2.5 × 10^5 cells/mL) was seeded into the apical side of sterile 12 mm insert (3.0 μm pore size, 1.12 cm^2 polyester membrane, Greiner bio-one, International GmbH) placed in a 12-well plate. 1 mL culture medium was added to the basolateral side of insert. Cells in insert were cultured under submerged condition for 7 days to achieve confluence. Apical medium was then removed, Calu-3 cells were cultured for an additional 7 days at ALI. Culture medium (only basolateral medium during ALI culture) was refreshed every 2-3 days. All supplements were purchased from Merck.

Chemicals: the respiratory sensitizers (platinum hydrochloride HClPt - CAS 12648-47-4, trimellitic anhydride TMA - CAS 552-30-7 and hexamethylen diisocianate HDI - CAS 822-06-0), the irritant (sodium dodecyl sulphate SDS - CAS 151-21-3) and the skin sensitizer (2,4-dinitrochlorobenzene - CAS 97-00-7) were purchased from Merck (Merck KGaA, Darmstadt, Germany) at the highest purity available. HClPt, SDS and TMA were dissolved in PBS, DNCB and HDI were dissolved in DMSO and diluted in PBS.

TEER Measurement: TEER of Calu-3 cells was measured by the Evom2 Voltohmmeter with a 4 mm chopstick electrodes (World Precision Instruments Inc., FL, USA).

Cloud exposure system: The VITROCELL® cloud exposure system (Vitrocell, Waldkirch, Germany) was used for ALI *in vitro* exposure at 37 C. Cells were then exposed to chemicals by sedimentation of the droplets. After exposure, all inserts were transferred to a new 12-wells plates with 1 mL fresh culture medium on the basolateral side and cultured in an incubator for 24 h.

Cell viability and LDH leakage: cell viability was measured by the MTT assay (Gerlier and Thomasset, 1986) and the leakage of total lactate dehydrogenase (LDH) in culture medium was measured with the LDH cytotoxicity detection kit (Takara Bio Inc). The absorbance was read at 595 and 450 nm respectively using a microplate reader (Molecular Devices) and CV80 and LDH leakage were calculated.

RNA extraction: the Rneasy Mini Kit (Qiagen) was used following the manufacturer's instruction. The quantification of the RNA was performed using a NanoReady (LifeReal Biotech). After RNA extraction, a miRNA reverse-transcription was carried out with miRCURY LNA RT kit (Qiagen) following the manufacturer's instruction.

miRNA expression: miRNA panel were assessed using miRCURY LNA miRNA Focus PCR Panels (Human Immunopathology Focus, YAHS-204Y, Qiagen) through a real-time PCR analysis (CFX Connect Real - BIO-RAD).

Data analysis: statistical analysis was performed using GraphPad InStat version 3.0a for Macintosh (GraphPad Software, San Diego, CA, USA).

CONCLUSIONS

- ALI airway model + miRNA profiling detected early, chemical-specific inflammatory and oxidative responses.
- HClPt, HDI, TMA induced distinct miRNA patterns and IL-6/HMOX modulation, highlighting different modes of action.
- No single signature separated respiratory sensitizers from irritants, but miRNA networks remained informative.
- Data support miRNA-based endpoints for sensitizer assessment.
- Future work: broader chemical panels, more reactive airway models, and clinical validation.

RESULTS

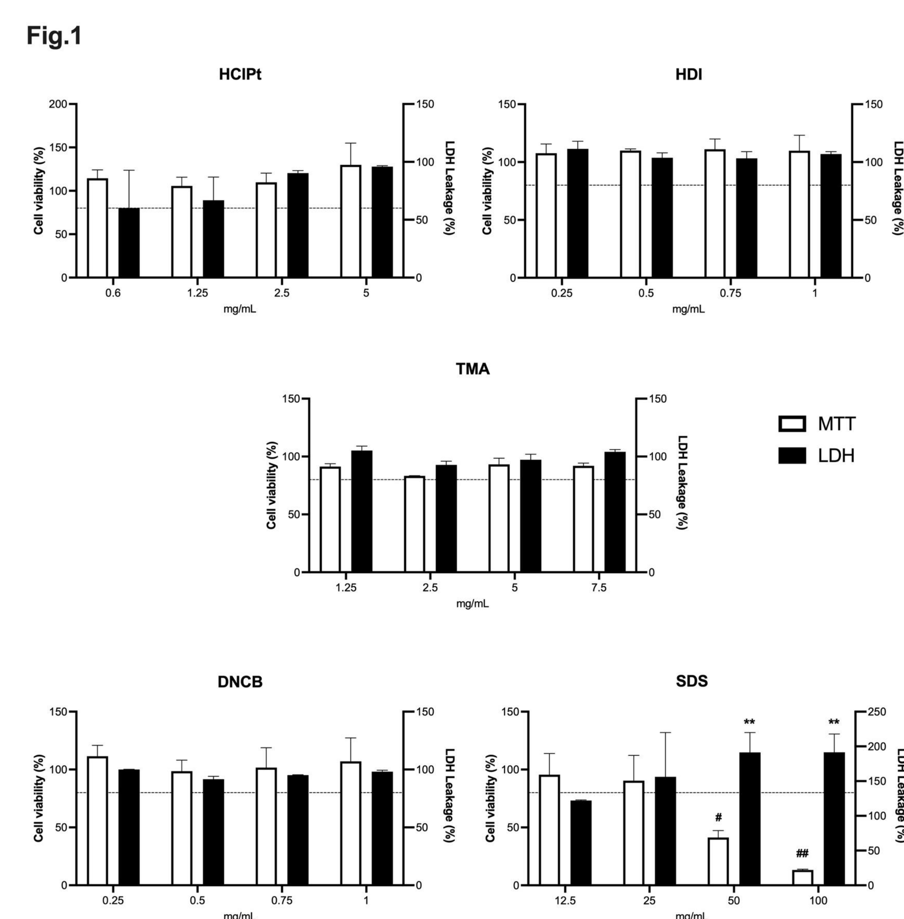


Figure 1: Evaluation of cell viability by LDH leakage and MTT test. Calu-3 cells were exposed to selected chemicals and then incubated for 24 h. MTT results are shown as white bars, whereas LDH release is shown as black bars. Controls (Ctrl-) are set at 100%. The dotted line corresponds to the CV80 (%). Statistical analysis was performed by two-way ANOVA for chemicals *vs*. Ctrl- conditions. The results are expressed as mean ± SEM and were considered statistically significant at $p \leq 0.05$, with $^{\#}p < 0.05$, and $^{\#\#,**}p < 0.01$ *vs*. Ctrl-.

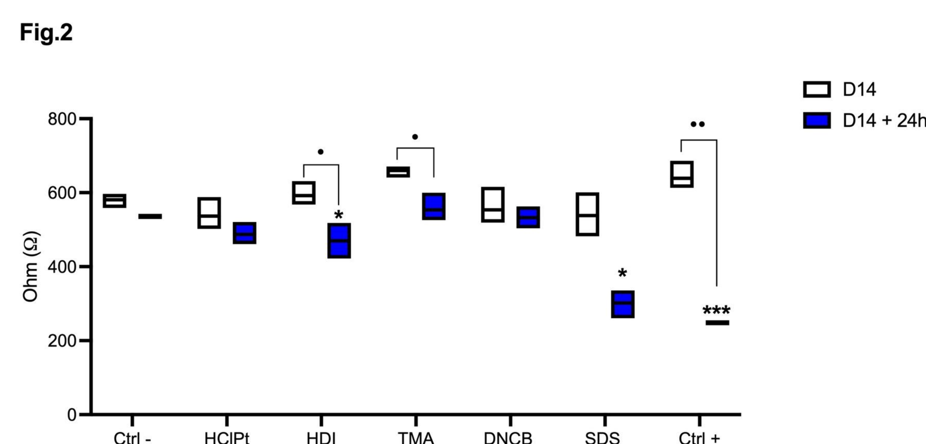


Figure 2: Effects of chemicals on membrane integrity (TEER). Calu-3 cells were cultured for 7 days in submerged conditions and subsequently cultured for an additional 7 days under ALI conditions. Chemical exposure was performed on day 14 (D14) using the VITROCELL® Cloud Alpha 6 System. TEER was measured at D14 (white boxes) and 24 hours after chemicals exposure (D14 + 24h, blue boxes). Results are expressed as floating bars (min to max) with the line at mean of n=3 measurements reported in Ohm (Ω). Statistical analysis was performed by paired Student's t-test for chemicals vs. Ctrl- and for post-exposure vs. pre-exposure conditions. Results were considered statistically significant at p ≤ 0.05, with *, • p < 0.05, •• p < 0.01, and *** p < 0.001 vs. Ctrl- (*) and vs. pre-exposure (•) conditions.

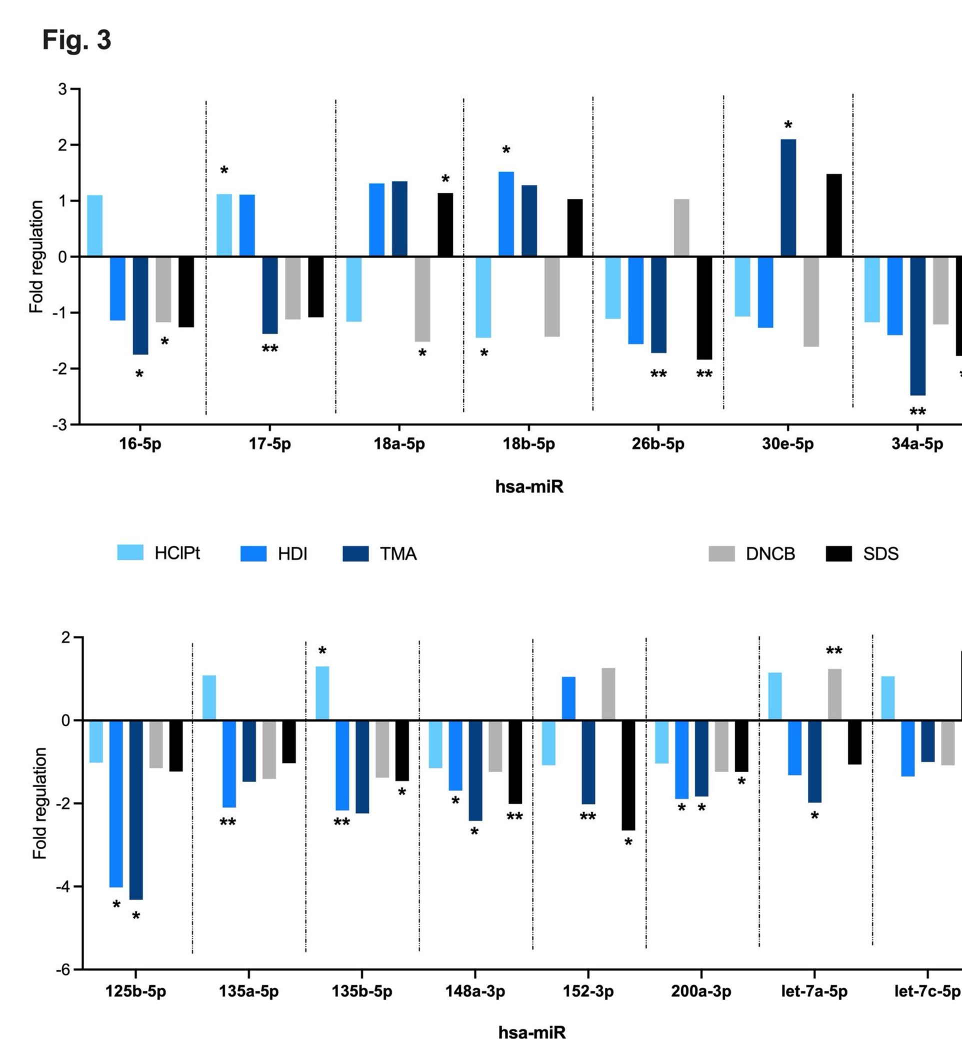


Figure 3: Analysis of the 15 miRNAs selected from the miRCURY LNA miRNA Focus PCR Panels (Human Immunopathology Focus, YAHS-204Y, Qiagen) after chemicals exposure. Results about miRNAs expression are expressed as fold regulation. Each value represents the mean of n=3 independent experiments. Statistical analyses were performed with a paired Student's t-test. Results were considered statistically significant at p ≤ 0.05, with * p < 0.05 and ** p < 0.01 vs. control vehicle treated cells.

	IL-6 release	miRNA expression	miRNA modulation
HClPt	⇧*	⇩ miR-18b-5p *	Mimic miR-18b-5p
		⇧ miR-135b-5p *	miR-135b-5p inhibitor
		⇧ miR-let7a-5p *	miR-let7a-5p inhibitor
HDI	⇩**	⇧ miR-18b-5p *	miR-18b-5p inhibitor Mimic mir-135b-5p Mimic-let-7a-5p
		⇩ miR-135b-5p **	
		⇩ miR-let7a-5p	
TMA	⇩*	⇧ miR-18b-5p	
		⇩ miR-135b-5p	
		⇩ miR-let7a-5p*	

	HMOX expression	miRNA expression	miRNA modulation
HClPt	⇧	⇩ miR-18b-5p *	Mimic miR-18b-5p
		⇩ miR-135b-5p *	miR-135b-5p inhibitor
		⇧ miR-let7a-5p *	miR-let7a-5p inhibitor
HDI	⇩***	⇧ miR-18b-5p *	miR-18b-5p inhibitor Mimic mir-135b-5p Mimic-let-7a-5p
		⇩ miR-135b-5p **	
		⇩ miR-let7a-5p	
TMA	⇩	⇧ miR-18b-5p	
		⇩ miR-135b-5p	
		⇩ miR-let7a-5p*	

Tables: Summary of IL-6 release, HMOX expression, and miRNA expression induced by the respiratory sensitizers, and the relative miRNA modulation approach used.

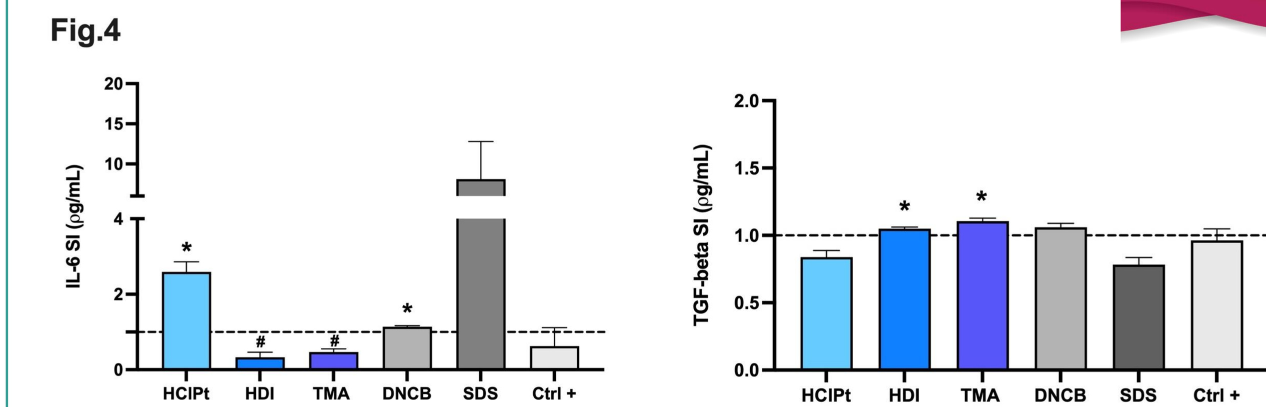


Figure 4: Effects of chemicals on the release of IL-6, IL-8, and TGF-β. Results are expressed as stimulation index (SI) compared to the Ctrl-. The dotted line corresponds to the Ctrl- set at 1. Results are expressed as mean ± SEM of n=3 replicates. Statistical analyses were performed with an unpaired Student's t-test, results were considered statistically significant at p ≤ 0.05, with # p < 0.05, **, ## p < 0.01.

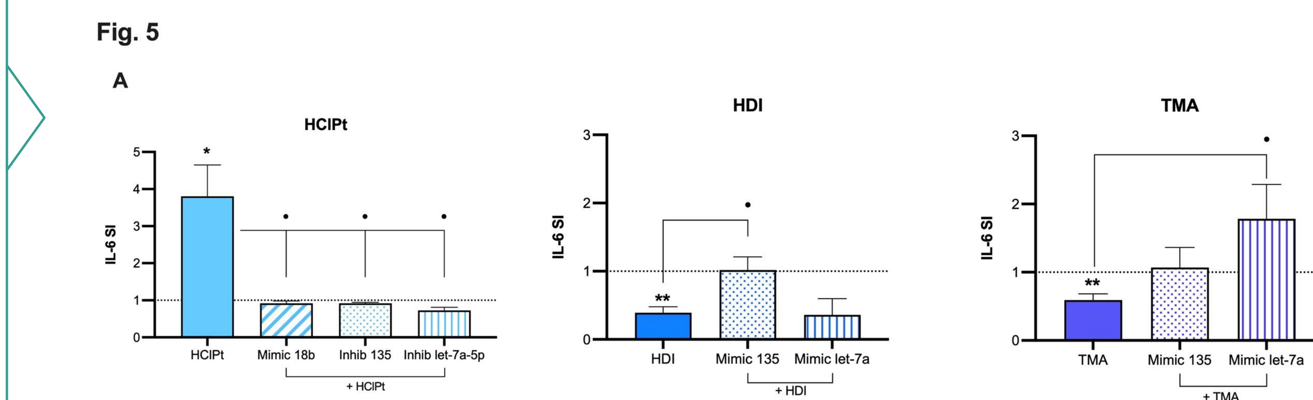


Figure 5: IL-6 release after pre-treatment with specific miRNA mimics or inhibitors and exposure to the selected respiratory sensitizers. IL-6 release is expressed as SI compared to Ctrl-. The dotted line corresponds to the Ctrl- set at 1. Each column represents the mean ± SEM of n=3 independent experiments. Statistical analysis was performed with paired Student's t-test, with * versus control, and • versus chemicals alone. Results were considered statistically significant at p ≤ 0.05, with *, • p ≤ 0.05, ** p<0.01 vs. control vehicle treated cells.

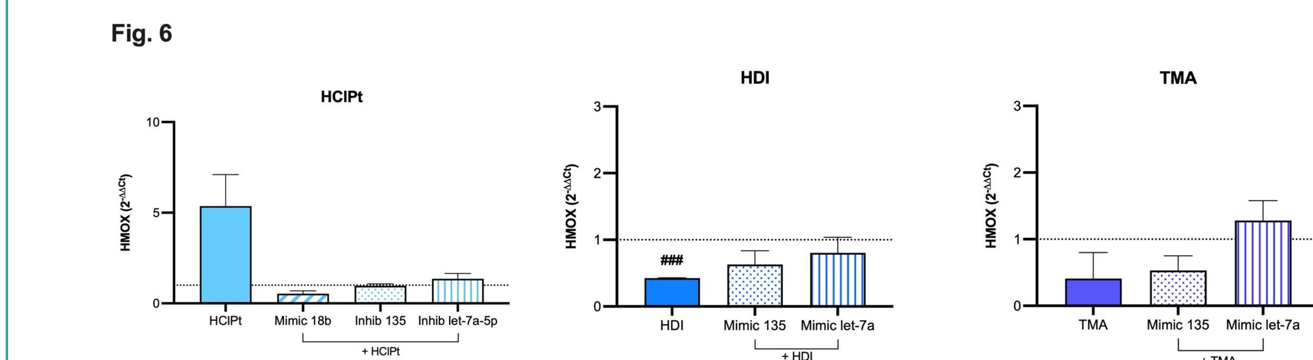


Figure 6: HMOX expression after pre-treatment with specific miRNA mimics or inhibitors and exposure to the selected respiratory sensitizers. HMOX gene expression is reported as fold change using 18s as housekeeping gene. The dotted line corresponds to the Ctrl- set at 1. Each column represents the mean ± SEM of n=3 replicates. Statistical analysis was performed with paired Student's t-test. Results were considered statistically significant at p ≤ 0.05 with ### p < 0.001 vs. control vehicle treated cells.

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© Conflict of Interest Statement: the authors declare no conflict of interest.

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